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A Novel Allosteric Inhibitor of Macrophage Migration Inhibitory Factor (MIF)*

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Background: MIF is a pro-inflammatory cytokine implicated in autoimmune diseases.
Results: A small molecule that binds to MIF and inhibits its cytokine activities was identified.
Conclusion: The inhibitor binds in a unique region on MIF and reveals a new way to block the cytokine activities of MIF.
Significance: The inhibitor is a valuable tool to design MIF-directed therapeutics for inflammatory diseases.

Macrophage migration inhibitory factor (MIF) is a catalytic cytokine and an upstream mediator of the inflammatory pathway. MIF has broad regulatory properties, dysregulation of which has been implicated in the pathology of multiple immunological diseases. Inhibition of MIF activity with small molecules has proven beneficial in a number of disease models. Known small molecule MIF inhibitors typically bind in the tautomerase site of the MIF trimer, often covalently modifying the catalytic proline. Allosteric MIF inhibitors, particularly those that associate with the protein by noncovalent interactions, could reveal novel ways to block MIF activity for therapeutic benefit and serve as chemical probes to elucidate the structural basis for the diverse regulatory properties of MIF. In this study, we report the identification and functional characterization of a novel allosteric MIF inhibitor. Identified from a high throughput screening effort, this sulfonated azo compound termed p425 strongly inhibited the ability of MIF to tautomerize 4-hydroxyphenyl pyruvate. Furthermore, p425 blocked the interaction of MIF with its receptor, CD74, and interfered with the pro-inflammatory activities of the cytokine. Structural studies revealed a unique mode of binding for p425, with a single molecule of the inhibitor occupying the interface of two MIF trimers. The inhibitor binds MIF mainly on the protein surface through hydrophobic interactions that are stabilized by hydrogen bonding with four highly specific residues from three different monomers. The mode of p425 binding reveals a unique way to block the activity of the cytokine for potential therapeutic benefit in MIF-associated diseases.

Macrophage migration inhibitory factor (MIF)[2] is a multifunctional protein that is both a cytokine and an enzyme possessing tautomerase activity (1). In the capacity of a cytokine, MIF functions as a central upstream mediator of innate immunity and a regulator of inflammation (2). MIF is believed to initiate inflammation by promoting the release of pro-inflammatory mediators, such as TNFα, interleukins (IL-1β, IL-6, and IL-8), and tissue-degrading matrix metalloproteinases (MMPs) (3–5). MIF also promotes inflammation by inducing a wide range of pro-inflammatory cellular processes, which include phagocytosis, T-cell proliferation, counter-regulation of immunosuppressive action of glucocorticoids, activation of macrophages, and promotion of their continued survival through suppression of p53-dependent apoptosis (2, 6–11). Although the molecular mechanism underlying the pleiotropic functions of MIF remains unresolved, it is known that MIF exerts at least part of its cytokine activity through signal transduction, initiated by direct binding to the CD74-CD44 extracellular receptor complex (12–14).

Given the broad immunomodulatory properties of MIF, dysregulation of its function has been implicated in the pathophysiology of a variety of diseases. Elevated circulating levels of MIF have been observed in rheumatoid arthritis, atherosclerosis, asthma, lupus, diabetes, colitis, sepsis, cancer, and cardiovascular and infectious diseases (15–20). Genetic evidence further supports the involvement of MIF in disease. Functional polymorphisms in the MIF gene affect disease severity, such that individuals inheriting a high expressor allele have increased levels of MIF in circulation and often show severe disease manifestation. Therapeutic relief achieved through blocking MIF activity by genetic, immunological, and pharmacological means further supports the involvement of MIF in disease pathology (17, 21–24).

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The atomic coordinates and structure factors (code 3U18) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[2] The abbreviations used are: MIF, macrophage migration inhibitory factor; HTS, high throughput screen; HPP, 4-hydroxyphenyl pyruvate; rhMIF, recombinant human MIF; PDB, Protein Data Bank; TPOR, thiol-protein oxidoreductase; MMP, matrix metalloproteinase.

In addition to its physiological and pathophysiological activities, MIF is known to act as a tautomerase capable of isomerizing substrates such as D-dopachrome and 4-hydroxyphenyl pyruvate (HPP) (26–28). No definite physiological role has been assigned to the catalytic activity of MIF, which is thought to be a vestige of its ancestral origin from bacterial isomerases involved in aromatic amino acid catabolism (25). Nevertheless, the uniqueness of an enzymatic activity associated with a cytokine led to studies elucidating the structure-activity relationship between the two activities. These studies revealed that *in vitro*, MIF exists as a 37.5-kDa homotrimer (26, 27). The three monomers that form the trimer exist as a β-α-β structure composed of two anti-parallel α-helices packed against a four-stranded β-sheet. The subunits interact via intersubunit β-sheet contacts to form a symmetrical β-barrel trimer with a solvent-exposed central channel. At the monomer interface within the trimer are three tautomerase active sites where the N-terminal catalytic proline (Pro^1) resides. The region encompassing the tautomerization site also makes critical contacts with the CD74 receptor, such that covalent modification of Pro^1 or its replacement with glycine abolishes tautomerase activity and impairs CD74 binding (12, 28–30).

Given the spatial relationship between the catalytic and receptor binding domains, targeting the tautomerase activity of MIF as a means to block its pro-inflammatory function has been extensively explored as a therapeutic strategy. To date, methods such as substrate analog screening, active-site targeting by computer-assisted modeling, and virtual high throughput screening have produced various classes of small molecule MIF tautomerase inhibitors (31). The majority of the inhibitors, which include derivatives of dopachrome, acetaminophen (*N*-acetyl-*p*-benzoquinone), phenylpyruvic acid (cinnamates), Schiff bases, isoxazolines (ISO-1), and isothiocyanates, act by either competing with the substrate for the catalytic site (ISO-1 and OXIMM11) or covalent modification of Pro^1 (*N*-acetyl-*p*-benzoquinone and isothiocyanates) (28, 29, 32). Many of these inhibitors have also been shown to inhibit the pro-inflammatory activities of MIF. For instance, ISO-1 and some of its derivatives block MIF-induced TNF secretion from LPS-stimulated macrophages and increase survival in a sepsis model (33, 34). Although these inhibitors provide a proof of concept for the therapeutic utility of small molecule MIF inhibitors, they are not ideal for pharmaceutical development. ISO-1 for instance was developed from a class of platelet glycoprotein IIb/IIIa antagonists (35) and displays only micromolar potency with respect to MIF inhibition (33, 36); its further development raises significant concerns about low potency and off-target effects. Covalent modifiers such as *N*-acetyl-*p*-benzoquinone are not desirable as pharmaceuticals due to lack of specificity (29, 37, 38).

