

Inhibition of MIF Bioactivity by Rational Design of Pharmacological Inhibitors of MIF Tautomerase Activity

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The pro-inflammatory mediator macrophage migration inhibitory factor (MIF) is produced by immune and endocrine cells and inhibits the antiinflammatory activities of glucocorticoids. MIF also catalyzes the tautomerization of the non-naturally occurring D-isomer of dopachrome, phenylpyruvate, and certain catecholamines, suggesting that MIF might exert its biological effects via enzymatic action on a substrate. However, no physiologically relevant substrate for MIF has been identified. Site-directed mutagenesis studies have not consistently supported a requirement for an intact, functional catalytic site as a prerequisite for MIF bioactivity. We hypothesized that the catalytically active site, but not the enzymatic activity per se, nevertheless plays a critical role in MIF pro-inflammatory activity. Accordingly, we designed small druglike molecules that bind at the catalytically active tautomerase site of MIF and tested the complex for MIF bioactivity. We describe herein the rational design and synthesis of a class of imine conjugates produced by coupling amino acids to a range of benzaldehyde derivatives that inhibit MIF tautomerase and biological activities. We found that aromatic amino acid Schiff bases were better inhibitors of MIF enzymatic and bioactivities compared to the aliphatic ones. For instance, the IC₅₀ inhibition of MIF tautomerase activity by aromatic amino acid Schiff base methyl esters was achieved at a concentration between 1.65 and 50 μM, suggesting a critical role for the additional binding of the aromatic residues within the vicinity of the active site. The most potent inhibitor of MIF tautomerase activity was 2-[(4-hydroxybenzylidene)amino]-3-(1*H*-indol-3-yl)propionic acid methyl ester (**8**), with an IC₅₀ of 1.65 μM. We found that compound **8** binding to MIF active site resulted in the inhibition of MIF bioactivity in three established bioassays: ERK-1/2 MAP kinase activation, p53-dependent apoptosis, and proliferation of serum-starved cells. Compound **8** inhibited MIF interaction with its as yet unidentified cognate cell surface receptor as shown by flow cytometry, concluding a critical role for the tautomerase active site in receptor binding. Thus the inhibitory effect of compound **8** on MIF bioactivities strongly correlated with the inhibition of MIF tautomerase activity, a connection not made previously through use of small-molecule MIF inhibitors. The inhibitory activity of amino acid–benzaldehyde Schiff base-type MIF antagonists is the first step toward a meaningful structure/function analysis of inhibitors of MIF cellular bioactivities.

Introduction

Macrophage migration inhibitory factor (MIF) has emerged as a potent pro-inflammatory factor, released from preformed stores in the pituitary gland and immune system, that acts in part by interference with the antiinflammatory activities of the glucocorticoids.¹ MIF is critically involved in the pathogenesis of a variety of inflammatory and autoimmune diseases, and neutralizing anti-MIF antibodies has proven extremely effective in several animal models such as Gram-negative and Gram-positive sepsis,^{2,3} delayed-type hypersensitivity,⁴ adjuvant-induced arthritis,⁵ and glomerulonephritis.⁶ Moreover, MIF ^{-/-} mice are resistant to the lethal

effects of high-dose bacterial endotoxin (lipopolysaccharide, LPS), confirming the role of MIF in animal models of sepsis.³ This suggests that an effective MIF antagonist might act as a potent antiinflammatory agent to neutralize MIF and restore the antiinflammatory benefits of endogenously expressed corticosteroids. In addition, MIF has recently been implicated as an important factor for tumor progression and angiogenesis.^{7–10}

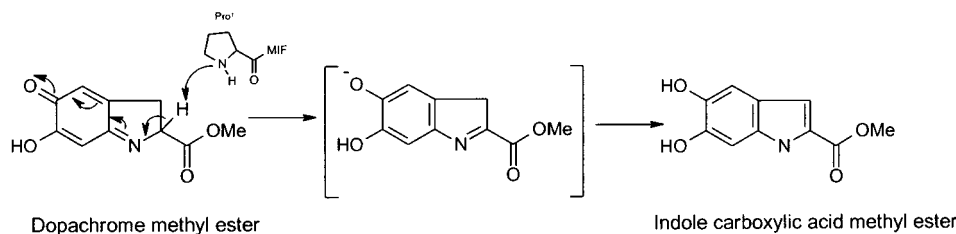
One unusual property uncovered for MIF is its ability to catalyze keto–enol isomerization (i.e., tautomerization) reactions. MIF tautomerizes D-dopachrome or L-dopachrome methyl esters to their corresponding indole derivatives (Scheme 1).¹¹ Because the enzymatic activity of MIF was first discovered in the context of a nonphysiological substrate (D-dopachrome), it has been hypothesized that the “true” substrate, and in turn the “true” enzymatic activity of MIF, remains to be discovered. Recently, phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid,¹² 3,4-dihydroxyphenylaminechrome, and nore-

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Scheme 1. MIF Tautomerizes L-Dopachrome Methyl Ester

pinephrinechrome¹³ also have been found to be MIF substrates. Corresponding Michaelis constant (K_m) values indicate that catalysis of these reactions is inefficient, suggesting that the above-listed substrates are unlikely to be definitive MIF enzymatic substrates.¹⁴ Elucidation of the three-dimensional crystal structure of human and rat MIF^{15–18} reveals significant structural (but no primary amino acid sequence) homology with the bacterial isomerases 4-oxalocrotonate tautomerase (4-OT) and carboxymethyl hydroxymuconate isomerase (CHMI).¹⁹ Both bacterial enzymes as well as MIF have been shown to act via a “one-base” mechanism and the catalytic residue has been identified to be a conserved N-terminal proline that is present at the base of a hydrophobic cavity.^{20–23}

The potential link between MIF cytokine activities and its catalytic site have remained unresolved. In three different studies utilizing mutant forms of MIF that lacked tautomerization catalytic activity, contradictory results were obtained.^{22–24} Recently, we have shown that the acetaminophen metabolite *N*-acetylbenzoquinonimine derivatizes MIF covalently at the active site and inhibits MIF bioactivities.²⁵ The possibility that the biological activity of MIF may be affected by chemical modification of the enzymatic active site suggests a potentially powerful approach to the rational design of small-molecule, pharmacological inhibitors of MIF based on structure–activity studies of substrate and transition-state analogues. Such inhibitors might act *in vivo* to block the counterregulatory effects of MIF, thereby maximizing the antiinflammatory efficacy of either endogenously released or exogenously administered glucocorticoids. In addition to their potential therapeutic applications, such inhibitors could prove to be extremely useful tools in unraveling the regulatory pathways of MIF action *in vivo*. Using available structural insights into the MIF catalytic site, we report herein the rational design and syntheses of *p*-hydroxyphenyl Schiff bases as potential inhibitors of MIF enzymatic and biological activity.

