**Highly selective inhibition of IMPDH2 provides the basis of antineuroinflammation therapy**

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Inosine monophosphate dehydrogenase (IMPDH) is an attractive target for immunosuppressive agents. Currently, small-molecule inhibitors do not show good selectivity for different IMPDH isoforms (IMPDH1 and IMPDH2), resulting in some adverse effects, which limit their use. Herein, we used a small-molecule probe specifically targeting IMPDH2 and identified Cysteine residue 140 (Cys140) as a selective druggable site. On covalently binding to Cys140, the probe exerts an allosteric regulation to block the catalytic pocket of IMPDH2 and further induces IMPDH2 inactivation, leading to an effective suppression of neuroinflammatory responses. However, the probe does not covalently bind to IMPDH1. Taken together, our study shows Cys140 as a druggable site for selectively inhibiting IMPDH2, which provides great potential for development of therapy agents for autoimmune and neuroinflammatory diseases with less unfavorable tolerability profile.

**IMP dehydrogenase-2 | druggable site | covalent binding | allosteric regulation | immunosuppression**

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**Results**

**IMPDH2 is Selectively Targeted by SA.** First, we found that sappanone A (SA) was a potent inhibitor of microbial activation. As shown in SI Appendix, Fig. S1 A and B, SA significantly suppressed the releases of NO, TNF-α, IL-6, and PGE2 and decreased the gene expressions of TNF-α, IL-6, IL-1β, MCP-1, iNOS, and COX-2 in BV-2 cells. Similarly, SA significantly suppressed the production of NO, TNF-α, and IL-6 in primary microglia (SI Appendix, Fig. S2).

**Significance**

Inosine monophosphate dehydrogenase (IMPDH) is an attractive target for immunosuppressive agents. Currently, small-molecule inhibitors do not show good selectivity for different IMPDH isoforms (IMPDH1 and IMPDH2), resulting in some adverse effects, which limit their use. Here, we identified Cys140 as an isoform-selective druggable binding site for IMPDH2 inhibition but not for IMPDH1. We found small-molecule sappanone A directly covalently targets Cys140 in IMPDH2 to block its activity, resulting in neuroinflammatory inhibition with less side effects than pan-IMPDH inhibitor. In summary, our findings reveal Cys140 is a druggable binding site for selectively inhibiting IMPDH2 for neuroinflammatory diseases with less unfavorable tolerability profile.


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To explore the pharmacological target of SA, we prepared chemical probes for affinity purification and fluorescent labeling. As shown in Fig. 1A, a biotin-tagged SA probe (Biotin-SA) was used to pull down the cellular target of SA, and a Cy3-tagged SA probe (Cy3-SA) was used for fluorescent labeling of the target protein. Both probes retained the ability to inhibit NO release, suggesting that the chemical modification did not influence the biological activity of SA (SI Appendix, Fig. S3). Pull-down assay coupled with stable isotope labeling with amino acids in cell culture (SILAC) revealed that the SILAC ratio (heavy/light) of IMP dehydrogenase-2 (IMPDH2) was much higher than other proteins. Therefore, we speculated that IMPDH2 might be as a key target protein (Fig. 1B, Top Right). Next, we performed pull-down assay coupled with shotgun proteomics to further confirm our findings. As presented in Fig. 1C, one obvious protein band could be observed between 50 and 60 kDa in the pull-down group with SA beads; however, a much weaker protein band was found in the group with an excess amount of SA for competition. LC-MS/MS analysis showed the protein was IMPDH2 (56 kDa, SI Appendix, Fig. S4). Western blot also verified this protein with a specific anti-IMPDH2 antibody (Fig. 1E). Other protein bands were identified as heat shock protein 90 and β-actin, which were excluded from the alternative targets of SA (SI Appendix, Fig. S4). We also confirmed IMPDH2 as the target of SA (Fig. 1H).
in primary cultured microglial lysates and the tissue lysate of LPS-injected mouse brains (SI Appendix, Figs. S5 and S6).

To verify the interaction of SA with IMPDH2, we incubated BV-2 cell lysates or recombinant IMPDH2 protein with SA beads in the absence or presence of an excess amount of SA for competitive binding. As shown in Fig. 1D, IMPDH2 was obviously pulled down by SA beads, which were detected by Western blot. Meanwhile, we did not detect obvious binding of SA with the type I isoform (IMPDH1) (Fig. 1D). SPR analysis revealed that the target affinity \( K_D \) (equilibrium dissociation constant) value] of SA binding to IMPDH2 was 3.944 nM, almost 10 times lower than the \( K_D \) of SA binding to IMPDH1 (29.44 nM, Fig. 1E). Fluorescent labeling assay also showed a specific fluorescent band around 56 kDa for Cy3-SA–labeled IMPDH2 protein (Fig. 1F).

