Published in final edited form as:

Biochemistry. 2012 September 25; 51(38): 7506–7514. doi:10.1021/bi3005494.

Structural Interactions Dictate the Kinetics of Macrophage Migration Inhibitory Factor Inhibition by Different Cancer-Preventive Isothiocyanates

Gregg V. Crichlow^{†,1}, Chengpeng Fan^{†,1}, Camille Keeler[‡], Michael Hodsdon[‡], and Elias J. Lolis^{*,†,‡}

[†]Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

[‡]Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut

^{*}Yale Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut

Abstract

Regulation of cellular processes by dietary nutrients is known to affect the likelihood of cancer development. One class of cancer preventive nutrients, isothiocyanates (ITCs) derived from consumption of cruciferous vegetables, is known to have various effects on cellular biochemistry. One target of ITCs is macrophage migration inhibitory factor (MIF), a widely expressed protein with known inflammatory, pro-tumorigenic, pro-angiogenic, and anti-apoptotic properties. MIF is covalently inhibited by a variety of ITCs, which in part, may explain how they exert their cancer-preventive effects. We report the crystallographic structures of human MIF bound to phenethylisothiocyanate and to L-sulforaphane (dietary isothiocyanates derived from watercress and broccoli, respectively), and correlate structural features of these two isothiocyanates with their second-order rate constants for MIF inactivation. We also characterize changes in the MIF structure using NMR HSQC spectra of these complexes and observe many changes at the subunit interface. While a number of chemical shifts do not change, many of those that change do not have similar features in magnitude or direction for the two isothiocyanates. The difference in the binding modes of these two ITCs provides a means of using structure-activity relationships to reveal insights into MIF biological interactions. The results of this study provide a framework for the development of therapeutics that target MIF.

Macrophage migration inhibitory factor (MIF) is a widely expressed pro-inflammatory protein that is under investigation as a potential target for interventions against sepsis, autoimmune diseases and cancer¹⁻³. The tautomerase activity of MIF (EC 5.3.2.1) has been used for drug screening and inhibitor design. Compounds that inhibit MIF tautomerase activity have been found also to inhibit MIF biological functions in cell-based and *in vivo* experiments.^{4, 5} However, there is a continuing debate as to whether inhibition of enzymatic activity or antagonism of MIF-receptor interactions is the mechanism that results in decreased biological activity.^{6, 7}

*Corresponding Author: Elias J. Lolis, Department of Pharmacology, Yale University School of Medicine, Box 208066, New Haven, CT 06520-8066, ph: (203) 785-6233 fax: (203) 785-5494, elias.lolis@yale.edu.

[†]These authors contributed equally to the publication.

Supporting Information

Table S1. List of residues from either sulforaphane or PEITC that do have large changes in the NMR HSQC compared to apo-MIF. This material is available free of charge online at <http://pubs.acs.org>.

Recently, proteomic studies to identify cellular proteins modified by isothiocyanates (ITCs) revealed MIF was specifically targeted.⁸⁻¹⁰ Isothiocyanates are a class of nutrients obtained through consumption of cruciferous vegetables, having well known cancer-preventive properties.^{11, 12} The inhibition of MIF by these compounds provides an explanation of some salubrious effects of ITCs that are not explained by its other known cellular targets and activities.¹⁰ Furthermore, oil containing phenylethylisothiocyanate (also known as phenethylisothiocyanate, PEITC) has been found to be active against ulcerative colitis.¹³ Although the target is not known in this study, it is interesting that MIF is implicated in colitis¹ as well as other pro-inflammatory diseases, and based on the proteomics experiments, is likely to be MIF.

It has been found that consumption of isothiocyanate nutrients derived from cruciferous vegetables may reduce the risk of having cancer.¹⁴⁻¹⁶ In particular the isothiocyanates L-sulforaphane, abundant in broccoli, and PEITC, abundant in watercress, have anti-myeloma properties.¹⁷ The mechanism of this protection has been found to be manifold including genotoxic effects on tumor cells,¹⁸ induction of redox-protective “phase 2” enzymes^{19, 20} and inhibition of cytochrome P450-mediated production of toxic carcinogenic metabolites.²⁰ ITCs also target tumor cells directly via glutathione depletion and increased oxidative stress.²¹ Although isothiocyanates have been found to be very beneficial nutritionally, not enough is known about proper dosage and timing of administration for them to be useful therapeutically. One group reported induction of bladder tumors in rats upon treatment with an isothiocyanate after induction of tumorigenesis via carcinogen administration,²² although isothiocyanate intake prior to, or along with, carcinogen administration reduced cancer risk in the same strain of rat.²³ Another group reported low concentrations of ITCs inhibit apoptosis, while high isothiocyanate dosage induces apoptosis.²⁴

MIF, which has been found to have pro-tumorigenic, pro-angiogenic and pro-inflammatory activities, is becoming widely recognized as a potential anti-cancer drug target. Small molecule inhibitors directed against MIF have been found useful in both animal models of sepsis,²⁵ and in culture against cellular proliferation and migration of lung cancer cells⁵ and restoration of contact inhibition in glioblastoma cells.² The reactivity of isothiocyanates against MIF therefore presents a natural nutritional targeting of an oncologically relevant protein, adding support to the rationale for development of anti-MIF compounds for cancer chemotherapy. Herein, we quantitate the kinetics of the reactions of MIF with the nutrient isothiocyanates PEITC and sulforaphane. We also report the crystal structures and NMR HSQC spectral changes of MIF complexed with these two ITCs and discuss their use for understanding the mechanism of MIF inhibition and further development of MIF inhibitors.

