Supporting Information

Crystallographic and Receptor Binding Characterization of *Plasmodium falciparum* Macrophage Migration Inhibitory Factor Complexed to Two Potent Inhibitors

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METHOD

Materials. All chemicals and media used in this study, including buffers and salts were of the high analytical grade (>98%). Compounds 1 and 2 were synthesized as previously described with purity >95 %. The biotin labeling kit, 3,3’-5,5’-tetramethylbenzidine (TMB), and Streptavidin HRP Conjugate were purchased from Roche.

Gene Expression and Protein Purification. To express the PfMIF gene, the full length nucleotide sequence was optimized for expression in E. coli, cloned into the pCRT7 expression vector (Invitrogen), and expressed using BL21(DE3) E. coli cells. Briefly, the cells were grown to an O.D. of 600 nm of 0.6 and induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C. After 4 h, the cells were harvested and resuspended into lysis buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl), supplemented with an EDTA-free protease inhibitor tablet, and lysed with sonication. The cell lysate was filtered and loaded onto a Q-Sepharose column (120 ml). The protein was eluted with a linear pH gradient from pH 8.0 in the lysis buffer to pH 6.8 in a buffer containing 30 mM Bis-Tris and 20 mM NaCl. The buffer was exchanged into lysis buffer and reloaded onto the Q-Sepharose column for salt gradient purification (from 20 mM to 1M NaCl in 20 mM Tris-HCl, pH 8.0). Pure PfMIF (>95 %) eluted at ~0.6-0.7 M NaCl. To remove other contaminants, PfMIF was exchanged into lysis buffer, loaded onto an SP-Sepharose column, and eluted in the flow-through. The pure PfMIF was concentrated using Amicon Ultra Centrifugal filter units with a 10,000 molecular weight cut-off.

For expression of the extracellular domain of CD74(his)114-232, the gene was cloned into the pET28a vector (Novagen) and expressed in BL21 (DE3) E. coli cells. The cell were grown at 37°C until the O.D. of 600 nm was 0.6 and induced with 0.4 mM of IPTG for 5 hours at 30 °C. The cells were resuspended into 20 mM Tris-HCl, 20 mM NaCl, 20 mM imidazole, pH 7.4, and lysed. The cell lysate was loaded onto a 5 ml HisTrap column (GE Healthcare). The pure protein (>95 %) was eluted using a gradient of 0-500 mM imidazole.

For expression of hMIF, pET11b-MIF expression vector was used as described previously. The cells were resuspended into 20 mM Tris-HCl, pH 7.4, and 20 mM NaCl, and lysed by sonication. The cell lysate was loaded onto a Q-Sepharose column connected in series to a SP-Sepharose column and the protein was eluted as the flow-through product. Small amounts of contaminants (~5%) were removed with Superdex 75 (size exclusion).

Crystallization and Structure Determination. To identify crystallization conditions, PfMIF was concentrated to 18 mg/ml and mixed with compound 1 or 2 at 1:3 MIF trimer:compound molar ratio followed by overnight incubation at 4°C. The next day each protein-inhibitor complex was centrifuged at 20 x g for 5 min and mixed with crystallization screening solutions (Hampton Research) at 1:1 ratio. The mixture was transferred to a 96-well round bottom COSTAR plate (Number 3795) with UV transparent plate seals. The whole procedure was carried out with a Mosquito (TTP LabTech) liquid handling robot. Each well contained 100 µl of crystallization condition. The plates were stored in a Rock Imager 1000 at 20°C and the drops were monitored on a regular basis. After the period of 8 days, crystals appeared in two crystallization conditions: i) 0.1 M Hepes, pH 7.5, 2M ammonium sulfate, 2% v/v PEG 400 (HR2-110), and ii) 0.1 M Hepes, pH 7.5, 1.0 M sodium citrate tribasic dehydrate (HR2-114). Due to poor diffraction, the crystallization conditions were optimized further. The 1:3 complex at 10 mg/ml of MIF was mixed with equal volumes of a well solution that contained 0.1 M Hepes
or Tris-HCl buffer pH 7.0-8.5, 2 M ammonium sulfate, and specific additives from the additive screening set (Hampton Research). Good quality crystals were grown via the hanging drop vapor diffusion method in a 24-well plate with 0.1 M Tris-HCl, pH 8.0, 2 M ammonium sulfate, 0.1 M NaCl. The crystals were flash frozen in the mother liquor containing 3.6 M ammonium sulfate as a cryoprotectant. The data sets were collected at the Yale School of Medicine Macromolecular Facility using a R-AXIS IV++ image plate detector (Rigaku, Tokyo, Japan) with a Rigaku rotating copper anode X-ray generator at -180°C, integrated, and scaled with HKL-2000. The PfMIF complexes with 1 and 2 diffracted to 3.02 Å and 2.87 Å in space group I222, respectively. The models of the PfMIF inhibitor complexes were solved by molecular replacement using PHASER. For PfMIF complexed to compound 1, the structure of Plasmodium berghei (Pb) MIF (PDB entry 2WKB) was used as the search model. The PfMIF complexed to compound 2 was solved with the protein coordinates from the PfMIF-compound 1 complex (without inhibitor) as the search model. Density modification was carried out with PARROT (CCP4) and refinement with REFMAC and COOT. Atomic models of the inhibitors were produced by the PRODRG server, fitted in electron density using COOT, and refined. The Fo-Fc maps of the compounds were generated by FFT (CCP4) and visualized in PyMOL. Interactions between PfMIF and the two compounds were calculated and visualized using CONTACTS (CCP4), LIGPLUS, and PyMOL. Hydrophobic interaction between the active site residues and compounds 1 and 2 were illustrated using LIGPLUS. The structures of apo-PfMIF (PDB entry 2WKF) and hMIF (PDB entry 3DJH) were superimposed using SUPERPOSE (CCP4). The electrostatic potential of PfMIF and hMIF was calculated PyMOL.

