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Structure–Activity Relationships and Anti-inflammatory Activities of N-Carbamothioylformamide Analogues as MIF Tautomerase Inhibitors

Yu Zhang,^{†,‡} Lei Xu,^{‡,§,‡} Zhiqiang Zhang,[†] Zhiyu Zhang,[†] Longtai Zheng,[†] Dan Li,[‡] Youyong Li,^{||} Feng Liu,[†] Kunqian Yu,[†] Tingjun Hou,^{*,†,‡,||} and Xuechu Zhen^{*,†}

[†]Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases & Department of Pharmacology, College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu 215123, China

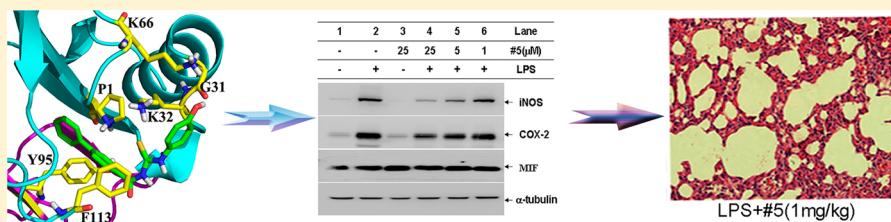
[‡]College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, China

[§]Institute of Bioinformatics and Medical Engineering, School of Electrical and Information Engineering, Jiangsu University of Technology, Changzhou 213001, China

^{||}Institute of Functional Nano & Soft Materials (FUNSOM), Soochow University, Suzhou, Jiangsu 215123, China

^{*}State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China

Supporting Information



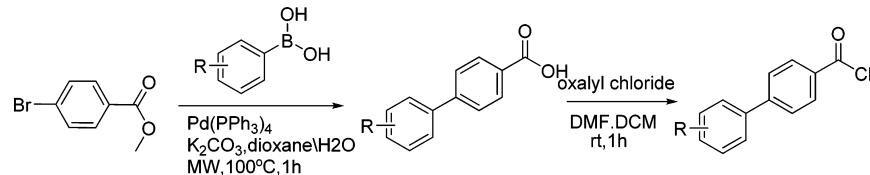
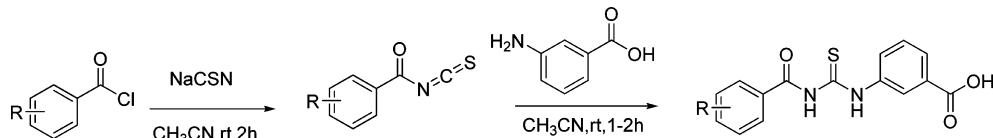
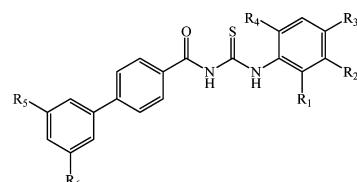
ABSTRACT: Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, is an attractive therapeutic target for the treatment of inflammatory diseases. In our previous study, 3-[(biphenyl-4-ylcarbonyl)carbamothioyl]amino benzoic acid (compound 1) was discovered as a potent inhibitor of MIF by docking-based virtual screening and bioassays. Here, a series of analogues of compound 1 derived from similarity search and chemical synthesis were evaluated for their MIF tautomerase activities, and their structure–activity relationships were then analyzed. The most potent inhibitor (compound 5) with an IC₅₀ of 370 nM strongly suppressed lipopolysaccharide (LPS)-induced production of TNF-α and IL-6 in a dose-dependent manner and significantly enhanced the survival rate of mice with LPS-induced endotoxic shock from 0 to 35% at 0.5 mg/kg and to 45% at 1 mg/kg, highlighting the therapeutic potential of the MIF tautomerase inhibition in inflammatory diseases.

INTRODUCTION

Macrophage migration inhibitory factor (MIF), a multifunctional protein,¹ has been regarded as an attractive therapeutic target for the treatment of sepsis² and other inflammatory diseases.^{3,4} Previous studies illustrated that the inhibition of MIF activity in vivo could attenuate the lethality of endotoxemia and sepsis in rodents.^{2,5,6} MIF exists as a homotrimer with three identical monomers, and the catalytic active site is located between two adjacent monomers of the homotrimer.⁷ In addition, MIF functions as a D-dopachrome tautomerase,⁸ a phenylpyruvate tautomerase,⁹ or a thiol–protein oxidoreductase.¹⁰ Despite relentless efforts, there is no clear-cut evidence between the catalytic activity and biological function of MIF.¹¹ However, targeting MIF tautomerase activity through small-molecule inhibitors has been proven to be an attractive strategy for inhibiting MIF proinflammatory activity and attenuating its biological activity in vitro and in vivo.^{11,12}

To date, a variety of small-molecule MIF tautomerase inhibitors have been reported by substrate analogue screening, structure-based virtual screening, and high-throughput screening.^{1,11,13–15} Though these inhibitors provide a proof of concept for therapeutic utility against MIF, most of them are not ideal for pharmaceutical development owing to low potency or irreversible inhibition on MIF tautomerase activity. For example, the prototypical MIF inhibitor ISO-1 shows only micromolar potency with respect to MIF inhibition.¹⁶ The first identified MIF inhibitor, NAPQI,¹⁷ a metabolite of acetaminophen, forms a covalent bond with the MIF N-terminal proline residue (Pro1), as well as 4-iodo-6-phenylpyrimidine (4-IPP)¹⁸ and isothiocyanates (ITC).¹⁹ Recently, by combining docking-based virtual screening and in vitro bioassays, 3-[(biphenyl-4-ylcarbonyl)carbamothioyl]amino benzoic acid (presented as compound 1 in

