ISO-1 Binding to the Tautomerase Active Site of MIF Inhibits Its Pro-inflammatory Activity and Increases Survival in Severe Sepsis

MIF is a proinflammatory cytokine that has been implicated in the pathogenesis of sepsis, arthritis, and other inflammatory diseases. Antibodies against MIF are effective in experimental models of inflammation, and there is interest in strategies to inhibit its deleterious cytokine activities. Here we identify a mechanism of inhibiting MIF pro-inflammatory activities by targeting MIF tautomerase activity. We designed small molecules to inhibit this tautomerase activity; a lead molecule, "ISO-1 ((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester)," significantly inhibits the cytokine activity in vitro. Moreover, ISO-1 inhibits tumor necrosis factor release from macrophages isolated from LPS-treated wild type mice but has no effect on cytokine release from MIF-deficient macrophages. The therapeutic importance of the MIF inhibition by ISO-1 is demonstrated by the significant protection from sepsis, induced by cecal ligation and puncture in a clinically relevant time frame. These results identify ISO-1 as the first small molecule inhibitor of MIF proinflammatory activities with therapeutic implications and indicate the potential of the MIF active site as a novel target for therapeutic interventions in human sepsis.

MIF is an important pro-inflammatory cytokine that has been implicated in the pathogenesis of inflammatory disorders (1–6). Administration of neutralizing anti-MIF antibodies has proven therapeutically effective in numerous animal models of systemic inflammation, including Gram-negative, Gram-positive, and polymicrobial sepsis, arthritis, and autoimmune diabetes (1–4, 7, 8). Circulating MIF levels are elevated in animals with sepsis and in patients with severe sepsis and septic shock (1). These and other results indicate that inhibiting MIF is a promising approach to develop new anti-inflammatory agents.

Three-dimensional x-ray crystallography of MIF shows that the molecule exists as a homotrimer (9–11). This trimer possesses the ability to catalyze the tautomeration of the non-physiological substrates dl-dopachrome methyl esters (supplemental Fig. 1) into their corresponding indole derivatives (11, 12). Crystallographic analysis of MIF complexed with p-hydroxyphenylpyruvic acid, a known MIF substrate (13), has revealed an active site which lies in a hydrophobic cavity formed between two adjacent subunits of the homotrimer (14). Tautomerase activity is an evolutionarily ancient phenomenon, which early life forms presumably utilized for synthesis, but there is no evidence that modern species use this in synthetic pathways. We reasoned that molecules that bind this site could be useful to target MIF function, because the tautomerase activity is expendable. We have designed a molecule to fit into the catalytic site and shown that (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1)" is a potent inhibitor of MIF tautomerase activity (15). The crystal structure of MIF complexed to ISO-1 reveals that ISO-1 binds to the enzymatic active site.

MATERIALS AND METHODS

All chemicals were obtained from commercial suppliers and used without further purification. Sucinimidyl ester of Rhodamine Red-X was purchased from Molecular Probes.

Methylene chloride (CH2Cl2) was distilled from phosphorous pentoxide. Dimethyl formamide (DMF) was stored under argon in capped DriSolv bottles and used without further purification. Aluminum-backed Silica Gel 60 with 254 nm fluorescent indicator TLC plates were used. Developed TLC plates were visualized under a short wave UV lamp, stained with an I2-SiO2 mixture, and/or by heating plates that were dipped in ninhydrin. Flash column chromatography (FCC) was performed using flash silica gel (32–63 μm) and usually employed a stepwise solvent polarity gradient, correlated with TLC mobility. All 1H and 13C spectra were recorded on a JEOL spectrometer at 270 MHz for the 1H NMR spectra and at 67.5 MHz for the 13C NMR spectra. Chemical shifts are relative to the deuterated solvent peak and are in parts per million. The coupling constants (J) are measured in Hertz. The signals are described as s (singlet), d (doublet), t (triplet), m (multiplet), and br s (broad singlet). Low resolution mass spectra were acquired using a liquid chromatography mass spectrometry detector.

Synthesis of ISO-1—ISO-1 was synthesized in three steps as described previously (16) and is presented in supplemental Fig. 1.

Synthesis of Fluorescent Derivatives of ISO-1—FL-ISO-1—Fluorescent derivatives of ISO-1 were synthesized as described in supplemental material.

