Inactivation of Tautomerase Activity of Macrophage Migration Inhibitory Factor by Sulforaphane: a Potential Biomarker for Anti-inflammatory Intervention

Zachary R. Healy, Hua Liu, W. David Holtzclaw, and Paul Talalay

Abstract

Background: Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine with keto–enol tautomerase activity, rises rapidly in response to inflammation and is elevated in many chronic diseases. Isothiocyanates, such as sulforaphane from broccoli, are very potent inactivators of MIF tautomerase activity. A simple rapid method for determining this activity in tissues and body fluids may therefore be valuable for assessing severity of inflammation and efficacy of intervention.

Methods: Existing spectrophotometric assays of MIF, based on conversion of methyl L-dopachrome to methyl 5,6-dihydroxyindole-2-carboxylate and associated loss of absorption at 475 nm, lack sensitivity. Assay sensitivity and efficiency were markedly improved by reducing the nonenzymatic rate, by lowering pH to 6.2, replacing phosphate (which catalyzes the reaction) with Bis-Tris buffer, and converting to a microtiter plate format.

Results: A structure-potency study of MIF tautomerase inactivation by isothiocyanates showed that sulforaphane, benzyl, n-hexyl, and phenethyl isothiocyanates were especially potent. MIF tautomerase could be readily quantified in human urine concentrated by ultrafiltration. This activity comprised: (i) a heat-labile, sulforaphane-inactivated macromolecular fraction (presumably MIF) that was concentrated during ultrafiltration; (ii) a flow-through fraction, with constant activity during filtration, that was heat stable and insensitive to sulforaphane. Administration of the sulforaphane precursor glucoraphanin to human volunteers almost completely abolished urinary tautomerase activity, which recovered over many hours.

Conclusion: A simple, rapid, quantitative MIF tautomerase assay has been developed as a potential biomarker for assessing inflammatory severity and effectiveness of intervention.

Impact: An improved assay for measuring MIF tautomerase activity and its applications are described.

Cancer Epidemiol Biomarkers Prev; 20(7); 1516–23. ©2011 AACR.

Introduction

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine expressed in many types of cells, including epithelial, endothelial, immune, and endocrine cells. It is an early mediator of both innate and acquired immune responses, and is rapidly upregulated in a variety of inflammatory conditions associated with septic shock, toxemias, development of neoplasia, atherosclerosis, autoimmune conditions, and hypoxia. For example, MIF levels in tissues and body fluids are elevated in acute inflammatory and in autoimmune conditions such as rheumatoid arthritis, glomerulonephritis, lupus erythematosus, asthma, and psoriasis. These diverse attributes of MIF have been reviewed comprehensively (1–4).

MIF is a homotrimer with each subunit (mol. wt. 12.5 kD, 115 amino acids for the human protein) containing 2 antiparallel α-helices and 6 β-pleated sheets (5, 6). On the basis of the topological (but not sequence) homology of MIF to the bacterial enzyme oxalocrotonate tautomerase (7, 8), it was speculated that mammalian MIF would possess enzymatic activity, and this was established by Rosengren (9, 10), who showed that MIF had dopachrome and phenyl pyruvate keto–enol tautomerase activity. In analogy to the bacterial oxalocrotonate isomerase (7, 8, 11, 12), the N-terminal proline of each subunit of MIF is the catalytic base (13). These proline residues reside in hydrophobic pockets, have very low pKₐ values, and are highly reactive with electrophiles such as isothiocyanates (14).

Our interest in MIF was stimulated by 3 recent independent reports that MIF enzymatic activity is irreversibly inactivated by a number of isothiocyanates, among which sulforaphane [1-isothiocyanato-4(R/methylsulfinyl)butane] was especially potent (14–16). Sulforaphane was isolated in this laboratory from broccoli (17), based
on its high potency as an inducer for the widespread cytoprotective gene response regulated by the Keap1-Nrf2-ARE signaling system (18–20). There is much current interest in the use of sulforaphane to attenuate the severity of oxidant, electrophile, and inflammatory stresses that contribute to the pathogenesis of many chronic diseases (18–20). We therefore considered the possibility of using MIF enzymatic activity and its modulation by isothiocyanates as a quantitative biomarker of inflammatory severity and as a measure of the effectiveness of anti-inflammatory intervention. This strategy is also supported by observations that elevated MIF levels in various body compartments correlate with the intensity of diverse chronic diseases, and tend to normalize with attenuation of disease severity. It is especially interesting that deletion of MIF (e.g., in mif−/− mice), or neutralization of MIF by anti-MIF antibodies, reduced the severity of some inflammatory conditions (21–23).