Recently, the identification of two new classes of MIF inhibitors revealed the feasibility of blocking MIF tautomerase activity by mechanisms other than active-site binding. An analog of the anti-inflammatory drug ibudilast binds to a region proximal to the catalytic site, whereas the anti-oxidant ebselen disaggregates MIF trimers (39, 40). Encouraged by these findings, we sought to obtain additional allosteric MIF inhibitors that neither compete for nor covalently modify the active site. We hypothesized that such inhibitors could not only block the tautomerase activity of MIF but also block its pro-inflammatory function. Herein, we report the identification, by high throughput screening, of an azo compound named p425 that blocks the tautomerase, CD74 receptor-binding, and pro-inflammatory activities of MIF. Through crystallographic studies, we show that the site of p425 binding to be at the interface of two MIF trimers. With a mode of binding unique among known MIF inhibitors, p425 reveals a new way to block the catalytic and cytokine activities of MIF. This molecule could prove invaluable as a tool to design new classes of therapeutic MIF inhibitors and to investigate the structural basis for the multifunctional nature of MIF.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Recombinant Human MIF (rhMIF)—The human *MIF* gene (GenBank™ accession number BU538576.1) was PCR-amplified from an EST cDNA clone purchased from Open Biosystems (Lafayette, CO) using primers (5′-CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAATGCCGATGTTCATCGTAAACACC-3′ and 5′-CCCAGATCTTATTAGGCGAAGGTGGAGTTGTTCCAG-3′) and inserted into the XbaI/BglII sites of pET32a vector (Novagen, Madison, WI). For protein production, *Escherichia coli* BL21-DE3 (New England Biolabs, Ipswich, MA) was transformed with the plasmid, grown in the presence of isopropyl 1-thio-β-D-galactopyranoside (0.1 mM), and harvested by sonication in 50 mM MES, pH 6.1. The cleared cell lysates were subjected to cation-exchange chromatography on an SP-Sepharose XL column (GE Healthcare). MIF was further purified by high resolution cation-exchange chromatography in 50 mM MES, pH 6.1, on a Mono S 5/50 GL column (GE Healthcare) under nondenaturing conditions. The composition of each fraction was determined by SDS-gel electrophoresis, and the MIF-containing fractions were dialyzed against 100 mM sodium citrate buffer, pH 5.0. The final yield was 20–25 mg of purified protein per liter of culture, with a purity of greater than 99%.

Compound Libraries and High Throughput Screen (HTS)—HTS was performed at the Institute of Chemistry and Cell Biology Longwood Screening Facility at Harvard Medical School. The libraries consisted of 230,000 small molecules, which included therapeutic compounds approved by the Food and Drug Administration and compounds purchased from the following: BioMol TimTec (Plymouth Meeting, PA); Prestwick (Ilkirch, France); ChemBridge (San Diego); ENAMINE (Kiev, Ukraine); Maybridge (Cornwall, UK); ChemDiv (San Diego); NINDS custom collection from MicroSource Diversity System (Gaylordsville, CT); and collections from the NCI, National Institutes of Health, and Harvard Medical School. The HTS assay was based on the keto-enol tautomerization of HPP (Sigma) catalyzed by rhMIF (12). The keto form of HPP was prepared by dissolving the HPP powder in 50 mM ammonium acetate, pH 6.0, and equilibrating it overnight at room temperature. For HTS, the assay was optimized in 384-well UV microplates from Corning (Corning, NY). A reaction mixture containing 0.37 M boric acid, pH 5.5, 78 nM rhMIF, and 8.9% DMSO in water was prepared, and 30 μl of this mixture was added to

each well of a reaction plate. Test compounds (12 μg/ml) were added to the wells, and the mixture was preincubated for 30 min. A substrate mixture containing 0.34 M boric acid and 1.5 mM keto-HPP was prepared, and 10 μl of this mixture was added to each well of the reaction plate. A_{306} was read after a 15-min incubation on a SpectraMax M5® plate reader (Sunnyvale, CA). The overall Z′ factor, a statistical measure of the effective separation of the high and low controls, for the screen was >0.7, a value suitable for HTS (41). Hits were classified as test compounds that inhibited the signal by >50% as compared with a no-inhibitor control.

Source of p425—For follow-up studies, p425 also known as Chicago Sky Blue 6B was purchased from Tocris Biosciences (catalog no. 0846, Bristol, UK).

MIF-CD74 Binding Assay—The MIF-CD74 receptor binding assay was performed as described previously (42, 43). Briefly, recombinant human sCD74(73–232) (recombinant soluble ectodomain of the receptor expressed and purified as described previously (43)) was immobilized in 96-well plates overnight at 4 °C. After washing, the plates were blocked with Superblock T20 (Thermo Scientific, Waltham, MA) for 2 h at room temperature. Biotin-labeled rhMIF (0.2 μM) was preincubated with either compounds or anti-human MIF antibody (R&D Systems, Goodhue, MN) for 30 min at room temperature. The mixture was added to plates and incubated overnight at 4 °C. An alkaline phosphatase-streptavidin conjugate (R&D Systems) and *p*-nitrophenyl phosphate substrate (Sigma) were added to each well, and A_{405} was read in a plate reader (BioTek, Winooski, VT). Percent inhibition at each inhibitor concentration was calculated with respect to a no-inhibitor control. The results were fitted onto a sigmoidal dose-response curve using GraphPad Prism 4.0 software (La Jolla, CA) to calculate the IC_{50} value (compound concentration that causes 50% inhibition).

