MIF as a disease target: ISO-I as a proof-of-concept therapeutic

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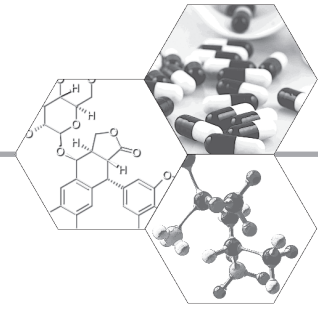

MIF as a disease target: ISO-1 as a proof-of-concept therapeutic

Macrophage migration inhibitory factor (MIF) is a pleiotropic proinflammatory cytokine that has been implicated as playing a causative role in many disease states, including sepsis, pneumonia, diabetes, rheumatoid arthritis, inflammatory bowel disease, psoriasis and cancer. To inhibit the enzymatic and biologic activities of MIF, we and others have developed small-molecule MIF inhibitors. Most MIF inhibitors bind within the hydrophobic pocket that contains highly conserved amino acids known to be essential for MIF's proinflammatory activity. The best characterized of these small-molecule MIF inhibitors, (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) has been validated in scores of laboratories worldwide. Like neutralizing anti-MIF antibodies, ISO-1 significantly improves survival and reduces disease progression and/or severity in multiple murine models where MIF is implicated. This MIF inhibitor, its derivatives and other MIF-targeted compounds show great promise for future testing in disease states where increased MIF activity has been discovered.

MIF, a pleiotropic mediator

Macrophage migration inhibitory factor (MIF) was discovered in 1966 as a T-cell factor that inhibited macrophage migration in delayed-type hypersensitivity responses [1,2]. Many years later, MIF was found to be an important pro-inflammatory cytokine produced and secreted locally from preformed stores by T cells, macrophages, eosinophils, granulocytes, B cells, CNS, epithelial cells and hypothalamic neurons, and systemically by the release of preformed stores from macrophages and pituitary adrenocorticotropic hormone ACTH cells (reviewed elsewhere [3]) [4,5]. It was subsequently found to be ubiquitously expressed and have an intracellular role in mediation of signaling and the G2M cell cycle checkpoint of DNA repair through an interaction with Jun activation domain binding protein-1 (Jab-1), a multifunctional signaling molecule [6]. Extracellularly, it functions through both autocrine and paracrine loops to induce itself and other proinflammatory cytokines including TNF-α, IL-β, IL-6, IL-8 and IFN-γ (reviewed elsewhere [7]) and matrix metalloproteases (MMPs) [8–9]. MIF release can be induced by lipopolysaccharide (LPS) as well as TNF-α and IFN-γ [4] and physiological concentrations of glucocorticoids. MIF is known to be secreted in a circadian fashion correlating with plasma cortisol levels [10]. MIF can also antagonize the anti-inflammatory activities of glucocorticoids (reviewed elsewhere [11]). A current hypothesis is that this antagonism occurs through a competition for downstream signaling molecules including Iκ-B [12] and MKP [13,14]. Additionally, physiological concentrations (in nonstressed, nondisease states) of steroids induce MIF secretion thereby allowing for potentiation of the pro-inflammatory response (most probably at local sites where other factors may be present). However, high concentrations of endogenous steroids (as in the stress response or an inflammatory challenge) inhibit MIF secretion allowing them to exert their dampening effect on the inflammatory response (an effective off switch once the immune response has been activated) [15]. These findings suggest a complex regulatory relationship between MIF and glucocorticoids in infection and inflammation [11].

A robust pro-inflammatory process is beneficial in many instances and is normally counter-regulated once recovery or wound healing is underway. Infectious disease models of sepsis and pneumonia understandably trigger an increase in systemic MIF and other pro-inflammatory proteins; however paracrine and autocrine feedback loops can further increase acute inflammation and related immune responses to a lethal degree. Results of experimental studies reveal that inhibition or antagonism of MIF and other pro-inflammatory cytokines in these infectious disease states can increase survival and improve outcomes [4,16,17]. Similarly, in other inflammatory disease states, including rheumatoid arthritis (RA), systemic lupus

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Key Terms

Macrophage migration inhibitory factor (MIF): Released systemically by macrophages and pituitary ACTH cells, locally by inflammatory cells, expressed intracellularly in a ubiquitous manner.

(*S,R*)-3(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1): Most studied small-molecule MIF inhibitor. It binds in the proinflammatory pocket and competitively inhibits MIF tautomerase and biologic activities.

Proinflammatory pocket: Binding site in MIF for which the natural ligand has not yet been described. This site was discovered to display tautomerase activity and inhibitors of MIF were designed to bind at or near this pocket and found to inhibit MIF's biologic activities to varying degrees.

erythematosis (SLE), psoriasis, inflammatory bowel disease (IBD) asthma, atherosclerosis, Type 2 diabetes (T2D)/obesity and cancer, several pro-inflammatory cytokines, including MIF, remain chronically elevated and their expression correlates with disease severity [18,19].

Unlike other pro-inflammatory cytokines, MIF has structural similarity to the bacterial tautomerase enzymes (4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase) [20,21] and has the ability to tautomerize the nonphysiological substrates D-dopachrome and L-dopachrome methyl ester into their indole derivatives [22]. A search for possible physiological substrates for the MIF catalytic site uncovered its ability to bind phenylpyruvic acid, p-hydroxyphenylpyruvic acid, 3,4-dihydroxyphenylaminechrome and norepinephrinechrome, but with high Michaelis Menton constant (Kms; mM range), it is unlikely that these are true physiological substrates of MIF. Mutational studies with this catalytic site have demonstrated its importance in some of the biological activities of MIF [8,23,24]. Our group has used inhibition of this catalytic site as the basis for rational drug design and development of a series of compounds, the lead of which was named **ISO-1** [25].

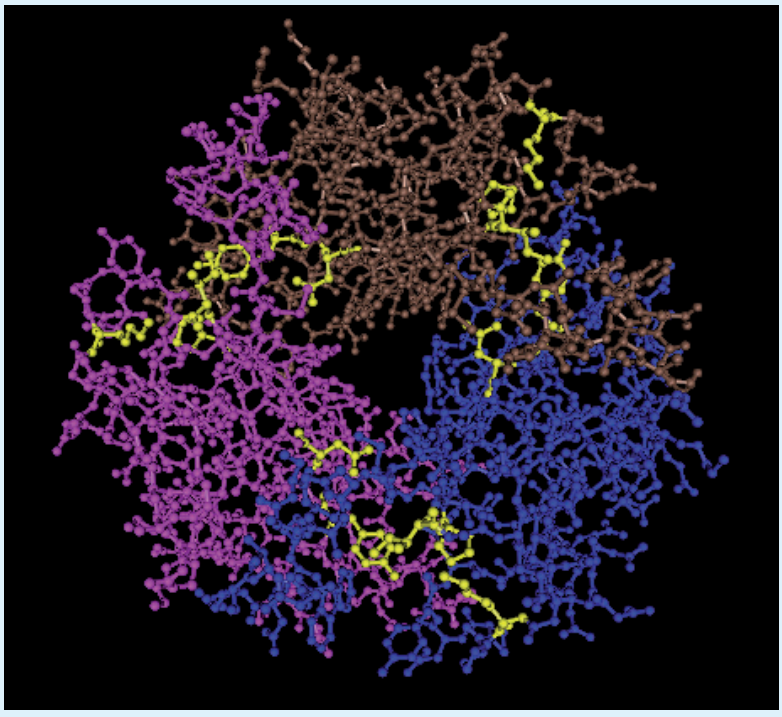

Figure 1. Crystal structure of macrophage migration inhibitory factor.
Amino acids associated with the proinflammatory pocket are highlighted in yellow.
Data from [20] and produced using [201].