Experimental Procedures

All solvents used were HPLC-grade from Fisher Scientific (Pittsburgh, PA). NMR spectra were recorded on a Jeol Eclipse 270 spectrometer. Coupling constants are reported in hertz (Hz), and chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS, 0.0 ppm) with CDCl₃, DMSO-*d*₆, or CD₃OD as solvent. Thin-layer (TLC) and flash column chromatography were performed on alumina B, F-254 TLC chromat.

Spectrophotometric Assay for Enzymatic Activity. A fresh stock solution of L-dopachrome methyl ester (2.4 mM) was generated by oxidation of L-3,4-dihydroxyphenylalanine methyl ester with sodium periodate, producing an orange-colored solution. The L-dopachrome methyl ester solution (0.3 mL) was diluted to 1 mL with assay buffer (50 mM potassium

phosphate, pH 6.0, and 1 mM EDTA) and 1 μ L of MIF solution (500–700 ng/mL) was added, followed immediately by 1 μ L of a dimethyl sulfoxide solution (1–100 mM) of the enzymatic inhibitor being tested. Spectrometric measurements at $\lambda = 475$ nm were used to monitor the rate of decolorization of L-dopachrome for 20 s.

General Condensation Procedure. Candidate inhibitors of MIF tautomerase activity were derived via a general condensation procedure, as previously described.²⁶ Briefly, triethylamine (1.8 equiv) was added to a suspension of amino acid methyl ester hydrochloride and MgSO₄ in CH₂Cl₂, followed by 4-hydroxybenzaldehyde (1 equiv). The reactions were left stirring for 12–16 h. The MgSO₄ was filtered off and the crude mixture was concentrated under partial vacuum. The crude preparation was redissolved in CH₂Cl₂ and washed twice with brine (2 \times 20 mL). The organic phase was dried over MgSO₄ and then purified by flash chromatography over basic alumina or silica pretreated with triethylamine to yield the desired product.

[(4-Hydroxybenzylidene)amino]acetic Acid Methyl Ester (1). Yield 0.28 g, 40%; ¹H NMR (270 MHz, CD₃OD) δ 3.74 (s, 3H), 4.36 (s, 2H), 6.83 (d, $J = 8.7$ Hz, 2H), 7.63 (d, $J = 8.7$ Hz, 2H), 8.21 (s, 1H); MS (ES) m/z 194 (M + H) base peak, 216 (M + Na).

(S)-2-[(4-Hydroxybenzylidene)amino]propionic Acid Methyl Ester (2). Yield 0.2 g, 67%; ¹H NMR (270 MHz, CD₃OD) δ 1.46 (d, $J = 6.4$ Hz, 3H), 3.72 (s, 3H), 4.13 (q, $J = 6.9$ Hz, 1H), 6.82 (d, $J = 8.7$ Hz, 2H), 7.63 (d, $J = 8.7$ Hz, 2H), 8.23 (s, 1H); MS (ES) m/z 208 (M + H) base peak, 230 (M + Na).

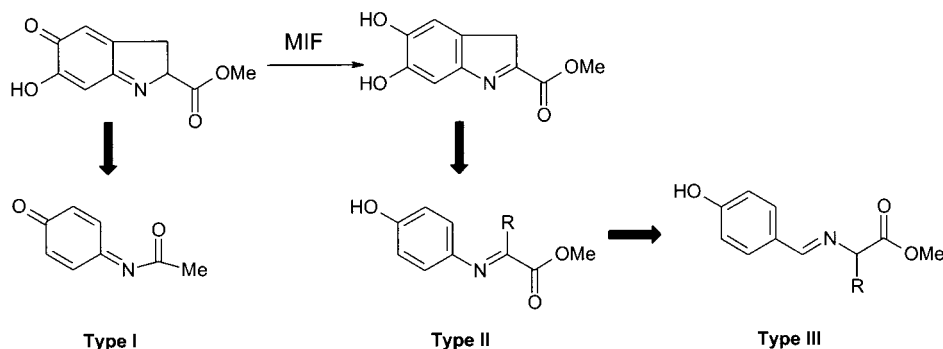
(S)-2-[(4-Hydroxybenzylidene)amino]-3-methylbutyric Acid Methyl Ester (3). Yield 0.2 g, 36%; ¹H NMR (270 MHz, CD₃OD) δ 0.87 (d, $J = 8.1$ Hz, 3H), 0.94 (d, $J = 6.9$ Hz, 3H), 2.23–2.36 (m, 1H), 3.63 (d, $J = 7.7$ Hz, 1H), 3.73 (s, 3H), 6.82 (d, $J = 8.6$ Hz, 2H), 7.65 (d, $J = 8.6$ Hz, 2H), 8.18 (s, 1H); MS (ES) m/z 236 (M + H) base peak, 258 (M + Na).

(S)-2-[(4-Hydroxybenzylidene)amino]-4-(methylsulfonyl)butyric Acid Methyl Ester (4). Yield 0.23 g, 43%; ¹H NMR (270 MHz, CD₃OD) δ 2.04 (s, 3H), 2.04–2.60 (m, 4H), 3.73 (s, 3H), 4.18 (dd, $J = 4.9, 8.9$ Hz, 1H), 6.83 (d, $J = 8.6$ Hz, 2H), 7.64 (d, $J = 8.6, 2H$), 8.24 (s, 1H); MS (ES) m/z 268 (M+H) base peak, 290 (M + Na).

