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INHIBITION OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) TAUTOMERASE ACTIVITY BY DOPACHROME ANALOGS

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Abstract: A macrophage migration inhibitory factor (MIF) dopachrome tautomerization assay was employed for identification of MIF inhibitors. One group of dopachrome analogs was identified which inhibits MIF tautomerase activity. In particular, the analogs with a leaving group at β position displayed activity at a concentration of tenfold less than that of the MIF-substrate. These findings could lead to a better understanding of MIF biological activities and the development of agents for the treatment of MIF related diseases.

Introduction

The protein mediator known as macrophage migration inhibitory factor (MIF) has emerged to play a central role in the control of the host inflammatory and immune response. Described originally as a lymphocyte "activity" responsible for inhibiting the random migration of macrophages in vitro,[1,2] MIF has been shown to be released from the anterior pituitary gland during stress and to be critically involved in the toxic response to septic shock.[3] Macrophages, T cells, and eosinophils release MIF in response to pro-inflammatory stimuli, and the expression of MIF is required for T-cell activation and antibody production by B cells.[4,5] More recent studies have shown that macrophages and T cells can secrete MIF in response to glucocorticoid stimulation, and that once released, MIF can "override" the immuno-suppressive effects of steroids on cytokine production and cellular activation.[4,5]

Studies performed in models of the inflammatory and immune response indicate that MIF acts extracellularly by stimulating the specific response of target cells. In 1996 however, Rorsman et al. reported that recombinant MIF (rMIF) catalyzes a tautomerization reaction, converting the non-naturally occurring D-isomer of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) to dihydroxyindole carboxylic acid (DHICA).[6] Intriguingly, subsequent elucidation of the three-dimensional crystal structure of MIF revealed it to exhibit significant three-dimensional (but not primary sequence) homology with two bacterial isomerases, 4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase.[7–9] These proteins share with MIF an apparently conserved active site structure that consists of a hydrophobic cleft bearing an active site proline. More recently, Rorsman and colleagues have proposed that MIF acts physiologically to catalyze the keto-enol tautomerization of *p*-hydroxyphenylpyruvate and phenylpyruvate.[10] Finally, the presence of the CXXC

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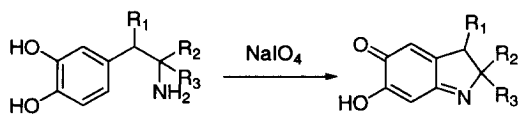
motif in MIF has prompted yet additional studies which suggest that MIF may function as a physiologically-relevant protein-thiol oxidoreductase.[11]

These observations notwithstanding, the precise relationship between MIF's enzymatic and immunological activities remain unclear. In two separate studies, the creation of an enzymatically-inactive form of MIF by site-directed mutagenesis shows the protein retains full glucocorticoid-regulatory activity,[12] but loses neutrophil priming activity.[13] In an effort to further explore the mechanism of action of MIF in biological systems, we have endeavored to identify potential small molecule inhibitors of MIF's isomerization activity. We report herein a study of MIF substrate analogs which inhibit dopachrome tautomerization at concentrations tenfold less than substrate. Such inhibitors may prove useful in the study of MIF structure function relationships and also assist in the development of small molecules that can be specifically targeted to MIF for pharmacological purposes.

Materials and Methods

Reagents. All chemicals were from Sigma (St. Louis, MA) or Aldrich (Milwaukee, WI). Pure, bioactive recombinant human MIF was prepared by expression in *E. coli* following methods described previously.[14]

Preparation of MIF Substrates and Analogs. The MIF-substrates **1b–4b** (Table 1) and the test inhibitors **5b–11b** were prepared by oxidizing their precursors **1a–4a** and **5a–10a** with sodium periodate,[7,8] as shown in Scheme 1. Compounds **7a** and **8a** were prepared from **5a** and **6a** by an esterification procedure.[8] The oxidation was performed 10 min prior to the tautomerization assay and the dopachrome compounds were tested without further purification. The test compounds **11–13** (Table 1) were obtained from Aldrich.



