Novel Anti-inflammatory Activity of Epoxyazadiradione against Macrophage Migration Inhibitory Factor

INHIBITION OF TAUTOMERASE AND PROINFLAMMATORY ACTIVITIES OF MACROPHAGE MIGRATION INHIBITOR FACTOR^{[3]}

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Athar Alam^{1}, Saikat Haldar^{2}, Hirekadathakallu V. Thulasiram^{1*}, Rahul Kumar^{3}, Manish Goyal^{4}, Mohd Shameel Iqbal^{5}, Chinmay Pal^{5}, Sumanta Dey^{5}, Samik Bindu^{4}, Souvik Sarkar^{4}, Uttam Pal^{5}, Nakul C. Maiti^{5}, and Uday Bandyopadhyay^{4,†}

From the ^{4}Division of Infectious Diseases and Immunology and ^{5}Department of Structural Biology and Bio-Informatics Division, Council of Scientific and Industrial Research (CSIR)-Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Kolkata 700032, West Bengal, India, ^{6}Chemistry-Biology Unit, Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune 411008, India, and ^{7}CSIR-Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110007, India

Background: Macrophage migration inhibitory factor (MIF) is responsible for proinflammatory reactions in many infectious and non-infectious diseases. We have investigated the mechanism of anti-inflammatory activity of epoxyazadiradione, a limonoid purified from neem (Azadirachta indica) fruits, against MIF.

Results: Epoxyazadiradione inhibited the tautomerase activity of both human and malarial MIF and prevents MIF-induced proinflammatory reactions.

Conclusion: Epoxyazadiradione bears therapeutic potential against MIF-induced proinflammatory reactions.

Significance: This novel molecule is a significant addition in the discovery of anti-inflammatory drugs.

Macrophage migration inhibitory factor (MIF) is responsible for proinflammatory reactions in various infectious and non-infectious diseases. We have investigated the mechanism of anti-inflammatory activity of epoxyazadiradione, a limonoid purified from neem (Azadirachta indica) fruits, against MIF. Epoxyazadiradione inhibited the tautomerase activity of MIF of both human (huMIF) and malaria parasites (Plasmodium falciparum (PfMIF) and Plasmodium yoelii (PyMIF)) non-competitively in a reversible fashion (K_{B}, 2.11–5.23 μM). Epoxyazadiradone also significantly inhibited MIF (huMIF, PyMIF, and PfMIF)-mediated proinflammatory activities in RAW 264.7 cells. It prevented MIF-induced macrophage chemotactic migration, NF-κB translocation to the nucleus, up-regulation of inducible nitric-oxide synthase, and nitric oxide production in RAW 264.7 cells. Epoxyazadiradione not only exhibited anti-inflammatory activity in vitro but also in vivo. We tested the anti-inflammatory activity of epoxyazadiradione in vivo after co-administering LPS and MIF in mice to mimic the disease state of sepsis or bacterial infection. Epoxyazadiradione prevented the release of proinflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α when LPS and PyMIF were co-administered to BALB/c mice. The molecular basis of interaction of epoxyazadiradione with MIFs was explored with the help of computational chemistry tools and a biological knowledgebase. Docking simulation indicated that the binding was highly specific and allosteric in nature. The well known MIF inhibitor (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) inhibited huMIF but not MIF of parasitic origin. In contrast, epoxyazadiradione inhibited both huMIF and plasmodial MIF, thus bearing an immense therapeutic potential against proinflammatory reactions induced by MIF of both malaria parasites and human.

Macrophage migration inhibitory factor (MIF)^{2} plays a pivotal role in the inflammatory process by affecting a number of immune cells, which produce inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-12, IL-18, and TNF-α (1–4). The MIF ortholog from malaria parasite Plasmodium yoelii (PyMIF) has also been reported to induce inflammatory cytokines such as IL-6, IL-10, IFN-γ, TNF-α, and macrophage chemotactic protein-1 (MCP-1) in BALB/c mice (5). Along with the cytokine activities, MIF also exhibits two distinct catalytic activities, i.e. tautomerase and oxidoreductase (2). MIF is ubiquitously expressed in various kinds of cells including monocytes, macrophages, neutrophils, eosinophils, basophils, blood dendritic cells, B cells, and mast cells, which are implicated in the pathogenesis of many inflammatory and autoimmune diseases (1, 6–8). Higher levels of MIF have been found both systemically and within affected tissues in patients with multiple sclerosis, sepsis, diabetes mellitus, glomerulonephritis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, inflammatory bowel disease, Guillain-Barré syndrome, neuro-Behçet disease, malarial anemia, gastric ulcer, Parkinson disease, hepatocellular carcinoma, breast cancer, and bladder urethelial cell carcinoma (9–17). MIF also has a role in deter-

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^{†} To whom correspondence should be addressed. Fax: 91-33-24730284; E-mail: ubandyo_1964@yahoo.com.

2 The abbreviations used are: MIF, macrophage migration inhibitory factor; NF-κB, nuclear factor-κB; iNOS, inducible nitric-oxide synthase; G-CSF, granulocyte colony-stimulating factor; hu, human; Pf, P. falciparum; Py, P. yoelii; MCP-1, macrophage chemotactic protein-1; ISO-1, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; Cl, chemotaxis index; Rt, retention time; Ni-NTA, nickel-nitritriacetic acid.
mining the outcome of infections caused by different parasites, bacteria, and viruses such as helminths (18), *Leishmania* (19), nematodes (20), malaria (21), *Streptococcus* (22), and dengue virus (23). Block of endogenous MIF by a small molecule such as (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) or sulforaphane and neutralization of MIF by anti-MIF antibodies or by plant-derived MIF inhibitors reduces the manifestations of immune inflammatory disorders in numerous preclinical models such as type II collagen-induced arthritis, immunologically induced kidney disease, experimental autoimmune encephalomyelitis, experimental allergic neuritis, immunoinflammatory diabetes, experimental autoimmune myocarditis, irradiation-induced acute pneumonitis, sepsis, and ischemia-reperfusion injury (14, 24–27). Likewise, MIF-deficient mice were more resistant than wild type to the development of sepsis, contact hypersensitivity, experimental autoimmune encephalomyelitis, inflammatory bowel disease, asthma, concanavalin A-induced liver injury, and allergic rhinitis (14, 28). Therefore, it is clear that MIF could be an effective therapeutic target against the above mentioned diseases.

Although the roles of MIF (host or parasitic origin) have been established in many diseases, studies showed the lack of MIF inhibitor, which could inhibit MIF activity irrespective of its origin. The well known inhibitor ISO-1 is very specific to the human MIF (huMIF) and unable to inhibit MIF of hookworm (29), *Leishmania* (19), and *P. yoelii* (5). Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (30). Unfortunately, these widely prescribed drugs have significant negative side effects, reducing their use in certain segments of the population (31). Hence, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Natural product-based anti-inflammatory agents with good efficacy and lower risk of side effects are in global demand to treat and prevent inflammation-related conditions. Traditional herbal remedies have been used for centuries to alleviate inflammatory ailments of many kinds of diseases (32). Neem (*Azadirachta indica* A. Juss) is a well known versatile medicinal plant that has a wide spectrum of biological activities including anti-inflammatory activity (33–37). The ethnopharmacological exploration and bioactivity testing of various neem compounds and extracts revealed its efficacy to prevent a wide variety of inflammatory disorders in infectious and non-infectious diseases (34, 37–45). It is interesting to note that neem compounds show a beneficial effect in pathological conditions in which MIF-mediated pathologies are prevalent (6, 10, 11, 14, 16, 17, 21).