Soluble CD74(his)\textsubscript{6}\textsuperscript{114-232} capture assay. Soluble CD74(his)\textsubscript{6}\textsuperscript{114-232} in PBS was used to coat a 96-well ELISA plate at a concentration of 26 ng/µl. After overnight incubation and washing with PBS/0.1% buffer Tween-20 (PBS-T), the plate was blocked with Superblock (Pierce) for 2 hours at room temperature. Recombinant PfMIF or hMIF was biotinylated using a Biotin labeling kit (Roche). Biotinylated-PfMIF or biotinylated-hMIF at a concentration of 5 ng/µl was incubated with increasing concentrations of compounds 1 and 2 (0.1, 0.25, 0.5, and 2.5 µM) or DMSO as vehicle control for 45 min with shaking at room temperature. After 45 min, the biotinylated sample (PfMIF-inhibitor complex or hMIF with inhibitor) was added to the plate and incubated overnight at 4°C. The next day the plate was washed with PBS-T, conjugated with Streptavidin HRP (Roche), and incubated at room temperature for one hour. After washing with PBS-T, the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB, from Roche) was added and the plate was incubated for 20 min. The reaction was terminated with 1N H\textsubscript{2}SO\textsubscript{4}/HCl. The results were expressed as the percentage of binding in the presence of compounds 1 or 2 compared with binding in the absence of inhibitors.

Molecular Docking. The graphical user interface Maestro (version 9.8, Schrödinger, LLC, New York, NY, 2014) provided through the Structural Biology Grid\textsuperscript{3} was used to access all of Schrödinger’s computational programs, including Glide\textsuperscript{4-6} (version 6.3, Schrödinger, LLC, New York, NY, 2014) implemented herein for docking. Docking was performed in successive standard precision (SP) and extra precision (XP) modes in Glide. Input protein and ligand structures used for docking were derived from their respective co-crystal structures, and prepared using Schrödinger’s Protein Preparation Wizard\textsuperscript{7} and LigPrep\textsuperscript{8} tools, respectively. Flexible ligand conformations were sampled while fixing the receptor to a single, rigid conformation. The selection of initial poses kept 50,000 poses per ligand within a scoring window of 100 kcal/mol,
but only the best 1000 poses per ligand were subject to energy minimization, which consisted of 250 minimization steps at a distance-dependent dielectric constant of 2. All van der Waals radii were scaled down by a factor of 0.8 for partial charges $<$0.15 in ligand atoms. In SP mode, a maximum of 500 poses were generated per input ligand, and a maximum of 1 pose was generated in XP mode from each SP-derived pose. Final poses were scored by Schrödinger’s GlideScore and model energy (E-model) scoring functions. Taking into consideration differences in residue environment between the three active sites of co-crystallized Pf MIF, each prepared ligand was docked exclusively within the protein structure and active site from which the ligand was derived. As such, receptor grid generation was performed by confining the central coordinates of each native ligand within an enclosing box having side lengths of 20 Å. Of note, native ligands were automatically excluded from the grid generation step to prevent occupancy bias in the docking. Poses for a given ligand entry were ranked and evaluated according to their E-model scores. Furthermore, as a quantitative measure of docking accuracy, RMSD calculations were performed in the docking step to measure heavy-atom displacements between generated poses and their corresponding native ligands in the crystal structure. All output poses were saved and documented, but only poses within an RMSD cutoff of 2 Å were deemed to be consistent with the co-crystal.

**Temperature-dependent circular dichroism (CD) study.** The samples were prepared in 20mM Tris, pH 8.0, and 20 mM NaCl at a concentration of 50 µg/ml. CD data were recorded using a Chirascan Applied Photophysics spectrometer with thermoelectric temperature control.