Determination of Intracellular MIF Tautomerase Activity—RAW 267.4 macrophages (1 × 106) were treated with various concentrations of ISO-1 (1–100 μM) for 30 min at 37 °C. Cells were washed repeatedly. Cells were lysed with 600 μl of ice-cold lysis buffer (Tris non-denaturing buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) with gentle rotation at 4 °C for 20 min. The supernatants were analyzed for the tautomerase activity of MIF using l-dopachrome methyl ester as follows (15, 17). l-dopachrome methyl ester was prepared at 2.4 mM through oxidation of l-3,4-dihydroxyphenylalanine methyl ester with sodium periodate. Activity was determined at room temperature by adding dopachrome methyl ester (0.3 ml) to a cuvette containing 0.7 ml of the above supernatants and measuring the decrease in absorbance from 2 to 20 s at 475 nm spectrophotometrically.

Intracellular Localization of Fluorescent ISO-1 Uptake by RAW 267 Macrophage—RAW 267.4 macrophages were plated on coverslips in a 24-well plate and treated with FL-ISO-1. After incubation with FL-ISO-1, the cells were washed five times with phosphate-buffered saline (PBS) (5 min each wash) and fixed using 4% formaldehyde (Ted Pella, Redding, CA) in PBS for 20 min at room temperature. Cells were washed and stained with DAPI (20 ng/ml) in PBS for 15 min at room temperature, mounted in 70% Vectashield.
ACCELERATED PUBLICATION: ISO-1 Protects against Severe Sepsis

FIGURE 1. a, intracellular localization of FL-ISO-1 uptake by RAW 267.4 macrophages. RAW 267.4 macrophages were plated on coverslips and treated with FL-ISO-1 (10 μM). After incubation with FL-ISO1, the cells were washed with PBS and fixed using 4% formaldehyde. Cells were washed and stained with DAPI (20 ng/ml) and mounted in 70% Vectashield mounting medium on a microscope slide. Observation and imaging of the cells were carried out on an Olympus IX70 microscope using a 40× oil immersion objective; b, inhibition of intracellular MIF tautomerase activity by ISO-1. RAW 267.4 macrophages (1 × 10^5) were treated with various concentrations of ISO-1 (1–100 μM) and then lysed using Tris buffer under non-denaturing conditions. The lysates were analyzed for the tautomerase activity of MIF using L-dopa-chrome methyl ester as we described previously (15, 17), and values represent mean ± S.D. of three separate experiments (*, p < 0.05 and **, p < 0.001).

mounting medium (Vector Laboratories, Burlingame, CA) in dH2O on a microscope slide and sealed using nail polish. Observation and imaging of the cells were carried out on an Olympus IX70 microscope using a 40× oil immersion objective and 568 nm (for FL-ISO-1) and 360 nm (for DAPI) excitation filters. Images were captured using a digital camera (Hamamatsu) and the iSee software program (Inovision).

Measurement of NF-κB Activation by Electrophoretic Mobility Shift Assay—RAW 267.4 macrophages were treated with various concentrations of ISO-1 (1–100 μM) 30 min prior to LPS (endotoxin, Escherichia coli 0111:B4, Sigma) addition. Macrophages were collected 2 h after activation with LPS and washed one time in PBS (pH 7.4). Nuclear extract was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer’s instructions (Pierce). For detection of NF-κB binding, nuclear extract from cells (~5 μg of protein) was incubated with 0.2 ng of ^32P-labeled double-stranded oligonucleotide sequence in a 10-μl reaction volume containing 5× gel shift binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 0.25 mg/ml poly(dI-dC)-poly(dI-dC)) for 30 min at room temperature. The samples were resolved on a 4% polyacrylamide gel and visualized directly by autoradiography after drying the gel. The NF-κB consensus sequence (Promega) was labeled with 10 units of T4 polynucleotide kinase (Promega) per 25 ng of oligonucleotide, 1× kinase buffer, and 5 μl of [γ-^32P]ATP (Amersham Biosciences, catalog no. PB10168, 10 mCi/ml) for 30 min at 37 °C. The bound NF-κB bands were quantified by scanning densitometry of a bio-image analysis system. The results for each treatment were expressed as relative intensity compared with the control (saline-treated) intensity.