Cytokine Luminex Assay and ELISA—Human foreskin fibroblasts (CRL-2522, ATCC, Manassas, VA) were cultured in minimum Eagle' medium with 0.5% FBS (Invitrogen). Approximately 1×10^4 cells/well were plated in 96-well plates and incubated overnight. The next day, 2.5 μM of each test compound was mixed with 200 ng/ml rhMIF in fresh culture medium and incubated for 30 min at room temperature. This mixture was added to cells, replacing the old medium. Controls included ISO-1, anti-MIF antibody, and compound vehicle (1% DMSO). After 6 h, the plates were centrifuged for 5 min at 200 × *g*. Culture supernatant was collected, and the amount of IL-6 and IL-8 in the medium was measured with a Bio-Plex kit (Bio-Rad) in a Luminex 200 system (Bio-Rad). To confirm the Bio-Plex results, test compounds (5-fold serially diluted for final concentrations of 2.5 μM to 0.4 nM) were incubated with rhMIF and fibroblast cells for 6 h as above. Cell culture media were collected and analyzed for individual cytokine production with ELISA kits (R&D Systems), and IC_{50} values were calculated.

MMP-3 ELISA—MMP-3 measurements were performed in primary human synovial fibroblasts-rheumatoid arthritis (Cell Applications, Inc., San Diego) using assay conditions similar to those used for cytokine measurements. Approximately 1×10^4 cells/well were plated in a 96-well plate overnight and then incubated with rhMIF 200 ng/ml either alone or mixed with the inhibitors (5-fold dilutions from 2.5 μM to 0.4 nM) for 24 h prior to the analysis. MMP-3 levels in the medium were measured with an ELISA kit (R&D Systems).

Apoptosis Assay—Apoptosis was measured in HeLa cells (CCL-2, ATCC). Briefly, 1×10^4 cells in DMEM containing 0.5% FBS were plated into each well of a 96-well plate and incubated overnight. To induce apoptosis, 1 μg/ml camptothecin (Sigma) was added to each well immediately followed by serially diluted rhMIF/inhibitor mixture (200 ng/ml MIF). The cells were incubated at 37 °C for 4 h. Apoptosis was measured with the Cell Death Detection ELISA^{plus} kit (Roche Applied Science), which is a photometric enzyme immunoassay for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments by induced cell death.

Cytotoxicity Assay—Compound-induced cytotoxicity was measured in human foreskin fibroblast cells. Briefly, 1×10^4 cells/well, grown in MEM containing 10% FBS, were plated in 96-well plates. Serially diluted compounds were added to each well, and the plates were incubated at 37 °C for 24 and 72 h. The level of compound-induced cytotoxicity was determined using a kit (Roche Applied Science) that measures the amount of lactate dehydrogenase released into the medium by lysed cells relative to an untreated control.

Crystallization, Structure Determination, and Refinement—Crystals of rhMIF-p425 complex were grown at 37 °C by vapor diffusion in sitting drops after mixing equal volumes of protein with precipitant solution. The protein solution consisted of 8 mg/ml rhMIF in 10 mM Tris, pH 7.2, and the precipitant solution consisted of a mixture of 100 mM p425 dissolved in 2.0 M ammonium sulfate, 4% 2-propanol, 0.1 M Tris, pH 8.0. The sitting drop was equilibrated against 0.5 ml of precipitant buffer without p425, in 24-well VDX plates (Hampton Research, Aliso Viejo, CA). Crystals grew within 2 weeks; they were dark blue in color and were of dimensions 0.5 mm on the largest face and 0.3 mm on the smallest face. Single crystals were soaked in cryoprotectant solution containing 15% ethylene glycol and 20% glycerol in precipitant solution for less than 5 min and directly cooled in a stream of liquid nitrogen prior to data collection. Data were collected using a 4-circle κ platform Xcalibur PX Ultra with a 165-mm diagonal Onyx CCD detector and a high brilliance sealed tube Enhance Ultra (copper) x-ray source (Oxford Diffraction, Oxford, UK), operating at 40 kV and 40 mA at a crystal-to-detector distance of 65 mm and exposure times of 120 s per 0.4° oscillations. The data sets were processed using the program Crysalis Pro (Oxford Diffraction). Crystallographic phases were determined by molecular replacement using the program PHASER (44). The search model used was the coordinates of MIF (PDB code 1MIF (26)) stripped of all heteroatoms. The initial MR solution revealed three monomers per asymmetric unit which, based on the volume of the unit cell being 398946 Å^3, corresponds to a Matthews' coefficient of 3.93 Å^3 Da^{-1} (68.7% solvent). MR was followed by several cycles of simulated annealing at 10,000 K to remove model bias. The ligand p425 was visible in the resulting unbiased Sigma weighted $2F_o - F_c$ omit electron density maps. The structural model was manually built using COOT (45) followed by refinement using both PHENIX (46) and REFMAC5 (47) with free-*R* (48) to yield a final model with statistics listed in Table 1. The

TABLE 1
Data collection and model refinement statistics
Values in parenthesis are for highest resolution shell.

Parameter	MIF-P425 complex basic
Data collection	
Space group	$P2_12_12_1$
Unit-cell parameters	$a = 67.83$, $b = 67.88$, $c = 86.63$ Å
Resolution limits (Å)	30.35 to 1.73 Å (1.82 to 1.73 Å)
$\langle I/\sigma(I)\rangle$	12.7 (1.5)
No. of reflections	163,225 (2,859)
No. of unique reflections	37,365 (2,354)
Multiplicity	4.3 (1.2)
R_{merge} [a]	7.1% (35.2%)
Completeness	89.3% (39.4%)
Refinement	
Resolution	30–1.8 (1.85–1.80)
Completeness (%)	95.95 (61.53)
R_{cryst} [b]/R_{free} [c]	15.4/19.4 (22.5/27.1)
Correlation coefficient	
$F_o - F_c$	0.965
$F_o - F_c$ (free)	0.942
Root mean square deviation	
Bond lengths	0.023 Å
Bond angles	2.211°
Model composition	
Monomers	3
Residues	114
Waters	314
Ligands/heteroatoms	
P425 (code Y0X)	1
Glycerol	9
Ethylene glycol	2
Isopropyl alcohol	2
Sulfate ions	2
Sodium	4
Ramachandran	
Preferred regions	100%
Outliers	0%

[a] $R_{merge} = S_{hkl}\, S_i |I_i(hkl) - (I(hkl))| / S_{hkl}\, S_i\, I_i(hkl)$, where $I_i(hkl)$ and $(I(hkl))$ are the intensity of measurement of I and the mean intensity of the reflection with indices hkl, respectively.
[b] $R_{cryst} = S||F_o| - |F_c||/S|F_o|$, where F_o is the observed and F_c is the calculated structure factor amplitudes.
[c] R_{free} set uses 5% of randomly chosen reflections.

coordinates corresponding to the different crystal structures were superposed using a least square alignment of all main chain atoms within PyMOL.