a b

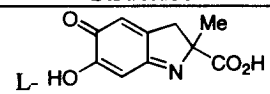
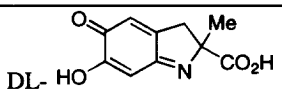
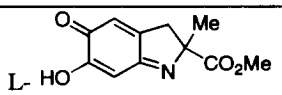
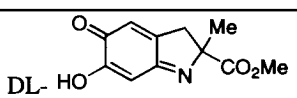
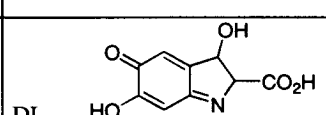
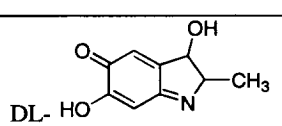
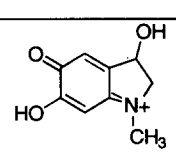
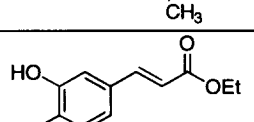
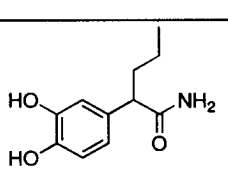
Compd	1	2	3	4	5	6	7	8	9	10
D/L	D	L	L	DL	L	DL	L	DL	DL	DL
R_1	H	H	H	H	H	H	H	H	OH	OH
R_2	H	H	H	H	Me	Me	Me	Me	H	H
R_3	CO_2H	CO_2H	CO_2Me	CO_2Me	CO_2H	CO_2H	CO_2Me	CO_2Me	CO_2H	CH_3

Scheme 1

Dopachrome Tautomerization Assay. The DOPA-related precursors (**1a–10a**) were prepared as 10 mM stock solutions in MIF assay buffer (10 mM sodium phosphate, pH 6.0). Thirty minutes before the tautomerization assay, 1.0 mL of each stock solution was diluted with 7 mL of assay buffer, and 1.0 mL of sodium periodate stock solution (20 mM in water) was added to initiate the oxidation of the precursors, thus generating each respective dopachrome derivative (**1b–10b**).[15] After 10 min, 1 mL of methionine (20 mM in

water) was added to react with the excess periodate. The dopachrome compounds were generated as orange solutions at a final concentration of 1 mM. Test inhibitors **11–13** were prepared as 10 μM stock solution and diluted to 1 mM solution with assay buffer before the assay. To perform the tautomerization assay, 0.5 mL of the 1 mM solution of the MIF-substrate (**1b**, **2b**, **3b**, or **4b**) was mixed with 0.5 mL of assay buffer. After the

Table 1. Inhibition of MIF tautomerase activity by test compounds

No.	Structure	Conc. of test compounds (mM)	% inhibition[a]
5b		0.5	29
6b		0.5	35
7b		0.5	45
8b		0.5	34
9b		0.5 0.25 0.125 0.06	100 98 41 9
10b		0.5 0.25 0.125 0.06	92 88 54 22
11		0.5 0.25 0.125 0.02	98 79 54 21
12		0.5	0
13		0.5	0

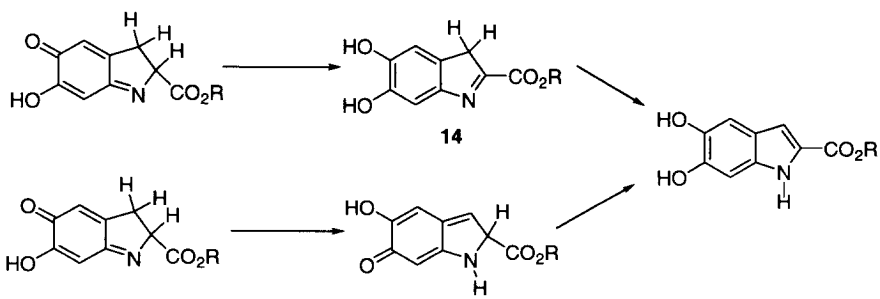
[a]0.5 mM of **3b** was used as MIF-substrate in this assay.