The use of MIF as a promising biomarker for inflammatory disease has been recently proposed by Grieb and colleagues (4). Fulfilling this goal requires simple, quantitative, and specific methods for determining MIF levels in a variety of biological compartments (cells, tissues, and biological fluids). The majority of current analytical methods depend on antibodies generated to a wide variety of epitopes, including a sandwich ELISA. Although these methods have high sensitivity, their ability to distinguish minor structural modifications of MIF that may affect its biological activity depends on the specific antibody used (14, 24). Since MIF has enzymatic activity, enzyme assays for MIF are more functionally specific. Although no natural substrate for MIF has been identified, and the relation between enzymatic activity of MIF and its immunomodulatory properties is incompletely understood, several studies have shown that inactivation of MIF tautomerase activity is sometimes, but not always, accompanied by attenuation of its biological activity in vitro and in vivo (24–30). Therefore, we chose to develop improved enzymatic assays for MIF since isothiocyanates, the inhibitors of potential therapeutic interest to us, inactivate MIF in a highly specific and chemically defined manner.

A suitable assay for determining MIF enzymatic activity depends on the spectrophotometric determination of the conversion of the methyl ester of 2-carboxy-2,3-dihydroxyindole-5,6-quinone (L-dopachrome) (i) to the methyl ester of 5,6-dihydroxyindole-2-carboxylate (ii) by monitoring the rate of decline in absorbance of the dopachrome at 475 nm.

In this article, we describe a highly quantitative and sensitive method for measuring the enzymatic (dopachrome tautomerase) activity of MIF, modified from Rosengren and colleagues (10), and adaptation of this assay to 96-well microtiter plate format. The sensitivity of the assay has been greatly increased by modifying the buffer and pH, and thus reducing the nonenzymatic reaction rate. We have compared the potencies of a variety of isothiocyanates in inactivating mouse (cell and recombinant) and human MIF, and confirmed the high potency of sulforaphane as an inactivator. We show the presence and describe the rapid determination of MIF activity in human urine, and its inactivation in the urine of human volunteers who consumed glucoraphanin (the naturally occurring glucosinolate precursor of sulforaphane) orally in the form of broccoli sprout extracts (31).

Materials and Methods

Expression and purification of murine recombinant MIF

The murine recombinant MIF coded by the pET11b plasmid vector (13, 32) was transfected into chemically competent BL21(DE3) E. coli (Invitrogen), and individual transformants were selected from LB plates containing ampicillin (100 μg/mL, Gibco) and used to inoculate overnight rotating cultures in LB broth with ampicillin (37°C, 225 RPM). These were used to inoculate fresh cultures at an initial OD_600 of 0.05, and were grown until mid-log phase (OD_600 0.4). Protein expression was induced with IPTG (0.5 mmol/L). Cultures were then grown for an additional 3 to 4 hours, and the cells were centrifuged for 15 minutes at 15,000 × g, and 4°C. Cells were lysed by sonication (50% power, 50% cycle, 5 min, 4°C, Branson Sonicator). Cell debris was removed by centrifugation (30 min, at 20,000 × g, 4°C), and the supernatant fraction was filtered and applied to a DEAE-cellulose column (20 mmol/L Tris, 20 mmol/L NaCl, pH 7.4). MIF does not adhere to this column, and flow-through fractions were assayed for MIF activity by use of the L-dopachrome methyl ester assay and pooled. The column purification was repeated, and the pooled MIF samples were assayed for enzymatic activity and protein concentration (33), and samples were stored at −80°C. The tautomerase activity of the purified recombinant mouse MIF was 200 to 300 μmol/min per mg protein when measured in 50 mmol/L Bis-Tris at pH 6.2.