Protein Data Base Accession Number—The atomic coordinates and structure factors of the p425-rhMIF complex are available from the Protein Data Bank (PDB code 3U18).

RESULTS

Identification of p425 as an Inhibitor of MIF Tautomerase and CD74 Receptor Binding Activities—rhMIF catalyzes the keto-enol tautomerization of HPP, a reaction that can be monitored spectrophotometrically as an increase in A_{306} (Fig. 1, *A* and *B*). We developed a 384-well plate format HPP tautomerase assay and screened 230,000 compounds for inhibitors of rhMIF HPP tautomerization. We identified 2,430 hits (1% hit rate), which inhibited the tautomerase activity of rhMIF by greater than 50% at 12 μg/ml (Fig. 1, *C* and *D*). The hit compounds were purchased and re-tested in the tautomerase assay, and 274 hits were shown to have confirmed activity.

Because our goal was to obtain MIF tautomerase inhibitors that also block its pro-inflammatory function, we proceeded to test the 274 confirmed hits in a CD74 receptor-binding assay to assess their ability to block the MIF-CD74 interaction. The ability of the hits to block the binding of biotinylated rhMIF with

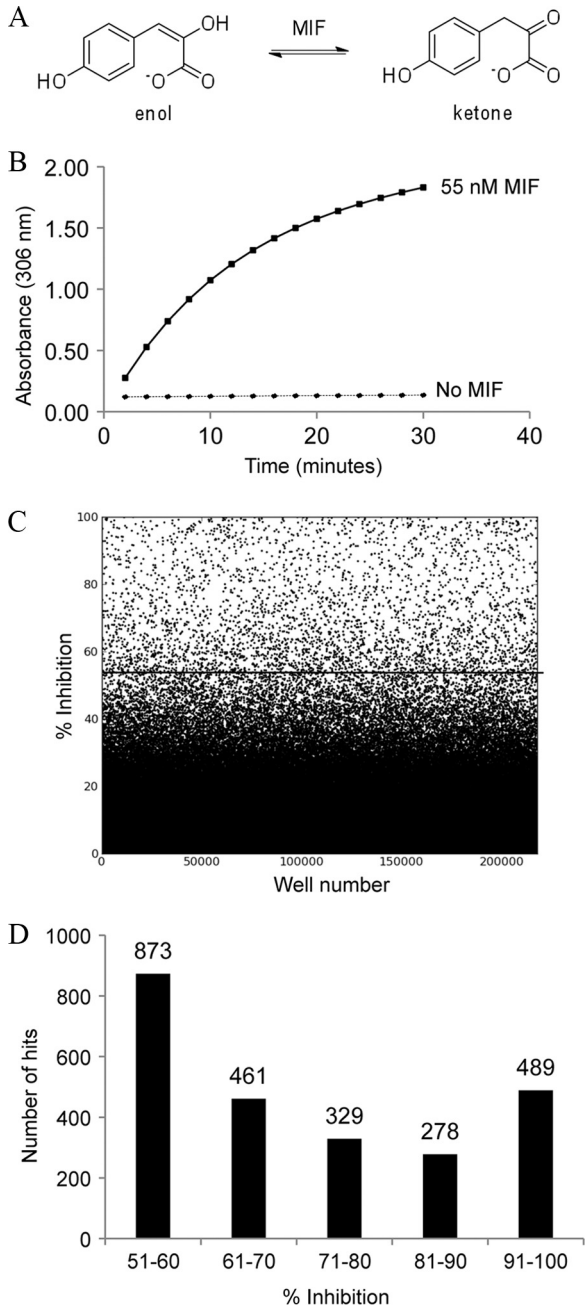

FIGURE 1. **High throughput screening for rhMIF HPP tautomerase inhibitors.** *A,* keto-enol tautomerization of HPP catalyzed by rhMIF. *B,* spectrophotometric detection of rhMIF-catalyzed HPP tautomerase reaction in a 384-well plate format. *C,* scatter plot of HTS outcome for one of the two replicates of 230,000 compounds. Compounds giving >50% inhibition above the line were classified as hits. *D,* distribution of the 2,430 hits, categorized into five groups with respect to percent inhibition.

recombinant soluble CD74 ectodomain (CD74(73–232)) was evaluated in a capture ELISA (42, 43). Of the 274 hits evaluated, four hits (p425, 1D2, 1H2, and 2F10) were inhibitory in the receptor-binding assay (Fig. 2*A*). The most potent of the four hits was p425, which displayed a dose-dependent inhibition with an IC_{50} of 0.81 μM. The structure of p425 is shown in Fig. 2*B*. It belongs to the azo family of large sulfonated organic acids that include Evans blue, furosemide, and bumetanide, all of which, at physiological pH, contain two centers of negative charge in close proximity to an electron-donating group (49,

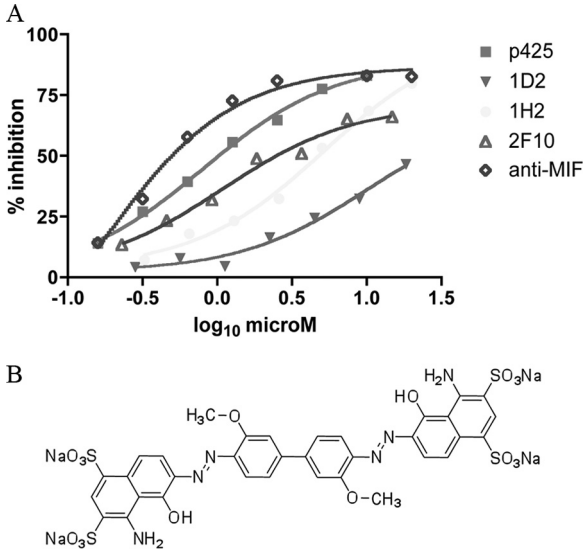

FIGURE 2. **Tautomerase inhibitors block rhMIF binding to CD74-receptor (*A*) and the structure of p425 (*B*).** *A,* inhibition of rhMIF-CD74 interaction by four tautomerase inhibitors (p425, 1D2, 1H2, and 2F10) identified from HTS. Biotinylated rhMIF binding to soluble CD74 was detected colorimetrically (A_{405}) with a streptavidin-alkaline phosphatase conjugate and *p*-nitrophenyl phosphate substrate. The most potent confirmed screening hit was p425 with an IC_{50} of 0.81 μM, ~10-fold higher than that of anti-MIF antibody (*anti-MIF*). *B,* chemical structure of p425 also known as Chicago Sky Blue 6B.