MIF structure & inhibitor design

The macrophage migration inhibitory factor is a trimeric protein composed of identical 12.5-kDa subunits. Each subunit contains two antiparallel β-helices and 6 α-sheets, which align to form a doughnut-like structure **(Figure 1).** The catalytic pocket, also referred to as the **proinflammatory pocket**, because inhibition of this site reduces some of the pro-inflammatory activities of MIF [16,26], is located near Pro-1, Lys-32, Ile-64, Tyr-95 and Asn-97.

After our initial screens searching for compounds that mimic the nonphysiologic MIF substrates and bind the catalytically active site in MIF, 2-[(4-hydroxybenzylidene)amino]-3-(1*H*-indol-3-yl-propionic acid methyl ester (an L-tryptophan Schiff base), was discovered and became a lead compound. It was chosen based on its ability to inhibit both the tautomerase activity (IC_{50} = 1.65 μM) and MIF biologic activities (ERK1/2, MAPK activation, p53-dependent apoptosis, proliferation of serum-starved cells (cell cycle progression) and surface binding to human acute monocytic leukemia [THP-1] cells) [27]. Further testing using phenylimine-based scaffolds culminated in ISO-1 **(Figure 2)**, an isoxazoline that bound stoichiometrically to each of the monomers without disrupting the 3D structure of MIF **(Figure 3).** ISO-1 is a competitive inhibitor of tautomerase activity (IC_{50} = 7 μM) as well as an antagonist of MIF-dependent arachidonic acid release from macrophages and glucocorticoid-mediated inhibition of cytokine production by LPS-stimulated macrophages (glucocorticoid antagonism) [25]. Additional testing of ISO-1 derivatives [28,29] by our group is currently underway.

Subsequently, we performed a **pharmacophore**-based screening method using a carbonyl-oxime scaffold (termed OXIM) to identify additional compounds that could inhibit MIF enzymatic and biologic activity. These experiments identified OXIM-11, which was unexpectedly found to bind the MIF pocket in the opposite orientation as ISO-1 [30]. OXIM-11 inhibited tautomerase activity (IC_{50} = 1.3 μM), attenuated MIF-mediated glucocorticoid antagonism, NF-κB activation, and when administered to mice protected against the lethality of cecal ligation and puncture (CLP)-induced sepsis.

Recently, we and other groups have used ISO-1 as a template to design and screen analogs that are more potent in inhibiting MIF's tautomerase and/or selected biological activities [28,31,32]. In addition, several groups have

used virtual-screening methods of chemical libraries and known drugs to search for compounds that bind MIF at or near the catalytic site [33–35]. These compounds exhibit a wide range of MIF binding affinities and effectiveness in MIF biological activity assays and tautomerase assays. Another group has discovered a known phosphodiesterase inhibiting drug that can bind and inhibit MIF allosterically. Ibudilast (AV411) and its phosphodiesterase-inhibition compromised analog (AV1013) were demonstrated to non-competitively inhibit tautomerase activity and MIF-induced chemotaxis. Their kinetic enzyme analysis compared the allosteric inhibitors AV-411 (K_d = 55.1 ± 3.4 μM; K_i = 30.9 ± 2.8 μM) and AV-1013 (K_d = 54.0 ± 3.1 μM; K_i = 74.9 ± 8.5 μM) to ISO-1 (K_d = 14.5 ± 2.3 μM; K_i = 24.1 ± 1.7 μM) [36].

Macrophage migration inhibitory factor inhibitors can be grouped into two major classes: 'pocket' inhibitors (that bind at or near the tautomerase active site) and allosteric inhibitors (that bind at sites other than the tautomerase site). Pocket inhibitors can be further divided into reversible (competitive) and irreversible (covalent or suicide) compounds. Multiple groups have reported the discovery of MIF inhibitors with efficacy in inhibition of MIF biological activity assays and the tautomerase assay and the most promising candidates are listed in **Table 1** [25,27–37]. ISO-1 remains the most widely tested MIF inhibitor and is often used as a reference for comparison to newer MIF inhibitors. A summary of ISO-1's *in vitro* efficacy is presented in **Table 2** [16,26,33,38–61] and highlights doses employed, MIF biological activities investigated and the associated citation.

Table 3 [16,17,29–31,39,45,48,61–74] lists the MIF inhibitors that have shown efficacy in animal models of disease. It remains to be determined which compounds will exhibit the least toxicity, the best bioavailability, highest specificity for MIF and the greatest effectiveness in each disease model. Because MIF has intracellular as well as extracellular functions and interacts with other proteins, compounds or combinations of compounds that are tailored to inhibit the desired biologic activity or antagonize/agonize the desired protein interaction will likely be developed and tested specifically for each disease state (e.g., ebselen [a nonspecific anti-inflammatory drug with peroxidase activity that has been found to bind the free cysteine residues in MIF and disrupt trimer formation] can inhibit some biological functions of MIF but agonizes chemotaxis [35]).

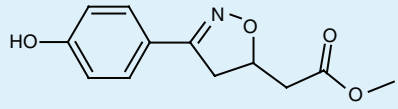

Figure 2. ISO-1. The small-molecule MIF inhibitor ISO-1 – (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (commercially available from Merck, USA and Calbiochem, USA).

Receptors & signaling

To date, several MIF receptors have been described, including CD74, CD44 and co-receptors CXCR2 and CXCR4. Current research suggests that CD74, a type II transmembrane protein, can associate with CD44 and signal through an ERK1/2 and PGE2 pathway (reviewed elsewhere [75]). CD74 can also undergo regulated intracellular proteolysis, releasing an intracellular fragment that travels to the nucleus and activates NF-κB/p65/Rel A homodimer and coactivator, transcription initiation factor TFIID subunit 2 (TAF_2)105 [76,77], in addition to undergoing serine phosphorylation [78]. The intracellular protein Jab1 has also been shown

Key Terms

IC_{50}: Concentration of a drug/compound that inhibits 50% of the activity measured in an assay (in this case the MIF tautomerase activity assay).