L-Dopachrome methyl ester tautomerase assay

The measurement of MIF dopachrome tautomerase activity was modified from Rosengren and colleagues (10). Extensive work on the optimization of sensitivity of this assay is described in Results and Discussion, and the final procedure is as follows. For reactions in 96-well microtiter plates, L-dopachrome methyl ester was prepared just before use by adding 500 μL of 12 mmol/L L-dopa methyl ester (L-3,4 dihydroxyphenylalanine methyl ester; Sigma) and 500 μL of 24 mmol/L sodium periodate to 19 mL of a reaction buffer (50 mmol/L Bis-Tris, 1 mmol/L EDTA, pH 6.2) and incubating at 25°C in the dark for 5 minutes. For reactions done in 1.0-cm
cuvettes, the volumes were adjusted proportionately to 2.0 mL. The dopachrome methyl ester solution was used immediately, and the rate of decrease in absorbance of L-dopachrome methyl ester at 475 nm was determined. Microtiter plate wells contained 200 µL of reaction buffer and 20 to 100 µL of the solution to be assayed. The rate was based on the maximum rate of initial decrease in absorbance in the first 5 minutes, and corrected for the nonenzymatic rate (usually 0.001 absorbance unit per minute). Assuming a molar absorption coefficient (ε) of 3,000 M⁻¹ cm⁻¹, the stoichiometric rate was calculated after adjusting for the light path length depending on the final reaction volume in the microtiter wells.

Purification of the L-dopachrome methyl ester was done in some cases by use of Sephadex C₁₈ reverse-phase columns (Waters; ref. 34). Briefly, the column was wetted with methanol, washed with deionized water, and the solution was applied with gentle vacuum. The column was washed with 10 volumes of water, and the purified product was eluted with methanol. The purified L-dopachrome methyl ester was either used directly or stored at −80°C.

Cell culture and preparation of cell lysates

RAW 264.7 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (Gibco) with 10% heat-inactivated FBS in humidified 5% CO₂ at 37°C. For preparation of cell lysates, cells were treated with lysis buffer (40 mmol/L HEPES, 50 mmol/L NaCl, 1 mmol/L EDTA, 1% CHAPS, and Protease Inhibitor Cocktail; Sigma) for 10 minutes on ice with gentle shaking, and the solution was centrifuged (10 min, 15,000 g, 4°C). The supernatant fraction was collected for MIF enzymatic assay. For cells cultured in microtiter plates, the centrifugation step was not done, as the debris did not interfere with the assay. All protein determinations were done with the bicinchoninic acid assay (33).

Inhibition of MIF in RAW 264.7 cells with sulforaphane

RAW 264.7 cells were seeded in 96-well plates and grown to 80% confluency as described above. The cells were then washed 3 times in Dulbecco’s PBS, and culture medium containing 10% FBS, and the indicated concentrations of sulforaphane in acetonitrile were added. The final concentration of acetonitrile did not exceed 0.1% (by volume). The cells were incubated (37°C, 5% CO₂) for the specified periods of time. The cells were then washed 3 times in Dulbecco’s PBS, and cell lysates were prepared by adding 40 µL of lysis buffer per well. Twenty microliters of lysate were transferred to a fresh 96-well plate for protein concentration determination. The remaining 20 µL were used for determination of MIF activity as described. MIF activity was normalized to total protein concentration (33).

Inhibition of recombinant mouse MIF with isothiocyanates

Serial dilutions of the individual isothiocyanates were prepared in the dopachrome reaction buffer (50 mmol/L Bis-Tris, pH 6.2) with a final volume of 100 µL (0.05% acetonitrile). Purified recombinant murine MIF was added to the wells of 96-well plates to yield a final concentration of 100 nmol/L MIF. The inhibitor-enzyme solution was incubated for 15 minutes at 25°C with a low-evaporation lid on an orbital shaker. To assay MIF activity, a twice-concentrated assay substrate was generated by mixing 500 µL of 24 mmol/L sodium periodate and 500 µL of 12 mmol/L L-dopa methyl ester with 9 mL of reaction buffer and adding 100 µL of this solution to each well. Control samples contained no enzyme. The activity was determined from initial rates as described, and the Median Effect concentrations (D₅₀) were determined by the Median Effect Equation (35).