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Scheme 1. General Procedure for the Synthesis of 12–14**Scheme 2.** General Procedure for the Synthesis of 35–42**Table 1.** Experimentally Determined Half-Maximal Inhibitory Concentrations (IC_{50}) of MIF Tautomerase Activity (TIC_{50}) for Biphenyl Series and NO Production of Compounds with $TIC_{50} < 10 \mu\text{M}$ in LPS-Activated RAW 264.7 Macrophage (NIC_{50})

no.	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	TIC ₅₀ (μM)	NIC ₅₀ (μM)
1	H	carboxy	H	H	H	H	0.72 ± 0.15	36.02 ± 1.56
2	H	carboxy		methyl	H	H	4.25 ± 0.63	49.93 ± 1.70
3	H	H	carboxy	H	H	H	11.66 ± 1.07	
4	H	H	acylamino	H	H	H	6.25 ± 0.80	33.51 ± 1.53
5	H	H	hydroxy	H	H	H	0.37 ± 0.09	4.26 ± 0.63
6	H	H	carbomethoxy	H	H	H	20.42 ± 1.31	
7	H	hydroxymethyl	H	H	H	H	8.42 ± 0.93	25.57 ± 1.41
8	H	carboxy	H	Cl	H	H	0.71 ± 0.15	23.77 ± 1.38
9	carbomethoxy	H	H	H	H	H	8.36 ± 0.92	26.57 ± 1.42
10	H	carboxy	H	methoxyl	H	H	5.53 ± 0.74	12.05 ± 1.08
11	H	acetyl	H	H	H	H	8.63 ± 0.94	13.10 ± 1.12
12 ^a	H	carboxy	H	H	trifluoromethyl	trifluoromethyl	1.48 ± 0.17	20.71 ± 1.32
13 ^a	H	carboxy	H	H	Cl	Cl	0.96 ± 0.02	23.24 ± 1.37
14 ^a	H	carboxy	H	H	F	F	2.48 ± 0.39	28.08 ± 1.45

^aSynthesized compounds.

this study) was identified to be a competitive inhibitor of MIF ($IC_{50} = 550 \text{ nM}$) and was found to effectively inhibit the biological functions of MIF.²⁰ However, the structure–activity relationships (SARs) of the analogues of compound 1 have not been characterized, and its *in vivo* potency has not been tested. Therefore, in this study, a number of analogues of compound 1 were purchased or chemically synthesized, and their MIF tautomerase activities were evaluated. In total, 31 compounds were found to be potent tautomerase inhibitors of MIF with IC_{50} values below $10 \mu\text{M}$ (six with $IC_{50} < 1 \mu\text{M}$). The strong anti-inflammatory potency of the most potent inhibitor, compound 5 ($IC_{50} = 370 \text{ nM}$), as evidenced by suppressing lipopolysaccharide (LPS)-induced macrophage activation *in vitro*, was verified by ELISA, quantitative real-time polymerase chain reaction (PCR), and Western blot. Furthermore, the therapeutic importance of the MIF inhibition by compound 5 was demonstrated by increasing the survival rate of mice with LPS-induced endotoxic shock from 0 to 35% at 0.5 mg/kg and to 45% at 1 mg/kg.

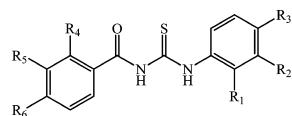
MATERIALS AND METHODS

Analogues Chosen by Similarity Search and Molecular Docking.

Because the commercial chemical databases are usually enriched with analogues by the nature of their synthetic routes, the analogues of compound 1 were identified from the ChemBridge and Specs databases by similarity search based on the Tanimoto similarity coefficients²¹ computed from the MACCS structural fingerprints in MOE.²² Tanimoto similarity coefficients higher than 0.85 and 0.80 were set as the cutoffs for the ChemBridge and Specs databases, respectively.

Then, the molecules selected by similarity search were processed with the program LigPrep in Schrödinger²³ to generate the tautomers and protonation states at pH 7.4 (up to 30 stereoisomers for each compound with chiral centers) and then docked into the binding site of MIF. The crystal structure of MIF complexed with *p*-hydroxyphenyl pyruvate (HPP) (PDB entry: 1CA7)²⁴ was selected as the template for molecular docking. The ligand located in the active site between chain A and chain B was retained, and the other two ligands were deleted. The N-terminal proline residue (P1) was protonated in terms of the experimental

Table 2. Experimentally Determined Half-Maximal Inhibitory Concentrations (IC_{50}) of MIF Tautomerase Activity (TIC_{50}) for Benzene Series and NO Production of Compounds with $TIC_{50} < 10 \mu\text{M}$ in LPS-Activated RAW 264.7 Macrophage (NIC_{50})



no.	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	TIC ₅₀ (μM)	NIC ₅₀ (μM)
15	H	carboxy	H	H	H	methyl	0.66 ± 0.18	12.61 ± 1.10
16	H	H	carboxy	H	H	methyl	1.33 ± 0.37	25.70 ± 1.41
17	methyl	carboxy	H	H	H	phenoxyethyl	6.67 ± 0.82	45.98 ± 1.66
18	H	H	carboxy	H	H	H	9.77 ± 0.99	59.74 ± 1.78
19	methyl	carboxy	H	H	H	isopropyl	8.63 ± 0.94	25.82 ± 1.41
20	H	H	carboxy	H	methyl	H	28.33 ± 1.45	
21	H	Cl	carboxy	H	H	F	39.06 ± 1.59	
22	H	acetyl	H	H	H	H	2.74 ± 0.44	16.39 ± 1.21
23	H	H	carbomethoxy	methyl	H	H	>100	
24	H	H	carbomethoxy	H	H	H	4.65 ± 0.67	40.35 ± 1.61
25	H	H	acetyl	H	H	H	2.73 ± 0.44	17.06 ± 1.23
26	H	H	carbomethoxy	H	methyl	H	64.94 ± 1.81	
27	H	H	benzamido	H	H	methyl	3.75 ± 0.57	32.52 ± 1.52
28	H	H	carbethoxy	H	H	H	13.18 ± 1.12	
29	H	H	acetylamino	H	methyl	H	10.39 ± 1.02	
30	H	H	carbamoyl	H	carbomethoxy	H	8.43 ± 0.93	17.13 ± 1.23
31	H	H	carbamoyl	H	H	carbomethoxy	5.00 ± 0.70	20.85 ± 1.32
32	H	H	acetylamino	H	H	methyl	10.58 ± 1.02	
33	carbomethoxy	H	H	H	H	methyl	0.81 ± 0.09	55.24 ± 1.74
34	carbomethoxy	H	H	H	methyl	H	0.77 ± 0.11	45.23 ± 1.66
35 ^a	H	carboxy	H	H	H	Br	16.69 ± 1.22	
36 ^a	H	carboxy	H	H	H	trifluoromethyl	21.01 ± 1.32	
37 ^a	H	carboxy	H	H	H	Cl	24.25 ± 1.39	
38 ^a	H	carboxy	H	H	H	F	26.26 ± 1.42	
39 ^a	H	carboxy	H	H	F	H	12.23 ± 1.09	
40 ^a	H	carboxy	H	H	Cl	H	28.71 ± 1.46	
41 ^a	H	carboxy	H	Cl	H	H	61.15 ± 1.79	
42 ^a	H	carboxy	H	F	H	H	>100	