Pharmacophores: Compounds designed using a chemical scaffold on which changes to the chemical moieties (R-groups) at specific sites are made to find a combination of R-groups which increase biologic potency while decreasing toxicity.

Figure 3. Crystal structure of MIF complexed with ISO-1.
ISO-1, represented in grey, green, and gold (ball and stick depiction), binds MIF (tubeworms) in a 'pocket' associated with tautomerase and pro-inflammatory activity, which is located near the monomer interfaces of the trimer.
Data from [25] and produced using [202].

Table 1. Overview of *in vitro* screening and testing of macrophage migration inhibitory factor inhibitors.

Screening/design approach	Class(es) of inhibitor identified	*In vitro* MIF antagonistic activities	Tautomerase Inhibition assay IC_{50} (dopachrome used as substrate unless specified otherwise [HPP])		Ref.
Substrate analogs (isoxalines, aromatic Schiff base methyl esters, phenolic hydrazones)	Active site-reversible	Glucocorticoid, antagonism, arachidonic acid release, Erk1/2 PO_4, LPS-induced cytokine release, NO production and CD-74 binding (max 40%)	7 μM	(ISO-1)	[25]
		MIF/MIF receptor binding (the not yet identified CD-74), Erk1/2 PO_4, fibroblast serum-induced proliferation and apoptosis protection in fibroblasts	1.65 μM	(Cmpd 8)	[27]
				(OXIM-11)	[30]
		Glucocorticoid antagonism and LPS-induced NF-κB activation	1.3 μM		
				(Cmpd 7)	[29]
		LPS-induced TNF release	130 nM	(Cmpd 17)	[28]
		Not yet reported	550 nM		
Synthesis and screening of ISO-1 derivatives	Active site-reversible	MIF-induced cytokine release	60 μM†	(Cmpd 7)	
			20 μM†	(Cmpd 25)	[32]
			5 μM = Kd (far UV-CD)	(ISO-F)	[31]
Virtual screen of zinc and Maybridge compound libraries analog screen	Active site-reversible	CD-74 binding	3 μM (HPP)	(Cmpd 23)	[34]
			0.5 μM (HPP)	(Cmpd 24)	
		CD-74 binding, ERK1/2 PO_4	10 nM (HPP)	(Cmpd 5)	[37]
Virtual screen of NINDS custom collection II and Maybridge compound libraries – initial screen with tautomerase assay	Active site-reversible and allosteric-irreversible	Glucocorticoid antagonism, AKT PO_4 and epithelial progenitor cell chemotaxis	16.9 μM	(Cmpd 9)	[35]
			2.4 μM	(Cmpd 11)	
			6 μM	(HCLP)	
			2.4 μM	(ebselen)	
Investigation of known drugs cross-reactivity (rational)	Allosteric-reversible	Monocyte chemotaxis	74.9 μM (HPP)	(AV-1013)	[36]
			30.9 μM (HPP)	(AV-411/ ibudilast)	
Virtual screen of ACD(MDL) library	Active site-irreversible	Lung adenocarcinoma cell chemotaxis and anchorage-independent growth	~5 μM	(4-IPP)	
			200–475 nM	(A1-A4‡)	[33]

†This group used a different concentration of MIF in the tautomerase assay, therefore results are difficult to compare with other reports.
‡4-IPP analogs.
4-IPP: 4-iodo-6-phenylpyrimidine; Cmpd: Compound; Far UV-CD: Far UV-circular dichroism; HCLP: Hexachlorophene; HPP: p-hydroxyphenylpyruvate; ISO-1: (S,R)-3(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; ISO-F: Fluorinated ISO-1; LPS: Lipopolysaccharide; MIF: Macrophage migration inhibitory factor; NINDS: National Institute of Neurological Disorders and Stroke.

to bind MIF and inhibit Jab1 related functions, including activation of JNK (c-Jun amino-terminal kinase) activity, $p27^{kip1}$ degradation and activation of the transcription factor AP-1 [79].

Chemokine receptors CXCR2 and CXCR4 have been demonstrated to bind MIF using competition studies with their cognate ligands. Direct binding between MIF and CXCR2 has also been reported [80]. CXCR2 binding may occur through a pseudo-(E)LR motif in MIF, which occurs in non-adjacent amino acids in neighboring loops of the MIF trimer [81]. Currently, the MIF/CXCR downstream signaling pathways implicated include induction of $G\alpha_{a1}$ and integrins (CXCR2 and 4), calcium influx (CXCR2 and 4) and AKT activation (CXCR4) [80,82]. In addition, the same group demonstrated that CD74 and CXCR4 can form a heteromeric receptor complex in doubly transfected HEK293 cells and also in untransfected (native) monocytes [82]. Another group has shown that RPS19(S19), a ribosomal protein released during apoptosis, can inhibit MIF binding to CD74 and disrupt MIF-induced CXCR2-dependent monocyte

Table 2. Summary of *in vitro* testing of ISO-1.