MIF tautomerase activity measurement in human urine

Freshly collected human urine was concentrated with Amicon Ultra cellulose filters (cutoff 3,000 mol. wt.; Millipore) usually from 30 mL to 250 µL (120-fold). Half of the concentrated urine was incubated with 100 µmol/L sulforaphane (10 mmol/L stock was prepared in water) at 4°C for 30 minutes. Both sulforaphane-treated and untreated urine aliquots (20 µL) were assayed in 96-well microtiter plates in triplicate. MIF tautomerase activity was calculated from the difference of total activity (sulforaphane untreated) and residual activity (sulforaphane treated), and finally normalized to the creatinine concentration in each original urine sample. All human study protocols were approved by our Institutional Review Board.

Results and Discussion

Optimization of enzymatic assay sensitivity and adaptation to microtiter plates

MIF catalyzes the keto–enol tautomerization of p-hydroxyphenylpyruvate and phenylpyruvate, in which the enols can be stabilized by chelation with borate. The enols absorb strongly at 300 nm, whereas the keto forms do not. Although this assay was adequate for measuring the enzymatic activity of purified or recombinant MIF, its use with cell lysates and tissue samples that absorb strongly in the 260 to 320 nm spectral region was difficult.

The spectrophotometric assay involving the tautomerization of L-dopachrome derivatives, permits measurements to be made at much longer wavelengths by monitoring the decline of the absorption of L-dopachrome methyl ester at 475 nm in 50 mmol/L sodium phosphate buffer at pH 7.0 (10, 36). The substrate is prepared just before use by oxidation of L-dopa methyl ester with an excess of sodium periodate. The reaction product, L-dopachrome methyl ester, has a molar absorption coefficient (ε) of 3,000 M⁻¹ cm⁻¹ at 475 nm and may be used directly as substrate, or after purification by adsorption on a reverse-phase microcolumn and elution with methanol (34).

The principal limitation to the sensitivity of this assay is the large nonenzymatic rate which is directly proportional to the concentration of substrate. In efforts to reduce the
nonenzymatic rate, we first observed that phosphate strongly catalyzes the reaction in a concentration- and pH-dependent manner (Fig. 1A). Thus, when using 200 μmol/L substrate, the nonenzymatic rate was reduced 30-fold by lowering the phosphate concentration from 50 to 10 mmol/L and the pH from 7.0 to 6.2. These modifications had little effect on the enzymatic rate. By using a smaller excess of NaIO₄ (2-fold instead of 8-fold during the preparation), the nonenzymatic rate was further decreased by 2- to 3-fold, because apparently the excess periodate also promotes the nonenzymatic reaction. Moreover, excess periodate inhibits the enzyme. When the phosphate buffer was replaced by 20 to 50 mmol/L Bis-Tris (bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane) buffer, the pH was lowered to 6.2, and the excess NaIO₄ was reduced, the nonenzymatic rate (at an initial 200 μmol/L substrate concentration) was decreased to 0.001 absorbance unit per minute at 475 nm in a 300 to 350 μL volume in a 96-well microtiter plate. For most experiments, the substrate was not purified, and similar nonenzymatic rates were observed. Notably, the low nonenzymatic rate observed in Bis-Tris was not affected by the concentration of the buffer. Moreover, a significant advantage of Bis-Tris buffer is that the enzymatic rates observed with recombinant mouse MIF were 1.5- to 2-fold higher in 50 mmol/L Bis-Tris than in 10 mmol/L sodium phosphate, irrespective of pH (5.8–7.0; Fig. 1B). By means of these modifications, the sensitivity of the assay was increased about 8- to 10-fold, making it possible to detect the tautomerase activity in body fluids such as urine.