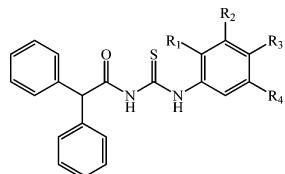
^aSynthesized compounds.

measurement and theoretical calculations.^{25,26} Hydrogen and other missing atoms were added, and the protein was energy-minimized using OPLS2005 force field²⁷ with the Protein Preparation Wizard module in Schrödinger (version 9.0). All structures were docked into the active site of MIF and scored by the extra precision (XP) scoring function of Glide. In the docking process of Glide, the protein conformation is fixed and each docked ligand is flexible. For the grid generation and ligand docking, the default Glide settings were employed. The top scored molecules were assessed by the REOS rules²⁸ and the drug-likeness filters developed in our group^{29–31} to remove compounds with toxic, reactive, or otherwise undesirable moieties, and finally, 46 compounds were chosen and purchased.

Synthetic Chemistry. The importance of the halogen substitution on MIF tautomerase activity was reported in a previous study, where the substitution of monofluorination onto the *ortho* position of the phenolic group of ISO-1 could improve the inhibition activity against MIF up to 41%.³² In order to study the effect of the halogen substitution on bioactivity, 11 analogues of compound 1 were synthesized. ¹H NMR and ¹³C NMR spectra were acquired on 400 MHz (Varian) spectrometers. Chemical shifts were given in parts per million with tetramethylsilane (TMS) as the internal standard. Finally, the compounds were purified using silica gel 100–200 mesh for column chromatography. The synthetic routes for the series are

depicted in Schemes 1 and 2. A mixture of 4-bromobenzoic acid methyl ester (645 mg, 3.0 mmol, 1.0 equiv), aryl boronic acid (3.6 mmol, 1.2 equiv), Pd(PPh₃)₄ (69 mg, 0.05 mmol, 0.02 equiv), and Na₂CO₃ (699 mg, 6.6 mmol, 2.2 equiv) in dioxane/H₂O (7:1, v/v) (16 mL) was stirred at 100 °C for 1 h. The solvent was removed under reduced pressure, and the residue was diluted with water (10 mL) and extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The residue was purified by flash column chromatography (hexane/EtOAc 50:1) to give the corresponding ester. To a solution of unsaturated acids (2 mmol) in dichloromethane (10 mL) was added oxalyl chloride (1.02 g, 8 mmol), followed by adding 2 drops of dimethylformamide. The mixture was stirred at room temperature for 1 h, and then excess oxalyl chloride and dichloromethane were removed in vacuo to provide crude acyl chloride. A suspension of benzoyl chloride (3 mmol) and sodium thiocyanate (486 mg, 6 mmol) in acetonitrile (9 mL) was stirred for 1 h at room temperature. Then, sodium chloride was filtered, and anthranilic acid was added to the filtrate. The mixture was stirred for 1–2 h; methanol (10 mL) was added to the solvent and stirred for 10 min, and then the solvent was filtered and the solid collected. The detailed spectral data are provided at Part 1 of the Supporting Information.

Table 3. Experimentally Determined Half-Maximal Inhibitory Concentrations (IC_{50}) of MIF Tautomerase Activity (TIC_{50}) for Diphenylmethane Series and NO Production of Compounds with $TIC_{50} < 10 \mu\text{M}$ in LPS-Activated RAW 264.7 Macrophage (NIC_{50})



no.	R ₁	R ₂	R ₃	R ₄	TIC ₅₀ (μM)	NIC ₅₀ (μM)
43	H	H	carboxy	H	13.55 ± 1.13	
44	H	carboxy	H	H	7.95 ± 0.90	59.74 ± 1.78
45	H	H	H	H	6.67 ± 0.82	22.50 ± 1.35
46	H	H	carbomethoxy	H	5.51 ± 0.74	13.43 ± 1.13
47	H	H	benzamido	H	2.74 ± 0.44	12.57 ± 1.10
48	H	H	formylamino	H	19.80 ± 1.30	
49	carbomethoxy	H	H	H	8.70 ± 0.94	18.66 ± 1.27
50	H	carbomethoxy	H	carbomethoxy	3.63 ± 0.56	9.33 ± 0.97
51	H	H	phenylamino	H	1.43 ± 0.15	15.51 ± 1.19

In total, there are 51 compounds in Tables 1–3. Compound 1 is the lead compound identified from our previous study;²⁰ compounds 2–11, 15–34, and 43–51 were purchased from the ChemBridge and Specs databases, and the others were chemically synthesized.

Assay for MIF Tautomerase Activity. The following protocol was adapted from Tao et al.³³ Fresh solution of L-dopachrome methyl ester was prepared by mixing equal volumes of L-3,4-dihydroxyphenylalanine methyl ester (6 mM) and sodium metaperiodate (12 mM) and incubated for 20 min at room temperature. The dopachrome solution was prepared before the inhibition assay due to its relative instability. Then, hMIF (120 nM) was added to a 96-well plate containing L-dopachrome (30 μL) in 10 mM potassium phosphate buffer and 0.5 mM EDTA at pH 6.2. The inhibitory effect of each compound against the tautomerase activity of MIF was measured by adding various concentrations of the compound to the 96-well plate containing hMIF (120 nM) and incubating for 30 min followed by the addition of L-dopachrome methyl ester. The absorbance was measured by monitoring the decrease in absorbance at 475 nm for 3 min with a Tecan Infinite M1000 microplate reader (Tecan Group Ltd.).

Cell Culture and Cell Viability Assay. The RAW 264.7 cell line was purchased from the American Type Culture Collection (ATCC). The cells were maintained in complete DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in a 37 °C, 5% CO₂ incubator. The cells were attached to the culture plate for at least 24 h prior to various experiments. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophages were seeded into 96-well plates (5×10^4 cells/well). After appropriate treatment, the cells were incubated in MTT solution at 37 °C for 4 h, and then 100 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the violet formazan crystals for 5 min as previously described.¹⁸ The optical density was measured at 540 nm on a microplate reader (Infinite M200 PRO, Tecan, Switzerland).