Small-molecule inhibitors can perturb protein function and increase the protein stability via forming a ligand–protein complex (14). Thus, we attempted to investigate whether SA could bind to IMPDH2 protein and increase its stability in intact cells or lysate using two target engagement assays (15, 16). From cellular thermal shift assay (CETSA), we found that SA treatment efficiently protected IMPDH2 protein from temperature-dependent degradation (Fig. 1G). Second, DARTS assay was used to monitor target engagement based on SA-induced stabilization of IMPDH2 protein. Our data also demonstrated a concentration-dependent reduced proteolysis with the incubation of SA (Fig. 1H).

**Cysteine 140 Is a Covalent Binding Site of IMPDH2.** To investigate the nature of SA interaction with IMPDH2, we first tested whether SA could covalently bind to IMPDH2. Western blot revealed that IMPDH2 was pulled down by SA beads, which were reversed by adding an excess amount of SA. However,
when IMPDH2 was preincubated with SA beads, posttreatment of an excess amount of SA could not prevent IMPDH2 binding to SA beads (Fig. 2A), indicating a covalent bond formation between SA and IMPDH2 protein. Because SA contains an α,β-unsaturated carbonyl group, which has a potential to react covalently with the thiol of cysteine on IMPDH2 (17), IMPDH2 was incubated with SA beads in the absence or the presence of β-mercaptoethanol (BME)/DTT for competitive binding to IMPDH2 via thiols (18). As shown in Fig. 2B, BME or DTT completely abolishes IMPDH2 binding to SA beads, suggesting that SA might covalently bind to the thiol of cysteine. Additionally, the SA-glutathione (GSH, a thiol donor) complex formed via Michael addition was also accurately confirmed using LC-pMRM analysis (SI Appendix, Fig. S7).

Next, we used BLAST analysis with full-length IMPDH2 protein sequence and found eight conserved cysteine residues (SI Appendix, Fig. S8). To determine which cysteine residue was attacked by SA, we incubated IMPDH2 protein with or without SA, followed by LC-MS/MS analysis. Tryptic peptides containing cysteine were evaluated, and Fig. 2C presents a peptide with a calculated mass of 1884.86 Da, which is 284.07 Da larger than the Cysteine140 (Cys140)-containing peptide ARHGFCGIPITDTGR, which has a calculated mass of 1600.79 Da. The mass difference of 284.07 Da was evaluated, and Fig. 2D presents a peptide with a calculated mass of 1600.79 Da. The mass difference of 284.07 Da exactly matches the molecular weight of an SA molecule. MS/MS spectrum of this peptide revealed that a 284.07 Da mass shift occurred starting from the b5 to the b6 fragment ions, indicating that the Cys140 residue was covalently modified by SA. This finding was also confirmed by synthetic peptides derived from human IMPDH2 containing Cys140 (PeptideC140, ARHGFCGIPIT-His) incubated with SA (SI Appendix, Fig. S9). Then, we mutated each cysteine residue of IMPDH2 into alanine. Pull-down assay with recombinant cysteine-mutated IMPDH2 proteins further supported that SA covalently modifies Cys140 but not the other cysteines of IMPDH2 (Fig. 2D).

Additionally, these observations were supported by fluorescent labeling experiments, which showed that only Cys140-mutated IMPDH2 (C140A) could not be labeled by Cy3-SA (Fig. 2E). We further investigated whether Cys140 mutation could impact the inhibitory effect of SA on IMPDH2. As shown in Fig. 2F, SA significantly inhibited WT IMPDH2 activity, which was markedly suppressed in Cys140-mutated IMPDH2. Meanwhile, SA did not show obvious inhibitory effect in WT IMPDH1 protein activity. A sequence comparison in Fig. 2G suggests that the Cys140 residue in IMPDH2 is conserved among various species. Interestingly, IMPDH1 does not possess a cysteine (Cys140) at the corresponding site, but a serine instead (Fig. 2G). Because hydroxyl in serine is a weaker nucleophilic group than the thiol in cysteine, this could explain why SA tends to preferably bind to IMPDH2 over IMPDH1.