**Inactivation of MIF tautomerase activity by sulforaphane and other isothiocyanates**

Exposure of RAW 264.7 macrophage-like cells for 60 to 240 minutes to 10 μmol/L sulforaphane completely abolished the enzymatic activity of MIF (Fig. 2). The D₅₀ were 0.6 to 2.0 μmol/L at the various time points, indicating powerful and rapid inactivation of the enzymatic activity. Purified recombinant mouse MIF was also potently inactivated by sulforaphane and other isothiocyanates (Fig. 2).

Figure 1. Effect of pH and concentration of various types of buffers on the rate of tautomerization of L-dopachrome methyl ester. A, nonenzymatic rates were measured in 25 and 50 mmol/L sodium phosphate, 50 mmol/L L-histidine, and 50 mmol/L Tris-Bis buffers at indicated pH values. B, comparison of enzymatic rates with mouse recombinant MIF (50 nmol/L) as a function of pH in 50 mmol/L Bis-Tris and 10 mmol/L sodium phosphate buffers. The measurements were made in cuvettes in final volumes of 2 mL by monitoring the initial rate of decrease in absorption at 475 nm of an L-dopachrome methyl ester prepared as described under Methods and are expressed as μmol per minute (corrected for optical path length and molar absorption coefficient). Enzymatic rates are corrected for the nonenzymatic contribution (usually 0.001 per minute).

Figure 2. Concentration and time dependence of the inactivation of MIF tautomerase activity in RAW 264.7 cells by sulforaphane. The cells were grown in 96-well microtiter plate wells to about 80% confluence under standard conditions. They were washed 3 times with Dulbecco’s PBS and then exposed at 37°C, 5% CO₂ for 60, 120, or 240 minutes to medium containing sulforaphane to give final concentrations of 0.2 to 10 μmol/L (0.1% acetonitrile). The cells were washed 3 times with Dulbecco’s PBS, treated with lysis buffer, and the protein concentration of each lysate and the initial rate of tautomerization of L-dopachrome methyl ester were measured. All measurements are normalized to protein concentration, and the specific enzyme activities so obtained were compared with the controls treated with only acetonitrile (0.1%) to which the value of 100 was assigned.
inhibited by sulforaphane and other isothiocyanates. The potencies of a range of concentrations of sulforaphane and benzyl, phenethyl, n-hexyl, 2-hexyl, n-nonyl, and n-decyl isothiocyanates on mouse recombinant MIF are shown as $D_m$ values in Table 1. The $D_m$ value for sulforaphane is 2.98 $\mu$mol/L and that for benzyl isothiocyanates is 2.27 $\mu$mol/L. Incubation of purified recombinant mouse MIF with 100 $\mu$mol/L sulforaphane for 15 minutes at 25°C inactivated the tautomerase activity of the concentrate by more than 95% (relative to 0.1% acetonitrile controls) independently of the buffer utilized.