Measurement of Intracellular Nitric Oxide (NO). RAW 264.7 macrophages were seeded into 96-well plates, and culture supernatants were collected after treatment with LPS or compounds. Concentration of NO was then determined by

using the Griess reagent as described previously.³⁴ Briefly, 50 μL of Griess reagent (containing 0.2% w/v N-(1-naphthyl)-ethylenediamine dihydrochloride and 2% w/v sulfanilamide in 5% v/v H₃PO₄) was mixed with the same volumes of cell culture conditioned medium and then incubated at room temperature for 10 min in the dark. The absorbance of mixture was measured by a 96-well microplate reader (Tecan Systems, Inc.) at 570 nm.

Enzyme-Linked Immunosorbent Assay (ELISA). The levels of TNF- α and IL-6 in the cell culture medium were determined by specific ELISA kits according to the manufacturer's instructions.³⁵

RNA Extraction and Real-Time Quantitative PCR (RT-qPCR). Total RNA was extracted using the TRIzol reagent according to its manufacturer's instructions (TaKaRa, Dalian, China). The obtained total RNA was used for synthesis of cDNA using the ImProm-II™ reverse transcription system (Promega, USA). Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR master mix. The specific primers used for cDNA amplification are as follows: iNOS forward, CAG GAG GAG AGA GAT CCG ATT TA, and iNOS reverse, GCA TTA GCA TGG AAG CAA AGA; TNF- α forward, CAT CTT CTC AAA ATT CGA GTG ACA A, and TNF- α reverse, TGG GAG TAG ACA AGG TAC AAC CC; IL-1 β forward, TCC AGG ATG AGG ACA TGA GCA C, and IL-1 β reverse, GAA CGT CAC ACA CCA GCA GGT TA; GAPDH forward, TGT GTC CGT CGT GGA TCT GA, and GAPDH reverse, TTG CTG TTG AAG TCG CAG GAG; MIF forward, CGT GCC AGA GGG GTT TCT GT, and MIF reverse, GTT CTG GGC ACC ACC GAT CT; HO-1 forward, CAC GCA TAT ACC CGC TAC CT, and HO-1 reverse, TCT GTC ACC CTG TGC TTG AC. The relative level of gene expression was normalized to the level of GAPDH and calculated using the $2^{-\Delta\Delta CT}$ formula.

Western Blot Analysis. After appropriate treatment, RAW 264.7 macrophages were collected and washed three times with ice-cold PBS. The cells were lysed with RIPA lysis buffer [50 mM Tris [pH 8], 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany)]. Protein concentration was measured with a protein extraction kit and BCA protein assay kit (Bio-Rad, Hercules, CA). The samples were boiled for 5 min, which contained 1% β -mercaptoethanol, followed by separation with SDS-PAGE, then

the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) and blocked in PBS-T (PBS plus 0.1% Tween-20) containing 5% skim milk at room temperature for 90 min. The membrane was probed with primary antibodies [polyclonal rabbit anti-mouse iNOS (Abcam, NT); polyclonal rabbit anti-mouse COX-2 (Santa Cruz Biotechnology); monoclonal anti-rabbit MIF (Abcam, NT); and monoclonal anti- α -tubulin mouse ascites fluid (Sigma)] at 4 °C overnight. After the membrane was washed with PBS-T, the membrane was incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich) at room temperature for 90 min, respectively. The protein bands were visualized using enhanced chemiluminescence (ECL) (Pierce Biotechnology Inc., Rockford, IL), with a ChemiScope 3300 mini (CLINX, Shanghai, China), and the relative signal intensity was quantified by densitometric analysis (Quantity One).

Experimental Endotoxic Shock Model. LPS (*Escherichia coli* 0111: B4, catalog no. L-2630, lot no. 78H4086, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline for administration to C57 BALB/c mice. Mice were injected intraperitoneally with LPS at a dose of 4.5 mg/kg and at a volume of 0.1 mL/10 g body weight to induce an endotoxin shock model. Mice received intraperitoneal administrations of compound 5 (0.5 or 1 mg/kg), which were prepared in 2% DMSO/2% Tween 80/2% Cremophor EL in saline or vehicle 1 h prior to LPS injection. Survival was monitored at 1, 2, 3, 4, 5, and 6 days post-LPS challenge. The number of animals used in each experimental group was 20. The mice that survived over 6 days were euthanized by cervical dislocation. All experimental procedures utilizing mice were in accordance with the National Institute of Health guidelines. The animal study was approved by the Institutional Review Board of Soochow University.

Histological and Statistical Analysis. The lung tissues were collected at 12 h after LPS challenge for histological assessment. Lung tissues were fixed in formalin and subsequently embedded in paraffin and then stained with hematoxylin and eosin (H&E) for histopathological evaluation under a light microscope (Olympus, Japan). Results were expressed as the mean \pm SD using GraphPad Prism 5.0. One-way ANOVA followed by Bonferroni tests was utilized for multiple-group comparisons. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

SAR Analyses for Analogues of Compound 1. The commercial chemical databases are usually enriched with analogues by the nature of their synthetic routes, and therefore, in this study, based on similarity search and molecular docking, 16 analogues from ChemBridge and 30 analogues from Specs were purchased for biological assays.

Seven out of the 46 compounds were not tested due to poor solubility in DMSO/ethanol/water, and the other compounds were then assayed for their MIF tautomerase activities with L-dopachrome as the substrate. The prototypical MIF inhibitor (compound 1), our previously reported tautomerase inhibitor of MIF,²⁰ was used as the reference control, and DMSO (1%, v/v) was used as the vehicle control. The *in vitro* MIF tautomerase assay showed that 28 analogues have IC₅₀ values below 10 μ M and 5 below 1 μ M. All of these compounds had one common key structural feature: two aromatic rings linked by *N*-carbamothioylformamide in the middle position. According to the structural differences, these analogues could be roughly classified into three categories (Tables 1–3): analogues with biphenyl (2–

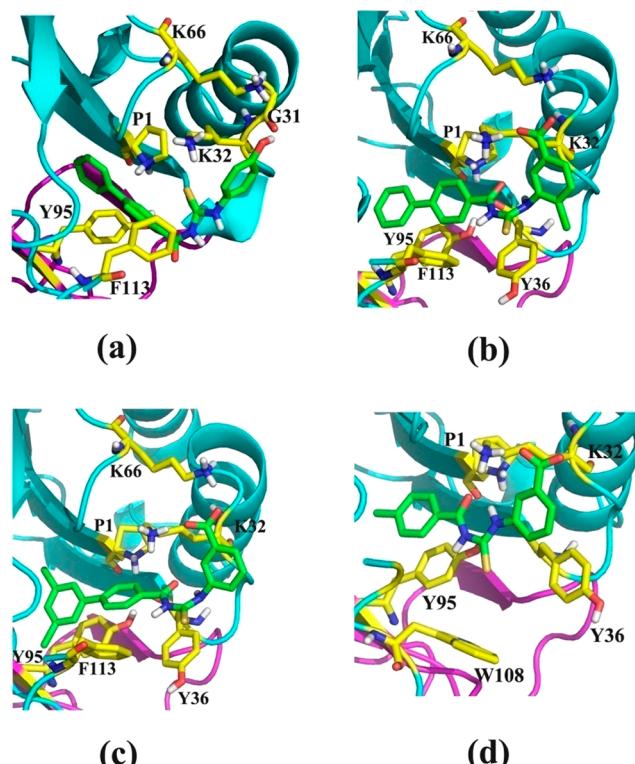


Figure 1. Binding pockets of MIF for compounds (a) 5, (b) 8, (c) 13, and (d) 15.