**Cysteine 140 Is Targeted via Michael Reaction.** To further explore the structure–activity relationship of SA, we synthesized several SA derivatives (represented by boldface numerals) including: 1 (esterification of phenolic hydroxyl), 2 (partial destruction of the α,β-unsaturated carbonyl), and 3 (complete destruction of the α,β-unsaturated carbonyl). NO assay demonstrated that SA and 1 exhibited similar inhibitory effects on NO release; however, 2 showed a weak inhibitory effect on NO production, and 3 almost lost its ability to suppress NO (Fig. 3A). Next, we investigated the binding capacities of SA derivatives to IMPDH2. As shown in Fig. 3B, SA
beads effectively pulled down IMPDH2 protein, which was markedly reversed by an excess amount of SA and I, but not 2 or 3. Additionally, a fluorescent labeling experiment showed that Cy3-SA–labeled IMPDH2 protein bands were significantly decreased by adding an excess amount of SA or I, but not 2 or 3 (Fig. 3C). To verify the functional significance of the α,β-unsaturated carbonyl in SA, we performed in vitro kinase assay (19, 20). As shown in Fig. 3D, both SA and I markedly inhibited IMPDH2 activity; however, 2 and 3 did not demonstrate any inhibitory effects on IMPDH2 activity. Overall, we demonstrated that SA directly targets and inactivates IMPDH2 protein via the Michael addition of thiol in cysteine to the α,β-unsaturated carbonyl (Fig. 3E).

Cysteine 140 Is an Allosteric Regulatory Site of IMPDH2. IMPDH2 protein has a two-domain structure: (i) a catalytic domain (amino acid residues 2–92 and 224–492) forming the core of the active enzyme; and (ii) a regulatory Bateman domain (amino acid residues 93–223) (Fig. 4A) (7, 9). Molecular dynamics simulation analysis reveals that SA is deeply embedded in the cleft of the IMPDH2 Bateman domain and further promotes its bending to catalytic domain (“head-lowering” conformation). Additionally, as shown in Fig. 4B, a strong hydrogen-bonding interaction exists between SA and the residues of IMPDH2, including Thr225, Arg224, and Arg226. Such noncovalent interactions can serve as an initial site-recognition step when SA binds to IMPDH2 and hence raises the probability of the covalent reaction. Upon SA binding to Cys140, a protein loop region containing 20 amino acids (amino acid residues 322–342) in catalytic domain moves into the substrate IMP-binding pocket (Fig. 4C), leading to the inactivation of IMPDH2. Moreover, the loop region interacts with the IMP-binding pocket via hydrogen bonds and enhances the conformational stabilization (Fig. 4D). The variations of hydrogen bond length in simulated movement locus were shown in SI Appendix, Fig. S10. Additionally, the interaction surface of the loop region contains several hydrophobic amino acids, tending to interact with the hydrophobic surface of the IMP catalytic pocket (SI Appendix, Fig. S11). These observations were also confirmed by pull-down assay using IMP-coupled beads. We found IMPDH2 protein could bind to IMP-coupled beads and was inhibited by adding an excess amount of SA (Fig. 4E).

IMPDH2 protein functions as a tetramer by clustering four monomers (7, 21). We tried to evaluate the effect of SA on IMPDH2 clustering by observing the colocalization of GFP-tagged IMPDH2 (green) and mCherry-tagged IMPDH2 (red). As shown in Fig. 4F, the overlap of green and red fluorescence (yellow) was obvious in control cells; however, SA treatment markedly suppressed the overlap of green and red fluorescence (Fig. 4F). Moreover, nondenaturing gel electrophoresis and cross-linked whole-cell extracts also showed that IMPDH2 tetramers were decreased by SA treatment (Fig. 4G).