background rate was monitored, 10 μL of recombinant MIF solution from a 20 mg/mL stock was added. The decrease in absorbance (λ = 475 nm) at 1 min following addition of MIF was measured as an index of tautomerase activity, For the inhibitory assay, 0.5 mL of the 1 mM solution of the MIF-substrate (**3b**) was mixed with 0.5 mL of the 1 mM solution of one of the test inhibitors (**5b–10b** and **11–13**), followed by addition of 10 mL of recombinant MIF stock solution (20 mg/mL). Compounds **9b**, **10b**, and **12** were tested at final concentrations of 0.25 mM, 0.125 mM, and 0.06 mM in addition to the standard 0.5 mM assay. The percent inhibition of the decrease in absorbance (λ = 475 nm) at 1 min in the presence of inhibitors was calculated from the spectrophotometric data by standard methods.[6]

Results and Discussion

Tautomerization of an orange colored solution of D-dopachrome (**1b**) by MIF produces the non-colored DHICA.[6] This conversion can be quantitated spectrophotometrically by measuring the rate of decrease of the iminochrome absorbance at a wavelength of 475 nm. The methyl ester derivatives of D- (or L-) dopachrome were found to be better substrates for this reaction than dopachromes (**1b** and **2b**). In our study L-dopachrome methyl ester **3b** was used as a test substrate for the identification of compounds with the ability to inhibit the tautomerization reaction.

The dopachrome tautomerization by MIF may occur by two different pathways as shown in Scheme 2. The first proceeds via the initial conversion of dopachrome to indolidine **14** and subsequent rearrangement to an indole. A possible alternative pathway involves the formation of quinone methide intermediate **15** which is then transformed further into an indole.



Scheme 2

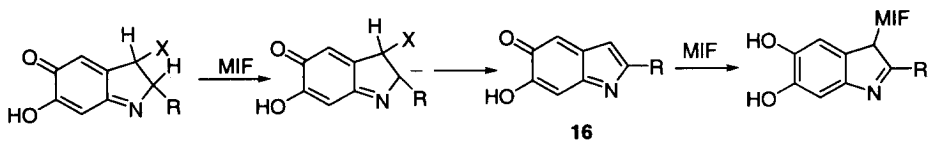
Understanding the mechanism of MIF catalyzed substrate tautomerization is helpful in the design and search of MIF inhibitors. Initial molecular modeling studies have suggested that the pathway through indolidine **14** might be favored, while comparison to structurally related tautomerases and more recent mechanistic data have favored the quinone methide pathway.[16,17] It is known that α-methyl quinone methide have UV and visible

spectra which are easily distinguished from the corresponding dopachrome spectra.[10] When **5b–8b** were exposed to MIF, no change of their spectra was observed. This observation supports the indolidine pathway described above and suggests that α-proton extraction is the initial step in dopachrome tautomerization by MIF.

L- and DL-α-methyl dopachrome (**5b** and **6b**) and their methyl esters (**7b** and **8b**), when present at the same concentration (0.5 mM) as the MIF-substrate **3b**, each displayed a moderate inhibitory effect on MIF tautomerase activity, with the esters showing slightly higher activity than the free acids as shown in Table 1. The moderate inhibitory activity of α-substituted dopachromes may result from the competition with MIF-substrate **3b** for the active site of MIF protein.

To identify inhibitors with higher activity, we needed to design compounds with stronger binding affinity or even ones capable of binding covalently to the MIF protein thereby blocking the active site and possibly changing the secondary structure of MIF.

As proposed in Scheme 3, dopachromes with a leaving group at β-position may form a very reactive intermediate **16** following α-deprotonation by MIF. Compound **16** may react with nucleophilic residues of the MIF protein to form a covalent bond with the protein. Our data have indeed shown that **9b**, **10b**, and **11** all displayed strong inhibition on MIF tautomerase activity (Table 1). Particularly, compounds **9b**, **10b**, and **11** all showed about 50% inhibition even at a concentration four times less than that of MIF-substrate. Further, **10b** and **11** showed activity at a concentration ten times less than that of MIF-substrate. Studies of the proposed MIF-covalent complexes (MIF-**10b** or **11**) by mass spectrometry were, however, inconclusive. Detailed elucidation of the nature of enhanced binding of these inhibitors to MIF requires further study.



16

Scheme 3

Finally, we examined acyclic compounds containing some structural features similar to the active MIF substrates/inhibitors. Thus, compounds **12** and **13** were tested and found to be inactive in the MIF tautomerization assay.

We have identified several inhibitors of MIF tautomerase activity. This discovery should lead to the development of inhibitors of MIF biological activities and the development of agents for the treatment of MIF related diseases.

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