Animal Experiments—All animal experiments were approved by the Institutional Animal Care and Use Committee of the North Shore-Long Island Jewish Research Institute. Male Balb/C mice, ~8 weeks old, were subjected to endotoxemia or cecal ligation and puncture (CLP).

Endotoxemia—Endotoxemia was induced by injection of a sublethal dose of LPS (5 mg/kg; intraperitoneally). Mice were treated with various concentrations of ISO-1 (3.5–35 mg/kg; intraperitoneally) or vehicle (aqueous 5% dimethyl sulfoxide) 30 min before and 6 h after LPS injection and then twice daily for 3 days. Animals were monitored for survival for 2 weeks.

Cecal Ligation and Puncture-induced Polymericial Sepsis—Mice were anesthetized (ketamine 100 mg/kg and xylazine 8 mg/kg administered intramuscularly), and abdominal access was gained via a midline incision. The cecum was isolated and ligated with a 6-0 silk ligature below the ileocecal valve and the cecum punctured once with a 22-gauge needle; stool (~1 mm) was extruded from the hole and the cecum placed back into the abdominal cavity. The abdomen was closed with two layers of 6-0 Ethilon sutures. Antibiotics were administered immediately after CLP (0.5 mg/kg Premaxin, subcutaneously, in a total volume of 0.5 ml/mouse) and a single dose of resuscitative fluid (normal saline solution administered subcutaneously (20 ml/kg body weight) immediately after CLP surgery (18). Control (aqueous 5% dimethyl sulfoxide as a vehicle) or ISO-1 (35 mg/kg; intraperitoneally) treatment was started 24 h after the induction of sepsis and repeated twice daily for 3 days. In one series of experiments, anti-MIF antibody (2.8 mg/kg; intraperitoneally) treatments were started 24 h after CLP-induced sepsis and then given once daily for 3 days. Animal survival was monitored for 2 weeks.

Statistical Analysis—Student’s t test, p < 0.05, was accepted as statistically significant and graphs show mean ± S.D. Data were analyzed with Fisher’s Exact Test using Prism software.

RESULTS AND DISCUSSION

Current data suggests that neutralization of the pro-inflammatory activity of MIF would be highly beneficial in the treatment of many diseases (5, 6). This assertion is supported by the substantial therapeutic effects of MIF-specific antibodies in several models of inflammatory and autoimmune diseases. Although anti-MIF antibody attenuates the inflammatory cascade in sepsis improving the survival rate, it is difficult to use anti-MIF antibodies as tools to delineate the mechanisms underlying MIF proinflammatory activity. Here we identify a method of inhibiting MIF pro-inflammatory activities by targeting MIF tautomerase activity, an evolutionary ancient activity of MIF. We reasoned that a more desirable approach than antibodies would be to develop non-toxic molecules that can specifically block the pro-inflammatory activities of MIF (15). We have designed small molecule inhibitors of MIF by targeting a unique, catalytically active site within the cytokine molecule. It is known that disruption of the active site by insertion of an alanine between Pro-1 and Met-2 abolishes the MIF tautomerase activity, and the resultant mutant is defective in the in vitro glucocorticoid counter-regulatory activity of MIF. Several other studies have supported the link between MIF bioactivity and this active site (19–21). Our first class of MIF inhibitors was a p-hydroxyphenyl derivative of amino acids, rationally designed based on the scaffold of dopachrome and p-hydroxyphenylimine derivative (13, 22). L-Trp Schiff base emerged as the most potent inhibitor of MIF tautomerase activity (17). Unfortunately, L-Trp Schiff base compounds lack long term stability. We also have shown that a P450-dependent metabolite of acetaminophen, N-acetyl-β-benzoquinone imine (NAPQI), covalently binds to MIF at its enzymatic site (23). The NAPQI adduct inactivates MIF cytokine activity in a number of in vitro bioassays, including interference with the anti-inflammatory effect of dexamethasone,
ISO-1 Protects against Severe Sepsis

**ACCELERATED PUBLICATION:**

**ISO-1 Protects against Severe Sepsis**

ISO-1 is a potential therapeutic agent for sepsis. It inhibits TNF secretion from LPS-treated macrophages (Fig. 2) and reduces TNF production in mice with endotoxin. ISO-1 also improves survival rates in a mouse model of sepsis (Fig. 3). This study demonstrates the potential of ISO-1 as an anti-inflammatory drug for sepsis treatment.