Experimental Procedures

Materials

Recombinant human MIF and ¹⁵N-MIF were expressed and purified as described.^{25, 26} L-sulforaphane (the naturally occurring *R* configuration) and PEITC were purchased from Sigma-Aldrich (St. Louis, MO). ¹⁵N-ammonium chloride was obtained from Cambridge Isotopes (Andover, MA).

MIF Derivatization Kinetics

Irreversible inhibition was observed by incubating MIF with inhibitor and diluting aliquots into assay buffer at various times to measure tautomerase activity as previously described for other covalent-based inhibitors.²⁵ Kinetics of isothiocyanate derivatization of MIF were measured using 15 μ M MIF incubated with or without 15 μ M inhibitor in 2.9% DMSO, 19.4 mM Tris (pH 7.5), 19.4 mM NaCl at room temperature (approximately 23°C). Aliquots

were assayed at 16 ($\pm$ 1), 90, 150, 240, 480 and 960 s of incubation time. The MIF concentration during tautomerase assays was 0.20 μ M, and the temperature of the spectrophotometer during the tautomerase assays was fairly stable (25-26°C when assaying tautomerase activity of the PEITC-derivatized MIF, 26-27°C for sulforaphane-MIF, and 24-25°C for the control incubation). Experiments were performed in triplicate for each inhibitor and for the control. Rate constants were calculated using Graph Pad Prism 4 software.

Crystallization

PEITC and L-sulforaphane stock solutions were prepared in DMSO. A solution of 0.2 mM MIF, 1.0 mM isothiocyanate, 10% DMSO, 18 mM Tris (pH 7.5), 18 mM NaCl was prepared and allowed to sit at ambient temperature on the bench for two minutes and 5 minutes for PEITC and L-sulforaphane, respectively. The ITC-MIF solution was subjected to a few rounds of concentration and re-dilution in 20 mM Tris (pH 7.5), 20 mM NaCl until the DMSO concentration was reduced to approximately 1% (for PEITC-MIF) or 0.5% (for sulforaphane-MIF). For crystallization, hanging drops were prepared by mixing 2 μ l of 0.96 mM PEITC-MIF or 2 μ l of 1.2 mM L-sulforaphane-MIF with 2 μ l of reservoir consisting of 2 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M Tris (pH 7.5), 3% isopropanol. Pyramid-like crystals, 0.3-0.4 mm on a side, were grown at 20°C.

Crystallographic Data Processing and Structure Determination

Diffraction data were collected on a Rigaku micromax 007 X-ray generator with an R-Axis IV++ image plate detector. The data were processed using HKL2000.²⁷ Structure determination for each complex was performed by molecular replacement with the program AMoRe in CCP4^{28, 29} using protein coordinates from native MIF (PDB 3DJH) as a search model.²⁵ Initial models for the inhibitors were generated using the Dundee ProdrG Server. Refinement was performed using CNS³⁰ and ligand placement and model building were performed using the programs O and COOT.³¹ Refinement statistics are listed in Table 1. Coordinates and crystallographic structure factors were deposited to the RCSB protein data bank (PEITC-MIF: PDB entry 3SMB, and sulforaphane-MIF: PDB entry 3SMC).

NMR HSQC Experiments

¹⁵N-labeled MIF was covalently modified as above and extensively dialyzed into 20 mM sodium phosphate buffer, pH 7.0 and 0.5 mM EDTA. Each NMR sample was prepared with 10% D₂O and protein concentrations of approximately 1mM. All NMR experiments were conducted at 25°C on a Varian INOVA 600 MHz spectrometer using a 5 mm triple-resonance probe equipped with triple-axis pulsed magnetic field gradients and utilized pulse sequences from the Varian BioPack User Library (Varian Inc., Palo Alto, CA). ¹H-¹⁵N HSQC NMR spectra were processed using NMRPipe³² with subsequent display and analysis in Sparky³³ and with chemical shifts referenced indirectly to external 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid at 0.00 ppm.

Results

Kinetics of MIF derivatization by PEITC and L-Sulforaphane

MIF was incubated with an equimolar concentration of PEITC or L-sulforaphane, and the rate of enzyme inactivation was determined by measuring tautomerase activity after various times of incubation. MIF is a homotrimer with the three active sites formed by subunit interfaces. ITCs react with the catalytic proline in MIF with a stoichiometry of one ITC to one MIF monomer (figure 1A, B). Therefore the concentration of MIF used in the experiments was calculated as the total concentration of MIF monomeric subunits. Enzyme

activity was fit to a hyperbolic curve (figure 1C) to determine second-order rate constants according to equation 1,

$$y = \frac{A}{Akt + 1}, \quad (\text{equation 1})$$

where A is the initial effective free MIF concentration in micromolar, t is time (in seconds) of MIF incubation with inhibitor prior to assaying enzymatic activity, and k is the second-order rate constant. PEITC covalently inhibits MIF with a rate constant of $3.1 (\pm 0.1) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$. The rate constant with sulforaphane is $1.3 (\pm 0.06) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$. As these compounds have the same reactive isothiocyanate group, the difference is due to the different R-groups attached to the functional isothiocyanate moiety (figure 1A,B).

Structure of the PEITC-MIF Complex

The 1.6 Å structure of the PEITC-MIF complex reveals that the phenylethyl group of the inhibitor occupies the hydrophobic active site of the protein (figure 2A,B). The form of the inhibitor-enzyme adduct was modeled as the thiocarbamoylated protein (with the nitrogen of PEITC protonated and the double bond maintained between the carbon and sulfur atoms) based on known chemistry of isothiocyanates with amines. This chemistry has often been employed in the Edman degradation³⁴ and occurs not only with primary, but with secondary amines³⁵ and therefore is applicable to the N-terminal proline of MIF.