1. Dahlgren, M. K.; Garcia, A. B.; Hare, A. A.; Tirado-Rives, J.; Leng, L.; Bucala, R.; Jorgensen, W. L. Virtual screening and optimization yield low-nanomolar inhibitors of the tautomerase activity of Plasmodium falciparum macrophage migration inhibitory factor. J Med Chem 2012, 55, 10148-59.
2. Sun, H. W.; Swope, M.; Cinquina, C.; Bedarkar, S.; Bernhagen, J.; Bucala, R.; Lolis, E. The subunit structure of human macrophage migration inhibitory factor: evidence for a trimer. Protein Eng 1996, 9, 631-5.
3. Morin, A.; Eisenbraun, B.; Key, J.; Sanschagrin, P. C.; Timony, M. A; Ottaviano, M.; Sliz, P. Collaboration Gets the Most out of Software. Elife 2013, 2, e01456.
4. Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A; Sanschagrin, P. C.; Mainz, D. T. Extra Precision Glide: Docking and Scoring Incorporating a Model of Hydrophobic Enclosure for Protein-Ligand Complexes. J. Med. Chem. 2006, 49, 6177–6196.
5. Halgren, T. A; Murphy, R. B.; Friesner, R. A; Beard, H. S.; Frye, L. L.; Pollard, W. T.; Banks, J. L. Glide: A New Approach for Rapid, Accurate Docking and Scoring. 2. Enrichment Factors in Database Screening. J. Med. Chem. 2004, 47, 1750–1759.
6. Friesner, R. A; Banks, J. L.; Murphy, R. B.; Halgren, T. A; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. Glide: A New Approach for Rapid, Accurate Docking and Scoring. 1. Method and Assessment of Docking Accuracy. J. Med. Chem. 2004, 47, 1739–1749.
7. Schrödinger Suite 2014-2 Protein Preparation Wizard; Epik version 2.8, Schrödinger, LLC, New York, NY, 2014; Impact version 6.3, Schrödinger, LLC, New York, NY, 2014; Prime version 3.5, Schrödinger, LLC, New York, NY, 2014.
8. LigPrep, version 3.0, Schrödinger, LLC, New York, NY, 2014.
**Supplementary Figure S1.** Alignment of PfMIF protein sequences. Comparison between the apo-PfMIF (2WKF) and PfMIF-inhibitor complexes (4P7M and 4P7S) revealed that the latter possess more C-terminal residues, especially in chain A. The missing residues are highlighted in grey. (*Chain C was not part of the asymmetric unit of the apo-PfMIF, which forms a trimer by crystallographic symmetry with an adjacent subunit. Therefore, it was not possible to compare the missing residues of this chain.)*. Protein alignment was carried out using SIM alignment tool (ExPASy).
Supplementary Figure S2. Temperature-dependent circular dichroism (CD) spectra of PfMIF. (A) Full CD spectra at different temperature. (B) Plot of molar ellipticity (θ at 212 and 222 nm) versus temperature. The T_m of PfMIF is 89°C.
Supplementary Figure S3. Examination of the inter-subunit interactions that stabilize the structure of hMIF. (A) Hydrogen bond interaction between the three subunits at one end of the solvent channel. (B) Each subunit forms one hydrogen bond with the adjacent chain. This is formed by His40 and Gln45. (C) The rotation axis and angle used to open two (of the three) subunits for examination of the subunit-subunit interface. (D) Electrostatic potential map of hMIF subunit-subunit interface. The white color of the subunit-subunit interface indicates that the monomers are stabilized mostly by hydrophobic interaction. Electrostatic interactions between the two subunits are shown by dotted circles. The positive and negative electrostatic potential are shown in blue and red, respectively.
Supplementary Figure S4. Electron density fitting of compounds 1 and 2 in an alternative orientation. Trials to fit the compounds in a different orientation rather than the one is shown in Figure 2 resulted in poor electron density fitting and clashes. A (i) Illustration of compound 1 fitting as this is reported in the manuscript. (ii), (iii) Fitting of compound 1 into the opposite orientation. B (i) Reported fitting of compound 2 into the experimental electron density map of chain B versus fitting in the opposite orientation (ii), (iii). C (i) Reported fitting of compound 2 into the experimental electron density map of chain C versus fitting in the opposite orientation (ii), (iii). The experimental electron density map (Fo-Fc) is shown in green, the reported orientation of either compound 1 or 2 in purple, same as the amino acid residues of PfMIF. The opposite orientation of either compound 1 or 2 in orange. Oxygen and nitrogen atoms are shown in red and blue respectively. The experimental electron density maps of compounds 1 and 2 counted at 3.0 σ.
**Supplementary Figure S5.** Hydrophobic interactions profile between compounds 1 and 2 and active site of PfMIF.  **(A)** Compound 1 is stabilized in the active site by a number of hydrophobic interactions that include residues from chain A and C.  **(B), (C)** Compound 2 was found in two conformations, 1 in the active site between subunits A and B (conformation 1) and conformation 2 for the active site between subunits B and C. Carbon, oxygen, and nitrogen atoms are shown as black, red, and blue balls while hydrophobic interactions are shown as red lines.
Supplementary Figure S6. Evaluation of the agreement between PfMIF-inhibitor crystal structures and docking results using either the fusion apo-PfMIF-PbMIF (A-C) or PfMIF-inhibitor crystal structures (D-F) as models. (A) Orientation of compound 1 as this appears in the crystal structure (green) versus the predicted orientation by docking (yellow). Orientation of compound 2 as it appears in the crystal structure (dark blue) versus the predicted orientation by docking (orange) in the active sites between (B) subunits A and B and (C) subunits B and C. (D) Docking of compound 1 using the PfMIF-inhibitor crystal structure as model results in significantly improved fitting. The color of the crystal structure and docked inhibitor are shown in green and yellow, respectively. For compound 2, (E) the docking model reveals significant agreement with the crystal structure in the orientation between subunits A and B. (F) The orientation between subunits B and C disagrees. The crystal structure and docked inhibitor are shown in dark blue and orange respectively.
Supplementary Figure S7. Structural basis of 1 and 2 selectivity for PfMIF over hMIF. (A) Sequence identity between hMIF and PfMIF. The two proteins share only 29% sequence identity with the identical residues to be highlighted in red. (B) Clashes between compound 1 and the active site residues of hMIF involved Met2, Ile64, Met101, and from an adjacent subunit, Tyr95. (C) Clashes between the active site residues of hMIF and compound 2 in orientation 1. The residues are Ile-64, Val106, and Phe113, and Tyr95 from an adjacent subunit. (D) Compound 2 in orientation 2 clashes with hMIF active site residues Ile-64, Val106, Phe113, and Tyr95 from an adjacent subunit.
Supplementary Figure S8. Compound 1 superimposed on the two conformations of compound 2. For both (A) and (B), green represents part of the ribbon for PjMIF bound to compound 1 with green carbon atoms. The solvent for both images is on the left and the active site residue Pro-1 is shown below the compounds. (A) Compound 1 and one conformation of compound 2 are both within the active site. (B) Part of the conformation for compound 2 faces outside the active site into the solvent.
**Supplementary Table S1.** Data collection, processing, and refinement statistics. *Values in parentheses are for highest-resolution shell. \( R_{\text{merge}} = \sum |I(h,i) - <I(h)>| / \sum I(h,i) \), where \(<I(h)\>\) is the mean intensity of reflections. \( R_{\text{work}} \) and \( R_{\text{free}} \) were calculated from working and test set reflections.