(S)-3-Hydroxy-2-[(4-hydroxybenzylidene)amino]propionic Acid Methyl Ester (5). Yield 0.1 g, 20%; ¹H NMR (270 MHz, CD₃OD) δ 3.74 (s, 3H), 3.79 (dd, $J = 11.0, 7.1$ Hz, 1H), 4.00 (dd, $J = 4.9, 11.0$ Hz, 1H), 4.10 (dd, $J = 7.2, 5.0$ Hz, 1H), 6.81 (d, $J = 8.9$ Hz, 1H), 7.65 (d, $J = 8.6$ Hz, 1H), 8.23 (s, 1H); MS (ES) m/z 224 (M + H), 246 (M + Na) base peak.

(S)-2-[(4-Hydroxybenzylidene)amino]-3-phenylpropionic Acid Methyl Ester (6). Yield 0.23 g, 58%; ¹H NMR (270 MHz, CD₃OD) δ 3.03 (dd, $J = 9.1, 13.3$ Hz, 1H), 3.32 (dd, $J = 5.2, 13.3$ Hz, 1H), 3.72 (s, 3H), 4.17 (dd, $J = 5.2, 8.9$ Hz, 1H), 6.78 (d, $J = 8.6$ Hz, 2H), 7.16–7.21 (m, 5H), 7.52 (d, $J = 8.7$ Hz, 2H), 7.8 (s, 1H); ¹³C NMR (67.5 MHz, CD₃OD) δ 39.3, 51.3, 73.0, 115.2, 126.3, 128.0, 129.4, 130.3, 165.1, 172.4; MS (ES) m/z 284 (M + H) base peak, 306 (M + Na).

(S)-2-[(4-Hydroxybenzylidene)amino]-3-(4-hydroxyphenyl)propionic Acid Methyl Ester (7). Yield 0.27 g, 61%; ¹H NMR (270 MHz, CD₃OD) δ 2.92 (dd, $J = 8.9, 13$ Hz, 1H), 3.25 (dd, 1H), 3.71 (s, 3H), 4.10 (dd, $J = 5.2, 8.9$ Hz, 1H), 6.63 (d, $J = 8.4$ Hz, 2H), 6.80 (d, $J = 8.6$ Hz, 2H), 6.96 (d, $J = 8.6$ Hz, 2H), 7.52 (d, $J = 8.7$ Hz, 2H), 7.82 (s, 1H); ¹³C NMR (67.5 MHz,

Scheme 2. Rational Design of MIF Inhibitors

CD₃OD) δ 38.6, 51.2, 74.9, 114.7, 115.2, 126.7, 127.9, 130.3, 130.5, 155.8, 160.8, 165.0, 172.7; MS (ES) m/z 299 (M + H), 322 (M + Na), base peak.

(S)-2-[(4-Hydroxybenzylidene)amino]-3-(1H-indol-3-yl)propionic Acid Methyl Ester (8). Yield 0.20 g, 53%; ¹H NMR (270 MHz, CD₃OD) δ 3.12 (dd, J = 8.9, 14.4 Hz, 1H), 3.50 (dd, J = 5.2, 14.4 Hz, 1H), 3.73 (s, 3H), 4.22 (dd, J = 5.2, 8.7 Hz, 1H), 6.70–7.54 (m, 9H), 7.76 (s, 1H); ¹³C NMR (67.5 MHz, CD₃OD) δ 29.3, 51.2, 73.6, 109.8, 110.9, 115.1, 118.0, 118.4, 121.0, 123.6, 127.2, 130.3, 136.7, 164.6, 173.1; MS (ES) m/z 323 (M + H) base peak, 3345 (M + Na).

Proliferation Studies. NIH/3T3 fibroblasts (1×10^5 cells/mL) were cultured until semiconfluent in 96-well plates containing Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated fetal bovine serum (FBS). The cells were then serum-starved overnight in 0.5% FBS-containing DMEM to synchronize the culture. The medium was replaced with 10% FBS-containing DMEM together with a neutralizing anti-murine MIF monoclonal antibody (mAb) (14.15.5, IgG₁ subclass),¹ an isotype control mAb, or MIF inhibitors. The 14.15.5 mAb has been shown previously to neutralize both endogenously released (native) MIF and rMIF (recombinant) in a variety of in vitro and in vivo studies.^{3–9} [³H]Thymidine (5 μ Ci/mL; Dupont NEN, Boston, MA) was added to each well and the cells were allowed to proliferate for 16 h. The cells then were washed and harvested, and the incorporation of [³H]-thymidine into DNA was quantified by liquid scintillation counting (Packard Instruments, Meriden, CT).

ERK-1/2 Assay. NIH-3T3 fibroblasts were cultured in DMEM/10% FBS³² and treated with MIF and candidate inhibitors. Whole-cell extracts were prepared from 2×10^6 cells. Cells first were washed in cold PBS, and then 250 μ L of ice-cold RIPA buffer [containing 1 mM NaVO₄, 2 mM NaF, and a protease inhibitor cocktail (Boehringer Mannheim)] was added. The cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 10 min and microcentrifugation at 3000 rpm for 15 min (4 $^{\circ}$ C), the supernatants were removed, the protein concentration was determined, and the lysates were stored at -80° C. Western blotting for ERK-1/2 and phospho-ERK-1/2 was performed according to the manufacturer's directions (New England Biolabs, Beverly, MA). Briefly, equal amounts of lysate (200 μ g in \sim 200 μ L) were incubated with 15 μ L of an immobilized anti-phospho-p44/p42 mitogen-activated protein kinase (MAP kinase) mAb and the samples were allowed to rotate overnight at 4 $^{\circ}$ C. The pellet was collected by centrifugation and washed with 500 μ L RIPA buffer followed by three washes with 1 \times kinase buffer. The pellet then was resuspended in 50 μ L of 1 \times kinase buffer supplemented with 200 μ M ATP and 2 μ g of Elk-1 fusion protein as substrate (New England Biolabs). After incubation at 37 $^{\circ}$ C for 30 min, the reaction was terminated by adding 25 μ L of 3 \times Laemmli sample buffer. Each sample (30 μ L) was electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and transferred to poly(vinylidene difluoride) (PVDF) membrane. The blot then was probed for phospho-Elk-1 protein by utilizing an anti-phospho-Elk-1 antibody.