The torsion angle between the inhibitor and Pro-1 is 9.7 ± 2.5 (mean $\pm$ S.D. among the three active sites of the trimer, figure 2B). Therefore this represents a *cis*-thiopeptide bond. Such a complex would be less likely with any other N-terminal residue due to the increased propensity for proline to form a *cis* peptide bond. Any energetic disadvantage of the *cis* bond formation is evidently compensated by the affinity of the PEITC R-group for the MIF active site, as the hydrophobic chain is buried in the cavity.

Structure of L-Sulforaphane-Bound MIF

The structure of L-sulforaphane bound to MIF was determined at 1.8 Å resolution. In contrast to filling the active site cleft by PEITC, the side chain of sulforaphane extends out of the binding site and resides at the surface of the protein (figure 2C). This would suggest a lower affinity for the active site cleft by the aliphatic side chain of sulforaphane relative to the aromatic side chain of PEITC and is consistent with the slower rate constant for sulforaphane than PEITC for reacting with MIF. The covalent modification still occurs, highlighting the inherent reactivity of MIF with the isothiocyanate group. With the sulforaphane extending towards the protein surface, the bond formed with Pro-1 is in the *trans* conformation ($\omega = 171.3 \pm 2.08$, mean $\pm$ S.D., fig 2C). One of the active sites lacks crystal contacts. At this site, although we see most of the sulforaphane, there is no electron density for the methylsulfinyl group of the inhibitor, indicating its mobility at the surface of the protein (figure 2D). In the other two active sites, electron density is present for the entire inhibitor, presumably due to symmetry-related copies of the protein restricting the motion of the sulforaphane.

Structural Changes of MIF upon Binding Isothiocyanates

Comparison of the X-ray structure of sulforaphane-bound MIF to unbound MIF reveals there are no large structural changes in neither the active site (figure 3A,B) nor trimer (figure 4A). Although the chemical reaction pathway from substrate transitioning to product cannot be determined in this study, the product of this chemical reaction suggests the sulforaphane isothiocyanate group is in an ideal position for covalent bond formation with the amine of Pro-1 because there no significant movement from Pro-1 in the apo-structure.

The structure of the MIF-PEITC complex, however, reveals a movement of Pro-1 within the active site (figure 3B), but still no significant change in the trimer (figure 4B). The position of the aromatic ring within the active site requires a conformational change in Pro-1 to make a covalent bond with PEITC. The disadvantage of the movement of Pro-1 and the *cis* conformation of the thiopeptide is overcome by the affinity of PEITC for the active site. The proline conformational change may explain why the kinetics of bond formation for PEITC, which makes more contacts with MIF in the active site and appears to have greater affinity than sulforaphane, is not greater than 2.5-fold relative to sulforaphane.

It is also informative to compare sulforaphane and PEITC to two other irreversible inhibitors, 4-iodophenylpyrimidine (4-IPP, figure 3C) and phenylmethylsulfonyl fluoride (PMSF, figure 3D).^{5, 25} A similar Pro-1 shift attributed to PEITC is seen when MIF binds 4-IPP, but is not seen when MIF binds to PMSF. The difference in Pro-1 movement in PEITC and 4-IPP complexes relative to PMSF is due to their larger size and favorable contacts in the active site. Comparison of sulforaphane and PMSF complexed to MIF reveals the ψ angle for Pro-1 in PMSF changes to accommodate the tetrahedral covalent bond that is formed. Neither inhibitor yields any significant conformational change elsewhere in the protein (figure 4).

NMR Studies of ¹⁵N-MIF with PEITC and Sulforaphane

A previous NMR study of the reaction of MIF and another isothiocyanate, benzyliothiocyanate (BITC), revealed large changes in ¹H-¹⁵N peak width in the subunit interface indicative of a major conformational change.⁸ We collected analogous ¹H-¹⁵N HSQC NMR peaks using the same buffer and temperature on the covalently modified forms of MIF presented here and compared them to the unliganded protein. As can be seen in Figure 5A, both of the covalent modifications significantly perturb the backbone amide chemical shifts for about 50% of the residues. However, unlike what was reported for BITC, there were no detectable changes in linewidths. Interestingly, the chemical shift changes induced by each of the covalent modifications were dissimilar in magnitude and direction. For the resonances that were not significantly perturbed by one covalent modification, they were also not affected by the other. In general, the pattern of backbone amide chemical shifts for the modified proteins remains consistent with a folded structural state. The distribution of backbone amide ¹H chemical shifts, with many values above 8 ppm, remains consistent with a high beta-sheet content. One possible explanation for all of these observations involves rearrangement of the structural interface between MIF monomers within the trimeric complex. We hypothesize that the interface residues within the MIF trimer are able to exist in multiple conformations, without any associated perturbations of the overall secondary, tertiary, or quaternary structure. Covalent modification of the N-terminus, which is located within the interface, may alter the population distribution of structural states for these interface residues. In the case of BITC, the observed line broadening for interface residue backbone amides could be explained by conformational exchange on the NMR timescale induced by the covalent modification, which was not observed with sulforaphane or PEITC. Interestingly, non-covalent inhibitors at the active site also cause chemical shift broadening and changes in the positions of the chemical shifts.⁷