50). Structure-based calculated properties of p425, including the size (M_r 904) and four highly polar sulfonic acid moieties, suggested that this molecule was unlikely to be an active-site binder. Anticipating that p425 might be a novel MIF inhibitor, we proceeded to examine this molecule in cell-based assays evaluating the pro-inflammatory activities of MIF and in structural studies elucidating the physical nature of inhibitor-MIF interactions.

p425 Impairs Pro-inflammatory Activities of MIF—We selected three MIF-driven cellular events that are hallmarks of inflammation to examine the inhibitory effects of p425 on the pro-inflammatory action of MIF. It is known that during inflammation, MIF promotes the following: (i) production of IL-6 and IL-8 inflammatory mediators (5, 51–53); (ii) secretion of MMPs (3, 4, 54, 55); and (iii) survival of cells through suppression of p53-dependent apoptosis (7, 10). We evaluated the ability of p425 to inhibit MIF activity in all three processes, and we compared its activity to the prototypical MIF inhibitor ISO-1.

It has been shown that pretreatment of fibroblasts with rhMIF increases IL-6 and IL-8 cytokine levels in the culture medium, and the presence of MIF-specific inhibitors, such as ISO-1 and anti-MIF antibody, diminishes this effect (5). To examine the effect of p425 on MIF-induced cytokine production, we measured the levels of IL-6 and IL-8 in the culture medium of human foreskin fibroblast cells, previously treated with rhMIF in the presence or absence of p425, ISO-1, or anti-MIF antibody. As shown in Fig. 3*A*, pretreatment of rhMIF with p425 causes a dramatic decrease in the cytokine levels, comparable with that observed for anti-MIF antibody, and it was more inhibitory than ISO-1. Fig. 3, *B* and *C,* shows the dose-dependent nature of the response and the higher potency of p425 in comparison with ISO-1 (IC_{50} of 0.6–0.7 μM *versus* >5 μM, respectively).

We proceeded to examine the effect of p425 on MIF-induced production of MMPs. MIF induces the expression of a number of MMPs (3, 4, 54, 55), which with their unique ability to degrade fibrillar collagens and other matrix components have been implicated in the pathology of rheumatoid arthritis (56, 57). Primary human synovial fibroblasts were pretreated with rhMIF alone or rhMIF in complex with p425 or ISO-1. The level of MMP-3 in the culture medium was measured, and the percent inhibition with respect to a no-inhibitor control is presented in Fig. 3*D*. The results show that p425 decreased the level of MMP-3 in the medium in a dose-dependent manner with an IC_{50} of ~0.1 μM. A similar, but less pronounced, effect was seen with ISO-1, which had an IC_{50} of >10 μM.

Next, we examined the effect of p425 on apoptosis, a normal cellular process that serves to limit activation of inflammatory cells to prevent excessive inflammation. MIF overrides this checkpoint and promotes cell survival by suppressing p53-dependent apoptosis (36). The effect of MIF inhibitors on apoptosis can be assessed following treatment of cells with camptothecin, a DNA-damaging agent that promotes cell death through p53-dependent pathways. HeLa cells were pretreated with camptothecin, followed by treatment with rhMIF either alone or complexed with p425 or ISO-1. The results, summarized in Fig. 3*E*, reveal that rhMIF dramatically reduces the extent of camptothecin-induced apoptosis and that this effect is impaired, albeit modestly, in the presence of 50 μM p425. No discernible difference in the extent of apoptosis was seen in the presence of 2, 10, or 50 μM of ISO-1 (data not shown).

We proceeded to examine toxicity of p425 on cells to ensure that the inhibitory effects observed on the pro-inflammatory activities of rhMIF are not due to inhibitor-induced cellular toxicity. Primary human foreskin fibroblasts were treated with varying concentrations of p425 for 24 and 72 h, and the extent of cytotoxicity, as measured by the levels of lactate dehydrogenase in the medium, was determined. As shown in Fig. 3*F*, 100 μM of p425 causes little or no cytotoxicity. Collectively, these results confirm that p425 not only inhibits the tautomerase and receptor binding activities of MIF, but also impairs the ability of MIF to mediate production of pro-inflammatory mediators and inhibit p53-dependent apoptosis.

Structure of rhMIF-p425 Complex Reveals a Novel Mode of MIF Inhibition—To understand the mechanism by which p425 interacts with rhMIF, we solved the co-crystal structures of the inhibitor-bound complex to 1.9-Å resolution. Co-crystallization was performed under both acidic (pH 6.3) and basic (pH 8) conditions, and crystals of identical morphology were obtained under both conditions. As expected from the previously published structure (30), rhMIF in the complex adopts a trimeric ring architecture. Each monomer is composed of a four-stranded mixed β-sheet and two antiparallel α-helices stacked against the β-sheets on the outside of the trimer. In the complex, p425 is found located at the interface of two adjacent rhMIF trimers (Fig. 4*A*). Binding of the inhibitor occurs on the protein surface, mainly through hydrophobic interactions