Keeping all the above mentioned findings in mind, we investigated the probable anti-inflammatory activities of neem limonoids *in vitro* and *in vivo* to evaluate their use as a possible lead inhibitor against proinflammatory conditions in which MIF is involved. It is noteworthy that an inhibitor that can inhibit the enzymatic and biological activities of MIF of both human (huMIF) and plasmodial (*Plasmodium falciparum* (*PfMIF*) and *Plasmodium yoelii* (*PyMIF*)) origin is lacking. It is worth mentioning that plasmodial MIF in combination with its human counterpart imparted the pathological manifestation of malarial anemia, cerebral malaria, and organ damage (46–48). Here we report that epoxyzadiradione, one of the six neem limonoids tested, significantly inhibited the tautomerase activity of MIF of human as well as plasmodial origin. Epoxyzadiradione also exhibited anti-inflammatory activity against MIF-induced proinflammation *in vitro* in a cellular model and *in vivo* in a mouse model.

### EXPERIMENTAL PROCEDURES

**Chemicals**—RP-MI 1640 medium, streptomycin, penicillin, sodium m-periodate, L-3,4-dihydroxyphenylalanine methyl ester hydrochloride, and the fluorometric nitric-oxide synthase detection kit were purchased from Sigma. pET21b was procured from Invitrogen. The Detoxi-Gel endotoxin-removing gel column was purchased from Pierce. RNase-free DNase and Ready-To-Go™ RT-PCR beads were purchased from GE Healthcare. Heat-inactivated fetal bovine serum and DMEM were procured from Invitrogen. Transwell plates were procured from Corning. The nuclear factor-κB (NF-κB) consensus sequence was procured from Promega. Ndel, Xhol, prestained and unstained protein ladders, and the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction kit were procured from MBI Fermentas. The mouse Multi-Analyte ELISAArray kit was purchased from SA Biosciences. All other chemicals were of analytical grade.

**General Procedure for the Isolation and Purification of Neem Limonoids**—For the extraction and purification, the required solvents were distilled prior to use. Solvents and chemicals were procured from Merck. Thin layer chromatography (TLC) was performed on precoated Merck silica gel plates (TLC Silica Gel 60 F254) using the solvent system dichloromethane:methanol (97:3), and the spots were visualized under a UV lamp and developed using anisaldehyde charring reagent (92% ethanol, 3.2% anisaldehyde, 2.8% H₂SO₄, and 2% acetic acid). For the preparative chromatography, 230–400 mesh silica gel was procured from Merck. Neem seeds for the isolation of the compounds were purchased locally. NMR spectra were recorded in Bruker AV-400 (400-MHz) spectrometer, and the residual solvent signal was used as the reference. The LC-electrospray ionization-MS run was carried out by a Waters Acquity Ultra Performance LC system fitted with photodiode array detectors and single quad detectors. HPLC runs were performed on a Waters HPLC system fitted with a Merck Chromolith RP-18e column (100 × 4.6 mm). For detection, a Waters 2489 UV/visible detector was used at 215 nm. A gradient program of water and methanol (0 min, 1% methanol and water; 15 min, 40% methanol and water; 30 min, 50% methanol and water, 45 min, 60% methanol and water; 50 min, 70% methanol and water; 55 min, 60% methanol and water; 60 min, 1% methanol and water) was used as the mobile phase with a flow rate of 1 ml/min throughout the run for analytical injections.

**Extraction of Neem Limonoids**—Neem limonoids (salannin, nimbin, and azadirachtins A and B) from the neem seed kernel were extracted as described earlier (49, 50) with slight modification. In brief, 1 kg of neem seed kernel was extracted with petroleum ether (4 × 5 liters) with stirring for at least 3 h in every turn. The combined petroleum ether layer was concentrated to obtain neem oil, which was further partitioned between the layer of methanol (1.7 liters) and petroleum ether
Anti-inflammatory Activity of Epoxyazadiradione

(1.5 liters). The petroleum ether layer containing residual oil was discarded, and the limonoid-rich methanol layer was concentrated under vacuum at 52 °C, producing 15 g of oily viscous liquid.

The defatted powder (neem cake) left after extraction of neem oil from seed kernel was further extracted with methanol (6 × 5 liters) in a similar fashion, and 153 g of oily viscous substance, which was further partitioned between ethyl acetate (2 liters) and water (2 liters) to remove water-soluble components, was obtained. This concentrated ethyl acetate layer (2 liters) and water (2 liters) to remove water-soluble components, was obtained. This concentrated ethyl acetate layer under reduced pressure at 50 °C (51 g) was washed with petroleum ether (3 × 1 liter) to remove the residual oil (28 g), and the removed residual oil fraction was combined with the previously extracted 15 g of oily viscous liquid for further processing. The residual powder (22 g) left after the neem oil extraction from the neem cake contained mainly limonoids, was dissolved in a minimal amount of ethyl acetate (180 ml), and was precipitated by adding 500 ml of n-hexane. The precipitate (11.6 g) contained mainly azadirachtins A and B, whereas the supernatant was enriched with salannin and nimbin.

The oily viscous liquid collected previously (15 + 28 = 43 g) was further partitioned between petroleum ether (700 ml) and methanol (400 ml). The petroleum ether layer was discarded, and the methanol layer was concentrated under reduced pressure at 52 °C, and 16 g of a relatively non-polar limonoid salannin- and nimbin-enriched fraction was obtained.

For the extraction and purification of azadiradione and epoxyazadiradione, neem fruits were used. Neem fruit powder (250 g) was extracted with methanol (5 × 2 liters), and the combined extract was concentrated under reduced pressure at 52 °C to 1 liter and washed with petroleum ether to remove the oil. After concentration to dryness under reduced pressure, 53 g of brown viscous solid was obtained. This solid was partitioned between a 1:1 mixture of ethyl acetate and water. The organic phase was concentrated under reduced pressure to dryness (24 g). The HPLC analyses indicated that the crude mixture contained two major limonoids.

The enriched limonoid fractions obtained by solvent partition were further subjected to column chromatography to obtain pure individual limonoids. The extracts were then purified by a flash column chromatographic technique. 20 g of extract of non-polar limonoid mixture (salannin- and nimbin-enriched fraction) was loaded onto a silica gel flash chromatography column (14 × 6 cm) equilibrated with petroleum ether. Initially, the column was eluted with petroleum ether (1 liter) to remove residual oil. The column was further eluted with a gradient mixture of dichloromethane and methanol (700 ml of 0.1%, 1 liter of 0.2%, and 4.3 liters of 0.4% methanol in dichloromethane) in a sequential manner. The fractions collected were analyzed by TLC and LC-MS. The fractions containing pure nimbin (0.92 g; Rf 0.65) and salannin (4.42 g; Rf 0.51) were pooled and concentrated under reduced pressure. The spectral data for these compounds matched well with an earlier report (51).