11), analogues with benzene (15–34), and analogues with diphenylmethane (43–51). The SAR analyses of these compounds could provide important insights of the essential structural features for effective MIF inhibitors.

Analyses of the 10 compounds in Table 1 illustrated some noteworthy observations of the SARs: (1) the substitution of the carboxy group on R₂ was critical, and the MIF inhibitory activity became worse when the position of carboxy was changed from *meta* to *para* (1 vs 3); (2) the introduction of a chlorine atom (R₄) slightly promoted the potency (1 vs 8), and the replacement of chlorine with the methyl or methoxyl group decreased the potency, which might be explained by the changes in the density distribution of the electron cloud of the benzene ring that resulted from an electron-withdrawing group in comparison with an electron-donating group (2, 8, and 10); (3) when only R₃ was changed by replacing the hydrogen atom with the hydroxy, acetylamino, carboxy, or carbomethoxy group, the activity decreased significantly (5, 4, 3, and 6); for instance, compound 5 showed the best MIF inhibitory potency. Thus, the introduction of hydroxy on R₂ could enhance the inhibitory activity on MIF.

In addition, the key observations of the SARs from the 20 compounds in Table 2 were summarized as follows: (1) Upon the replacement of hydrogen with a methyl group on R₆, improvement of the inhibitory activity was observed (16 vs 18). Moreover, the position of methyl had a substantial influence on the inhibitory activity, and the potency increased from *meta* to *para* (16 vs 20) and strongly decreased to *ortho* (23 vs 24). (2) When only R₃ was changed by replacing hydrogen with the acetyl, carbomethoxy, carboxy, or carbethoxy group, the activity decreased gradually (25, 24, 18, and 28), and thus the introduction of acetyl on R₃ might be beneficial to the potency on MIF inhibition. (3) The substitution of carbomethoxy on R₁

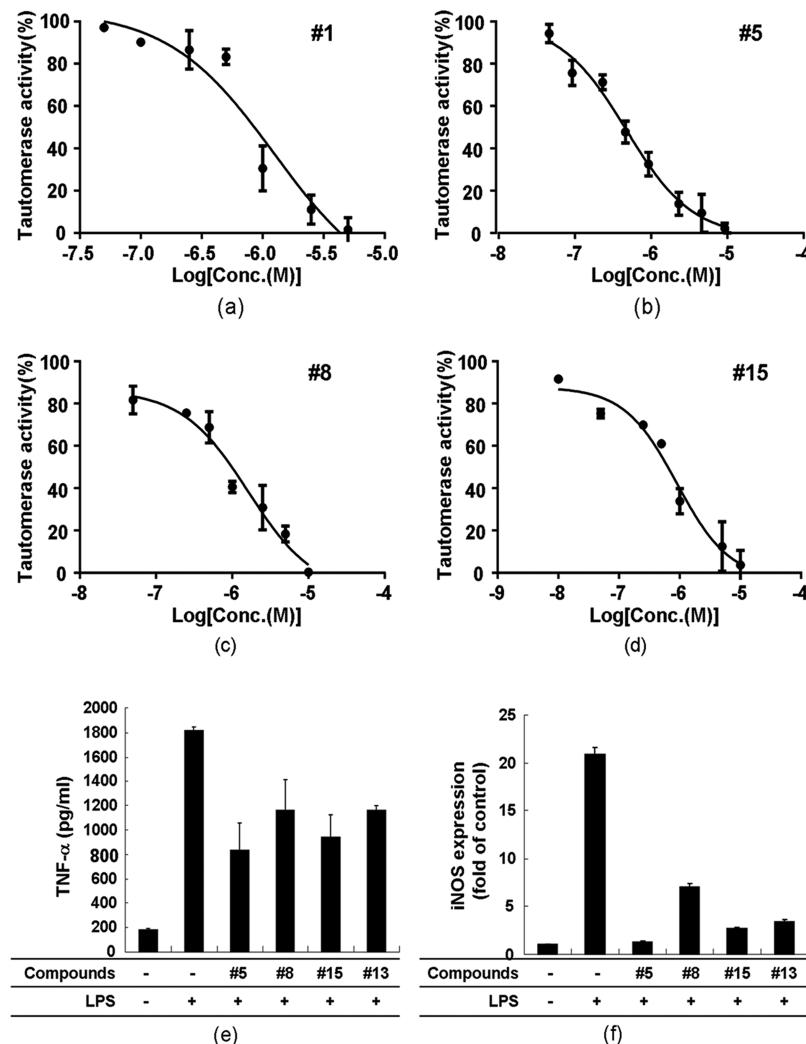


Figure 2. (a–d) Concentration-dependent inhibition of MIF tautomerase activity by three active molecules. (e,f) Three compounds were chosen to measure iNOS expression and TNF- α release based on the inhibitory effect on both tautomerase activity and NO production. All compounds show a protective effect in LPS-stimulated RAW 264.7 macrophages, with compound 5 exhibiting the highest potency.

was important for the inhibitory activity (compounds 33 and 34 with IC₅₀ values below 1 μ M), and when only R₃ was changed, the potency increased upon the replacement of carboxy with the carbomethoxy group (18 vs 24). Generally, the introduction of the carbomethoxy group was beneficial to the potency, in particular on R₁.

Analyses of the nine compounds in Table 3 exhibited some interesting observations of the SARs: (1) the substitution of carbomethoxy was beneficial, and compound 46 with carbomethoxy at the *para* position showed better inhibition than the corresponding analogue 49 with carbomethoxy at the *ortho* position; (2) when only R₃ was changed by replacing hydrogen with the phenylamino, benzamido, carbomethoxy, carboxy, or formylamino group, the activity decreased gradually (51, 47, 46, 43, and 48). Generally, the substituents of the aromatic group on R₃ were critical to the potency (47 and 51).