We used Sparky to overlay the peaks from both ITCs to those assigned from apo-MIF in order to produce a qualitative assessment of which residues moved upon formation of a covalent bond with the ITCs. More than 10% of the peaks from both ITCs superimposed very well with the apo-MIF peaks (e.g., see Val-41 at 8.77 ppm, 127.61ppm). We also included chemical shifts that were not perturbed significantly upon formation of the covalent bond with ITCs those that partially overlapped or moved slightly relative to the apo-MIF peaks (Table S1). We highlighted on the ribbon structure of apo-MIF all other residues

corresponding to chemical shift due to ITC covalent bond formation that were significantly perturbed or were absent relative to the apo-MIF peaks (figure 5B). Over half the residues are perturbed and are mostly located around the active site at the subunit interface. These residues include the β -sheet and the hydrophobic residues from the α -helices that abut against the β -sheet. A large portion of the C-terminal region, for example, interdigitates into the adjacent subunit and contributes one of the six strands in the β -sheet for each monomer. Perturbation of these C-terminal β -strands results in changes to the hydrophobic residues of the helix (helix II: residues 69-87) in the adjacent subunit. The lower half of other helix (helix I: residues 18-29) is perturbed because it is adjacent to β -strand I, directly following Pro-1. The loop (residues 30-37) that connects helix I to β -strand II possesses residues that are part of the active site and is also perturbed. The absence of chemical shift changes for more than half of the solvent-exposed helical residues distal to Pro-1 is an indication that the secondary and tertiary structure remains similar to the apo-MIF structure. The absence of chemical shift changes at this location also indicates that a large number of chemical shifts are due to subtle effects that are observed due to the sensitivity of NMR. However, this does not exclude changes in the conformation of MIF side chains due to covalent binding of the two ITCs.

Discussion

The well-studied cancer preventive effects of isothiocyanates are consistent with inhibition of the pro-inflammatory, pro-tumorigenic, and pro-angiogenic protein MIF. Covalent modification by ITCs obtained through the diet represents a natural mechanism for curtailing MIF-mediated cellular proliferation. Our observation that MIF is covalently modified by an ITC such as sulforaphane that extends out of the pocket is consistent with previous studies demonstrating that MIF is inhibited by a wide variety of ITCs, some containing aromatic rings that are commonly seen binding in the MIF binding pocket, and others that do not contain such moieties. This supports the theory that MIF inhibition plays a relevant role in the anti-tumorigenic effects of ITCs.^{11, 12} The more efficient *in vitro* derivatization of MIF by PEITC relative to sulforaphane contrasts with previously reported cellular activities. Cross *et al* working with HeLa cells, and Brown *et al* working with Jurkat cells found sulforaphane inhibited tautomerase activity at lower concentrations than PEITC when added to cells.^{8, 9} In these previously reported experiments exogenous MIF was not added to the cells and it is suggested that intracellular MIF was inhibited, as only lysates were assayed. The relative membrane permeabilities of sulforaphane and PEITC are not known and could explain the difference between the *in vitro* and cellular results, with sulforaphane penetrating the cellular membrane more efficiently than PEITC. There are other possible explanations that have yet to be studied. Among them are binding to other proteins with aromatic sites that sequester PEITC without any covalent modification and the half-life of PEITC and sulforaphane in cells.

ITCs demonstrate a similar kinetic profile to that of MIF inhibition by PMSF²⁵ except that the rates of covalent complex formation are much faster. (PMSF is so slow in modifying Pro-1 that a second order rate constant could not be calculated but pseudo-first order kinetics experiments were performed). PMSF binds within the active site whereas sulforaphane extends outward. Therefore, the improved kinetics of sulforaphane over PMSF must be due to the greater reactivity towards MIF of the ITC functional group relative to a fluorinated sulfonyl moiety. The MIF-binding R-group of PEITC in the active site allows faster condensation with the Pro-1 after the required proline movement is achieved relative to sulforaphane.

Condensation of an isothiocyanate with the N-terminus of a protein is the first step in the Edman degradation. However, Edman performed his original procedure at pH 8.6 in order to

deprotonate the free amino terminus to allow the protein to react³⁴ PEITC, however, reacts with MIF at near-neutral pH values. Complex formation was carried out in pH 7.5 buffer in both the crystallization and kinetic experiments. The irreversible inhibition of MIF by PEITC is therefore dependent on the uniquely low pKa of Pro-1,³⁶ which makes this protein a specific target of covalent ITC modification. Cross *et al.* found that MIF is the predominant protein modified by PEITC in HeLa cells by using a whole-cell proteomic labeling method.⁹ Likewise, Brown *et al.* discovered that MIF is uniquely targeted by PEITC in Jurkat T-cells using a similar whole-cell lysate proteomic pull-down study.⁸

It is surprising no large regional or global conformational changes were observed in the crystal structures upon binding of these two isothiocyanates. NMR HSQC experiments to probe changes upon binding MIF of BITC resulted in changes in peak width due to exchange between multiple conformational species that are observed on the NMR times scale that mapped to a large region of the subunit-subunit interface indicative of a conformational change.¹⁰ It must be also added that other non-covalent inhibitors at the active site also had major HSQC changes but their X-ray structures were never solved.⁷ The HSQC changes of PEITC and sulforaphane were examined under similar conditions. Although the NMR experiments with these two compounds did not lead to changes in peak width, most of the chemical shifts indicated a change in the subunit interface with no detectable changes in the secondary, tertiary, or quaternary structure of MIF (Figure 5). Given the sensitive nature of NMR experiments, these changes may not lead to a large rearrangement of the MIF structure but may indicate the existence of other, subtly different conformational states captured by each isothiocyanate in solution.

Although there are no major global changes of MIF modified by either ITC examined in this study, the structure of the PEITC-derivatized MIF indicates a local conformational change in Pro-1 (figure 4). *In silico* modeling by Brown *et al.* suggested a change in backbone conformation in the vicinity of Pro-1 and Lys-32 in the presence of PEITC.⁸ There is a movement of Pro-1 in the crystal structure but not nearly as large as predicted by modeling. There are no significant changes in Lys-32. Brown *et al.* found that PEITC reduced the affinity of a monoclonal antibody for MIF, which was interpreted as evidence for a protein conformational change even though the exact epitope was not known.⁸ The same study, however, found a polyclonal serum to recognize PEITC-derivatized MIF equally as well as free MIF. In light of our results, the epitope recognized by the monoclonal antibody could include the subunit interface or active site, which is modified by PEITC and thereby alters the epitope.