Dose(s) employed	ISO-1 *in vitro* effects on MIF-mediated activities	Ref.
0.1–100 μM	Inhibited glucocorticoid antagonism (TNF-α PGE_2, Cox-2) and MIF-induced arachidonic acid release	[25]
10 μM	Decreased constitutive expression of CXCL8 and IL-8 from influenza A-infected human lung epithelial cell line (A549)	[38]
1–100 μM	Decreased endogenous MIF tautomerase activity, NF-κB activation and fluorescent ISO-1 taken up by RAW 264.7	[16]
0.1–100 μM	Decreased cell proliferation, MIF protein secretion and invasion in androgen-independent prostate cancer cells (DU-145)	[39]
Tenfold excess	Did not affect hookworm (rACE MIF), MIF tautomerase activity or rACE MIF-mediated chemotaxis of human PBMC's	[40]
10 and 100 μM	Decreased invasion and anchorage-independent growth of human lung adenocarcinoma cells (A549)	[41]
20 μM	Decreased MIF induced increases in Bcl-2 and IL-8 mRNA and protein in *ex vivo* B-CLL cells	[42]
10–100 μM	ISO-1 compared with 4-IPP – decreased tautomerase activity, lung adenoma cell motility, migration and anchorage-independent growth (A549 cells)	[33]
25–200 μg/ml	Decreased nitrate accumulation from L929 cells, primary murine fibroblasts and endothelial cells, RIN and MIN cells (insulinoma cell lines), and murine islet β-cells, and decreased iNOS expression in RIN cells; decreased TNF-α production from RIN and MIN cells, and increased viability of IL-1β/IFN-γ-treated RIN and MIN cells	[43]
50 μM	Decreased TLR-4-induced proinflammatory cytokine production in monocytes (via ERK1/2) and decreased LPS-induced activation of monocyte/epithelial cell co-cultures	[44]
10–100nM	Decreased colon carcinoma cell (CT26) proliferation and endogenous MIF tautomerase activity	[45]
2.5, 3 μM/ 30 ng/ml MIF (~3000-fold M excess)	Decreased MIF induced (rat) cardiomyocyte apoptosis (via caspase-3 and Bcl-xL/Bax), decreased MIF-induced activation of pJNK1/2 and ERK1/2	[46]
0.1 μM	Decreased NF-κB in CD-74 transfected 293 cells; decreased MIF-stimulated entry into S-phase, Bcl-2 transcription, and measures of apoptosis in B220+ murine B-cells (via CD74-CD44)	[47]
100 μM	Decreased MIF-induced MIP2 in RAW 264.7 cells (via p44/42 MAPK); and decreased BAL neutrophil accumulation, MIP-2 and KC release.	[48]
500 or 1000 μg ISO-1/ μg MIF (80,00 or 160,000 M excess)	Increased migration of mesenchymal stem cells to bronchial epithelial cells (override of suppressive effect of MIF)	[49]
100 and 1000 μM	Decreased migration and invasion of Hs683 glioma cells and enhanced dexamethasone inhibitory effect on migration and invasion	[50]
100 and 1000 μM	Decreased proliferation and mitogenic signaling (ERK1/2, pAKT) in glioblastoma cells (LN229, LN18)	[51]
50 μM	Decreased COX-2 mRNA in *ex vivo* human endometrial cells	[52]
85 μg/ml (360 μM)	Restored MIF-induced reduction of mesenchymal stem cells and chemokinesis to control (no MIF) levels	[53]
200 nM 50–100 μM	Decreased Aβ toxicity in neuroblastoma (hu-SHSY) and microglial (mu-BV2) cell lines (Alzheimer's model)	[54]
20 μM	Decreased TAp63 mRNA in B-CLL cells (Tap63 involved in B-CLL survival pathway)	[55]
100 μM	Decreased TNF-α, IL-6 secretion and MMP-1, and MMP-3 expression in *ex vivo* Sindbus infected human macrophages (viral arthritis model)	[56]
10 and 100 μM	Decreased invasion index and growth of drug-resistant (CXCR4 upregulated) colon cancer cells (HT-29)	[57]
40 μM	Decreased $CD4{-}8^-$ T-cell regulation of IFN-γ produced by mixed $CD4{-}8^-/CD4^+8$ T-cell cultures	[58]
40 μM	Decreased glucose-induced apoptosis (via caspase-3 and JNK) in AC16 human cardiac myocytes	[59]
50 μM	Decreased MIF-induced VEGF, IL-8 and MCP-1 mRNA expression and protein secretion from human ectopic endometrial stromal cells	[60]
5–100 μM	Decreased TNF-α, IL-6, IL-8 production from whole blood cultures and PBMC (via Iκ-B, NF-κB)	[61]

B-CLL: B-cell chronic lymphocytic leukaemia; iNOS: Inducible nitric oxide synthetase; JNK: c-Jun N-terminal kinase; KC: Keratinocyte-derived cytokine; ISO-1: (S,R)-3(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; LPS: Lipopolysaccharide; L929; Murine fibrosarcoma cell line; Min: Mouse insulinoma cell line; PBMC: Peripheral blood mononuclear cells; rACE: Recombinant Ancylostoma ceylanicum; *RAW 264.7: Mouse macrophage cell line; RIN: Rat insulinoma cell line; 4-IPP: 4-iodo-6-phenylpyrimidine.*

adhesion [83], providing further evidence that MIF signals through a CD74/CXCR2 receptor complex. These MIF receptors and co-receptors seem to be fairly ubiquitous and several groups are investigating specific downstream signaling in various cell types. Of note, proinflammatory pocket

Table 3. Overview of *in vivo* testing of macrophage migration inhibitory factor inhibitors.

Drug/compound	*In vivo* disease model efficacy (murine)	Dose/route of administration	Ref.
ISO-1	Sepsis	3.5–35 mg/kg x 3 day/intraperitoneal	[16]
	Type I diabetes	1 mg/mouse/day x 14 day/intraperitoneal	[62]
	Prostate cancer xenografts	20 mg/kg^{-2} – 2x/week x 5 weeks/intraperitoneal	[39]
	Experimental allergic neuritis (EAN) Guillian–Barré model	1 mg/mouse day 12–28 p.i./intraperitoneal	[63]
	Cardiocirculatory depression in sepsis (rats)	16 mg/rat at 6,18 and 24 h/intraperitoneal	[64]
	Survival in west nile virus-infected mice	1 mg/mouse from 1–7 day p.i./intraperitoneal	[65]
	Experimental colitis (IBD model)	20 mg/kg daily/intraperitoneal	[66]
	Pneumonia (H5N1)	3.5 mg/kg x 7 day/intraperitoneal	[17]
	Hyperalgesia (pain) model	120 µg/kg/intrathecal	[67]
	Colorectal cancer	20 mg/kg – 2x/week x 4 weeks/intraperitoneal	[45]
	Airway inflammation (neutrophil accumulation)	0.5 ug/intratracheal	[48]
	Asthma (airway remodeling)	35 mg/kg before each challenge/intraperitoneal	[68]
	Hippocampal associated learning, anxiety, and depression (ISO-1 blocks positive effect of MIF)	7 mg/kg x 14 day/intraperitoneal	[69]
	Bladder inflammation (cystitis)	20 mg/kg x 2 day/intraperitoneal	[70]
	Bone marrow mature B cells (IBD model)	20 mg/kg x 6 day/intravenous	[71]
	Vibrio vulnificus infection (gram-negative sepsis model)	25 mg/kg pretreatment (-0.5 hr)/intraperitoneal	[61]
OXIM-11	Sepsis	3.5 mg/kg – 2 x day for 3 day/intraperitoneal	[30]
Cmpd 7 (Al-Abed)	Sepsis	4 mg/kg – 2 x day for 2 day/intraperitoneal	[29]
ISO-F (fluorinated ISO-1 analog [28])	DSS-colitis	25 mg/kg x 5 day/oral	[31]
CPSI-1306/2705 (ISO-1 derivatives)	EAE (multiple sclerosis)	1.0 mg/kg x 21 day/oral	[72]
	Type II diabetes	0.1 and 1.0 mg/kg x 30 day/oral	[73]
4-IPP	*Pseudomonas aeruginosa*-induced ocular keratitis	~~50 µ~~g/mouse x 5 days/intraperitoneal (+ gentamycin drops [control])	[74]

4-IPP: 4-iodo-6-phenylpyrimidine; Cmpd: Compound; DSS-colitis: Dextran sodium sulfate-induced acute experimental colitis EAE: Experimental autoimmune encephalomyelitis; EAN: Experimental allergic neuritis; IBD: Inflammatory bowel disease; ISO-1: (S,R)-3(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; MIF: Macrophage migration inhibitory factor p.i.: Post-infection.

