Highly selective inhibition of IMPDH2 provides the basis of antineuroinflammation therapy

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Inosine monophosphate dehydrogenase (IMPDH) of human 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.

Inosine monophosphate dehydrogenase (IMPDH) is a major rate-limiting enzyme involved in guanosine and deoxyguanosine biosynthesis and widely expressed in immunocytes (1). There exist two IMPDH isoforms (IMPDH1 and IMPDH2), which are encoded by distinct genes (2, 3). Many inflammation-relevant diseases have been specially characterized by the high expression of isoform II of IMPDH (IMPDH2) in rapidly proliferating immunocytes, rather than the “housekeeping” type I isoform (IMPDH1) in normal human leukocytes and lymphocytes (4, 5). Therefore, selective targeting of IMPDH2 with small molecules is an attractive topic for development of anti-inflammation agents with low side effects.

Both IMPDH isoforms contain two major domains: the catalytic domain for substrate interaction and the Bateman domain, which is not required for catalytic activity but exerts an important allosteric regulation effect on IMPDH activity by communicating with the catalytic domain (6, 7). By influencing catalytic domain activity, the Bateman domain can regulate IMPDH function and further blocks the downstream-of-inflammation signaling pathways (8, 9). Currently, IMPDH inhibitors are divided into two major categories. One kind of inhibitor, including 6-chloropurine riboside ribavirin and mizoribine, targets the binding pocket of the natural substrate, inosine monophosphate (IMP). Another kind of inhibitor (e.g., mycophenolic acid and thiazole-4-carboxamide adenine dinucleotide) targets the site of the cofactor, NAD+/NADH, which usually leads to low selectivity or even side effects in clinical trials, such as diarrhea and leukopenia (10, 11). Moreover, a third ligand has been speculated to bind to a possible site far from the IMP and NAD+ pockets as an allosteric inhibitor. However, an allosteric site for designing selective IMPDH2 inhibitors has been largely unexplored.

Natural small molecules remain promising drug sources (12, 13). In the present study, we report that a natural small-molecule probe, sappanone A (SA, Fig.1A), demonstrated significant inhibitory effects on neuroinflammation by directly targeting the conserved cysteine residue 140 (Cys140) in the noncatalytic Bateman domain of IMPDH2. Interestingly, SA selectively targets and inactivates IMPDH2 but not IMPDH1. The selectivity is explained by differential substituent groups of amino acids in two IMPDH isoforms. The thiol in cysteine 140 of IMPDH2 can lead to irreversible covalent binding via the Michael addition to the α,β-unsaturated carbonyl in SA. However, the corresponding amino acid in IMPDH1 is serine with a weaker nucleophilic group hydroxyl, resulting in a weakened covalent binding effect of SA. The selective modification of SA on IMPDH2 caused an allosteric effect on its catalytic domain to narrow the substrate combination space in the catalytic pocket, which led to a suppression of IMPDH2 activity and IMPDH2-dependent neuroinflammatory response without obvious hemato-logical side effects.

These findings indicate that cysteine 140 is a druggable binding site for selectively targeting IMPDH2. Small molecules binding to cysteine 140 of IMPDH2 can exert an effective antineuroinflammation therapy in clinical trials with fewer side effects.

Results

IMPDH2 Is Selectively Targeted by SA. First, we found that sappanone A (SA) was a potent inhibitor of microglial 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 drugbinding 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 drugbinding 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), which is a global protein assay, 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. 1D). 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. 1G, Top Right).
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. Western blot and silver staining. Moreover, an excess amount of SA effectively blocked the binding of IMPDH2 to SA beads. 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 abolished 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 accurately confirmed using LC-pMRM analysis (SI Appendix, Fig. S9).

Then, we mutated each cysteine residue of IMPDH2 containing Cys140 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 preferentially 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

![Image](https://example.com/image1.png)

**Fig. 3.** Cysteine 140 is targeted via Michael reaction. (A) α,β-unsaturated carbonyl is the major pharmacophore of SA for its inhibitory effect on NO production. BV-2 cells were treated with LPS in the absence or presence of different concentrations of SA and its derivatives (1, 2, and 3) for 24 h. (B) The α,β-unsaturated carbonyl is a major site for IMPDH2 binding with SA (pull-down assay). SA beads were incubated with recombinant IMPDH2 proteins in the presence of SA and its derivatives (1, 2, and 3) at 4 °C for 12 h. (C) α,β-unsaturated carbonyl is a major site for IMPDH2 binding to SA (Cy3-SA labeling assay). Recombinant IMPDH2 proteins were incubated with Cy3-SA (20 μM) in the absence or presence of SA and its derivatives (1, 2, and 3) at 4 °C for 12 h, and then the products were resolved by SDS-PAGE for detecting Cy3-SA-labeled IMPDH2 protein (red). (D) α,β-unsaturated carbonyl contributes to the inhibitory effect of SA on IMPDH2 activity. Recombinant IMPDH2 protein was used as kinase, and IMP was used as the substrate. The mixtures were incubated with SA and its derivatives (1, 2, and 3) for 3 h at 37 °C for the IMPDH2 activity assay. (E) The proposed covalent binding mode of SA to IMPDH2.
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 222–342) 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. S12 A and B). We found that SA-dependent down-regulation of the phosphorylation of IKKβ, IκBα, and NF-κB, as well as NF-κB-responsive luciferase activity, were 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. 5 E, 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. 5 E), 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. 5 F). Additionally, we detected the effects of different SA derivatives on IMPDH2 activity.
As expected, 4, 5, and 6 significantly suppressed IMPDH2 activity, and the IC50 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).
acid (MPA), which is a non-isom-selective pan-IMPDH inhibitor. Furthermore, immunohistochemical staining showed that activated microglia (indicated by specific Iba-1, CD11b, and CD68 antibodies) in the cortex and hippocampal CA1 regions 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 significant hematological side effects; however, the same dose of MPA caused significant decrease in several hemogram indexes, including 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 immunosuppressive agents in clinical trials (24). Thus, there has been a concerted effort to identify small-molecule inhibitors of IMPDH for inflammation-related diseases (3). However, current small-molecule inhibitors do not show good selectivity for different IMPDH isoforms (IMPDH1, IMPDH2), resulting in some adverse 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 structure 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 to be 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) simulation analysis indicated that the covalent binding of SA to Cys140 induced an allosteric effect on IMPDH2 by promoting the Bateman domain to bend to the catalytic 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 occupied 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 inflammation 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 nucleotide 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 resultant guanine nucleotide biosynthesis stagnation, which is important for the tyrosinase genetic transcription process.

In summary, we discovered Cys140 as a covalent allosteric regulatory 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 antiinflammation 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 covalently 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 identification, 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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