It was determined by Ouertatani-Sakouhi *et al.* that various ITCs interfere with MIF binding to CD74.¹⁰ This was attributed to the conformational change of MIF upon binding BITC. Another possibility is that ITCs interfere with MIF-CD74 binding due to the covalent inhibitor physically blocking a portion of the CD74 binding site. This demonstrates the importance of the MIF tautomerase active site in its interaction with CD74, whether due to direct binding to the active site or to other residues in the subunit interface that are affected by binding of an inhibitor to the active site.

The efficiency with which isothiocyanate groups react with the active site of MIF provides a useful tool for deciphering structure-activity relationships (SAR). ITCs can be used to investigate how inhibitors with different functional groups affect MIF activity. Namely, inhibition of MIF biological activity conferred by ITCs that protrude from the active site can be compared to functional inhibition derived from those that bind in the active site. Such SAR data would provide information as to how MIF interacts with receptors. One MIF-interacting compound has already been reported to enhance MIF biological activity.³⁷ Another example has to do with a parasitic (*Ancylostoma ceylanicum*) MIF (AceMIF) that

interacts with human CD74.^{38, 39} Characterization of inhibitors identified by targeting the *AceMIF* active site revealed that the diuretic drug furosemide also inhibits *AceMIF*-CD74 interactions. Removal of furosemide's sulfonamide and chloride groups protruding out of the active site and making no interactions with the protein retains similar inhibition of tautomerase activity but fails to block *AceMIF*-CD74 interactions. These examples involve compounds that interact with MIF non-covalently. An advantage of covalent inhibitors, such as ITCs, is that R-groups can be modified while still maintaining irreversible binding to MIF. Once complete derivatization is achieved, any difference in receptor interaction of MIF modified by one ITC versus another is likely due to their respective effects on MIF-receptor interaction. The ITC scaffold therefore provides a convenient tool for attachment of various chemical moieties that extend into or out of the active site to different degrees and in different conformations. These potential reagents can be used to determine the range of enhancement or inhibition of MIF receptors CD74, CXCR2 or CXCR4 binding and signaling.⁴⁰ Therefore, this structural manipulation of the tautomerase active site, and presumably the subunit interface, can lead to insights regarding the nature of ligand-receptor interactions in MIF signaling.

Although we focus on the MIF interactions with cell surface receptors there is substantial evidence that MIF is also an intracellular enzyme with an unknown substrate and product. It is ubiquitously expressed in every nucleated human cell and resides in the cytosol until the cell is stressed and exported in a signal sequence-independent pathway.⁴¹ Human MIF has homologues with ~30% sequence identity in organisms that normally do not express cytokines such as *A. thaliana*, *C. elegans*, and the marine cyanobacterium *P. marinus* and certainly do not express any of the known MIF receptors. Among the 30% identical residues is Pro-1 and some of the other human active site residues. All of this points to an enzymatic activity that may contribute to MIF's known biology. The modification of Pro-1 by isothiocyanates will certainly disrupt the any enzymatic activity.

The NMR studies highlight the change in conformational states at the subunit interface upon inhibitor binding. The structural and kinetic aspects of this study underscore that MIF inhibition can be optimized to design more effective covalent inhibitors. The reactive ITC moiety can be coupled to MIF active site-binding groups that have increased affinity, thereby increasing potency and selectivity for covalent inhibitors. Structures of competitive inhibitors complexed to the active site may provide further insight on designing more effective R-groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge Dr. Richard Bucala for helpful discussion.

This work was supported by the National Institutes of Health Grants 5R01AI065029 and 5R01AI082295.

References

1. de Jong YP, Abadia-Molina AC, Satoskar AR, Clarke K, Rietdijk ST, Faubion WA, Mizoguchi E, Metz CN, Alsahli M, ten Hove T, Keates AC, Lubetsky JB, Farrell RJ, Michetti P, van Deventer SJ, Lolis E, David JR, Bhan AK, Terhorst C, Sahli MA. Development of chronic colitis is dependent on the cytokine MIF. *Nat Immunol.* 2001; 2:1061–1066. [PubMed: 11668338]