MIF inhibitors designed by our group, including ISO-1, inhibit MIF's ability to bind its surface receptor in THP-1 cells [Al-Abed Y; Unpublished Data], with an association seen between potency of tautomerase inhibition and interference with receptor binding [27]; however, further investigation is needed. Additionally, Cournia *et al*. found that several inhibitors that blocked the MIF–CD74 interaction also inhibited MIF tautomerase activity to various degrees [34], making it likely that the proinflammatory pocket and endogenous proteins, which bind to it, could play a major role in MIF function.

Elevated MIF expression in disease

To date, increased circulating MIF levels have been described in many human disease states and models, including infectious diseases such as pneumonia [17], sepsis [84] and viral arthritis [56]; autoimmune disease such as RA [85,86], SLE [87,88], multiple sclerosis (MS) [89,90] and psoriasis [91,92]; and inflammatory diseases including IBD [66] and endometriosis [52,93]. Other diseases, including metabolic syndrome (obesity)/T2D [94,95], atherosclerosis [96], polycystic ovary syndrome [97,98], asthma [99,100] and cancer [101,102], which are likely the result of a combination of genetic, inflammatory and/or environmental factors, have also been associated with increased serum MIF and proinflammatory cytokine levels.

In an effort to discover whether *mif* gene polymorphism was a factor in genetic susceptibility to human disease, the gene and promoter region were examined in control and disease populations. The MIF promoter region

contains two polymorphisms of note, a variable number of CAAT repeats (five to eight repeats at -825) and a single nucleotide polymorphism (at -173). These two promoter polymorphisms have been linked to several of the diseases listed above including pneumonia [103], arthritis (RA [85,104]), psoriasis [92], IBD [105], MS [90], obesity [106], SLE [88], asthma [107] and cancer [108–110], wherein the genetic variation that was associated with higher MIF expression (higher number of CAAT repeats at -825 and/or G > C at -173) correlated with susceptibility to disease. It is likely that *mif* polymorphisms will continue to be discovered and associated with various inflammatory diseases.

MIF inhibition studies: animal models

■ Autoimmune & autoimmune-associated diseases

Inflammatory bowel disease (or experimental colitis)

Inflammatory bowel disease encompasses diseases of the intestine including Crohn's disease and ulcerative colitis. It can be triggered in genetically susceptible individuals by a chemical or pathological insult and result in a breakdown of the intestinal epithelial barrier and T-cell apoptosis. As in other autoimmune-associated disease, macrophage activation and proinflammatory cytokine production (TNF-α, IL-1 and IL-6) are increased (reviewed elsewhere [111]).

Preliminary data in a small study demonstrated that MIF mRNA is elevated in patients with refractory ulcerative colitis [112]. Additionally, in murine dextran sodium sulfate-induced acute experimental colitis (DSS-colitis), evident increases in colonic mRNA and plasma MIF concentrations were noted [113]. Furthermore, MIF transgenic mice exhibit increased severity of colitis, as measured by body weight, colon thickening and rectal bleeding and show decreased survival [114]. By contrast, MIF knockout (k.o) mice were shown to be more resistant to experimentally induced acute colitis and had decreased plasma concentrations of proinflammatory (TH1) cytokines, TNF-α and IFN-γ and increased concentrations of IL-4, an anti-inflammatory (TH2) cytokine [115,116]. Treatment of T-cell-transferred/Rag2 k.o mice (another colitis model) with anti-MIF or anti-TNF-α antibodies (Abs) could effectively suppress colitis in mice, demonstrating the crucial role of these proinflammatory cytokines in this disease model [116]. As such, it is not surprising that MIF inhibitor ISO-1 (20 mg/kg intra-peritoneal [i.p.] daily) suppressed experimentally induced colitis in genetically manipulated mice (intestinal epithelial cell conditional von-Hippel-Lindau factor k.o mice) as demonstrated by improvements in rectal bleeding, body weight, diarrhea and colon length [66]. Proinflammatory cytokine (TNF-α, IFN-γ and IL-6) and mediator (iNOS, ICAM-1, COX-2) expression were also significantly reduced by ISO-1 in the aforementioned study. A fluorinated analog of ISO-1 (Cmpd 17 [28] aka ISO-F) was also tested in an animal model of DSS-induced colitis [31]. This ISO-1 derivative, when administered orally (25mg/kg; days 5–10) was found to improve measures of body weight, colon length and weight, colon and rectal bleeding, blood hemoglobin levels and stool consistency leading to overall improvements in the disease activity index in murine DSS-colitis. Additionally, it improved DSS-induced histological abnormalities including colonic edema, inflammatory cell infiltration, crypt damage and decreased colonic expression of TNF-α, IL-6 and IL-1β.

Type I diabetes

Initiation of Type 1 diabetes mellitus (T1D) is believed to be the result of destruction of β-cells from pancreatic islets by autoreactive T cells and macrophages leading to lack of insulin production and overt diabetes. MIF mRNA and protein levels are increased in murine models of T1D including the spontaneously diabetic non-obese diabetic (NOD) mouse [117] and the multiple low dose-streptozotocin (MLD–STZ) model [62], respectively, however these associations have yet to be confirmed in human studies.

Studies on MIF k.o mice demonstrate the importance of MIF in T1D as development of MLF–STZ diabetes is markedly suppressed in these mice [118]. Furthermore, anti-MIF Ab administration significantly attenuated markers of diabetes progression in NOD mice [118]. Administration of either anti-MIF Ab or ISO-1 (1mg/mouse/day i.p. × 14 days) significantly inhibited hyperglycemia and insulitis, decreased pancreatic MIF staining, reduced adhesive and proliferative properties and also reduced proinflammatory cytokine production (IFN-γ and TNF-α) of spleen mononuclear cells in the mouse model of MLD–STZ-induced T1D, recapitulating the phenotype observed in MIF k.o mice [62]. Additionally, MLD–STZ diabetic mice treated with anti-MIF Ab or ISO-1 (1mg/mouse/day i.p. × 14 days) showed decreased *ex vivo* pancreatic islet production of IL-12, IFN-γ, IL-1β,

IL-17 and NO, iNOS expression, NO accumulation [43]. Nitrate accumulation (NO production) was inhibited in fibroblasts, endothelial cells, insulinoma cell lines and pancreatic islets by ISO-1 (100–200 μg/ml) and TNF-α protein expression was inhibited by ISO-1 at doses between 50–100 μg/ml [43]. MIF inhibitors had no effect on the chemically ablative (non-autoimmune) high-dose streptozotocin-induced T1D model suggesting that the primary role of MIF may be in the very early autoimmune stages of this disease [62]. Taken together, these results demonstrate that MIF may be an important target for inhibition during the early stages of T1D disease development in animal models. Further research is needed to investigate the involvement of MIF in the first stages of human autoimmune T1D as studies in advanced stages of the disease have shown a negative association of serum MIF with islet auto-Ab levels (an indicator of disease progression) [119].