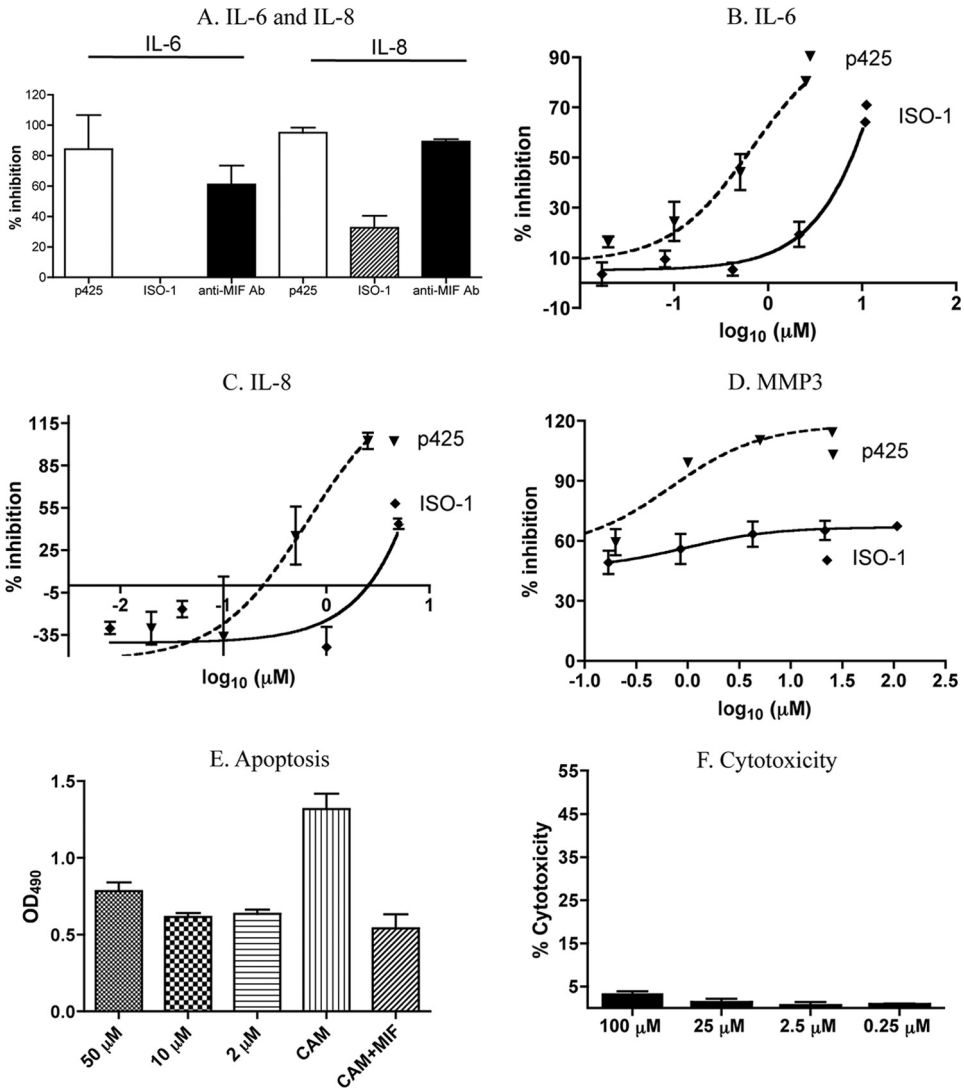

FIGURE 3. **p425 impairs MIF-mediated production of IL-6, IL-8, and MMP-3 and inhibition of p53-dependent apoptosis.** *A,* inhibition of rhMIF-induced IL-6 (*left panel*) and IL-8 (*right panel*) production in human foreskin fibroblast cells in the presence of p425 (*white*), ISO-1 (*stipple*), and anti-MIF antibody (*black*). IL-6 and IL-8 levels in the culture medium were determined by ELISA, and the percent inhibition was calculated with respect to a no-inhibitor control. Dose-dependent inhibition of rhMIF-induced IL-6 (*B*) and IL-8 (*C*) production in the presence of p425 and ISO-1. *D,* inhibition of MMP-3 production in the presence of p425 and ISO-1 in primary human synovial fibroblast cells. MMP-3 levels in the culture medium were determined by ELISA, and the percent inhibition was calculated with respect to a no-inhibitor control. *E,* p425 impairs the ability of rhMIF to inhibit p53-dependent apoptosis. HeLa cells were exposed to camptothecin (*CAM*) and then treated with rhMIF or rhMIF plus p425. Apoptosis was measured by ELISA. The plot shows the level of apoptosis in untreated (*CAM*), treated with rhMIF alone (*CAM*+*MIF*) and rhMIF in the presence of 2, 10, and 50 μM p425. *F,* p425 does not cause significant cytotoxicity in human foreskin fibroblast cells.

along one side of the trimer. In the crystal structure, the inhibitor also forms highly specific hydrogen bonds with four residues, Lys^{32} and Asn^{110} from one monomer, Glu^{54} from a second monomer, and Ser^{53} from a monomer of an adjacent trimer (Fig. 4*B*). These interactions appear to give stability and specificity to the complex.

To compare the mode of binding of p425 with that of HPP or the active-site inhibitor, coumarin, we compared the co-crystal structures of the three complexes. Fig. 4, *C* and *D,* shows the superimposition of the co-crystal structures rhMIF-p425 with rhMIF-HPP (PDB 1CA7 (58)) and rhMIF-coumarin derivative (PDB 1GCZ (59)), respectively. Structural comparisons revealed the binding of p425 distal to the active sites and at the interface of two MIF trimers to be very different from that of HPP or coumarin. Thus, p425 belongs to a new class of MIF antagonists that do not engage Pro^1 but instead occupy the interface of MIF trimers.

DISCUSSION

Recognized as a major regulator of inflammation and a central upstream mediator of innate immunity, MIF is considered an attractive therapeutic target for multiple inflammatory and autoimmune disorders. To date, almost all MIF-directed small molecule drug discovery efforts have targeted the catalytic tautomerase site with the majority of the identified inhibitors functioning by either competing with the substrate or covalently modifying the catalytic Pro^1. Allosteric MIF inhibitors are rare. With the exception of AV411 and ebselen, no other MIF inhibitors of this class have been described. Herein, we report p425 as a novel class of allosteric inhibitors that occupies the interface