Apoptosis Assay. Primary murine embryonic fibroblasts were plated at 2×10^5 cells/well in 6-well plates. Cells were

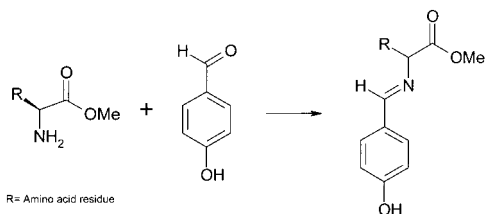
subsequently cultured overnight in 10% serum containing DME medium or serum-free DME medium in the absence or presence of recombinant human MIF, vehicle (Me₂SO), and/or Schiff base inhibitors. Cell lysates were normalized for equal protein from parallel samples and analyzed for apoptosis by the cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Biochemicals, Indianapolis, IN) according to the manufacturer's directions. Results shown are expressed as optical density at 405 nM \pm SD of triplicate samples and are representative of two experiments.

MIF Binding Assay. THP-1 cells were induced with interferon γ (1 ng/mL) in 10% serum containing RPMI at 37 $^{\circ}$ C for 3 days. After being washed with PBS, pH 7.4, 5×10^5 cells were resuspended in 0.1 mL of PBS and (a) were incubated with 200 ng of Alexa 488 rMIF conjugates at 4 $^{\circ}$ C for 45 min or (b) were incubated with a mixture of 200 ng of Alexa 488–rMIF conjugates and 10 μ M compound **8** at 4 $^{\circ}$ C for 45 min (the mixture of Alexa 488–MIF and compound was preincubated at 4 $^{\circ}$ C for 30 min before incubating with cells). After unbound human Alexa 488–rMIF conjugates were washed out, the cells were subjected to flow cytometry analysis.

Results and Discussion

A logical approach to discover novel inhibitors of the enzymatic activity of MIF (tautomerase) is to synthesize compounds whose structural units resemble the main features of the known MIF substrates dopachrome and *p*-hydroxyphenylpyruvate. Recently, we have discovered that *N*-acetylbenzoquinonimine (NAPQI), an acetaminophen metabolite (Scheme 2, type I), derivatizes MIF at the enzymatic site. MIF's enzymatic activity, per se, does not appear necessary to convey its pro-inflammatory activity by inhibition of the anti-inflammatory activities of glucocorticoids. However, it is clear that covalent binding of NAPQI inactivates MIF cellular activity with respect to a number of in vitro bioassays.²⁵ Unfortunately, the toxicity of NAPQI prevents its use as a systemic MIF antagonist. We hypothesized that nontoxic compounds that bind MIF's active site will be efficient and effective inhibitors of the cytokine's pro-inflammatory properties. To achieve this design goal, Schiff base adducts were synthesized via coupling of various *L*-amino acid methyl ester with *p*-hydroxybenzaldehyde (amino acid Schiff bases, AASB) (Scheme 2, type III).

As a first approach, Schiff base adducts were synthesized via coupling of 4-hydroxyaniline with glyoxalic acid and α -ketoacid methyl ester (Scheme 2, type II). However, these compounds proved to be weak inhibitors of MIF tautomerase activity ($IC_{50} > 300 \mu$ M when R = H). Subsequent derivatization with a methyl group weakened the inhibitory effect further ($IC_{50} > 500 \mu$ M

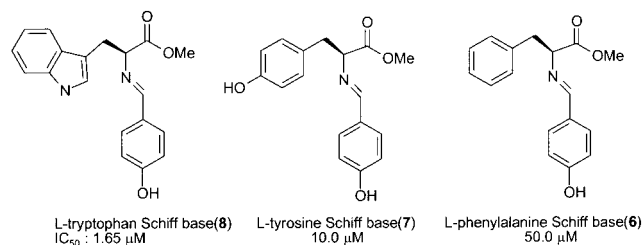
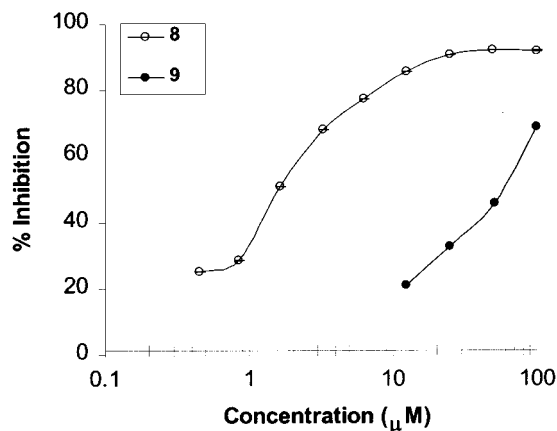
Scheme 3. Synthesis of Amino Acid Methyl Ester Schiff Bases**Table 1.** Schiff Base Derivatives of L-Amino Acid Methyl Esters

compd	R	IC ₅₀ (μM)
1	H	> 100
2	CH ₃	70.0
3	CH(CH ₃) ₂	> 100
4	CH ₂ CH ₂ SCH ₃	100.0
5	CH ₂ OH	100.0
6	Ph	50.0
7	4-OH-Ph	10.0
8	1-H-indolyl	1.65

when R = CH₃) likely because of steric effects in the functionalized system. We therefore coupled L-amino acid methyl ester with *p*-hydroxybenzaldehyde to furnish an AASB-type compound (Scheme 3).²⁶ These compounds were first tested for their inhibitory effects in the dopachrome tautomerization assay. The aliphatic amino acid Schiff bases (**1–5**; Table 1) displayed weak to moderate inhibitory effects (IC₅₀ ~70–100 μM) while the aromatic amino acid methyl ester Schiff bases (**6–8**) were found to be better inhibitors (Table 1). The most potent inhibitors were found to be the tryptophan (**8**) and tyrosine (**7**) Schiff bases, with IC₅₀ values of 1.65 and 10.00 μM, respectively. The other aromatic amino acid adduct, phenylalanine Schiff base (**6**), was less potent, exhibiting an IC₅₀ of 50.0 μM (Table 1).