Rheumatoid arthritis & psoriasis

Rheumatoid arthritis is an autoimmune disease characterized by symmetrical joint inflammation and damage and is known to be triggered by both genetic and environmental factors. High levels of proinflammatory cytokines at diseased joints lead to permanent damage to cartilage and bone and current treatments include cytokine inhibition (TNF-α and IL-1) and methotrexate (reviewed elsewhere [120]).

Psoriasis is the most common of the autoimmune diseases and is defined by erythrosquamous lesions of the skin and nails and can manifest as several different clinical types. Approximately 20% of patients with psoriasis also develop psoriatic arthritis where joint involvement is usually asymmetric. Patients with psoriasis also have an increased rate of accompanying conditions such as metabolic syndrome, cardiovascular disease and stroke (reviewed elsewhere [121]).

Both RA and psoriasis patients exhibit increased serum MIF levels that correlate with disease activity [85,91,122]. Likewise, disease sites in both RA and psoriasis have shown increases in MIF (synovial fluid [123] and skin [124]). The CAAT repeat and the single nucleotide polymorphism (173 G > C) promoter polymorphisms of the *mif* gene have also been associated with disease in both RA and psoriasis [85,92,104]. Currently, anti-MIF Ab therapy has only been tested in rodent adjuvant- and collagen-induced arthritis models and has shown effectiveness in significantly decreasing disease severity [125,126]. Another study found that MIF k.o mice had reduced MMP-2 expression (responsible for matrix degradation of the joints) in antigen-induced arthritis models and that MIF-induced MMP-2 expression through signaling molecules PKC, JNK and Src [127]. So far, we know of no published studies examining the effectiveness of ISO-1 or other small-molecule MIF inhibitors in RA or psoriasis disease models.

Multiple sclerosis & Guillain–Barré Syndrome

Like other autoimmune associated diseases, MS is suspected to have a genetic component(s) that may be triggered by an environmental insult. Although the causative agent is unknown, MS is characterized by autoreactive $CD4^+$ T cells that enter the CNS and target the myelin sheath of axons. This results in demyelination and axon loss leading to progressive deterioration of motor and sensory function. MIF was shown to be elevated in the cerebrospinal fluid of relapsing MS patients [128], which led to a further study in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). Dekinger *et al.* demonstrated that anti-MIF Ab treatment decreased the expression of CNS VCAM-1, impaired targeting of neuroantigen-specific T cells to the CNS and reduced the clonal size of autoantigen-specific T cells leading to a reduced severity and accelerated recovery in EAE mice [129].

In addition, a recent study compared the effects of a genetic deletion of MIF and MIF reduction with ISO-1 analogs in the EAE mouse model. MIF k.o mice are susceptible to EAE-induced disease but demonstrated reduced disease severity [130], fewer inflammatory CNS lymphocyte infiltrates and reduced expression of VCAM-1 in the CNS [72]. Although in MIF inhibitor CPSI-1306 (orally dosed)-treated mice VCAM-1 expression was not reported, as predicted these mice displayed reduced numbers of F4/80+ macrophages in the perivascular space compared with vehicle-treated controls, leading to the conclusion that ongoing or previous CNS immune cell migration was not reversed but that new infiltration was prevented by CPSI-1306. In a relapsing/remitting EAE model, an orally dosed structurally related small-molecule MIF inhibitor (CPSI-2705) was able to protect animals from relapse and reduce the disease severity index compared with controls. Lastly, an increase in FoxP3 and $CD4^+/CD25^+$ lymphocytes was found in both MIF k.o and CPSI-1306-treated mice. They proposed that these regulatory

subsets of T cells could be controlled by MIF and involved in CNS inflammation or selective lymphocyte trafficking to the CNS [72].

Guillan–Barré Syndrome (GBS) is an inflammatory demyelinating disease of the peripheral nervous system. It presents with multifocal T cell and monocyte/macrophage infiltration around roots and nerves with secondary axonal degeneration in severe cases. Inflammatory cell infiltration and damage is mediated by pro-inflammatory cytokines/chemokines and MMPs [131]. As GBS shares features of MS, an investigation was launched investigating the role of MIF in this disease. Patients with GBS were found to have significantly elevated plasma MIF levels, which correlated with the disease severity score [63]. Furthermore, in experimental allergic neuritis, an animal model of the disease, mice receiving either anti-MIF mouse Abs or ISO-1 (1 mg/mouse – days 12–28/i.p.) were observed to have significantly reduced cumulative disease scores (grade of paralysis) and shortened duration of disease. They hypothesized that MIF antagonistic therapies were interfering with the MIF-mediated amplification of proinflammatory cytokine responses and the homing of T cells and macrophages to disease sites [63].

■ Infectious disease

Sepsis

Sepsis can occur as the result of infection, shock, trauma and/or injury. Proinflammatory mediators are induced and undergo paracrine and autocrine loops resulting in an immune system that is overactive and destructive to the organism. Symptoms of sepsis include altered body temperature, tachycardia, tachypnea, lactic acidosis, hypotension and hypoperfusion. Sepsis in humans is often lethal even with therapeutic intervention and current treatment modalities are multifaceted and include intravenous fluids, broad-spectrum antibiotics, glucocorticoids, insulin and human-activated protein C (sepsis reviewed elsewhere [132]).

Two widely used experimental models of sepsis include endotoxemia and CLP (sepsis). In the endotoxemia (i.e., endotoxic shock) model, LPS, an inflammatory bacterial cell wall component, is injected directly into mice and the inflammatory response is triggered exclusively by this molecule. In CLP-sepsis, the intestine is punctured leading to a polymicrobial stimulated septic response. Both models induce increases in plasma proinflammatory cytokine levels, including MIF [133], as seen in human sepsis [134].

In experimental murine CLP-sepsis, treatment with anti-MIF Ab increased survival by almost 50% [4,84]. MIF k.o mice also display reduced susceptibility to the lethality of endotoxic shock [135]. It has been proposed that the mechanism for this protection from sepsis is through early signaling intermediaries [136] and/or negative transcriptional regulation of the LPS co-receptor, TLR-4 [137,138]. As expected, administration of the MIF inhibitor, ISO-1, significantly improved survival in experimental models of endotoxemia (threefold) and polymicrobial sepsis (twofold), when given concurrently (endotoxemia) or up to 24 h after induction of disease (CLP-sepsis) at a dose of 35 mg/kg, twice daily for 3 days [16].