**NF-κB and p38 MAPK Pathways Contribute to IMPDH2-Dependent Neuroinflammation.** GTP is a key cellular metabolite of IMPDH2 (22). Fig. 5A shows that SA markedly reduced GTP level in BV-2 cells by about 50%. We next sought to elucidate whether the function of IMPDH2 is required for SA to inhibit microglial activation. As shown in Fig. 5B, blockage of IMPDH2 gene expression using a specific IMPDH2 siRNA significantly reversed SA-mediated inhibition of NO production. Moreover, we found...
similar inhibitory effects of SA on TNF-α and IL-6 release, which was abolished in IMPDH2 siRNA-transfected BV-2 cells. Although the detailed molecular pathway remains unknown, our present observation implies that NF-κB inflammation pathway activation is preferentially susceptible to suppression by SA (SI Appendix, Fig. S12A and B). We found that SA-dependent down-regulation of the phosphorylation of IKKβ, IκBα, and NF-κBα, as well as NF-κB-responsive luciferase activity, was markedly reversed when IMPDH2 expression was knocked down (Fig. 5 C and D). Moreover, mitogen-activated protein kinases (MAPKs) also regulate microglial activation (23). As shown in Fig. 5E, SA significantly inhibited p38 MAPK phosphorylation, but not on JNK or ERK. Moreover, a specific IMPDH2 inhibition via siRNA substantially reversed the SA-dependent down-regulation of p38 phosphorylation (Fig. 5E), demonstrating that p38 MAPK inflammatory signal might function as an essential component downstream of IMPDH2.

Based on rational drug structure optimization, we next synthesized several SA derivatives (4, 5, and 6) by inducing an electron-withdrawing group such as -F, -Br, and -NO2 to elevate the activity of α,β-unsaturated carbonyl. NO assay demonstrated that 4, 5, and 6 exhibited stronger inhibitory effects on NO release than SA. Notably, the IC50 of 5 is around 620 nM (Fig. 5F). Additionally, we detected the effects of different SA derivatives on IMPDH2 activity.

**Fig. 5.** NF-κB and p38 MAPK pathways contribute to IMPDH2-dependent neuroinflammation. (A) Intracellular GTP concentration was detected by HPLC analysis. (B) IMPDH2 is necessary for SA-mediated antiinflammatory activity. IMPDH2 siRNA-transfected BV-2 cells were treated with LPS in the absence or presence of SA for antiinflammation assay. (C) IMPDH2 is involved in SA-mediated NF-κB pathway suppression. BV-2 cells were treated with LPS in the absence or presence of SA for 30 min. Additionally, BV-2 cells transfected with IMPDH2 siRNA were treated with LPS in the absence or presence of SA for 30 min. (D) IMPDH2 is necessary for SA-dependent NF-κB inactivation. BV-2 cells transfected with IMPDH2 siRNA were further transfected with NF-κBα and Renilla reporter plasmids for 24 h, and then the cells were treated with LPS in the absence or presence of SA for 12 h and subjected to luciferase assay. (E) IMPDH2 is involved in SA-mediated p38 pathway suppression. BV-2 cells were treated with LPS in the absence or presence of SA for 30 min. Additionally, BV-2 cells transfected with IMPDH2 siRNA were treated with LPS in the absence or presence of SA for 30 min. (F) SA derivatives show a stronger inhibitory effect on NO production. (G) SA derivatives show a stronger inhibitory effect on IMPDH2 activity. The values represent the means ± SD of triplicates in an independent experiment. *P < 0.05, **P < 0.01, compared with the control group.

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As expected, 4, 5, and 6 significantly suppressed IMPDH2 activity, and the IC_{50} of 5 is around 750 nM (Fig. 5G).

SA Shows Inhibitory Effects on Neuroinflammation with Low Side Effects in Vivo. We next assessed the in vivo antineuroinflammatory effect of SA in LPS-stimulated BALB/c mouse model (Fig. 6A). We found that oral administration of SA effectively inhibited the inflammatory macrophage infiltration in the brains of mice that received LPS (SI Appendix, Fig. S13). Moreover, as shown in Fig. 6B, several inflammatory-related proteins in the brain tissue, including iNOS, COX-2, TNF-α, IL-β, and IL-6, were significantly decreased following the administration of SA or mycophenolic acid (MPA).