For the purification of azadirachtins A and B, the polar limonoid mixture (11.6 g) (azadiradione- and epoxyazadiradione-enriched fraction) obtained from the methanol extract precipitated with n-hexane was subjected to column chromatography (13 × 6 cm) over silica gel (230–400 mesh) and eluted with a gradient mixture of methanol and dichloromethane. The fractions obtained by eluting with 1.25% methanol in dichloromethane contained a single compound (0.60 g; Rf 0.34). Based on the spectral characteristics, the compound was identified as azadirachtin B (52). Further elution of the column with 1.5% methanol in dichloromethane yielded a fraction containing a compound that was identified as azadirachtin A (2.04 g; Rf 0.46) based on the spectral characteristics, which matched well with that of reported (51). The above dried crude extract (24 g) from neem fruit was then subjected to column chromatography (14 × 6 cm) over silica gel (230–400 mesh), and the compounds were eluted using a gradient mixture of methanol in dichloromethane. Elution of the column with dichloromethane yielded a compound that was identified as epoxyazadiradione (4.31 g; Rf 0.82) based on various spectral studies and comparison with an earlier report (53, 54). Further elution of the column with 0.1% methanol in dichloromethane yielded a fraction containing a compound (5.88 g; Rf 0.66) that was identified as azadiradione by comparing the spectral data with earlier reports (53, 54).

Experimental Animals and Parasites—Male BALB/c mice 6–8 weeks of age (~20–25 g) were used for all experiments. Mice were housed in the experimental animal center of the Indian Institute of Chemical Biology, Kolkata, India under specific pathogen-free conditions. Mice were infected intraperitoneally with 1 × 10^3–1 × 10^6 P. yoelii (multidrug-resistant strain)-parasitized red blood cells (RBCs). Infections were monitored by counting parasitized RBCs in thin smears of tail blood stained with Giemsa stain and expressed as percent parasitemia as described earlier (55–57).

Cloning, Overexpression, and Purification of MIFs—PmMIF was cloned, overexpressed, and purified as reported earlier (58). For the cloning of PyMIF and huMIF, the same protocol was followed as described earlier (5, 59) with slight modification. In brief, for the cloning of PyMIF, P. yoelii-infected BALB/c mice with 40–50% parasitemia were sacrificed, blood was collected for RNA extraction, and cDNA was synthesized using Ready-To-Go RT-PCR beads (GE Healthcare). The coding sequence of PyMIF was amplified by polymerase chain reaction (PCR) using PyMIF-specific forward primer (5’-GGAATTCATCCATATGCCACTGCTTGCTGCGAATTAAT-3’) (Ndel restriction site is underlined) and reverse primer (5’-CCCTCAGGGCCAAATAGTGAAAACCTAAAGC-3’) (Xhol restriction site is underlined). The PCR product was then cloned in-frame into pET21b (Invitrogen) using the Ndel and Xhol restriction sites.

For the cloning of huMIF, full-length huMIF cDNA cloned into pCMV6-XL5 cloning vector was obtained from OriGene. The complete ORF from the clone pCMV6-XL5 was PCR-amplified with forward primer 5’-CTTCTGCCATAATGGCCATG-3’ (Ndel restriction site is underlined) and reverse primer 5’-CTCGAGATGAGGCAAGTTG-3’ (Xhol restriction site is underlined). The PCR product was then cloned in Escherichia coli expression vector pET21b (Invitrogen). Cloning of the complete ORFs of PyMIF and huMIF was finally confirmed by sequencing of the clones. For the overexpression and purification of PyMIF and huMIF, essentially the same procedure was
adopted as reported earlier for PyMIF (58). These recombinant proteins were used in all experiments.

Preparation of L-Dopachrome Methyl Ester and Tautomerase Assay—Tautomerase activity of MIFs was determined as described earlier (60, 61). Briefly, activity was determined at 25 °C by adding L-dopachrome methyl ester (0.25–1.5 mM) to a cuvette containing 60 mM huMIF or PyMIF, or PyMIF in 10 mM potassium phosphate buffer, pH 6.2 containing 0.5 mM EDTA. The activity of each MIF was monitored by measuring the conversion of L-dopachrome methyl ester (colored) to indole carboxylic acid methyl ester (colorless) at 475 nm.

To test the inhibitory activity of all six neem limonoids extracted, limonoids were freshly dissolved in DMSO and added at different concentrations to the cuvette containing huMIF, PyMIF, or PyMIF and incubated for 20 min prior to the addition of the L-dopachrome methyl ester. The IC_{50} (the concentration required of the given compound to achieve 50% inhibition) was obtained with different concentrations of the limonoids using nonlinear regression analysis using Prism4 (GraphPad Prism). K_{i} (the inhibition constant) was determined by nonlinear regression against a non-competitive equation using Prism4 (GraphPad Prism).

Macrophage Cell Culture—The RAW 264.7 cell line was maintained in DMEM supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum. The cells were kept in a 37 °C incubator with 5% CO_{2}. Cells were subcultured by scraping when plates reached 80–90% confluence in fresh medium. To follow NF-κB translocation, 5 × 10^{5} cells ml^{-1} were seeded in 6-well plates 12 h prior to treatment, whereas to measure inducible nitric-oxide synthase (iNOS) induction and nitric oxide (NO) production, cells were seeded at a density of 2.5 × 10^{4} cells ml^{-1} (viability of cells was checked by trypan blue staining using a hemocytometer).

Chemotaxis Assays—RAW 264.7 cells (2 × 10^{5} cells/well) were loaded into the upper chamber of a Transwell system (Corning) using 5-μm polycarbonate membrane. The chemoattractants huMIF, PyMIF, and PyMIF (100 ng ml^{-1}) were loaded into the lower chambers with/without increasing concentrations of epoxyazadiradione (5–20 μM). RAW 264.7 cells without any treatment were used as a control. Heat-inactivated MIFs (treating MIFs at 65 °C for 30 min) and medium alone (DMEM) were used as negative controls. MCP-1 (50 ng ml^{-1}) was used as a positive control. Epoxyazadiradione (20 μM) was added to a separate chamber loaded with MCP-1 to test the specificity of the compound. The Transwell plates were incubated for 4 h at 37 °C in 5% CO_{2}. Macrophages that had migrated through the filter were dislodged using 2 mM EDTA. Following staining with trypan blue (Fluka/Sigma-Aldrich), migrated cells were counted by light microscopy. To compare the results from different experiments, a chemotaxis index (CI) (CI = number of RAW 264.7 cells migrating toward the MIF or MIF plus epoxyazadiradione-loaded wells/number of RAW 264.7 cells migrating toward control medium) was calculated. The number of cells represents the mean of five high power fields for each condition. The results are reported as the mean CI ± S.E. of at least three independent experiments.

Anti-inflammatory Activity of Epoxyazadiradione

Electrophoretic Mobility Shift Assay (EMSA) for NF-κB Translocation—To determine NF-κB translocation, essentially the same protocol was followed as reported earlier (62). RAW 264.7 cells (5 × 10^{5} cells ml^{-1}) were treated with different concentrations of epoxyazadiradione 30 min prior to the addition of huMIF, PyMIF, or PyMIF (100 ng ml^{-1}) or left untreated. A low concentration of LPS (10 ng ml^{-1}) was added to all cultures except control (RAW 264.7 cells without any treatment) along with MIFs to induce endogenous MIF (63, 64). After 4 h of treatment, cells were washed twice with PBS, pH 7.4, and nuclear extracts from treated or untreated RAW 264.7 cells were prepared using the ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas) according to the manufacturer’s instructions. RAW 264.7 cells without any treatment were used as a control. The NF-κB consensus sequence (Pro-mega) was annealed to create a double strand and 5’-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to standard protocols. Unincorporated [γ-32P]ATP was removed by ethanol precipitation at −20 °C (overnight) followed by washing with 70% ethanol. Reactions were performed in a volume of 20 μl containing 32P-labeled double-stranded NF-κB consensus sequence (50 nm) incubated with nuclear extract from treated and untreated cells (80 μg) in binding buffer (10 mM HEPES buffer, pH 7.6, 50 mM NaCl, 1 mM EDTA, 5 mM MgCl_{2}, 0.1 mM dithiothreitol, 1 mg ml^{-1} BSA, and 0.05% Triton X-100) for 30 min on ice. NF-κB-Boligo complexes were then separated by native gel electrophoresis (5%) run at 10 V/cm at 4 °C with 0.5× TBE (Tris borate/EDTA; 1× TBE = 45 mm Tris borate and 1 mm EDTA) running buffer. The gel was dried and exposed to an x-ray film overnight at −80 °C before film development.