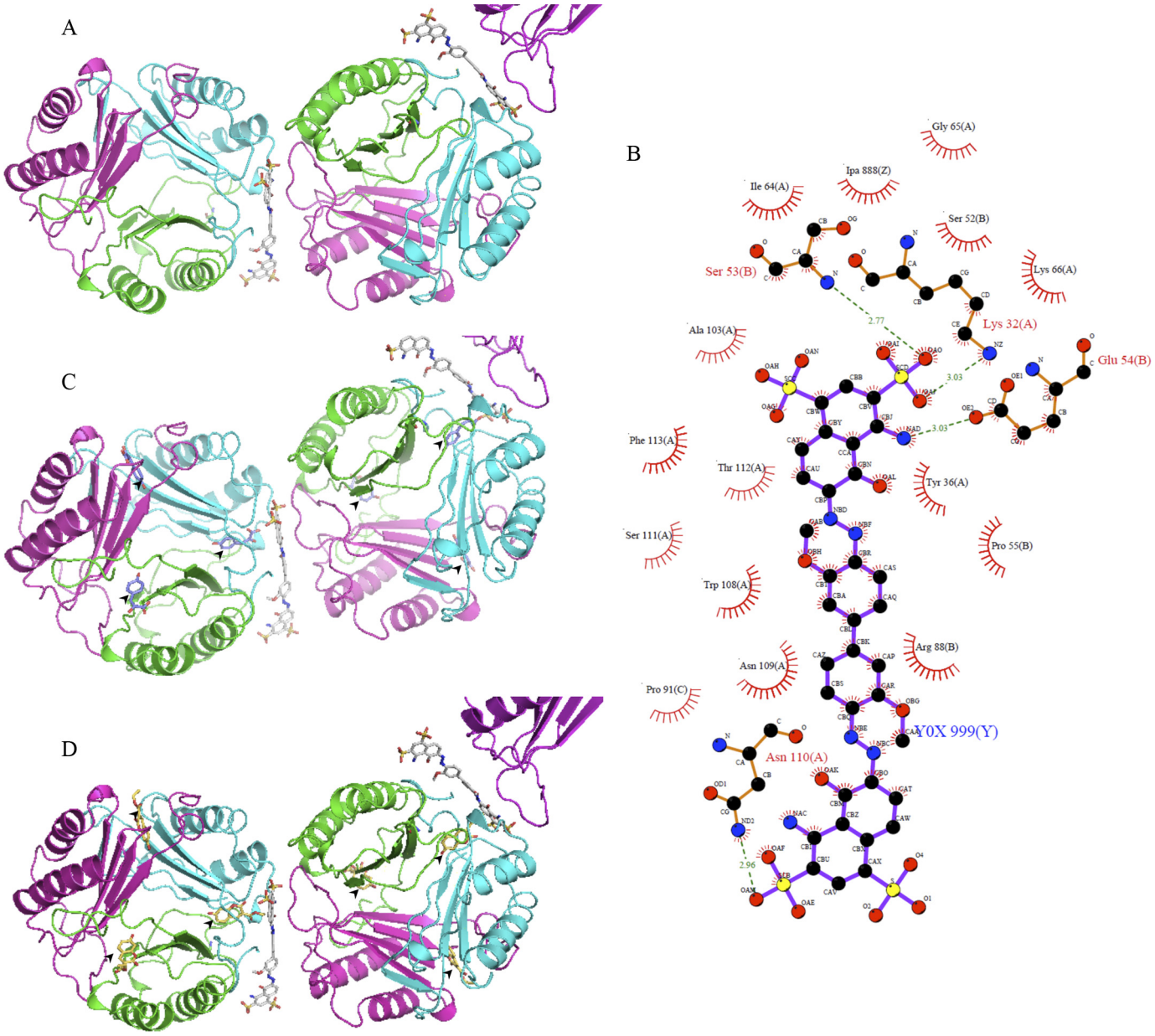

FIGURE 4. **Crystallographic analysis of rhMIF-p425 complex.** *A, ribbon* diagram depicting the unique binding of the p425 at the interface of two of the tightest packed rhMIF crystallographic trimers. The ligand makes contacts with monomer A (*blue*) and monomer C (*cyan*) of one trimer and with monomer B (*magenta*) of an adjacent trimer. *B,* ligplot representation of rhMIF-p425 interaction reveals hydrogen bonding (*dashed lines*) with four residues, Lys^{32} and Asn^{110} from one monomer, Glu^{54} from a second monomer, and Ser^{53} from a monomer of an adjacent trimer. *C* and *D,* superimposition of p425-rhMIF complex with HPP-rhMIF and coumarin derivative-rhMIF, respectively. *Arrowheads* indicate the respective ligands in the active site.

of MIF trimers, leading to the loss of its tautomerase, receptor binding, and pro-inflammatory activities.

The binding of p425 to rhMIF is unique as it engages residues from two adjacent trimers. p425 forms hydrogen bonds with Lys^{32} and Asn^{110} from one monomer, Glu^{54} from a second monomer, and Ser^{53} from an adjacent trimer. Loss of tautomerase activity could be a direct consequence of p425 bonding with Lys^{32} as this residue is important for HPP catalysis. A previously reported rhMIF/HPP co-crystal structure reveals that Lys^{32} forms hydrogen bonding with the carboxylate group of HPP and that during catalysis this interaction is necessary to properly orient the substrate within the active site (58). In the rhMIF/p425 complex, Lys^{32} is likely inaccessible for linkage with HPP, resulting in inefficient substrate binding. An analogous scenario has been predicted for aryl-1,2,3-triazole derivatives, a set of potent tautomerase inhibitors, which, by molecular docking studies, were modeled to engage the ammonium group of Lys^{32} (60).

Additionally, p425-Lys^{32} interaction could alter the electrostatic potential of the active site, a feature thought to impact the tautomerase activity of MIF (61). There are three active sites in a MIF trimer, and these sites form well defined cavities at the interface of the monomer subunits (26). Each site contains six core residues in a unique arrangement, with five residues, Lys^{32}, Ile^{64}, Tyr^{95}, Asn^{97}, and Val^{106}, surrounding Pro^{1} (26). In this hydrophobic environment, Pro^{1} has an unusually low pK_a of

5.6, a value that is almost 4 pH units lower than the pK_a of free prolines (61). Under physiological conditions, the increased nucleophilicity of Pro^1, due partly to the influence of the positively charged Lys^{32}, allows it to function as a general base catalyst in the tautomerase reaction (62). It has been suggested that the role of Pro^1 is to provide structural rigidity to the catalytic site to maintain a low pK_a micro-environment, and it is the acidic nature of the active site and not Pro^1 *per se* that is necessary for catalytic activity (58). Accordingly, structural alterations in the active site caused by a Pro^1 to Gly^1 substitution or spatial shift of Pro^1 due to an insertion of an adjacent alanine, severely impair the catalytic activity (58). In the case of p425, it is conceivable that hydrogen bonding between the inhibitor and Lys^{32} could perturb the spatial orientation of Pro^1 and consequently the pK_a of the catalytic site, leading to inefficient or loss of tautomerase activity.