### MIF tautomerase activity in human urine

In the urine of normal human volunteers, the tautomerase activity can be measured easily after suitable concentration by centrifugal ultrafiltration. When 30 mL of urine were concentrated to 250 mL (120-fold), addition of a 20-$\mu$L aliquot of the concentrated urine to 200 $\mu$L of assay buffer in 96-well microtiter plates gave a range of absorbance changes of 0.01 to 0.04 per minute (corrected for water blank rate of about 0.001 per minute) at 475 nm for various urine samples. In contrast, 20 $\mu$L of the filtration flow-through fraction under similar conditions gave a corresponding rate of absorbance change greater than 0.001 per minute (0.002–0.004). However, the tautomerase activity in the flow-through fraction and the concentrated urine displayed very different properties: (i) Activity of the flow-through fraction remained constant during the course of concentration by ultrafiltration, whereas that of the concentrated fraction rose with the degree of concentration, suggesting that the activity of the flow-through fraction was promoted by a low molecular weight component that passed through the filtration membrane easily. In contrast, the concentrated fraction contained a macromolecular component, presumably MIF, which did not pass through the filter. (ii) Treatment with sulforaphane (100 $\mu$mol/L at 4°C for 30 min) almost completely inactivated the tautomerase activity of the concentrate, but had no effect on the activity of the flow-through fraction. Residual activity still existed in the concentrate after treatment with sulforaphane, however, which indicated that the tautomerase activity of urine was not entirely enzymatic. To distinguish the fraction of the total activity arising from the presence of enzyme, we treated preparations to be tested with 100 $\mu$mol/L sulforaphane for 30 minutes at 4°C. A variety of experiments with longer incubation times, higher concentrations of sulforaphane, and different temperatures between 4 and 25°C showed that the above conditions invariably led to the almost total inactivation of the enzymatic activity. (iii) When the flow-through fraction of freshly concentrated urine was heated at 65°C for 30 minutes, the activity remained unchanged. The concentrate, however, lost much of its activity when heat treated under similar conditions. Specifically, when the concentrate was treated with 100 $\mu$mol/L sulforaphane for 30 minutes at 4°C, the residual activity was not heat sensitive, and correction for this activity indicated that more than 95% of the enzymatic activity was heat labile. (iv) In contrast to the concentrated urine, the flow-through fraction showed no reaction in an ELISA assay. The simplest explanation for these observations is that urine contains a heat-stable component that catalyzes the tautomeration. Phosphate is a logical candidate for the nonenzymatic isomerization catalyst, and exists in human urine at about 10 to 40 mmol/L level, which can promote the reaction (see Fig. 1A).

Figure 3 shows the MIF tautomerase activity of a single subject determined on 5 random urine samples obtained

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### Table 1. Inactivation of recombinant mouse MIF tautomerase activity by sulforaphane and other isothiocyanates

<table>
<thead>
<tr>
<th>Isothiocyanate</th>
<th>$D_m$ ($\mu$mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulforaphane</td>
<td>2.98</td>
</tr>
<tr>
<td>Phenethyl</td>
<td>5.00</td>
</tr>
<tr>
<td>Benzyl</td>
<td>2.27</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>1.35</td>
</tr>
<tr>
<td>2-Hexyl</td>
<td>50.2</td>
</tr>
<tr>
<td>n-Nonyl</td>
<td>182</td>
</tr>
<tr>
<td>n-Decyl</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

**NOTE:** Purified recombinant murine MIF (100 nmol/L) was incubated with serial dilutions of the individual isothiocyanates in 100 $\mu$L 50 mmol/L Bis-Tris buffer, pH 6.2, at 25°C for 15 minutes. MIF tautomerase activity was assayed in 96-well microtiter plates. $D_m$ values were determined by the Median Effect Equation (33).
on different days during a 33-day period. Each sample was concentrated and measured soon after collection and the MIF activity values were normalized to per milligram of urinary creatinine concentration, to compensate for variations in urine volume. Each sample was also tested after treatment with 100 \( \text{mmol/L} \) sulforaphane for 30 minutes at 4°C. The residual sulforaphane-resistant activity amounted to about 0.12 to 0.14 nmol/min per mg of creatinine, whereas the total activity was about 0.40 to 0.60 nmol/min per mg of creatinine, which when corrected for the nonenzymatic activity, gave values of the enzymatic activity of 0.28 to 0.46 nmol/min per mg of creatinine.

### Temperature stability of MIF tautomerase activity in human urine

The experiments on human urine described above were all done with freshly voided human urine, or urine that had been stored at 4°C for at most 24 hours. Storage of unconcentrated urine at 4°C for up to 8 days, followed by concentration by ultrafiltration and direct assay for tautomerase activity or assay after treatment with sulforaphane, gave the results shown in Table 2. Whereas the residual activity remaining after sulforaphane inhibition showed only a small decline (about 15%) during storage, the sulforaphane-inhibited component lost about 36% of its tautomerase activity. These findings are consistent with the interpretation that the sulforaphane-sensitive activity is enzymatic, but the sulforaphane-resistant activity is not. In contrast, conventional slow freezing at −20°C of fresh urine before concentration resulted in a substantial loss of MIF enzymatic activity, and the concentrated urine frozen in this way lost its MIF activity to a similar extent. The MIF tautomerase activity was partially protected when the concentrated urine was quickly frozen in liquid nitrogen, however, or when a small amount of glycerol (10%) was added to the urine before freezing. These properties suggested that MIF might be a cold-sensitive enzyme (37).