Assay of iNOS—To assay iNOS, RAW 264.7 cells were seeded overnight in a 24-well plate at a density of 2.5 × 10^{4} cells ml^{-1} in serum-free medium. The cells were pretreated with increasing concentrations of epoxyazadiradione (5–20 μM) or left untreated for 30 min. These pretreated and untreated cells were then stimulated with 100 ng ml^{-1} huMIF, PyMIF, or PyMIF for 24 h, and the inhibition of iNOS induction in the cells was quantitatively assayed using a fluorometric nitric-oxide synthase detection kit (Sigma) according to the manufacturer’s instructions. RAW 264.7 cells without any treatment were used as a control. The assay was monitored in a Hitachi F-7000 spectrofluorometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The data were expressed as relative fluorescence units. The inhibition of MIF-mediated iNOS induction was calculated by comparing the change in average relative fluorescence units between the groups of epoxyazadiradione-untreated and -pretreated RAW 264.7 cells.

Measurement of Nitric Oxide—Direct measurement of NO production is difficult. Therefore, nitrite (NO_{2}^{-}) and nitrate (NO_{3}^{-}), the stable products of NO oxidation, were measured in cell culture medium for NO estimation. The culture supernatants of control RAW 264.7 cells (without epoxyazadiradione or MIF treatment), RAW 264.7 cells treated with MIFs, or RAW 264.7 cells pretreated with different concentrations of epoxyazadiradione (5–20 μM) prior to the induction with MIFs were collected and stored quickly at −80 °C. The released NO_{2} in
the medium was measured using Griess reagent (1:1 mixture of 0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% H₃PO₄) as an indicator of NO formation (65). Aliquots of cell-free culture supernatants were mixed with an equal volume of Griess reagent, and after 10 min, the absorbance at 570 nm was determined in a microplate reader. The data were expressed as a percentage of NO production relative to the control (MIFs).

**Inflammatory Cytokine Profile in Vivo in BALB/c Mice—**
BALB/c mice (6–8 weeks old, ~20–25 g) were injected intraperitoneally with 25, 50, or 100 mg/kg of body weight epoxyazadiradione dissolved in peanut oil. Recombinant PyMIF (5 μg) mixed with LPS (400 μg/kg of body weight) were injected intraperitoneally once at 2 and 8 h after the administration of epoxyazadiradione. LPS was mixed with PyMIF to induce endogenous MIF to closely mimic the pathogenic condition of sepsis (63, 64). Groups of mice (n = 6/group) were euthanized 24 h after the last injection, blood samples were collected from the mice via cardiac puncture, and serum was collected from the mice. Collected serum was stored at −80 °C for further examinations. As a control, peanut oil instead of PyMIF was given to mice intraperitoneally. The levels of four cytokines, IL-1α, IL-1β, IL-6, and TNFα, were measured in mouse serum. The levels of cytokine IL-1α and IL-1β were determined using a mouse Multi-Analyte ELISArray kit (SABiosciences) following the manufacturer’s instructions. In brief, 50 μL of serum or standard antigens (IL-1α and IL-1β) was added to each well in a 96-well enzyme-linked immunoassay plate (each well already contained 50 μL of assay buffer) and incubated for 2 h at room temperature. Then 100 μL of each detecting antibody was added and incubated at room temperature for 1 h. 100 μL/well avidin-horseradish peroxidase solution was added to the well, and the plate was incubated for 30 min at room temperature. The plate was washed three times with washing buffer between each step. Afterward, 100 μL of developing solution was added to each well, and the plate was incubated in the dark for an additional 30 min at room temperature. The reaction was stopped by the addition of 50 μL/well stopping solution and analyzed using a BioTek microplate reader at 450 nm, and the minor optical imperfections in the ELISA plate were corrected by subtracting the value at the 570-nm wavelength. The mean absorbance for each set of duplicate standards, controls, and samples was calculated after subtracting the absorbance of the blank.

For the measurement of the levels of IL-6 and TNFα from serum, a mouse-specific ELISA kit (RayBiotech) was used following the manufacturer’s instructions. Briefly, samples of 100 μL of serum or standard were incubated on ELISA plates at 4 °C overnight with gentle shaking. The next day, the solution was discarded, and the wells were washed four times with 300 μL of 1× wash solution. After washing, 100 μL of 1× prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature with gentle shaking. After incubation, the solution was discarded, and the wells were washed three times with 300 μL of 1× wash solution. 100 μL/well prepared streptavidin solution was added to the wells, and the plate was incubated for 45 min at room temperature with gentle shaking. The plate was washed three times with washing buffer between each step. Afterward, 100 μL of 3,3′,5,5′-tetramethylbenzidine one-step substrate reagent was added to each well and incubated for 30 min at room temperature in the dark with gentle shaking. The reaction was stopped by the addition of 50 μL/well stopping buffer and analyzed using a BioTek microplate reader at 450 nm, and the minor optical imperfections in the ELISA plate were corrected by subtracting the value at the 570-nm wavelength. The mean absorbance for each set of duplicate standards, controls, and samples was calculated after subtracting the absorbance of the blank. Data were plotted as the -fold increase in cytokine levels over the control.

**Binding Analysis by Molecular Docking—**
Epoxyazadiradione (CID 122801), azadiradione (CID 363978), nimbin (CID 108058), azadirachtin A (CID 5281303), and azadirachtin B (CID 6436601) conformations were obtained from the PubChem Comounds database. The salannin structure was drawn in GaussView. The conformations were relaxed with steepest descent and then conjugate gradient algorithms in universal force field until the energy change was less than 10⁻⁶ kJ mol⁻¹. huMIF (Protein Data Bank code 1GD0; 1.5-Å resolution), huMIF-ISO-1 complex (Protein Data Bank code 1LIJ; 2-Å resolution), ideal coordinates for ISO-1 (Protein Data Bank code HDI), and PyMIF (Protein Data Bank code 3GAD; 1.8-Å resolution) were obtained from the Protein Data Bank. The PfMIF sequence (UniProtKB accession number Q815C5) was obtained from the UniProt Knowledgebase. Because the PfMIF structure available in the Protein Data Bank is truncated and could not be used as a template, it was modeled on its nearest ortholog, PyMIF, with which it shows 76.52% sequence similarity. Modeling was accomplished at the SWISS–MODEL homology modeling server using 3GAD as the explicit template. This server uses the QMEAN server for the model quality estimation. AutoDock 4.2 (66) along with MGLTools (67) of The Scripps Research Institute was used for the docking calculations following a protocol elaborately described in our previous work (68). The system was verified by docking HDI with 1GD0 to reproduce the bound conformation as in 1LIJ. The output was rendered by the PyMOL molecular viewer and MGLTools. Least energy docked conformations were analyzed for structural insight. The PoseView server at The Center for Bioinformatics (ZBH) of the University of Hamburg generated the two-dimensional representations of the ligand/protein interactions. The accessible surface areas of the ligands, proteins, and complexes were computed using a 1.4-Å probe size in MGLTools.