To determine the activity affected by the substitution of the halogen atom, 11 compounds were synthesized and assayed for the MIF tautomerase inhibitory activities. As shown in Tables 1 and 2, the introductions of the fluorine atoms on R₅ and R₆ showed unfavorable effect on the inhibitory activity in comparison with the chlorine atoms (13 vs 14), as well as the trifluoromethyl group (12 vs 13). Accordingly, we speculated

that the introduction of the trichloromethyl group on R₅ and R₆ might be favorable to the inhibitory activity. In addition, the position of the fluorine atom had significant impact on the MIF inhibitory activity, and the potency dramatically increased from *ortho* to *para* to *meta* (42, 38, and 39). Consequently, the introduction of the fluorine atom on R₅ might be beneficial, whereas the introduction of the fluorine atom on R₄ resulted in a dramatic loss of the inhibitory activity. The potency gradually increased with the substitution of the fluorine, chlorine, or bromine atom, respectively (38, 37, and 35), which might be explained by the inductive effect that leads to the changes in the density distribution of the electron cloud of the benzene ring, thereby making a strong π – π interaction with Tyr95 and Phe113.

Predicted Binding Poses of the Four Most Potent Inhibitors. The most potent inhibitor (compound 5) possesses a hydroxyl group at the *para* position, in comparison with the carboxyl group at the *meta* position of the other three compounds (8, 13, and 15). As shown in Figure 1a, the biphenyl group of compound 5 could form the aryl–aryl interaction with Tyr95 and Phe113 and the cation– π interaction with the protonated Pro1 (Figure 1a). In addition, the hydroxyl group of compound 5 could form the H-bonds with Gly31 and Lys66 and

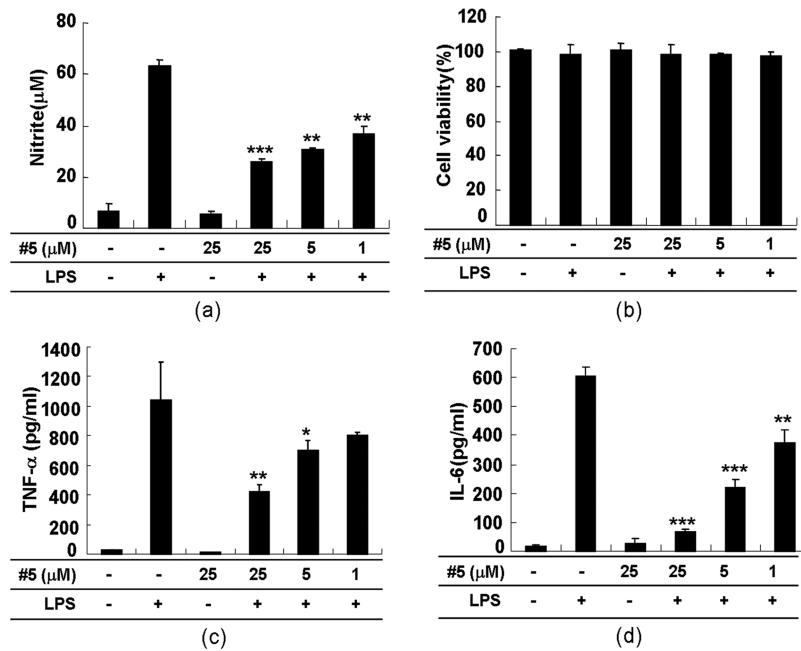


Figure 3. Effects of compound 5 on the production of nitric oxide (NO), TNF- α , and IL-6 in LPS-activated RAW 264.7 macrophage cells. RAW 264.7 macrophage cells were pretreated with or without the indicated concentration (1–25 μ M) of compound 5 for 30 min, followed by LPS (50 ng/mL) treatment for 24 h. (a) Amounts of NO in the culture supernatants were measured using Griess reagent. (b) Cell viability was determined by MTT assay. Amounts of (c) TNF- α and (d) IL-6 in the supernatants were measured using ELISA. The data were expressed as percentage of surviving cells over control cells. The data are the mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the LPS-only group.

the hydrophobic interaction with Pro1, Trp108, and Phe113. Compounds 8 and 13 were the chlorine derivatives of compound 1, though one substitutes in the benzoic acid side chain and the other in the biphenyl group. The modeling results suggested that their docked poses were quite similar, and their biphenyl rings were stabilized in a T-shaped geometry by forming a network of the π – π interactions with Tyr95 and Phe113. The carboxyl group of compound 8 was predicted to form the H-bonds with Lys32 and Lys66, and compound 13 formed the cation– π interaction with the protonated Pro1. Compared with the other three compounds (5, 8, and 13), compound 15 had a phenyl group rather than a biphenyl side chain. However, its activity ($IC_{50} = 660$ nM) was improved slightly in comparison with that of compound 1 with the biphenyl group ($IC_{50} = 720$ nM), and its carbonyl group was predicted to form the H-bonds with the basic residues Lys32 and Lys66. Accordingly, we speculated that if the biphenyl group of compound 5 was substituted by the benzyl group, and the chlorine atom was introduced at the *para* position of the hydroxyl group, its potency might be promoted.

Biological Activities. As an important proinflammatory cytokine, MIF can induce the secretion of itself and other proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 and stimulate NO production.^{36,37} Expression studies also identified that MIF secretion could be induced by LPS, TNF- α , and IFN- γ in RAW 264.7 macrophages.³⁸ A separate line of investigation implicated a strong relationship between the specific inhibition of MIF tautomerase activity and suppression of MIF proinflammatory activities, such as ISO-1 that could reduce inflammation induced by MIF.^{11,16} It was also reported that inhibition of MIF tautomerase activity not only suppressed MIF-induced production of proinflammatory cytokines but also reduced LPS-triggered inflammatory responses in vitro and in vivo.^{6,39} Therefore, we investigated the anti-inflammatory effects of MIF tautomerase inhibitors on the LPS-induced macrophage. Overproduction of NO has been used as a hallmark of

inflammatory activation in LPS-treated macrophages.⁴⁰ Thus, we examined the effect of inhibitors of MIF enzymatic activity on NO production in LPS-induced RAW 264.7 cells. All compounds with potent tautomerase activities ($IC_{50} < 10$ μ M) could attenuate LPS-stimulated NO production, and three of them with $IC_{50} < 720$ nM exhibited the highest potency (Table 1 and Figure 2). LPS markedly increased TNF- α and iNOS expression, which was significantly inhibited by the respective compounds among them, and compound 5 exhibited the most potent activity (Figure 2e,f). Therefore, we focused on the anti-inflammatory activity of compound 5 in the following experiments.