In exploring these structure–activity relationships, we found that the location of the *p*-hydroxyl group in compound **8** was required to maintain the inhibitory activity and that relocation or replacement by a halide group resulted in complete loss of inhibitory activity in compound **8** analogues (data not shown). Reduction of the Schiff base by sodium borohydride produced the *N*-(4-hydroxybenzyl) amino acids, which lack inhibitory activity compared to the parent compound **8**. With regard to the specificity of MIF toward different chiral amino acids, we found that the D-aromatic amino acids (compound **9**) were 30-fold (IC₅₀ > 50 μM) less efficient than their L-amino acid counterparts (Figure 2). The discrimination between L- and D-amino acids was not surprising, since MIF has been known to discriminate between L- and D-dopachrome since the earliest descriptions of MIF tautomerase activity.¹¹

Recently, crystal structure analysis of MIF complexed with (*p*-hydroxyphenyl)pyruvate revealed that MIF exists as a trimeric protein carrying three molecules of (*p*-hydroxyphenyl)pyruvate.²⁷ The hydroxyl group of the substrate forms a hydrogen bond with Asn-97 of MIF, which is located at the bottom of the active-site cleft,

**Figure 1.** Comparison of the inhibitory effect of aromatic amino acid Schiff base compounds **6–8**.**Figure 2.** Inhibition of MIF dopachrome tautomerase activity by L- and D-tryptophan:hydroxybenzaldehyde Schiff bases. A fresh stock solution of L-dopachrome methyl ester (2.4 mM) was generated as an orange solution through oxidation of L-3,4-dihydroxyphenylalanine methyl ester with sodium periodate. The L-dopachrome methyl ester solution (0.3 mL) was diluted to 1 mL with assay buffer (50 mM potassium phosphate, pH 6.0, and 1 mM EDTA) and 1 μL of MIF solution (500–700 ng/mL) was added, followed immediately by 1 μL of a dimethyl sulfoxide solution (100 mM) of the enzymatic inhibitor being tested. Spectrometric measurements were made at λ = 475 nm by monitoring the rate of decolorization of L-dopachrome in comparison to a standard curve.

and the carboxylate forms a hydrogen bond with Lys-32 located at the top of the cleft. Similar interactions were revealed by cocrystallization analysis of MIF and (*E*)-2-fluoro-*p*-hydroxycinnamate, a competitive inhibitor of MIF tautomerase activity.^{28,29}

We expected compound **8** to bind MIF via similar interactions, on the bases of our structure–activity relationships and the observation that the inhibitory effect of AASB-type compounds was lost upon methylation of the substrate *p*-hydroxyl group. Figure 3 (top) illustrates the hypothetical interactions between compound **8** and MIF active site based on published data that describe the cocrystallization of MIF with (*p*-hydroxyphenyl)pyruvate²⁷ and (*E*)-2-fluoro-*p*-hydroxycinnamate²⁸ and finally our enclosed SAR. Further, hydrogen-bond formation between Lys-32 and the carboxylate methyl ester of AASB type inhibitors was not only expected but also determined to be critical, as reduction of the ester to alcohol resulted in complete loss of the inhibitory effect. Of note, the hydrophobicity of the active site is also extended to the cleft as it is rich with aromatic amino acid residues such as Phe 49, Tyr 36, Tyr 95, Trp 108, and Phe 113, offering an explanation for the superior inhibitory activity of compound **8** over compound **1** due to additional hydrophobic interactions between the indolyl functionality and the

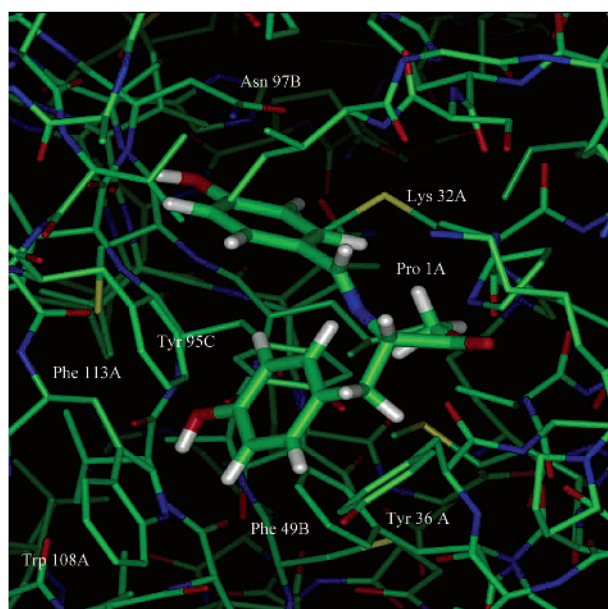
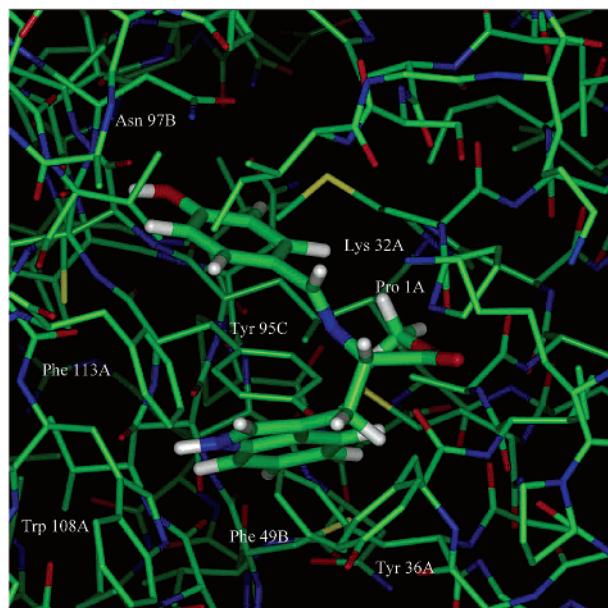