**Statistical Analysis—**
All the data were expressed as mean ± S.E. The levels of significance were calculated by performing unpaired Student’s t test and one-way analysis of variance. A p value less than 0.05 (p < 0.05) was taken as statistically significant.

**RESULTS**

**Purification of Epoxyazadiradione and Other Limonoids from Neem Plant—**
We extracted and purified six limonoids (azadirachtin A, azadirachtin B, nimbin, salannin, azadiradione, and epoxyazadiradione) from neem plant to test their possible effect on tautomerase and anti-inflammatory activity against MIFs in vitro in a cellular model and in vivo in BALB/c
mice. Azadirachtin A, azadirachtin B, nimbin, and salannin were extracted from the seed kernel, whereas azadiradione and epoxyazadiradione were extracted from the neem fruits. The purity of azadirachtin A (retention time (Rt) = 28.3 min), azadirachtin B (Rt = 29.7 min), nimbin (Rt = 47.9 min), salannin (Rt = 52.2 min), azadiradione (Rt = 46.0 min), and epoxyazadiradione (Rt = 56.2 min) was 98.8, 99.1, 99.1, 98.2, 98.9, and 99.6%, respectively, as determined by HPLC (Fig. 1).

Each limonoid was further characterized by LC-electrospray ionization-MS and 1H and 13C NMR studies (supplemental Figs. 1–24).

Purification of Human, P. falciparum, and P. yoelii MIF—MIFs were cloned, overexpressed, and purified using Ni-NTA affinity column chromatography. Because Ni-NTA affinity chromatography purification co-purifies some contaminating proteins along with the desired MIFs, we further subjected Ni-NTA-purified MIFs to Sephadex 75 gel filtration (Amersham Biosciences), contaminating protein-free fractions of MIFs were collected, and purity was confirmed by silver staining (Fig. 2, A, B, and C, insets). The molecular weights of the purified huMIF, PfMIF, and PyMIF were confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy (Applied Biosystems, Foster City, CA), which showed exact molecular masses (m/z) of 13,430, 13,266, and 13,251 kDa, respectively (Fig. 2, A, B, and C). These purified MIFs were then used for further studies.

Epoxyazadiradione Inhibits Tautomerase Activity of Both Human and Plasmodial MIFs—The tautomerase activity of MIFs was monitored by following the tautomerization of L-dopachrome methyl ester by the recombinant MIFs in the presence or absence of increasing concentrations of all six limonoids extracted from neem. The data indicated that among these six limonoids epoxyazadiradione showed the highest tautomerase inhibitory activity against MIFs (IC50 = 6.4 μM (huMIF), 9.5 μM (PfMIF), and 9.8 μM (PyMIF)) (Table 1). Nimbin (IC50 = 17.5 (huMIF), 22.5 μM (PfMIF), and 22.4 μM (PyMIF)) and azadiradione (IC50 = 35.2 (huMIF), 45.6 μM (PfMIF), and 43.0 μM (PyMIF)) also inhibited tautomerase activity, but they were less potent than epoxyazadiradione but more potent than salannin (IC50 = 85.3 (huMIF), 89.5 μM (PfMIF), and 95.2 μM (PyMIF)) (Table 1). In contrast, azadirachtin A and azadirachtin B did not inhibit tautomerase activity of MIF of both human and plasmodial origin even at high concentration (200 μM). Because epoxyazadiradione...
showed the most potent tautomerase inhibitory activity, we therefore selected only epoxyazadiradione for further studies.

Epoxyazadiradione inhibited almost 85–90% of the tautomerase activity at a concentration of 15–20 μM. However, when epoxyazadiradione-inhibited MIFs were diluted 10 times, almost 80–90% of the activity was regained, indicating that epoxyazadiradione inhibited the tautomerase activity in a reversible fashion (Fig. 3). To further verify the mode of inhibition, we performed MALDI-MS of epoxyazadiradione-inactivated MIFs. As expected, we did not find any adduct (epoxyazadiradione-MIF complex) formation in MALDI-MS spectroscopy (data not shown). Inhibition by epoxyazadiradione was also not due to protein denaturation as evident from fluorometric studies (data not shown). We further analyzed the type of inhibition and determined the inhibition constant (K_i). A Lineweaver-Burk plot of concentration-response curves revealed the non-competitive type of inhibition of MIFs by epoxyazadiradione (Fig. 4, A–F) and supported the previous result of a gain of activity and the MALDI-MS observation. Epoxyazadiradione inhibited the tautomerase activity of huMIF with a K_i of 2.01 μM, whereas the K_i values for PfMIF and PyMIF were 5.11 and 5.23 μM, respectively.

**Anti-inflammatory Activity of Epoxyazadiradione**

Epoxyazadiradione Inhibits MIF-mediated Macrophage Chemotactic Migration—MIF promotes macrophage chemotactic migration and plays an important role in leukocyte recruitment and inflammation (69). Inhibition of the tautomerase activity of MIF reduces inflammation induced by MIF (70, 71). Therefore, inhibition of the tautomerase activity of MIFs could inhibit the recruitment of leukocytes and consequently inflammation. To address any potential inhibitory effect of epoxyazadiradione on MIF-mediated chemotactic activity, we performed a chemotactic migration assay using RAW 264.7 cells in the presence or absence of increasing concentrations of epoxyazadiradione. Epoxyazadiradione showed a potent inhibitory effect on the MIF-induced chemotactic migration of RAW 264.7 cells against MIFs of human (CI = 1.12 compared with CI = 3.78 for cells without epoxyazadiradione) and plasmodial origin (CI = 1.28–1.45 compared with CI = 3.43–3.54 for cells without epoxyazadiradione) (Fig. 5). We validated the assay of macrophage chemotactic migration using heat-inactivated MIFs and DMEM; no significant migration was evident (Fig. 5). MCP-1 used as a macrophage chemoattractant led to significant chemotactic migration (CI = 4.67), and it is interesting to note that epoxyazadiradione even at a high concentration (20 μM) failed to inhibit the MCP-1-induced chemotactic migration of RAW 264.7 cells (Fig. 5).