**Fig. 6.** SA shows inhibitory effects on neuroinflammation with low side effects in vivo. (A) Schedule of animal treatments. BALB/c mice were orally treated with 0.5% sodium carboxyl methyl cellulose (vehicle), SA (50 or 100 mg per kg body weight), and MPA (50 or 100 mg per kg body weight). One hour later, the inflammatory responses were activated by one i.p. injection of LPS (2 mg/kg). (B) Differential inflammatory protein expressions in the brain were measured 24 h after LPS challenge by Western blot. (C) Representative sections (cortex and CA1 region) prepared from brain tissues were stained with the specific microglia marker Iba-1, CD11b, and CD68. Arrows indicate the activated microglia. (D) Representative sections (cortex and CA1 region) prepared from the brain tissues were stained with Nissl solutions. (E) Analysis of hematological side effects. BALB/c mice were orally treated with SA or MPA (100 mg per kg body weight) for continuous 10 d (n = 10). **P < 0.01, compared with the control group.
acid (MPA), which is a non-isofrom-selective pan-IMPDH inhibi-
tor. Furthermore, immunohistochemical staining showed that
activated microglia (indicated by specific Iba-1, CD11b, and CD68 antibodies) in the cortex and hippocampal CA1 re-
 gions were effectively inhibited by SA or MPA (Fig. 6C).
Meanwhile, SA or MPA effectively protected neurons against
microglia-mediated neuroinflammatory injuries by Nissl’s staining
assay (Fig. 6D). It is noteworthy that SA did not show signifi-
cant hematological side effects; however, the same dose of MPA
caus ed significant decrease in several hemogram indexes, in-
cluding whole-blood-cell counts, lymphocyte counts, and monocyte
counts, suggesting that specific IMPDH2 inhibitor SA can inhibit
neuroinflammatory responses with high drug safety and less side
effects (Fig. 6E).

Discussion
Over the past decade, IMPDH has been viewed as an attractive
drug target for the chemotherapy for autoimmune disorders,
and IMPDH inhibitors appeared to act as effective immunosup-
 pressive agents in clinical trials (24). Thus, there has been a
concerted effort to identify small-molecule inhibitors of IMPDH
for inflammation-related diseases (5). However, current small-
molecule inhibitors do not show good selectivity for different
IMPDH isoforms (IMPDH1, IMPDH2), resulting in some ad-
verse effects, which limit their clinical use.

The druggable target identification is extremely important for
seeking therapy drugs (25–27). To explore the direct cell target of
SA (28), we designed a small-molecular probe based on SA struc-
ture and found it selectively targets IMPDH2, but not IMPDH1.
Notably, IMPDH2 contains a critical cysteine residue (Cys331) in
its catalytic domain that was targeted by several current inhibitors (29,
30). Interestingly, SA is selective toward Cys140 in regulatory
Bateman domain but not Cys331 in catalytic domain. Hitherto,
Cys140 has not been reported as a druggable site for IMPDH2
inhibition. Notably, SA showed selectivity to IMPDH2 rather than
IMPDH1. We assume that the molecular geometry of SA and the
chemical environment surrounding the binding pocket might be the
key factors. The covalent binding site of SA in IMPDH2 is the thiol
of Cys140, which supplies a structural specificity for the recognition
and binding of SA. However, the corresponding site in IMPDH1 is
serine, which contains a hydroxyl group and is reactionless to SA.

Molecular dynamics (MD) stimulation analysis indicated that the
cova lent binding of SA to Cys140 induced an allosteric effect on
IMPDH2 by promoting the Bateman domain to bend to the cata-
lytic domain. To our surprise, we found that the IMP-binding site
in the catalytic domain was also affected by the SA-induced allosteric
effect. In the SA-IMPDH2 complex, the IMP-binding site was oc-
cupied by the neighboring loop region and caused a dysfunction of
substrate processing as well as IMPDH2 inactivation. We found
that SA-induced serpentine flow mainly passes from the Bateman
domain to the IMPDH domain and arrives in the catalytic domain.
Notably, Ile461, Leu235, Ser237, and Ala236 play important roles in
serpentine flow passing (SI Appendix, Fig. S14). Thus, we speculated
that IMPDH2 activity might be subject to distinct regulation by SA
in the Bateman domain and further contribute to conformational
changes of the catalytic domain. Collectively, these observations
suggest a physiologically important role in the regulatory region
outside of the catalytic site of IMPDH2. To our knowledge, SA
represents the first small-molecule allosteric inhibitor that blocks
IMPDH2 by directly targeting Cys140 residue in the regulatory
Bateman domain.

Notably, S. Lee et al. have reported that SA could inhibit in-
flammation response on murine periphery macrophages via
Nrf2 and NF-κB pathways (31); however, the direct target of SA is
largely unexplored. Here, we showed SA directly targeted Cys140 in
IMPDH2 to block IKKβ kinase activity, leading to an effective
suppression of the NF-κB pathway. Moreover, we revealed that SA
showed an inhibitory effect on IMPDH2-mediated guanine nucleo-	ide biosynthesis, which was important for DNA or RNA synthesis.
This could cause the blockade of various inflammation-associated
gene expressions. Thus, we speculate that SA might also inhibit
the expression of heme oxygenase (HO)-1, which was revealed in S. Lee
et al.’s work (31) by direct inhibition of IMPDH2. Interestingly, SA
was also found to inhibit cellular tyrosinase activity via repressing
tyrosinase gene expression in mouse B16 melanoma cells (28). This
could be explained by SA-mediated IMPDH2 inhibition and re-
sultants guanine nucleotide biosynthesis stagnation, which is impor-
tant for tyrosinase genetic transcription process.