### Data collection

|                    | PfMIF-1 | PfMIF-2 |
|--------------------|---------|---------|
| Space group        | I222    | I222    |
| Cell dimensions    |         |         |
| \(a, b, c\) (Å)    | 79.03, 79.75, 97.08 | 79.55, 80.13, 97.66 |
| \(\alpha, \beta, \gamma\) (°) | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Resolution (Å)     | 50.00-3.02(3.07-3.02) * | 50.00-2.88(2.93-2.88) * |
| \(R_{\text{merge}}\) | 0.078(0.301) | 0.059(0.416) |
| \(I / \sigma I\)   | 16.9 (4.9) | 24.5(2.7) |
| Completeness (%)    | 96.8 (98.7) | 98.7(95.9) |
| Redundancy          | 5.0(5.1)  | 5.3(4.8)  |

### Refinement

|                    | PfMIF-1 | PfMIF-2 |
|--------------------|---------|---------|
| Resolution (Å)     | 25.02-3.02(3.09-3.02) | 48.83-2.87(2.95-2.88) |
| No. reflections    | 6077    | 7270    |
| \(R_{\text{work}} / R_{\text{free}}\) | 0.24/0.32 | 0.28/0.34 |
| No. atoms          |         |         |
| Protein            | 2091    | 2086    |
| Ligand/ion         | 21      | 50      |
| Water              | -       | -       |
| \(B\)-factors      |         |         |
| Protein            | 52      | 45      |
| Ligand/ion         | 87      | 41      |
| Water              | -       | -       |
| R.m.s. deviations  |         |         |
| Bond lengths (Å)   | 0.012   | 0.012   |
| Bond angles (°)    | 1.467   | 1.473   |
| Ramachandran plot (%) | 98.85, 0 | 98.15, 0 |
| PDB entries        | 4P7M    | 4P7S    |