### TABLE 1

| Compounds      | Inhibition of tautomerase activity of MIFs (IC_{50} (μM)) | huMIF | PfMIF | PyMIF |
|----------------|----------------------------------------------------------|-------|-------|-------|
| Epoxyazadiradione | 6.4 ± 0.8                                               | 9.5 ± 1.7 | 9.8 ± 1.9 |
| Azadiradione    | 33.2 ± 5.6                                               | 45.6 ± 6.4 | 43.0 ± 6.7 |
| Salnin         | 85.2 ± 5.1                                               | 89.5 ± 6.9 | 95.2 ± 7.4 |
| Nimbin         | 17.5 ± 0.7                                               | 22.5 ± 1.9 | 22.4 ± 1.3 |
| Azadiradione-A | Not active                                               | Not active | Not active |

**FIGURE 2.** Purification and MALDI-TOF mass analysis of MIFs. A, MALDI-TOF mass spectrum of purified huMIF. The inset shows silver-stained huMIF purified sequentially through Ni-NTA and then a Sephadex 75 column and electrophoresed in a 4–20% gradient gel. B and C, MALDI-TOF mass spectrum of purified PfMIF and PyMIF, respectively. The insets in B and C show silver-stained PfMIF and PyMIF, respectively, purified through Ni-NTA and then a Sephadex 75 column and electrophoresed in a 4–20% gradient gel. Arrows indicate the bands of the purified proteins. Detailed descriptions for SDS-PAGE, silver staining, and MALDI-TOF mass spectroscopy are given under “Experimental Procedures.”
Epoxyazadiradione Inhibits MIF-mediated NF-κB Translocation, Induction of iNOS, and Nitric Oxide Production in Vitro in RAW 264.7 Cells—The NF-κB pathway has been considered as a typical proinflammatory signaling pathway largely based on the role of NF-κB in the expression of proinflammatory genes including cytokines and chemokines (72). NF-κB translocation to the nucleus by different proinflammatory cytokines including huMIF has already been reported (24, 73–77). To further evaluate the effect of epoxyazadiradione on MIF-induced translocation of NF-κB to the nucleus, an EMSA was performed. The isolated nuclear extracts (proteins) from control, MIF-induced, and epoxyazadiradione-treated MIF-induced RAW 264.7 cells were checked by EMSA. No shifting was observed when only labeled DNA (NF-κB consensus sequence) was used (Fig. 6, A–C, lane 1). The translocation of NF-κB was not observed when nuclear extract from control RAW 264.7 cells was used as no complex was visible (Fig. 6, A–C, lane 2). However, increased NF-κB translocation was observed in nuclear extracts of RAW 264.7 cells treated with huMIF (Fig. 6A, lane 3), PyMIF (Fig. 6B, lane 3), or PyMIF (Fig. 6C, lane 3) as evident from the shifting of the band due to protein-DNA complex formation. Nuclear extracts from epoxyazadiradione-treated RAW 264.7 cells showed concentration-dependent inhibition of MIF-mediated translocation of NF-κB as evident from reduced complex formation (Fig. 6, A, B, and C, lanes 4–7).

Because NF-κB translocation is one of the important factors for up-regulation of the proinflammatory molecule iNOS (78–80), we further evaluated the effect of epoxyazadiradione on MIF-mediated iNOS induction and production of its stable product, nitrite (NO₂⁻), in RAW 264.7 cells. Data indicated that huMIF (100 ng ml⁻¹) induced an ~12-fold iNOS induction in RAW 264.7 cells, whereas PyMIF and PyMIF induced an ~8–9-fold induction at the same concentration (100 ng ml⁻¹) (Fig. 7). When the effect of epoxyazadiradione was evaluated against MIF-induced iNOS induction, epoxyazadiradione significantly inhibited the MIF-induced up-regulation of iNOS concentration-dependently with almost 85–95% inhibition at 20 μM (Fig. 7). Epoxyazadiradione exhibited concentration-dependent inhibition of MIF-mediated iNOS induction with EC₅₀ values of 9.4 μM for huMIF, 10.9 μM for PyMIF, and 11.6 μM for PyMIF (Fig. 7).

NO is the product of iNOS. When measured in supernatants of culture media, the same pattern of NO production induced by MIFs and concentration-dependent inhibition by epoxyazadiradione was observed as that of iNOS. NO release was effectively inhibited by epoxyazadiradione with an EC₅₀ of 7.3 μM for huMIF, whereas the EC₅₀ values for PyMIF and PyMIF were 10.1 and 9.8 μM, respectively. At 20 μM, epoxyazadiradione caused about 85–90% inhibition of NO production induced by MIFs (Fig. 8) without affecting the cell viability (data not shown). NO production induced by MIFs was considered as 100%, and the relative change in NO production due to the inhibitory effect of epoxyazadiradione was expressed as percent change of NO production.

Epoxyazadiradione Inhibits the Release of Proinflammatory Cytokines in BALB/c Mice Exposed to LPS and MIF—To further explore the anti-inflammatory effect of epoxyazadiradione in vivo, BALB/c mice were co-injected with PyMIF (5 μg) and LPS (400 μg/kg of body weight) in epoxyazadiradione (25–100 mg/kg of body weight)-pretreated mice. LPS was used to induce the endogenous secretion of MIF, which in turn enhances the lethality (64). Mice without any treatment were used as a control, whereas mice treated with only peanut oil were used as a negative control. We measured four inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and tested the ability of epoxyazadiradione to inhibit the release of these cytokines. The data indicated that the levels of all four of these cytokines (IL-1α, IL-1β, IL-6, and TNF-α) were elevated significantly in serum of PyMIF plus LPS-co-injected mice compared with the control (Fig. 9, A–D). Epoxyazadiradione dose-dependently inhibited the release of IL-1α with 95% inhibition at a dose of 100 mg/kg of body weight (Fig. 9A). Epoxyazadiradione also prevented the release of the other cytokines, IL-1β (Fig. 9B), IL-6 (Fig. 9C), and TNF-α (Fig. 9D), in a dose-dependent fashion with almost 90–95% inhibition at 100 mg/kg of body weight. However, peanut oil did not show any proinflamma-
binding/anti-inflammatory effect because the cytokine levels in peanut oil-treated mice were almost the same as those of the control mice (Fig. 9, A–D).

**Binding Analysis of Epoxyazadiradione with MIFs by Molecular Docking**—We have presented evidence that epoxyazadiradione inhibits the tautomerase activity of MIFs non-competitively and reversibly. We were interested to further explore the interaction between MIFs and epoxyazadiradione with the help of molecular docking and computational chemistry. Structural details for huMIF (Protein Data Bank code 1GD0; 1.5-Å resolution) and PyMIF (Protein Data Bank code 3GAD; 1.8-Å resolution) were obtained from the Protein Data Bank; however, the PfMIF structure available in the Protein Data Bank is a truncated monomer and could not be used for docking. The lack of PfMIF structural information led us to homology modeling. Our PfMIF model was a very good model based on a QMEAN4 score (81) of 0.79 and a QMEAN Z-score (82) of $-0.095$ (supplemental Fig. S1, A and B) as well as comparative analysis of the QMEAN Z-score of the model with that of the Protein Data Bank non-redundant data set solved by x-ray crystallography at high resolution (supplemental Fig. S1, A–C). For molecular docking studies, the homotrimer was docked with the compound epoxyazadiradione, and probability statistics was applied extensively to the outputs to rule out any computational errors. Each docking experiment was a composite of 100 independent runs. All the binding interactions and the bound conformations of the best ligand, epoxyazadiradione, are shown (Fig. 10). Molecular docking outputs showed epoxyazadiradione in complex with huMIF at an allosteric site with a binding stoichiometry of 1. This binding of epoxyazadiradione...
Anti-inflammatory Activity of Epoxyazadiradione

at the allosteric site of MIF could change the conformation of the enzyme, and therefore the affinity of the substrate for the active site and MIF/CD74 interaction are reduced. The intrinsic tautomerase activity of MIF is not essential for biological activity, but the MIF/CD74 interaction is required for the downstream activation responses (83). The binding site of ligand is located at the pole toward the N- and C-terminal sides of huMIF (Fig. 10A). However, for PyMIF (Fig. 10C) and PyMIF (Fig. 10E), epoxyazadiradione binds to a site located on the opposite side of the protein with respect to its binding site on huMIF (Fig. 10, C and E). Fig. 10, right, shows the correspond-

FIGURE 5. Concentration-dependent inhibition of MIF-mediated chemotactic migration of RAW 264.7 cells by epoxyazadiradione. Increasing concentrations of epoxyazadiradione (5–20 μM) and 100 ng ml⁻¹ huMIF, PiMIF, or PyMIF (MIFs) were loaded into the lower chambers of a Transwell system (Corning) 30 min prior to the addition of RAW 264.7 cells (2 × 10⁵ cells/well) into the top of the well. The migration of RAW 264.7 cells across the Transwell membrane after 4-h incubation was quantitated, and the chemotactic index was plotted with the number of cells migrated toward the medium (DMEM) versus 0.05. Data are the mean ± S.E. of three independent experiments (*, p < 0.001 versus DMEM; **, p < 0.05 versus MIFs).