Figure 3. Results of docking of compound **8** (top panel) and compound **7** (bottom panel).

cleft environment. We expect that hydrophobicity of the aromatic amino acid residue is not the only factor that enhances binding to the cleft and that a role for hydrogen-bond interactions is indicated by the difference in inhibitory activity between compounds **7** (IC_{50} 10 μ M) and **6** (IC_{50} 50 μ M) (Figure 1). Additionally, we modeled a hypothetical interaction between compound **7** and MIF as shown in Figure 3 (bottom) and found that a potential hydrogen-bond formation between the hydroxyl group of compound **7** and indolyl residue of Trp 108 may explain the better inhibitory effect of compound **7** compared to compound **6**. Taken together, we conclude that the AASB-type inhibitors bind to the cleft of MIF analogously to (*p*-hydroxyphenyl)pyruvate, and that the amino acid component furnishes additional intermolecular interactions that enhance binding affinity at the active site. Of note, this explanation also was supported by the recent report by Orita et al.,³⁰ which

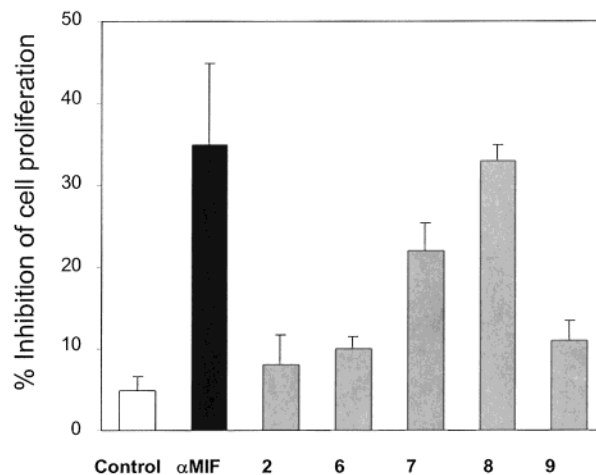


Figure 4. Inhibitors of MIF suppress serum-induced cell proliferation. DME medium (supplemented with 10% FBS) was added to quiescent NIH/3T3 fibroblasts together with a neutralizing anti-MIF mAb or compound **2**, **6**, **7**, **8**, or **9** at 100 μ M. Proliferation was assessed by the incorporation of [³H]-thymidine into DNA, and percent inhibition of proliferation was calculated. The results shown are the mean of triplicate assays and are representative of two separate experiments.

identified a second hydrophobic region at the rim of the active site.

Since no cellular receptor for MIF has been identified, we screened for cellular binding of MIF by flow cytometry. We have developed a fluorescently tagged MIF reagent (FL-MIF) in which Alexa dye (Molecular Probes, Inc. Eugene, OR) modifies primary amines within MIF by a covalent modification in 1:1 molar ratio of Alexa dye to MIF (data not shown). Of note, in our laboratory, we have shown that FL-MIF retains both dopachrome tautomerase activity and MIF pro-inflammatory activity, and preincubation of THP-1 cell cultures with excess unlabeled rMIF inhibits the binding of FL-MIF. Compound **7** or **8** decreased the number of THP-1 cells that bound fluorescent MIF by 45% respectively at 50 and 10 μ M. In contrast, compound **6** or **9** did not inhibit the MIF binding at a 100 μ M concentration, suggesting a correlation between the potency of the tautomerase active site inhibitors and receptor binding interference.

In addition to a mitogenic activity for recombinant MIF, our previous studies revealed that endogenous, cellular MIF regulates cellular proliferation,³¹ because serum-induced cell cycle progression requires secretion and autocrine action of preformed cellular MIF.³¹ To determine the ability of amino acid methyl esters to inhibit the cell cycle effects of MIF, potential MIF inhibitors were added to cultures of quiescent fibroblast upon stimulation with serum. As shown in Figure 4, serum stimulation of quiescent fibroblasts was inhibited in the presence of compounds **7** and **8**. No inhibition was detected in the presence of **2**, **6**, or **9**. The level of inhibition by compound **8** was similar to that of the well-characterized MIF-neutralizing monoclonal antibody.³¹ This suggests that cellular activities of MIF, likely to require a cell surface receptor for the cytokine, are susceptible to inhibition by the amino acid-hydroxybenzaldehyde Schiff base-type inhibitors identified here.

Although the requirement for a cell surface localized receptor for MIF remains ambiguous, it is clear that MIF stimulates the proliferation of murine fibroblasts,

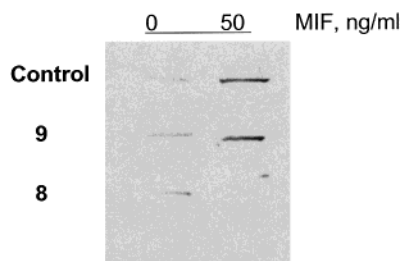


Figure 5. MIF inhibitors prevent MIF-dependent p44/p42 ERK MAP kinase phosphorylation. Quiescent NIH/3T3 cells (2×10^5 cells/mL) were washed and stimulated at an MIF concentration of 50 ng/mL in the presence of vehicle (Me_2SO) (control, top panel), L-tryptophan Schiff base (**8**), or D-tryptophan Schiff base (**9**) at 100 μM . Western blot analysis was performed on whole-cell lysates and with specific anti-phospho-p44/p42 ERK or total p44/p42 ERK antibodies.