Through a large naphthyl group, p425 also makes contact with Asn^{110}, a C-terminal residue located farther away from the active site. Although the role of Asn^{110} in HPP catalysis is not well defined, it is possible that the linkage between p425 and this residue could impair substrate binding. Previous NMR spectroscopic studies have shown that HPP binding to the active site affects not only the N terminus but a much larger set of residues, including Asp^{102}, Gly^{107}, Trp^{108}, Phe^{113}, and Ala^{114} at the C terminus (63). Truncation of C-terminal residues 110–114 causes significant structural changes in the active site and abolishes tautomerase activity, suggesting a possible role for this region in regulating the conformational flexibility and fine-tuning the catalytic activity of MIF (63). The large naphthyl group of p425 in hydrogen bonding with Asn^{110} could impose structural rigidity on the MIF trimer rendering it less flexible and thus impairing proper orientation of the substrate within the active site.

Our results also show that p425 severely impairs the ability of rhMIF to bind the purified CD74 receptor. Because CD74 binding on MIF occurs in the vicinity of the active site (43), it is likely that p425 linkage with Lys^{32}, an active-site residue, could account for the loss in receptor binding. There is evidence that the binding of substrate or an inhibitor to the tautomerase sites of MIF induces conformational changes sufficient to impair antibody recognition (29). In an analogous manner, binding of a large molecule such as p425 with Lys^{32} in the vicinity of the active site could disrupt the conformational integrity of an epitope that is critical for receptor binding.

In studies evaluating the pro-inflammatory activities of MIF, p425 proved to be a much superior inhibitor as compared with the prototypical MIF inhibitor ISO-1. Our results revealed that pretreatment of rhMIF with p425 significantly reduced the ability of the cytokine to promote secretion of IL-6 and IL-8, production of MMP-3, and inhibition of p53-dependent apoptosis, as compared with ISO-1 treatment. The mode of binding of p425 at the interface of two MIF trimers likely accounts for its enhanced activity as compared with ISO-1, which simply binds to the catalytic site (64). It has been suggested that inhibitors that exclusively target the tautomerase site may not be effective at inhibiting the entire spectrum of MIF-mediated pro-inflammatory activities. This is due in part to reports linking several of the cytokine functions of MIF to a second catalytic domain with a thiol-protein oxidoreductase (TPOR) activity (65). In MIF, the Cys^{56}-Ala^{57}-Leu^{58}-Cys^{59} sequence represents the TPOR consensus motif (*CXXC*) necessary for the ability of MIF to catalyze the reduction of insulin and small molecular disulfide compounds (66–68). The TPOR region also defines part of a structural epitope recognized by a neutralizing antibody that is therapeutic in mouse models of sepsis, highlighting the importance of this region for the *in vivo* activity of MIF (69). p425 forms hydrogen bondings with Ser^{53} and Glu^{54}, two residues in close proximity to the TPOR sequence. This interaction could likely alter the conformation of the "therapeutic" epitope, which could account for the enhanced activity of the inhibitor in assays evaluating the pro-inflammatory function of MIF as compared with ISO-1. p425 binding to Ser^{53} and Glu^{54} could also impact the chemokine activity of MIF, which orchestrates cell migration processes, including leukocyte recruitment during inflammation (70). The chemokine activity of MIF is mediated through direct binding to chemokine receptors CXCR2 and CXCR4 (70), which on the MIF monomer occurs at an N-like loop that spans residues 47–56 (71). If the effects of p425 on the oxidoreductase activity of MIF, recognition of neutralizing antibodies, and chemokine receptor binding were experimentally confirmed, p425 could define a new way to block both the catalytic and receptor binding activities of MIF that result in a more pronounced therapeutic effect.

In the p425-rhMIF crystal structure, the inhibitor is found located at the interface of two adjacent trimers. It is not apparent at this point whether p425 inserts into a pocket that forms between two adjacent rhMIF trimers during crystallization, or the inhibitor, upon binding to one trimer, brings an adjacent trimer into close apposition due to its "sticky" nature. Nevertheless, the possibility that p425 could "dimerize" MIF trimers in solution could prove valuable in studies interrogating possible roles of MIF oligomerization as a structural basis for the pleiotropic activities of this cytokine.

MIF was originally discovered as a T-cell factor that inhibited the random migration of macrophages and leukocytes (72). Contrary to its historical and eponymous function, MIF is now recognized as a key modulator of innate immunity involved in a wide range of cellular processes. Neither the molecular mechanism nor the structural basis underlying each function of MIF is well understood. The tautomerase activity has been the prime lead in the search to elucidate the molecular basis of MIF action, but to date, there are no compelling experimental data linking enzymatic activities of MIF with its immunological function. In fact, the catalytic functions of MIF have been relegated to be vestigial (1), partly due to lack of *bona fide* substrates and partly to the existence of other immune mediators with catalytic activities of no known biological relevance, such as the adult T-cell leukemia-derived factor with oxidoreductase activity (73) and the macrophage-derived factor cyclophilin with prolyl-peptidyl isomerase activity (74).

MIF is classified as one of five members of the tautomerase superfamily, a group of structurally related proteins that are characterized by a β-α-β building block and a catalytic N-terminal proline (Pro^1). The other four members of the tautomerase superfamily are bacterial enzymes involved in the degradation of aromatic hydrocarbons and amino acids (75). Three of

these enzymes are trimers, and one is a hexamer (a trimer of homodimers), but all are made up of the same β-α-β structural unit. Like MIF, the bacterial enzymes can catalyze other reactions in addition to tautomerization. Shared catalytic promiscuity in the tautomerase superfamily points to divergent evolution of these proteins from a common ancestor (75). In the tautomerase superfamily, it seems nature has fashioned new activities and structures by "stitching" together various combinations of the same simple structural unit. Still confounding, however, is how a MIF trimer, made up of three seemingly simple β-α-β structural units, can modulate such complex array of biological activities. Recently, the notion that MIF may employ different states of oligomerization as a molecular switch for modulating its various functions has been evoked (28). Although recombinant MIF purifies as a trimer, native MIF from bovine brain has been shown to display considerable size heterogeneity, ranging from 12 to 80 kDa (76). It is conceivable then that MIF, in different oligomeric states, such as monomers, dimers, trimers or even dimers of trimers, could carry out distinct functions. Studies to elucidate the structural basis for the diverse biological activities of MIF could significantly benefit from small molecules that can trap the different oligomeric species. p425, which bridges two MIF trimers, could prove central to these efforts.

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