FIGURE 6. Protective effect of epoxyazadiradione on MIF-induced NF-κB translocation from cytosol to nucleus in RAW 264.7 cells. Nuclear fractions isolated from RAW 264.7 cells (5 × 10⁵ cells ml⁻¹) treated with or without epoxyazadiradione (5–20 μM) 30 min prior to co-stimulation with huMIF, PiMIF, or PiMIF (100 ng ml⁻¹) plus LPS (10 ng ml⁻¹) or left unstimulated were used for EMSA. RAW 264.7 cells without any treatment were used as a control. EMSAs were performed on nuclear extracts using radiolabeled oligonucleotides containing the NF-κB binding site. Details of the methodology are described under “Experimental Procedures.” Lanes 1, only labeled DNA; lanes 2, labeled DNA plus nuclear fraction isolated from RAW 264.7 cells co-stimulated with LPS plus huMIF (A), PiMIF (B), or PyMIF (C), respectively; lanes 4–7, labeled DNA plus nuclear fraction isolated from RAW 264.7 cells pretreated with epoxyazadiradione (Epoxy) (at different concentrations as mentioned) and co-stimulated with LPS plus huMIF (A), PiMIF (B), or PyMIF (C), respectively. Arrows indicate DNA-bound NF-κB complexes.
Anti-inflammatory Activity of Epoxyazadiradione

FIGURE 7. Concentration-dependent inhibition of MIF-mediated induction of iNOS in RAW 264.7 cells by epoxyazadiradione. RAW 264.7 cells seeded in a 24-well plate at a density of 2.5 × 10^6 cells ml^(-1) were pretreated with increasing concentrations of epoxyazadiradione (5–20 μM) 30 min prior to the addition of MIFs or left untreated. The treated and untreated cells were then co-stimulated with 100 ng ml^(-1) MIFs to activate iNOS. RAW 264.7 cells without any treatment were used as a control. Induction of iNOS in the RAW 264.7 cells was quantitatively assayed using nitric-oxide synthase detection system (fluorometric kit, Sigma) according to the manufacturer’s instructions. Data are presented as the iNOS induced in RAW 264.7 cells in relative fluorescence units and are the average of three experiments. The details of the methodology for iNOS induction are described under “Experimental Procedures.” Data are presented as mean ± S.E. of three independent experiments (**, p < 0.01; ###, p < 0.05 versus MIFs).

FIGURE 8. Concentration-dependent inhibition of MIF-induced NO production by epoxyazadiradione in RAW 264.7 cells. RAW 264.7 cells seeded in a 24-well plate at a density of 2.5 × 10^6 cells ml^(-1) were pretreated with increasing concentrations of epoxyazadiradione as indicated 30 min prior to the addition of MIFs or left untreated. The treated and untreated cells were then co-stimulated with 100 ng ml^(-1) MIFs to activate iNOS and eventually NO release. The supernatants of each well were collected after 24 h, and the released NO_2, the stable product of NO oxidation, in the medium was measured using Griess reagent. The details of the methodology are described under “Experimental Procedures.” Data are presented as the percentage of NO produced by epoxyazadiradione-treated RAW 264.7 cells compared with that of only MIF-stimulated cells. Data are the average of three independent experiments and are presented as mean ± S.E. of three independent experiments (**, p < 0.01; ***, p < 0.05 versus MIFs).

successfully generated the conformation of ISO-1 (Protein Data Bank code HDI) with a 0.83-Å root mean square deviation docked to the crystal (Protein Data Bank codes 1LJT and HDI) and placed it in the active site of huMIF (1GD0) with a 1.83-Å root mean square deviation (as obtained after aligning the 1GD0 ISO-1 complex with 1LJT). The ISO-1 binding site is narrow and deep, and it lies between two subunit junctions near the N termini. When bound in the site between A and B subunits, ISO-1 forms hydrogen bonds with Asn-97 of chain B and Lys-32 of chain A. π/π interaction between the phenolic ring of ISO-1 and Tyr-95 of chain B also stabilized the binding. Hydrophobic interaction between ISO-1 and amino acids Pro-1, Tyr-36, and Val-106 of chain A has also been reported (see Protein Data Bank code 1LJT). There are similar binding sites in plasmoidal MIFs, but ISO-1 binding to these sites is energetically less favored (supplemental Fig. S2). Thus, the molecular docking simulation experiments suggest favorable binding of epoxyazadiradione with MIF of all three experimental species, whereas interaction with ISO-1 is restricted to human MIF.

DISCUSSION

In this study, we report that epoxyazadiradione inhibits the tautomerase activity of MIF of both human and plasmoidal origin and offers anti-inflammatory activity against proinflammatory MIF both in vitro in a cellular model and in vivo in BALB/c mice. MIF, a pleotropic proinflammatory cytokine, has been implicated to play a causative role in many disease states, and the inhibition of MIF activity by either a small molecule inhibitor or neutralization with anti-MIF antibody significantly improves survival and reduces disease progression and/or severity. To search for a novel anti-inflammatory molecule against proinflammatory MIF, six limonoids were extracted and purified from neem because neem extract is known to possess an anti-inflammatory property (33–37). These compounds were tested first for their ability to inhibit the tautomerase activity of MIFs and finally evaluated for their effect on MIF-induced inflammation and cytokine production.

Like host MIF, parasitic MIF also plays a pivotal role in host pathology by modulating the host immune response (5). We cloned and overexpressed MIF from both the host (human; huMIF) and its parasite P. falciparum (PMIF). We also cloned the MIF from P. yoelii (PyMIF), a rodent parasite, to test the compound in a more natural condition in a mouse model.

Dopachrome tautomerization, a well established method to assess tautomerase activity of MIF (1, 24, 83), was followed to screen the inhibitory activity of all six purified neem limonoids. We found epoxyazadiradione to be the most potent inhibitor among the six limonoids followed by nimbin, azadiradione, and salannin. Interestingly, azadiractins A and B were found incapable of inhibiting the tautomerase activity of MIFs even at a very high concentration (200 μM; data not shown). However, azadirachtin B has been reported as an anti-inflammatory compound that acts via interaction with retinoic acid receptors and suppresses all-trans-retinoic acid binding, inhibits falling off the receptors, and activates transcription factors like CAMP-response element-binding protein, Sp1, and NF-κB (84).

Epoxyazadiradione shares the structural scaffold of azadiradione but differs in that it has an epoxide group instead of the...
alkenyl in azadiradione (Table 1). Therefore, it appears that the difference in activity between epoxyazadiradione and azadiradione probably arises from the epoxide group present in epoxyazadiradione. The epoxide group is very reactive due to the strain in the oxirane ring and can readily undergo bond formation with the NH group of amines (85), which could not be explained by molecular mechanics because of its intrinsic limitations but might be elucidated by dedicated Raman spectroscopy or crystallography. Besides, the presence of the oxirane ring altered the three-dimensional geometry of the ligand itself (Fig. 12); for example, a hydrogen bond acceptor got shifted about a bond length away.