In summary, we discovered Cys140 as a covalent allosteric regu-
 latory site for selective IMPDH2 inhibition. The small molecules
targeting Cys140, such as SA, can induce an allosteric effect on
catalytic pocket and suppress IMPDH2 activity, leading to antiin-
flammation and immunosuppressive action. Therefore, Cys140 may
represent a promising drug-binding site of IMPDH2 inhibitors to
accelerate clinical drug development for neuroinflammation with
low side effects.

Methods
Cell Survival Assay. The cell survival assay was performed using the
MTT method. The detailed protocol is found in SI Appendix, SI Methods.

Identification of SA Target Proteins. Identification of SA target proteins
was based on pull-down technology coupled with SILAC and shotgun proteomics
analysis. The methods are found in SI Appendix, SI Methods.

Determination of the SA-Binding Site on IMPDH2. The SA-binding site on
IMPDH2 was detected using LC-MS/MS analysis on LTQ-Orbitrap. The detailed
protocol is found in SI Appendix, SI Methods.

Molecular Dynamics Simulation. The force field parameters for inhibitor co-
valently bonded to Cys140 residue of IMPDH2 protein were generated by the
General AMBER Force Field (GAFF) and Restrained Electrostatic Potential (RESP).
The detailed methodologies for MD are provided in SI Appendix, SI Methods.
All other methods, including cell culture, chemical synthesis, target identi-
fication, enzyme activity, gene or protein expression, animal experiments, data
collection, and so forth, are described in detail in SI Appendix, SI Methods.

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**IV. Supplementary references**
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 H$_2$SO$_4$ to pH = 2. The mixture was extracted with ethyl acetate. The combined organics were washed with brine and dried over Na$_2$SO$_4$ 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-yne (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-yl)oxy) chroman-4-one. 7-(prop-2-yn-1-yl)oxy) 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 (finial 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) δ_H 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) δ_H 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, 12H), 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) δ_H 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) δ_H 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®
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®
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:

\[
\text{Relative mRNA level of target gene (folds of control)} = 2^{-\Delta\Delta CT}
\]

**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 C140-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 (Ubria 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**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
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| Tnf-α  | F: 5’-AAGCAAGCAGCCAACCAG-3’  
|        | R: 5’-CCACAAGCAGGAATGAGAAAGA-3’              |
| Il-1β  | F: 5’-TGGAGAAGCTGTCAGCTACCT-3’ 
|        | R: 5’-GAACGTACACACACCAGCAGTT-3’             |
| Il-6   | F: 5’-ACAAGCCAGAGTCCCTTGAGAGA-3’            |
|        | R: 5’-ACAAGCCAGAGTCCCTTGAGAGA-3’            |
| Mep-1  | F: 5’-CTTCTGGCGCTGTACACAGTT-3’              |
|        | R: 5’-TTCTTTAGGTCTGTCCG-3’                  |
| Impdh2 | F: 5’-ATGGCTTCTGTGATAC-3’                   |
|        | R: 5’-TTCTTTAGGTCTGTCCG-3’                  |
| Gapdh  | F: 5’-GGTGAAGGTCGGTGTGAACG-3’               |
|        | R: 5’-CTCGCTCTGGAAGATGGTG-3’                |
Supplementary Table 2. siRNA sequences for transfection

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<th>5’-3’ (antisense)</th>
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<td>UUGGAAGAUGGAGUUUCCCTT</td>
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<tr>
<td>Negative</td>
<td>GCGAUUCGAUCUGCCUAAGAUTT</td>
<td>AUCUUAGGCAGAUCGUCGCTT</td>
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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$^{C140}$-SA complex by LC-MS. MS/MS analysis of Peptide$^{C140}$ 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 Cys331locates.

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).
1 Supplementary Fig. 22. $^1$H NMR spectra for Cy3-SA.

2

Supplementary Fig. 23. MS spectra for Cy3-SA.

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Supplementary Fig. 23. MS spectra for Cy3-SA.

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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