### Table 2. Stability of MIF tautomerase activity in human urine stored at 4°C

<table>
<thead>
<tr>
<th>Storage time (d)</th>
<th>Total activity</th>
<th>Sulforaphane-treated residual activity</th>
<th>Sulforaphane-sensitive (by difference) activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.458</td>
<td>0.138</td>
<td>0.320</td>
</tr>
<tr>
<td>5</td>
<td>0.368</td>
<td>0.126</td>
<td>0.242</td>
</tr>
<tr>
<td>8</td>
<td>0.322</td>
<td>0.118</td>
<td>0.204</td>
</tr>
</tbody>
</table>

NOTE: Aliquots of a freshly collected single human urine sample were stored at 4°C for 8 days. After ultrafiltration, half of the concentrated urine was treated with 100 \( \mu \text{mol/L} \) sulforaphane at 4°C for 30 minutes. MIF tautomerase activity was then assayed in 96-well microtiter plates.

### Figure 4. A, inactivation of MIF tautomerase activity in human urine by consumption of glucoraphanin

Urine samples were collected from 4 healthy human volunteers at 8 a.m., immediately before treatment (predosing), and the entire urine was collected from 8 a.m. to 4 p.m. (8 hours after dosing), and from 4 p.m. to 8 a.m. the following morning (24 hours after dosing) after they consumed 200 \( \mu \text{mol} \) of glucoraphanin in the form of broccoli sprout extracts. After concentration by centrifugation (120-fold), the MIF activity of the concentrated urine was measured in 96-well microtiter plates, and was normalized to creatinine concentration in each urine sample. MIF activity shown in the graph was the sulforaphane-sensitive activity (total activity minus sulforaphane-treated residual activity). B, correlation of MIF tautomerase activity measured in the 4 predosing human urine samples and their MIF levels determined by ELISA, both were normalized to the creatinine concentration in each original urine sample.

### Inactivation of urinary MIF tautomerase activity by glucoraphanin/sulforaphane consumption

Four healthy volunteers (all female, age range 32–61 years, 1 African American, 3 Caucasian) adhered to a...
correlated (human urine samples was proportional to and well
dopachrome tautomerase activity measured in 4 different
these experimental conditions. More importantly, MIF
change the binding of MIF to its antibody, at least under
which indicated that sulforaphane treatment did not
reflect the active MIF in biological fluids.

In summary, the sensitivity of a MIF L-dopachrome
methyl ester tautomerase activity assay has been signifi-
cantly increased, and the assay has been successfully
adapted to microtiter plates. Although no natural sub-
strates of MIF have been recognized, this does not affect
the value of using the enzymatic activity to measure MIF
levels as biomarkers. This made possible the direct mea-
urement of MIF activity in human urine. In view of our
interest in the inactivation of MIF activity by direct
reaction of its N-terminal proline with isothiocyanates,
such as sulforaphane, the enzymatic activity provides a
direct index of active MIF levels. Nevertheless, sulfora-
phane-inactivated MIF retains reactivity with anti-MIF
antibody in an ELISA assay. By use of the microtiter plate
assay for MIF tautomerase activity, a large-scale screen
for MIF inhibitors could lead to the discovery of potential
novel anti-inflammatory agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Dr. C. P. Whitman, University of Texas, Austin, Texas, for
providing the plasmid containing recombinant mouse MIF which origi-
nated from the laboratory of Dr. R. Bucala. We also thank Dr. J.T. Stivers,
Johns Hopkins School of Medicine, for valuable advice.

Grant Support

This work was supported by NIH grants R01CA93780 and R01CA49076, and
by American Institute for Cancer Research, Washington DC.
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Received March 17, 2011; revised April 21, 2011; accepted May 8, 2011;
published OnlineFirst May 20, 2011.

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Cancer Epidemiol Biomarkers Prev 2011;20:1516-1523. Published OnlineFirst May 20, 2011.

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