To further test the anti-inflammatory effect of compound 5, the production of NO, TNF- α , and IL-6 was measured from LPS-induced in RAW 264.7 macrophage in the presence or absence of compound 5. As shown in Figure 3, compound 5 strongly inhibited NO production in LPS-stimulated RAW 264.7 in a dose-dependent manner (Figure 3a). To exclude the possibility that the decrease of NO was due to cell death, the cell viability was measured by MTT assay. Results showed that compound 5 at the indicated concentrations (1–25 μ M) did not alter the cell viability (Figure 3b). TNF- α and IL-6 are proinflammatory cytokines that can initiate the inflammatory response and mediate the development of chronic inflammatory diseases.^{41,42} The productions of TNF- α and IL-6 in the culture supernatants were measured by ELISA. In agreement with the results of NO, compound 5 markedly inhibited LPS-induced production of TNF- α and IL-6 in a dose-dependent manner (Figure 3c,d).

Next, we measured the gene and protein expression of those proinflammatory cytokines in RAW 264.7 macrophages. As shown in Figure 4a–e, compound 5 could dramatically inhibit the gene expression of iNOS, IL-6, TNF- α , COX-2, and IL-1 β in a dose-dependent manner in LPS-stimulated RAW 264.7 macrophages. Recent study suggested that HO-1 was one of

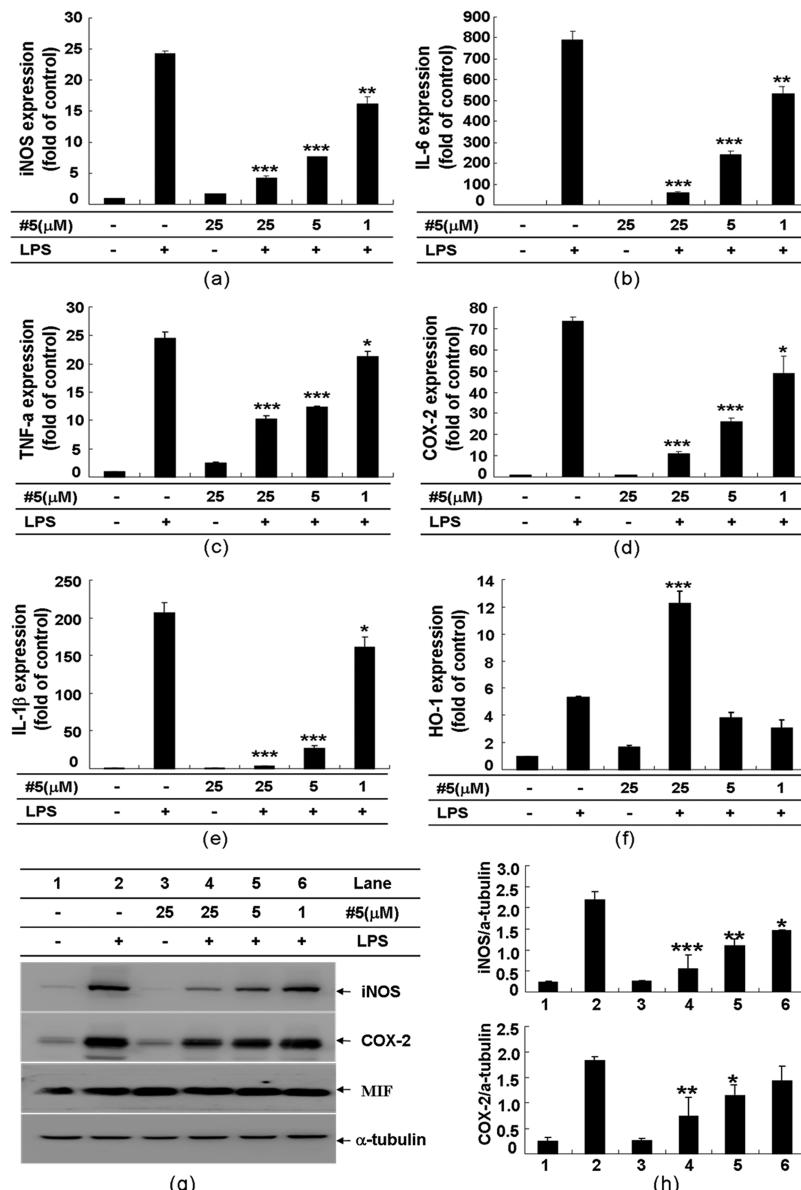


Figure 4. Effects of compound 5 on gene expression of proinflammatory factors and HO-1 in LPS-treated RAW 264.7 macrophage cells. RAW 264.7 macrophage cells were treated with compound 5 (1–25 μM) for 30 min, followed by LPS (50 ng/mL) treatment. (a–f) After 6 h of LPS stimulation, the mRNA levels of iNOS, IL-6, TNF-α, COX-2, IL-1β, and HO-1 were determined by quantitative real-time PCR assay. After 16 h of LPS stimulation, the protein levels of iNOS, COX-2, and MIF were determined by Western blotting using respective primary antibodies. (g) α-Tubulin was used as an internal control. Quantification of protein expression was performed by densitometric analysis. (h) Data are the mean ± SD of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with the LPS-only group.

the key defense molecules against LPS-induced sepsis, and HO-1 overexpression could protect against the systemic inflammatory response.^{43–46} To determine whether compound 5 could attenuate inflammatory response by inducing HO-1 expression, the HO-1 mRNA expression levels were also measured. LPS resulted in an increase in HO-1 mRNA levels, and pretreatment with compound 5 (25 μM) for 30 min before LPS further increased HO-1 protein levels (Figure 4f). In accordance with the mRNA expression, compound 5 also suppressed LPS-induced iNOS and COX-2 expression in a dose-dependent manner in RAW 264.7 macrophages without altering the MIF protein level (Figure 4g).