**FIGURE 2.** ISO-1 inhibits TNF secretion from LPS-treated macrophages. RAW 267.4 macrophages (10^6) were treated with various concentrations of ISO-1 (1–100 μM) 30 min prior to LPS (100 ng/ml) addition. After 16 h of incubation, cells culture supernatants were collected for determination of TNF concentration by an ELISA. Data are presented as mean ± S.D. (n = 3; *, p < 0.05 and **, p < 0.001). b. ISO-1 inhibits NF-κB activation from LPS-treated macrophages. Macrophages were treated with various concentrations of ISO-1 (1–100 μM) 30 min prior to LPS addition. The nuclear extract was isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents. For detection of NF-κB binding, nuclear extract from cells (~5 μg of protein) was incubated with 0.2 ng of 32P-labeled double-stranded oligonucleotide sequence, and the samples were resolved on a 4% polyacrylamide gel and visualized directly by autoradiography after drying the gel. The results for each treatment were expressed as relative intensity compared with the control (saline-treated) intensity.

**FIGURE 3.** ISO-1 is protective agent in mouse model of endotoxemia. a. ISO-1 inhibition of TNFα production is specific to MIF. As described previously (25), C57Bl/6 MIF^+/+ and MIF^-/- mice (each n = 7) were injected intraperitoneally with LPS (10 mg/kg) and either ISO-1 (1 mg/mouse) or vehicle. Peritoneal macrophages were cultured ex vivo for an additional 24 h, at which time supernatants were collected for determination of TNF concentration by ELISA; b, male Balb/c mice were injected intraperitoneally with LPS (10 mg/kg) and followed by different doses of ISO-1 (3.5–35 mg/kg) or vehicle twice a day for 3 days. Mice were observed for 2 weeks. Data points are from three independent experiments (n = 33; **, p < 0.001).

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FIGURE 4. ISO-1 treatment is protective when initiated 24 h after induction of polymicrobial sepsis. Mice were injected intraperitoneally with ISO-1 (35 mg/kg) (n = 34, ** p < 0.001) (a) or 100 μg of mouse monoclonal antibody against MIF (XIV.15.5; Anti-MIF) or IgG (control antibody) or vehicle 24 h after CLP (n = 15) (b). An additional two injections were given on days 2 and 3.

significantly improves survival in septic mice (1). We examined the time course of MIF release in mice with CLP-induced peritonitis, a widely used model of sepsis. We found that serum MIF levels increased to 70% of maximum levels within 24 h post-CLP and peaked at 36 h (data not shown). This identified MIF as a late mediator in sepsis, with potential to be inhibited in a clinically relevant time frame. Based on these results we reasoned that a delayed treatment with ISO-1, consistent with the kinetics of MIF release, could be successfully applied to improve survival. ISO-1 treatment (35 mg/kg) initiated 24 h after CLP surgery and continued for 3 days resulted in survival of 77% (** p < 0.001) compared with 38% in the control (vehicle-treated) group (Fig. 4b).

These data indicate that MIF activity can be therapeutically regulated with a molecule that specifically targets the tautomerase active site, a non-essential enzymatic function. It is not likely that the tautomerase activity is directly involved in the proinflammatory action of MIF, because no physiological substrates of the D-series of molecules have been identified in vertebrates. Rather, it is much more plausible that ISO-1 interacts specifically with MIF at the tautomerase site, resulting in altered binding of MIF to other cellular signaling protein partners. Previously we have shown that inhibition of the MIF tautomerase active site abolishes the ability of the molecule to regulate glucocorticoid activity in vitro (15). Here we demonstrate the importance of ISO-1 inhibition of this MIF catalytic site in the suppression of the cytokine pro-inflammatory activities and identify potential therapeutic implications.

ISO-1 significantly increases the survival rate in severe sepsis induced by endotoxin or CLP. The successful treatment of sepsis in mice by ISO-1 in a clinically relevant time frame indicates that this strategy to design MIF inhibitors can be developed further. These studies also raise the possibility that the deleterious sequence of MIF in diseases of MIF excess may be abrogated by treatments with ISO-1 or related, specific, small molecule MIF inhibitors.

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