Pneumonia

Pneumonia is the end result of infection with one or more of many possible bacteria [139] or viruses (e.g., H5N1, H1N1, coronaviruses, hantavirus, influenza A or B, parainfluenza 12 and 3, respiratory syncytial virus and adenovirus) and key features include inflammation of the lung parenchyma with abnormalities in gas exchange. X-ray or CT scans can be used to detect inflammatory infiltrates in the lungs. Research has indicated that elevated levels of serum proinflammatory cytokines and chemokines frequently accompany symptoms in some forms of pneumonia [140–142]. In general, lung inflammation during either viral or bacterial pneumonia is mediated by neutrophil and macrophage recruitment. Insufficient neutrophil recruitment during pneumonia may lead to decreased pathogen clearance and conversely, excessive neutrophil recruitment can cause excessive inflammation, both resulting in irreversible lung damage and morbidity [139].

Some studies do not support the negative role of MIF in models of lung injury or pneumonia. Analysis of the MIF high expression promoter polymorphism (G/C at -173) in pneumonia patients actually revealed a beneficial effect on 90-day mortality rates [103]. Additionally, although ISO-1 reduced cytokine levels of IL-1β, IL-6, TNF-α and chemokine IP-10 and lung pathology in H5N1-infected mice, it had no effect on their ultimate survival rates [17]. Takahashi *et al.* used recombinant MIF (rMIF) in mice to successfully induce alveolar neutrophil recruitment and lung inflammation [48]. They found that anti-MIF therapies including anti-CD74 Ab and ISO-1 could significantly reduce chemokine release and alveolar neutrophil accumulation and that signaling occurred

via the p44/42 MAPK pathway. The effect of rMIF or anti-MIF therapies on pneumonia recovery or survival was not examined. These results suggest that MIF may be necessary for an effective response in lung inflammation and pneumonia and that a MIF-induced cytokine storm in the lungs is not necessarily the cause of lethality.

Inflammatory (viral) & reactive (bacterial) arthritis

Viral and bacterial arthritic diseases are the result of previous or concurrent infection with a microorganism, which results in asymmetric (distinguishable from RA), nonpurulent joint inflammation and possible other extra-articular manifestations. Each type has specific diagnostic criteria that allow it to be discerned from other forms of arthritis, however, there remains some controversy with regards to whether active microorganisms or simply its antigen, or DNA/RNA are present in the infected joint. There is also evidence that infectious arthritis may lead to rheumatologic arthritis and disease due to molecular mimicry or other pathways (reviewed elsewhere [143]). Antibiotic treatment differs depending on the type of infecting organism and may resolve symptoms, but some patients go on to develop chronic arthritis. Standard therapies include NSAIDs, articular steroid injections and physical therapy [144,145]. Features common to these diseases, their murine models and RA are immune cell activation in the affected joint (either via antigen stimulation or immune complex activation) and increased proinflammatory cytokine and MMP production by synovial macrophages (TNF-α, MIF) [146–148]. The ability of the virus to replicate in and mediate inflammation in the articular macrophages has been demonstrated by several groups, underscoring the key role of this immune cell in viral arthritis [149–151].

In an effort to link macrophages and their immune products to the pathogenesis observed in viral arthritis, Assuncao-Miranda *et al.* recently showed that Sindbis virus (known to cause arthritis in humans) could replicate in and stimulate the release of proinflammatory proteins including MIF, TNF-α, IL-1β, IL-6 and MMPs from human and mouse monocyte-derived macrophages *in vitro* [56]. MIF secretion was found to be stimulated threefold when compared in Sindbis-infected macrophages versus controls. Furthermore, inhibition of MIF using either anti-MIF Ab or ISO-1, attenuated TNF-α and IL-6 secretion by 50% and blocked MMP-1 and MMP-3 gene expression by more than half. Additional evidence for the role of MIF in the cytokine, MMP cascade in virally infected macrophages was provided by testing macrophages from MIF k.o mice. Murine MIF k.o macrophages infected with Sindbis virus produced extremely low levels of TNF-α and IL-6 [56], suggesting that MIF is critical in macrophage-mediated pathways of joint inflammation and destruction (MMPs).

■ Other diseases

Type 2 diabetes

Type 2 diabetes is considered a multifactorial disease induced by environmental (diet or oxidative stress), genetic (gene polymorphisms) and/or epigenetic factors that manifest in insulinemia, insulin resistance, hyperglycemia and dyslipidemia. This form of diabetes is often associated with obesity, cardiovascular disease and metabolic syndrome. Metabolic syndrome can be characterized by a cluster of abnormalities including glucose intolerance (insulin resistance/T2D), obesity, hypertension and dyslipidemia (high triglycerides, low high-density lipoprotein cholesterol) [152]. There is a general state of inflammation in T2D and metabolic syndrome and elevations in plasma MIF and proinflammatory cytokines (TNF-α) have been described [73,153–157]. There is some variation in the literature with regards to association of obesity/T2D with elevated plasma MIF levels but factors such as gender, use of hormone replacement therapy in women, diurnal cycle and gene polymorphisms may not have been taken into account (reviewed elsewhere [158]).

In vitro studies have shown that glucose can stimulate MIF secretion from rat pancreatic β-cells in a dose- and time-dependent manner, in addition, an autocrine role for MIF in regulating glucose-induced insulin secretion from pancreatic β-islets was observed [159]. This stimulation of insulin secretion could be inhibited by anti-MIF Abs underscoring MIF's role in endocrine and carbohydrate metabolism [159]. Animal models of T2D generally have obesity as a component (spontaneous or diet-induced), however a single low dose of streptozotocin can also be used to induce a state similar to T2D. Sanchez-Zamora *et al.* induced T2D with STZ in balb/c mice and compared disease indicators with STZ-treated MIF k.o mice. They found STZ-treated MIF k.o mice were resistant to STZ–T2D (no measurable clinical disease)

and had measurably reduced glycemia, serum proinflammatory cytokines and resistin levels and polyuria. They also used the STZ–T2D model in outbred ICR mice and discovered an ISO-1 analog (CPSI-1306) could reduce measures of T2D including hyperglycemia, serum IL-6 and TNF-α levels [73]. Efficacies of other small-molecule MIF inhibitors in T2D have yet to be described.

■ Cancer: a special case

The macrophage migration inhibitory factor has been implicated in several preclinical cancer models and many forms of human cancer. It plays a role in many aspects of cancer progression including angiogenesis [160], metastasis [161], apoptosis [162] and cancer cell growth [163], in addition to immune system mediation [164]. The pro-neoplastic activities of MIF are thought to be the result of a chronic activation of the gene and may be different from its acute proinflammatory properties. The association between chronic inflammation and cancer has long been recognized and has been reviewed elsewhere [165–167].