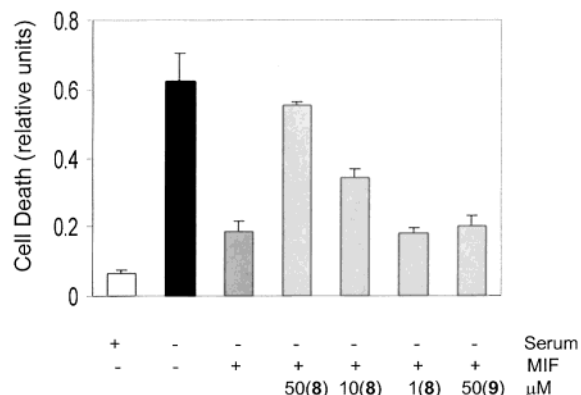


Figure 6. Inhibition of MIF-mediated protection from apoptosis by L-tryptophan Schiff base. Primary murine embryonic fibroblasts were plated at 2×10^5 cells/well in six-well plates. Cells were subsequently cultured overnight in 10% FBS containing medium or serum-free medium in the absence or presence of rMIF at 50 ng/mL. Vehicle alone or including compound **8** at 1–50 μM or compound **9** at 50 μM were preincubated with MIF in serum-free medium 5 min prior to addition to cells. Cell lysates were analyzed for apoptosis by ELISA as described under Experimental Procedures. Results shown are \pm SD of triplicate samples and are representative of two experiments.

and this appears to be linked to the activation of the ERK family of MAP kinases.³¹ Figure 5 shows that compound **8** inhibited the ability of recombinant MIF to induce ERK MAP kinase phosphorylation in NIH3T3 fibroblasts, while the corresponding D-isomer (**9**) or vehicle control (Me_2SO) had no effect. We conclude that inhibitors of MIF tautomerase activity can also block the MIF signaling pathway upon binding to the active site of MIF, suggesting an important role for the active site in MIF/receptor-induced signal transduction.

Recently, it was reported that MIF had the unique ability to inhibit the transactivation properties of the tumor suppressor protein p53.³² Specifically, Hudson et al.³² showed that MIF could suppress p53-dependent apoptosis, cell cycle arrest, and cell senescence. We have confirmed that exogenously applied recombinant MIF will inhibit apoptosis associated with growth factor deprivation in primary murine embryonic fibroblasts, and this assay serves as a useful tool to determine the relative inhibitory properties of potential MIF antagonists. To assess the activity of the L- and D-aromatic amino acid inhibitors of the anti-apoptotic action of recombinant MIF, we treated cells with an optimal

concentration of MIF (50 ng/mL) in the presence of vehicle (Me_2SO), and compound **8** or **9** during incubation of cells in serum-free medium for 24 h. Compound **8** dose-dependently inhibited the anti-apoptotic effect of MIF in concentration between 1 and 50 μM , while compound **9** had no effect at high concentrations (Figure 6).

These results demonstrate that rationally designed substrate mimetics that bind and inactivate the tautomerase active site of MIF are able to block MIF's bioactivity in a panel of in vitro assays. The effectiveness of these novel, amino acid Schiff base-type inhibitors of MIF validates the approach of targeting the MIF tautomerase active site, despite the fact that tautomerase activity of MIF per se is not required for the pro-inflammatory and proliferative activities of MIF. Anti-inflammatory MIF antagonists such as these hold promise as therapeutic agents for an assortment of inflammatory diseases including rheumatoid arthritis, inflammatory bowel disease, and sepsis.

References

- Calandra, T.; Bernhagen, J.; Metz, C. N.; Spiegel, L. A.; Bacher, M.; Donnelly, T.; Cerami, A.; Bucala, R. MIF as a glucocorticoid-induced counter-regulator of cytokine production. *Nature* **1995**, *377*, 68–71.
- Bernhagen, J.; Calandra, T.; Mitchell, R. A.; Martin, S. B.; Tracey, K. J.; Voelter, W.; Manogue, K. R.; Cerami, A.; Bucala, R. Macrophage migration inhibitory factor (MIF) is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **1993**, *365*, 756–759.
- Bozza, M.; Satoskar, A. R.; Lin, G.; Lu, B.; Humbles, A. A.; Gerard, C.; David, J. R. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. *J. Exp. Med.* **1999**, *189*, 341–346.
- Bernhagen, J.; Bacher, M.; Calandra, T.; Metz, C. N.; Doty, S. B.; Donnelly, T.; Bucala, R. An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. *J. Exp. Med.* **1996**, *183*, 277–82.
- Leech, M.; Metz, C.; Santos, L.; Peng, T.; Holdsworth, S. R.; Bucala, R.; Morand, E. F. Involvement of macrophage migration inhibitory factor in the evolution of rat adjuvant arthritis. *Arthritis Rheum.* **1998**, *41*, 910–917.
- Yang, N.; Nikolic-Paterson, D.; Ng, Y.-Y.; Mu, W.; Metz, C.; Bacher, M.; Meinhardt, A.; Bucala, R.; Atkins, R.; Lan, H. Reversal of established rat crescentic glomerulonephritis by blockade of macrophage migration inhibitory factor (MIF): potential role of MIF in regulating glucocorticoid production. *Mol. Med.* **1998**, *4*, 413–424.
- Chesney, J.; Metz, C.; Bacher, M.; Peng, T.; Meinhardt, A.; Bucala, R. An essential role for macrophage migration inhibitory factor (MIF) in angiogenesis and the growth of a murine lymphoma. *Mol. Med.* **1999**, *5*, 181–191.
- Takahashi, N.; Nishihira, J.; Sato, Y.; Kondo, M.; Ogawa, H.; Ohshima, T.; Ue, Y.; Todo, S. Involvement of macrophage migration inhibitory factor (MIF) in the mechanism of tumor cell growth. *Mol. Med.* **1998**, *4*, 707–714.
- Shimizu, T.; Abe, R.; Nakamura, H.; Ohkawara, A.; Suzuki, M.; Nishihira, J. High expression of macrophage migration inhibitory factor in human melanoma cells and its role in tumor cell growth and angiogenesis. *Biochem. Biophys. Res. Commun.* **1999**, *264*, 751–758.
- Onodera, S.; Suzuki, K.; Kaneda, K.; Fujinaga, M.; Nishihira, J. Growth factor-induced expression of macrophage migration inhibitory factor in osteoblasts: relevance to the plasminogen activator system. *Semin. Thromb. Hemost.* **1999**, *25*, 563–568.
- Rosengren, E.; Bucala, R.; Aman, P.; Jacobsson, L.; Odh, G.; Metz, C. N.; Rorsman, H. The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol. Med.* **1996**, *2*, 143–149.
- Rosengren, E.; Aman, P.; Thelin, S.; Hansson, C.; Ahlfors, S.; Bjork, P.; Jacobsson, L.; Rorsman, H. The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase. *FEBS Lett.* **1997**, *417*, 85–88.
- Matsunaga, J.; Sinha, D.; Pannell, L.; Santis, C.; Solano, F.; Wistow, G. J.; Hearing, V. J. Enzyme activity of macrophage migration inhibitory factor toward oxidized catecholamines. *J. Biol. Chem.* **1999**, *274*, 3268–3271.
- Stamps, S. L.; Fitzgerald, M. C.; Whitman, C. P. *Biochemistry* **1998**, *37*, 10195–10202.