The non-linear regression analysis of concentration-response curves reveals that epoxyazadiradione is a non-competitive inhibitor of MIF. MALDI-MS analysis indicates that the epoxyazadiradione inhibited MIF non-covalently (reversibly) as we did not find any epoxyazadiradione/H18528MIF adduct or complex when MIF was treated with epoxyazadiradione. The details of the methodology for the cytokine analysis are described under “Experimental Procedures.” Data are the mean ± S.E. from three individual experiments and are presented as the -fold increase over control (*, p < 0.05 versus control; #, p < 0.01; ##, p < 0.05 versus LPS plus PyMIF).

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attracted the RAW 264.7 cells chemotactically, but when MIFs were treated with different concentrations of epoxyazadiradione, concentration-dependent inhibition was observed with almost 80–85% inhibition at 20 μM. The inhibition of RAW 264.7 cell chemotaxis by epoxyazadiradione was specific as no inhibition was found when MCP-1 was used as the chemoattractant, thereby confirming the specificity of these inhibitors against MIF. Epoxyazadiradione is not very toxic as epoxyazadiradione showed a very mild effect on cell viability at 20 μM (the maximum concentration used in vitro). Epoxyazadiradione showed some cytotoxicity in vitro in RAW 264.7 cells at high concentration (100 μM). However, at this high concentration, it did not show any significant toxicity in primary cultured gastric mucosal cells in vitro as evident from a cell viability assay. No lethal effect of epoxyazadiradione was found in vivo in BALB/c mice at a dose of 200 mg/kg of body weight.

It is reported that binding of MIF to CD74/CD44 complex leads to the release of the cytosolic domain of CD74 (CD74 ICD), which initiates the signaling pathway, and consequently to activation of NF-κB (73). Treatment of cells with ISO-1, an antagonist of human MIF, reduced NF-κB activation, indicating a role of MIF in NF-κB (73) activation. As NF-κB activation is responsible for the regulation of a plethora of genes involved in inflammation (87), we investigated the inhibitory effect of
epoxyazadiradione on MIF-induced translocation of NF-κB to the nucleus. Data clearly indicate that MIFs induce NF-κB translocation to the nucleus in RAW 264.7 cells, whereas the nuclear extract from the control (without MIFs) cells did not as evident from EMSA. When cells were treated with epoxyazadiradione, concentration-dependent inhibition of NF-κB translocation to the nucleus was observed. As LPS induces endogenous MIF (63, 64), we added a small amount of LPS along with MIF to follow the anti-inflammatory activity of epoxyazadiradione in RAW 264.7 cells.

Among the proinflammatory genes up-regulated by NF-κB during an inflammatory response is iNOS, which produces NO, a highly reactive free radical with important second messenger functions involving the mediation of inflammatory events (88). Increased expression of iNOS and concomitant NO levels have been reported in several inflammatory diseases such as Crohn disease, asthma, and rheumatoid arthritis (89). We further evaluated the effect of epoxyazadiradione on MIF-induced up-regulation of iNOS and NO because if the data obtained for NF-κB is true then it must also affect iNOS and NO. As expected, MIFs induce iNOS and NO in RAW 264.7 cells, and epoxyazadiradione down-regulates the production of iNOS and NO concentration-dependently.

After finding evidence that epoxyazadiradione possesses anti-inflammatory activity in vitro, we next tested its effect in an in vivo condition as there are many factors such as absorption, distribution, concentration in fluids and tissues, protein binding, rate of metabolism, and excretion that reduce the efficacy of drugs in vivo (90). Also, inflammation is a complex physiological process, and the inflammatory response cannot be extrapolated from an in vitro study alone, and the data obtained in vitro are not always conclusive. To further test the anti-inflammatory effect of epoxyazadiradione in vivo, PyMIF was co-injected with LPS in epoxyazadiradione pretreated BALB/c mice. MIF is a major secreted protein released in response to LPS stimulation (63) and greatly enhances the lethality of LPS when MIF is co-injected with LPS via up-regulation of Toll-like receptor 4, the signal-transducing molecule of the LPS receptor complex (64). Immunoneutralization of MIF or deletion of the
Anti-inflammatory Activity of Epoxyazadiradione

Mif gene was shown to protect animals from lethal endotoxemia, staphylococcal toxic shock, and septic shock in experimental models of bacterial peritonitis (91). We found that the levels of proinflammatory cytokines tested (IL-1α, IL-1β, TNF-α, and IL-6) decreased dose-dependently with increasing doses of epoxyazadiradione. These cytokines are mainly produced by macrophages (92) where MIF receptor (CD74) is prevalent. Neutrophil and endothelial cells where CD74 is present could also be possible sources for these cytokines. Because the cytokine network is very complex and regulation and expression of one cytokine by other cytokine is tightly controlled, the identification of the exact source of cytokines released in vivo is very difficult. Although the exact source of these cytokines released after induction with co-injected LPS plus PyMIF could not be determined decisively in vivo, epoxyazadiradione treatment prevented the release of all four cytokines (IL-1α, IL-1β, TNF-α, and IL-6). It is to be noted that these cytokines actively participate in inflammatory disease states such as arthritis, inflammatory bowel disease, shock, atherosclerosis, allergy, and some types of cancer (93, 94). Therefore, epoxyazadiradione is a candidate molecule against an array of MIF-induced proinflammatory diseases.

We further explored the reason why epoxyazadiradione inhibits MIF of both human and plasmodial origin and why ISO-1, a well known huMIF inhibitor, failed to inhibit plasmodial MIF (PfMIF or PyMIF). To compare epoxyazadiradione and ISO-1, we docked ISO-1 with MIF of both human and plasmodial origin. AutoDock 4 predicted ISO-1 binding with the plasmodial MIF at topologically similar binding sites, but the binding specificity was found to be very poor as rendered in the clustering information (supplemental Fig. S2). Low energy clusters are the major clusters in the case of ISO-1/huMIF interaction, indicating high specificity in binding, whereas the bell-shaped clustering distributions over the energy axis for the ISO-1 binding with plasmodial MIF suggest mostly stray binding. It is evident from the results that the interactions of ISO-1 with plasmodial MIFs are energetically less favored than its interaction with huMIF. Besides, more solvent exposure is apparent from the lesser decrease in accessible surface area of the ligand in the complexes of ISO-1 with plasmodial MIF. Unlike ISO-1, epoxyazadiradione was found to be active against both human and plasmodial MIFs, although its specificity was much greater for huMIF (supplemental Fig. S2). Despite the stray binding with plasmodial MIF, the best clusters are the major clusters, and interactions are implicated to be favorable. van der Waals attractions, hydrogen bond formation, and hydrophobic interactions are the major contributors in this binding. However, π/cation interaction was found to be involved in the binding with the plasmodial MIF. The active moieties in the ligand and the amino acid residues in the proteins involved in the binding and the interactions between them are illustrated in Fig. 10.

Hence, the molecular docking simulation experiments suggest favorable binding of epoxyazadiradione with MIF of both experimental species, whereas the reach of ISO-1 is restricted to human MIF. In conclusion, data support the potential value of epoxyazadiradione as a lead compound for the development of a novel anti-inflammatory drug against MIFs.

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Anti-inflammatory Activity of Epoxyazadiradione

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