2. Schrader J, Deuster O, Rinn B, Schulz M, Kautz A, Dodel R, Meyer B, Al-Abed Y, Balakrishnan K, Reese J, Bacher M. Restoration of contact inhibition in human glioblastoma cell lines after MIF knockdown. *BMC Cancer*. 2009; 9:464. [PubMed: 20038293]
3. Zhang Y, Zeng X, Chen S, Zhang Z, Li P, Yi W, Huang H, Yao J, Li S, Hu C. Characterization, epitope identification and mechanisms of the anti-septic capacity of monoclonal antibodies against macrophage migration inhibitory factor. *Int Immunopharmacol*. 2011; 11:1333–1340. [PubMed: 21571101]
4. Crichlow GV, Cheng KF, Dabideen D, Ochani M, Aljabari B, Pavlov VA, Miller EJ, Lolis E, Al-Abed Y. Alternative chemical modifications reverse the binding orientation of a pharmacophore scaffold in the active site of macrophage migration inhibitory factor. *J Biol Chem*. 2007; 282:23089–23095. [PubMed: 17526494]
5. Winner M, Meier J, Zierow S, Rendon BE, Crichlow GV, Riggs R, Bucala R, Leng L, Smith N, Lolis E, Trent JO, Mitchell RA. A Novel, Macrophage Migration Inhibitory Factor Suicide Substrate Inhibits Motility and Growth of Lung Cancer Cells. *Cancer Res*. 2008; 68:7253–7257. [PubMed: 18794110]
6. Fingerle-Rowson G, Kaleswarapu DR, Schlander C, Kabgani N, Brocks T, Reinart N, Busch R, Schutz A, Lue H, Du X, Liu A, Xiong H, Chen Y, Nemajerova A, Hallek M, Bernhagen J, Leng L, Bucala R. A Tautomerase-null MIF Gene Knock-in Mouse Reveals that Protein Interactions and not Enzymatic Activity Mediate MIF-dependent Growth Regulation. *Mol Cell Biol*. 2009; 29:1922–1932. [PubMed: 19188446]
7. Ouertatani-Sakouhi H, El-Turk F, Fauvet B, Cho M-K, Pinar Karpinar D, Le Roy D, Dewor M, Roger T, Bernhagen J, Calandra T, Zweckstetter M, Lashuel HA. Identification and Characterization of Novel Classes of Macrophage Migration Inhibitory Factor (MIF) Inhibitors with Distinct Mechanisms of Action. *J Biol Chem*. 2010; 285:26581–26598. [PubMed: 20516071]
8. Brown KK, Blaikie FH, Smith RA, Tyndall JD, Lue H, Bernhagen J, Winterbourn CC, Hampton MB. Direct modification of the proinflammatory cytokine macrophage migration inhibitory factor by dietary isothiocyanates. *J Biol Chem*. 2009; 284:32425–32433. [PubMed: 19776019]
9. Cross JV, Rady JM, Foss FW, Lyons CE, Macdonald TL, Templeton DJ. Nutrient isothiocyanates covalently modify and inhibit the inflammatory cytokine macrophage migration inhibitory factor (MIF). *Biochem J*. 2009; 423:315–321. [PubMed: 19723024]
10. Ouertatani-Sakouhi H, El-Turk F, Fauvet B, Roger T, Le Roy D, Karpinar DP, Leng L, Bucala R, Zweckstetter M, Calandra T, Lashuel HA. A new class of isothiocyanate-based irreversible inhibitors of macrophage migration inhibitory factor. *Biochemistry*. 2009; 48:9858–9870. [PubMed: 19737008]
11. Wattenberg LW. Inhibition of carcinogen-induced neoplasia by sodium cyanate, tert-butyl isocyanate, and benzyl isothiocyanate administered subsequent to carcinogen exposure. *Cancer Res*. 1981; 41:2991–2994. [PubMed: 6788365]
12. Yang MD, Lai KC, Lai TY, Hsu SC, Kuo CL, Yu CS, Lin ML, Yang JS, Kuo HM, Wu SH, Chung JG. Phenethyl isothiocyanate inhibits migration and invasion of human gastric cancer AGS cells through suppressing MAPK and NF-kappaB signal pathways. *Anticancer Res*. 2010; 30:2135–2143. [PubMed: 20651362]
13. Dey M, Kuhn P, Ribnicki D, Premkumar V, Reuhl K, Raskin I. Dietary phenethylisothiocyanate attenuates bowel inflammation in mice. *BMC Chem Biol*. 2010; 10:4. [PubMed: 20423518]
14. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res*. 2007; 55:224–236. [PubMed: 17317210]
15. Tang L, Zirpoli GR, Guru K, Moysich KB, Zhang Y, Ambrosone CB, McCann SE. Intake of cruciferous vegetables modifies bladder cancer survival. *Cancer Epidemiol Biomarkers Prev*. 2010; 19:1806–1811. [PubMed: 20551305]
16. Tang L, Zirpoli GR, Jayaprakash V, Reid ME, McCann SE, Nwogu CE, Zhang Y, Ambrosone CB, Moysich KB. Cruciferous vegetable intake is inversely associated with lung cancer risk among smokers: a case-control study. *BMC Cancer*. 2010; 10:162. [PubMed: 20423504]
17. Jakubikova J, Cervi D, Ooi M, Kim K, Nahar S, Klippel S, Cholujova D, Leiba M, Daley JF, Delmore J, Negri J, Blotta S, McMillin DW, Hideshima T, Richardson PG, Sedlak J, Anderson KC, Mitsiades CS. Anti-tumor activity and signaling events triggered by the isothiocyanates,