- (15) Sun, H. W.; Bernhagen, J.; Bucala, R.; Lolis, E. Crystal structure at 2.6-Å resolution of human macrophage migration inhibitory factor. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5191–5196.
- (16) Sugimoto, H.; Suzuki, M.; Nakagawa, A.; Tanaka, I.; Nishihira, J. Crystal structure of macrophage migration inhibitory factor from human lymphocyte at 2.1 Å resolution. *FEBS. Lett.* **1996**, *389*, 145–148.
- (17) Suzuki, M.; Sugimoto, H.; Nakagawa, A.; Tenaka, I.; Nishihira, J.; Sakai, M. Crystal structure of the macrophage migration inhibitory factor from rat liver. *Nat. Struct. Biol.* **1996**, *3*, 259–266.
- (18) Kato, Y.; Muto, T.; Tomura, T.; Tsumura, H.; Watarai, H.; Mikayama, T.; Ishizaka, K.; Kuroki, R. The crystal structure of human glycosylation-inhibiting factor is a trimeric barrel with three 6-stranded β -sheets. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3007–3010.
- (19) Subramanya, H. S.; Roper, D. I.; Dauter, Z.; Dodson, E. J.; Davies, G. J.; Wilson, K. S.; Wigley, D. B. Enzymatic ketonization of 2-hydroxybutyrate: specificity and mechanism investigated by the crystal structures of two isomerases. *Biochemistry* **1996**, *35*, 792–802.
- (20) Stivers, J. T.; Abeygunawardana, C.; Mildvan, A. S.; Hajjipour, G.; Whitman, C. P. 4-Oxalocrotonate tautomerase: pH dependence of catalysis and pK_a values of active site residues. *Biochemistry* **1996**, *35*, 814–823.
- (21) Stivers, J. T.; Abeygunawardana, C.; Mildvan, A. S.; Hajjipour, G.; Whitman, C. P.; Chen, L. H. Catalytic role of the amino-terminal proline in 4-oxalocrotonate tautomerase: affinity labeling and heteronuclear NMR studies. *Biochemistry* **1996**, *35*, 803–813.
- (22) Swope, M.; Sun, H. W.; Blake, P. R.; Lolis, E. Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor. *EMBO J.* **1998**, *17*, 3534–3541.
- (23) Bendrat, K.; Al-Abed, Y.; Callaway, D. J. E.; Peng, T.; Calandra, T.; Metz, C. N.; Bucala, R. Biochemical and mutational investigations of the enzymatic activity of macrophage migration inhibitory factor (MIF). *Biochemistry* **1997**, *36*, 15356–15362.
- (24) Hermanowski-Vosatka, A.; Mundt, S. S.; Ayala, J. M.; Goyal, S.; Hanlon, W. A.; Czerwinski, R. M.; Wright, S. D.; Whitman, C. P. Enzymatically inactive macrophage migration inhibitory factor inhibits monocyte chemotaxis and random migration. *Biochemistry* **1999**, *38*, 12841–12849.
- (25) Senter, P.; Al-Abed, Y.; Metz, C. N.; Benigni, F.; Mitchell, R. A.; Chesney, J.; Han, J.; Gartner, C. G.; Nelson, S. D.; Bucala, R. Inhibition of macrophage migration inhibitory factor (MIF) tautomerase and biological activity by acetaminophen metabolites. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 144–149.
- (26) Stork, G.; Leong, A. Y. W.; Touzin, A. M. Alkylation and Michael addition of glycine ethyl ester. Use in amino acid synthesis and as acyl carbanion equivalent Schiff bases. *J. Org. Chem.* **1976**, *41*, 3491–3493.
- (27) Lubetsky, J. B.; Swope, M.; Dealwis, C.; Blake, P.; Lolis, E. Pro-1 of macrophage migration inhibitory factor functions as a catalytic base in the phenylpyruvate tautomerase activity. *Biochemistry* **1999**, *38*, 7346–7354.
- (28) Taylor, A. B.; Johnson, W. H., Jr.; Czerwinski, R. M.; Li, H. S.; Hackert, M. L.; Whitman, C. P. Crystal structure of macrophage migration inhibitory factor complexed with (*E*)-2-fluoro-*p*-hydroxycinnamate at 1.8 Å resolution: implications for enzymatic catalysis and inhibition. *Biochemistry* **1999**, *38*, 7444–7452.
- (29) Pirrung, M. C.; Chen, J.; Rowley, E. G.; McPhail, A. T. Mechanistic and stereochemical study of phenylpyruvate tautomerase. *J. Am. Chem. Soc.* **1993**, *115*, 7103–7110.
- (30) Orita, M.; Yamamoto, S.; Katayama, N.; Aoki, M.; Takayama, K.; Yamagiwa, Y.; Seki, N.; Suzuki, H.; Kurihara, H.; Sakashita, H.; Takeuchi, M.; Fujita, S.; Yamada, T.; Tanaka, A. Coumarin and chromen-4-one analogues as tautomerase inhibitors of macrophage migration inhibitory factor: Discovery and X-ray crystallography. *J. Med. Chem.* **2001**, *44*, 540–547.
- (31) Mitchell, R. A.; Metz, C. N.; Peng, T.; Bucala, R. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A_2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J. Biol. Chem.* **1999**, *274*, 18100–18106.
- (32) Hudson, J. D.; Shoaibi, M. A.; Maestro, R.; Carnero, A.; Hannon, G. J.; Beach, D. H. A proinflammatory cytokine inhibits p53 tumor suppressor. *J. Exp. Med.* **1999**, *190*, 1375–1382.

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