Compound 5 Elicits Protection from LPS-Induced Endotoxic Shock in Mice. Many studies have demonstrated that anti-MIF antibodies or MIF inhibitors could attenuate an

inflammatory cascade and improve the survival rate in sepsis.^{2,38,47,48} To determine whether compound 5 also produced anti-inflammatory activity in vivo, a LPS-induced experimental shock mouse model was employed. As shown in Figure 5a, application of compound 5 could protect the BALB/c mice from endotoxic shock, as evidenced by increasing the survival rate of mice from 0 to 35% at 0.5 mg/kg and to 45% at 1 mg/kg. It is known that LPS induces dramatic increases in inflammation cytokines, such as TNF-α and IL-6, and results in severe tissue damage, multiple organ failure, and eventually leading to death in sepsis.^{49,50} We found that compound 5 significantly decreased the serum levels of TNF-α and IL-6 in the LPS-induced endotoxin model. These results clearly indicated that compound 5 attenuated inflammatory responses in LPS-induced sepsis mice by inhibiting the secretion of proinflamma-

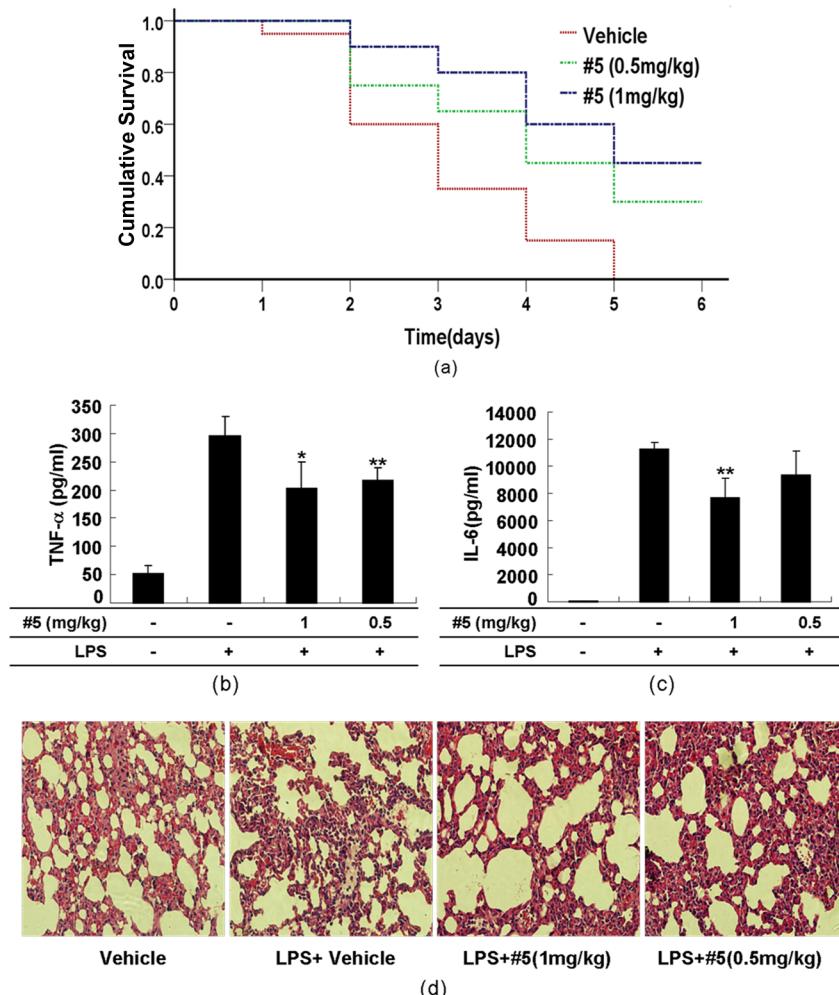


Figure 5. Effects of compound 5 on LPS-induced endotoxic shock. (a) Male BALB/c mice were injected with compound 5 [intraperitoneal (i.p.) injection] at indicated doses or vehicle 1 h prior to administration of LPS (4.5 mg/kg, i.p.) for 3 days. Survival was monitored for 6 days ($n = 20$ for each group). (b) Mice were pretreated with compound 5 (1, 0.5 mg/kg, i.p.) or vehicle 1 h prior to the administration of LPS (4.5 mg/kg, i.p.). Serum was collected at 12 h postinjection of LPS. The serum levels of TNF- α and IL-6 were measured using ELISA. Data are presented as means \pm SD ($n = 6$ for each group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the LPS-only group. (c) Mice were pretreated compound 5 (1, 0.5 mg/kg, i.p.) or vehicle 1 h prior to the administration of LPS (4.5 mg/kg, i.p.). Twelve hours after the LPS challenge, mice were sacrificed to obtain lung tissue for histological analysis. (d) H&E staining was performed using lung tissue specimens from vehicle LPS + vehicle, LPS + 1 mg/kg compound 5, LPS + 0.5 mg/kg compound 5 (400 \times magnification).

tory cytokines. Tissue injury, especially lung, is a main cause of mortality in LPS-induced sepsis.⁵¹ Therefore, the lungs were examined histologically at the points of 12 h after LPS treatment with or without compound 5 (1, 0.5 mg/kg, i.p.) 1 h pretreatment. Histopathology of the lung sections is illustrated in Figure 5d. Vehicle group showed integrated and clear alveolar spaces, while LPS-only treatment showed an acute inflammatory response including infiltration of inflammatory cells, alveolar septum thickness, and swelling of the alveolar wall. However, the lung injury was significantly alleviated in the mice with compound 5 (1, 0.5 mg/kg) pretreated group. These findings highlighted that compound 5 reduced the mortality by improving inflammatory responses.

CONCLUSION

In this study, the SARs for the 50 analogues of N-carbamothioylformamide were analyzed. The results provided valuable information about the essential structural features of effective MIF inhibitors. We further studied the biological functions of compound 5, the most potent MIF inhibitor with

$IC_{50} = 370$ nM, in anti-inflammatory responses both in vitro and in vivo. The present data illustrated that targeting the tautomerase active site of MIF by small-molecule inhibitors was an attractive strategy for therapeutic interventions in the inflammatory diseases such as sepsis.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.5b00445.

Detailed spectral data for compounds 12–14 and 35–42; number, docking score, source of database, and IDs for the compounds shown in Tables 1–3 from similarity search (PDF)

AUTHOR INFORMATION

Corresponding Authors

*(T.H.) E-mail: tingjunhou@zju.edu.cn or tingjunhou@hotmail.com. Phone: +86-517-8820-8412.

^{*}(X.Z.) E-mail: zhenxuechu@suda.edu.cn. Phone: +86-512-6588-0369.

Author Contributions

[#]Y.Z. and L.X. contributed equally.

Notes

The authors declare no competing financial interest.

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