- sulforaphane and phenethyl isothiocyanate, in multiple myeloma. *Haematologica*. 2011; 96:1170–1179. [PubMed: 21712538]
18. Musk SR, Astley SB, Edwards SM, Stephenson P, Hubert RB, Johnson IT. Cytotoxic and clastogenic effects of benzyl isothiocyanate towards cultured mammalian cells. *Food Chem Toxicol*. 1995; 33:31–37. [PubMed: 7821874]
 19. Dinkova-Kostova AT, Fahey JW, Wade KL, Jenkins SN, Shapiro TA, Fuchs EJ, Kerns ML, Talalay P. Induction of the phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts. *Cancer Epidemiol Biomarkers Prev*. 2007; 16:847–851. [PubMed: 17416783]
 20. Guo Z, Smith TJ, Wang E, Sadrieh N, Ma Q, Thomas PE, Yang CS. Effects of phenethyl isothiocyanate, a carcinogenesis inhibitor, on xenobiotic-metabolizing enzymes and nitrosamine metabolism in rats. *Carcinogenesis*. 1992; 13:2205–2210. [PubMed: 1473225]
 21. Trachootham D, Zhang H, Zhang W, Feng L, Du M, Zhou Y, Chen Z, Pelicano H, Plunkett W, Wierda WG, Keating MJ, Huang P. Effective elimination of fludarabine-resistant CLL cells by PEITC through a redox-mediated mechanism. *Blood*. 2008; 112:1912–1922. [PubMed: 18574029]
 22. Okazaki K, Umemura T, Imazawa T, Nishikawa A, Masegi T, Hirose M. Enhancement of urinary bladder carcinogenesis by combined treatment with benzyl isothiocyanate and N-butyl-N-(4-hydroxybutyl)nitrosamine in rats after initiation. *Cancer Sci*. 2003; 94:948–952. [PubMed: 14611670]
 23. Okazaki K, Yamagishi M, Son HY, Imazawa T, Furukawa F, Nakamura H, Nishikawa A, Masegi T, Hirose M. Simultaneous treatment with benzyl isothiocyanate, a strong bladder promoter, inhibits rat urinary bladder carcinogenesis by N-butyl-N-(4-hydroxybutyl)nitrosamine. *Nutr Cancer*. 2002; 42:211–216. [PubMed: 12416262]
 24. Zanichelli F, Capasso S, Cipollaro M, Pagnotta E, Carteni M, Casale F, Iori R, Galderisi U. Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect. *Age (Dordr)*. 2012; 34:281–293. [PubMed: 21465338]
 25. Crichlow GV, Lubetsky JB, Leng L, Bucala R, Lolis EJ. Structural and kinetic analyses of macrophage migration inhibitory factor active site interactions. *Biochemistry*. 2009; 48:132–139. [PubMed: 19090677]
 26. Cho Y, Crichlow GV, Vermeire JJ, Leng L, Du X, Hodsdon ME, Bucala R, Cappello M, Gross M, Gaeta F, Johnson K, Lolis EJ. Allosteric inhibition of macrophage migration inhibitory factor revealed by ibudilast. *Proc Natl Acad Sci USA*. 2010; 107:11313–11318. [PubMed: 20534506]
 27. Otwinowski Z, Minor W. Processing of X-Ray Diffraction Data Collected in Oscillation Mode. *Methods In Enzymology*. 1997:307–325.
 28. Project CC. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr*. 1994; 50:760–763. [PubMed: 15299374]
 29. Navaza J. Implementation of molecular replacement in AMoRe. *Acta Crystallogr D Biol Crystallogr*. 2001; 57:1367–1372. [PubMed: 11567147]
 30. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr*. 1998; 54:905–921. [PubMed: 9757107]
 31. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:486–501. [PubMed: 20383002]
 32. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
 33. Goddard, TD.; Kneller, DF. Sparky 3. University of California; San Francisco:
 34. Edman P. A method for the determination of amino acid sequence in peptides. *Arch Biochem*. 1949; 22:475. [PubMed: 18134557]

35. Le ZG, Chen ZC, Hu Y, Zheng QG. Organic reactions in ionic liquids: ionic liquid-promoted efficient synthesis of disubstituted and trisubstituted thioureas derivatives. *Chin Chem Lett.* 2005; 16:201–204.
36. Swope M, Sun HW, Blake PR, Lolis E. Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor. *EMBO J.* 1998; 17:3534–3541. [PubMed: 9649424]
37. Jorgensen WL, Gandavadi S, Du X, Hare AA, Trofimov A, Leng L, Bucala R. Receptor agonists of macrophage migration inhibitory factor. *Bioorg Med Chem Lett.* 2010; 20:7033–7036. [PubMed: 20971005]
38. Cho Y, Jones BF, Vermeire JJ, Leng L, DiFedele L, Harrison LM, Xiong H, Kwong YK, Chen Y, Bucala R, Lolis E, Cappello M. Structural and functional characterization of a secreted hookworm Macrophage Migration Inhibitory Factor (MIF) that interacts with the human MIF receptor CD74. *J Biol Chem.* 2007; 282:23447–23456. [PubMed: 17567581]
39. Cho Y, Vermeire JJ, Merkel JS, Leng L, Du X, Bucala R, Cappello M, Lolis E. Drug repositioning and pharmacophore identification in the discovery of hookworm MIF inhibitors. *Chem Biol.* 2011; 18:1089–1101. [PubMed: 21944748]
40. Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med.* 2007; 13:587–596. [PubMed: 17435771]
41. Merk M, Baugh J, Zierow S, Leng L, Pal U, Lee SJ, Ebert AD, Mizue Y, Trent JO, Mitchell R, Nickel W, Kavathas PB, Bernhagen J, Bucala R. The Golgi-Associated Protein p115 Mediates the Secretion of Macrophage Migration Inhibitory Factor. *J Immunol.* 2009; 182:6896–6906. [PubMed: 19454686]
42. Engh RA, Huber R. Accurate bond and angle parameters for X-ray protein structure refinement. *Acta Crystallographica Section A.* 1991; 47:392–400.

Abbreviations

MIF	macrophage migration inhibitory factor
ITC	isothiocyanate
PEITC	phenethyl isothiocyanate
4-IPP	4-iodo-6-phenylpyrimidine
PMSF	phenylmethylsulfonyl fluoride
PMS	phenylmethylsulfonyl group
HPP	3-(4-hydroxyphenyl)pyruvate

Figure 1.

Kinetics of MIF reactivity with PEITC and L-sulforaphane. A,B, Structures of PEITC (A) and L-sulforaphane (B) and the reactions they undergo with MIF, according to the known product of the reaction of isothiocyanates with proteins.³⁴ C, 15 μM MIF was incubated with an equal concentration of either PEITC or L-sulforaphane, or with vehicle (DMSO). At indicated times, the amount of remaining unreacted MIF, “effective free MIF”, was assessed by measuring its tautomerase activity as described in *Methods*. Data are plotted as mean $\pm$ S.E.M. In some cases, the error bars are smaller than the data symbols. The data were fit to a hyperbolic curve as described in the text. The calculated second-order rate constants for MIF derivatization are $3.1 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ with PEITC and $1.3 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ with L-sulforaphane.

Figure 2.

Structures of PEITC and sulforaphane bound to MIF. In all panels, both the inhibitor and Pro-1 were omitted from the electron density map calculations in order to remove crystallographic bias. A and B, PEITC bound to Pro-1 in the active site of MIF. The 1.6 Å $F_o - F_c$ simulated annealing omit map is contoured at 4σ . Views were chosen to highlight the electron density of the PEITC aromatic ring (A) and the bond with Pro-1, (B). The active site shown is representative of all three active sites. C and D 1.8 Å $F_o - F_c$ simulated annealing omit map of L-sulforaphane bound to MIF, contoured at 3σ . The electron density in panel C is representative of two of the active sites, in which crystal contacts place the inhibitor close to other MIF molecules in the crystal. The active site shown in D does not have nearby symmetry-related MIF molecules. The incomplete electron density for the inhibitor in D reveals the flexibility of sulforaphane when bound to MIF in the absence of crystal contacts. The views in B–D show the conformation of the inhibitor-protein bonds: *cis* when Pro-1 is bound to PEITC (B) and *trans* when bound to sulforaphane (C and D).

Figure 3.

Effects of ITCs on MIF active site residues. All overlays were performed using a least-squares superposition of α -carbon atoms corresponding to residues 3-27, 37-50, 52-65 and 67-114. A, Sulforaphane-derivatized MIF overlaid upon unliganded MIF (PDB 3DJH) reveals that there are no major conformational changes in the protein backbone. There are two conformations for Met-2 in the unliganded MIF. The side chain conformational difference seen in Tyr-36 is due to crystal contacts made by Tyr-36 in the active site shown of sulforaphane-MIF. These Tyr-36 crystal contacts are not present in the unliganded MIF crystal form shown in the figure, which crystallized in a different space group. B, Superposition of sulforaphane-MIF onto PEITC-MIF shows that PEITC displaces Pro-1 from its native position. C, The Pro-1 displacement observed in the PEITC-MIF complex is similar to that observed upon MIF reaction with 4-IPP (which leaves 4-phenylpyrimidine, 4-PP, bound to the protein).⁵ D, ITC derivatization of MIF produces a planar proline imine nitrogen, and therefore does not cause the bending in Pro-1 that is observed when MIF reacts with PMSF (which leaves a phenylmethylsulfonyl group, PMS, attached to the protein) that yields a tetrahedral imine.

Figure 4. Binding to ITCs does not cause a conformational change in MIF. Superposition of sulforaphane-MIF (A) and PEITC-MIF (B) onto unliganded MIF is shown. The upper panels show the full trimeric protein, and the lower panels show a close-up view of a representative active site, highlighting Lys-32. The position and orientation of Lys-32 do not undergo large changes upon ITC binding, and the global MIF tertiary structure remains unchanged. Overlays were performed as in Figure 3.

Figure 5.

Analysis of NMR HSQC data. (A) Overlaid ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC NMR spectra of ${}^{15}\text{N}$ -MIF (black contours) and L-sulforaphane (blue contours)- and PEITC-derivitized ${}^{15}\text{N}$ -MIF (red contours). All spectra were collected on a 600MHz Varian Inova NMR spectrometer at 25°C as described in *Methods*. The dispersion of peaks in all three spectra is indicative of a high beta-sheet content protein while the observed peak shifts upon derivitization are consistent with changes in the subunit interfaces of MIF. Unchanged NMR peak widths suggest a paucity of conformational and quaternary structural changes upon derivitization with PEITC and sulforaphane. (B) Ribbon diagram of the MIF trimer with backbone chemical shifts that are perturbed (magenta) or not perturbed (green), relative to the assigned apo-MIF chemical shifts, upon formation of a covalent bond with both sulforaphane and PEITC based on NMR HSQC experiments. Pro-1 and the C-terminus of the same subunit are labelled.

Table 1

Crystallographic Statistics

	PEITC-MIF	L-sulforaphane-MIF
<i>Integration and Scaling</i>		
space group	P2 ₁ 2 ₁ 2 ₁	P3 ₁ 2 ₁
unit cell	a = 67.49 Å, b = 67.67 Å, c = 87.77 Å, $\alpha = \beta = \gamma = 90^\circ$	a = b = 95.64 Å, c = 103.52 Å, $\alpha = \beta = 90^\circ, \gamma = 120^\circ$
resolution, Å (highest shell)	50-1.6 (1.63-1.6)	50-1.8 (1.83-1.8)
unique reflections	52,662	51,163
completeness, %	97.9 (98.8)	99.9 (100)
redundancy	4.4 (4.3)	10.8 (10.4)
avg I/avg σ	20.31 (4.13)	25 (4.2)
R _{merge}	0.080 (0.277)	0.104 (0.502)
<i>Refinement (F = 0)</i>		
reflections	52,638	51,159
atoms in asymmetric unit	3,074	3,103
R (work), %	18.1	17.2
R-free, %	19.7	18.6
rms deviations from ideality ^a		
bond lengths, Å	0.007	0.005
bond angles, °	1.3	1.3
average B-factors, Å ²		
overall	19.52	16.30
protein (number of residues)	17.75 (114 × 3)	14.18 (114 × 3)
water	29.76 (434)	27.75 (446)
ions	36.52 (12)	30.67 (12)
inhibitor	17.53 (3)	23.43 (3)

^aCompared to ideal bond lengths and angles as determined by Engh and Huber.⁴²