Macrophage migration inhibitory factor mRNA has been shown to be increased in prostate, colon and hepatocellular cancer, as well as, adenocarcinoma of the lung, melanoma and glioblastoma (reviewed elsewhere [168]). Addition of MIF to endothelial cells was shown to enhance angiogenesis [169,170], thus potentially leading to enhanced tumor growth. In addition to the angiogenic effect of MIF, it also exhibits antiapoptotic effects on cells through inhibition of the p53 gene via the PI3k/Akt pathway [170,171]. Hindrance of contact inhibition and stimulation of cell cycle progression have also been linked to the tumor-promoting activities of MIF [51,163].

Studies with prostate cancer cell lines have shown that ISO-1 and other anti-MIF therapies (RNAi, anti-MIF Ab and anti-CD74 Ab) were effective in reducing cell proliferation, MIF protein secretion and invasion only in an androgen-independent cell line (DU-145). Further studies using DU-145 xenografts in mice established that ISO-1 treatment could significantly reduce mean tumor volume, weight and neovascularization [39].

Additional studies using MIF-directed small-interfering RNA and ISO-1 have implicated MIF's involvement in the migration and invasive potential of human lung adenocarcinoma cells [41]. Furthermore, inhibition studies with ISO-1 and anti-CD74 Ab provided evidence that MIF secretion from human chronic lymphocytic leukemia B-cells regulated IL-8 secretion. In turn, this CD74-stimulated IL-8 secretion then activated survival pathways by regulating Bcl-2, potentially linking MIF to an anti-apoptotic pathway [42].

Another group has shown that anti-MIF therapies, including anti-MIF Ab and ISO-1, could decrease tumor volume, weight and incidence of hepatic metastasis in a mouse model of colon carcinoma. Human colorectal cancer cell line CT26 was also found to have greatly reduced cell proliferation after ISO-1 treatment [45].

Shrader *et al.* provided evidence that ISO-1, as well as antisense MIF-directed RNA and anti-MIF Ab could reduce the growth rate of glioblastoma cells lines. In this study ISO-1 was also demonstrated to inhibit MIF mitogenic signaling through MAPK/Erk and PI3K/Akt pathways [51]. Finally, ISO-1 was demonstrated to inhibit the stimulatory effect of exogenous MIF on U373 MG glioblastoma cell proliferation and to reduce migration and invasion potential of HS683 glioma cells. In HS683 cells, ISO-1 could induce sensitivity to the inhibitory effects of dexamethasone on migration and invasion, and evidence suggested this occurred through competition between glucocorticoid receptor and MIF receptor signaling pathways for Erk 1/2 and MAPK [50].

In summary, MIF inhibition in cancer may require extracellular and intracellular targeting due to its multiple roles in this disease. A fluorescent derivative of ISO-1 has been shown to be internalized by cells (RAW 264.7) and localizes to the cytoplasm and nuclei [16]. Furthermore, pretreatment of RAW cells with ISO-1 significantly decreased endogenous MIF tautomerase activity [16], providing evidence that nonderivatized ISO-1 is also capable of entering cells. Anti-MIF therapies and drugs, which can enter the cell and access the desired cellular compartment will be an active area of research.

Past perspective

Research on the macrophage migration inhibitory factor has taken many paths over the years. After its initial discovery as a mediator of macrophage migration in the 1960s, research on its other immune functions and role in disease was not reinitiated until the 1990s. Its intracellular, extracellular, local and systemic roles make it a complex drug target, yet inhibition studies (using both neutralizing Abs and

ISO-1 or other small-molecule inhibitors) have shown profound results in many of the disease models tested. Perhaps this is the result of reducing the elevations of MIF found in each disease to a normal level, turning off the autocrine/paracrine loops and allowing the immune system to reset. ISO-1 continues to be the MIF inhibitor used the most in preclinical studies, as it was one of the first to be tested and found effective in multiple disease models. This drug represents an effective positive control for comparison to newer and more potent MIF inhibitors. Recent studies with ISO-1 have shown that it can also block the MIF-induced expression of COX-2 in human ectoptic endometrial cells [52], decrease pain-associated flinching in a formalin-induced hyperalgesia model (likely via a downstream effect of MIF on NMDA receptors) [67], enhance the migration of mesenchymal stem cells to epithelial cells in a model of lung repair [53], reduce the toxic effect of Alzheimer's β-peptides on SHSY neuroblastoma cell lines [54], abrogate measures of lung injury and reduce TGF-β in the ovalbumin-induced mouse asthma model [68], inhibit proliferation of neurogenic hippocampal cells (a negative effect in anxiety/depression syndromes) [69] and inhibit the proinflammatory responses of macrophages infected with dengue virus [9]. These, as well as previous studies provide strong support for the rationale of the 'pocket inhibitor' design of compounds to antagonize MIF biological activities.

Future perspective

In light of the extensive research taking place on small-molecule MIF inhibitors, it is likely to continue to be a competitive field as lead compounds with the most biological inhibition, least toxicity and best bioavailability (delivery, stability and clearance) are narrowed down. ISO-1 and its derivatives will undoubtedly be tested in animal models of RA, psoriasis, sarcoidosis, MS, SLE, obesity and atherosclerosis. As more is discovered about the different biological roles of MIF, research into proteins that bind and interact with MIF will lead to the design and development of compounds that specifically agonize or antagonize those interactions. We can foresee numerous compounds making it to clinical testing based on their specific ability to inhibit or enhance the different biological effects known to be mediated by MIF thus far. ISO-1 and derivatives that inhibit the catalytically active site (proinflammatory pocket) seem the most effective in the disease models studied so far. The natural ligand for the proinflammatory pocket of MIF has yet to be described and will further elucidate the complex nature of this multifunctional protein.

TNF-α is another proinflammatory cytokine that plays a crucial role in many of the disease states and models described herein. Its inhibition in disease models using Abs, k.o strategies and RNAi is well documented and has led to the current anti-TNF therapies used in autoimmune disease (reviewed elsewhere [172,173]). Clinically, anti-TNF therapies have been shown to produce some undesirable side effects in certain individuals, leaving room for the development of drugs targeting upstream, downstream and parallel pathways. The reciprocal paracrine loops existing between TNF-α and MIF [4] represents a novel target for inhibition of these cytokines. Evidence for this theory was demonstrated by Wijbrandts *et al.* when sustained plasma reductions of MIF were noted in RA patients undergoing anti-TNF Ab treatment (adalimumab) compared with those not receiving anti-TNF treatment [122]. It is likely that small-molecule MIF inhibitors could be used to replace the anti-TNF therapies to modulate proinflammatory cytokines levels.

We believe there is compelling evidence for the effectiveness of anti-MIF therapies in animal disease models and the potential for MIF-targeted therapies to be just as effective in the respective human diseases where MIF is elevated. ISO-1, its derivatives and other small-molecule compounds targeting MIF will continue to serve as attractive candidates for further testing and study in diseases where MIF has been implicated.

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Yousef Al-Abed is the inventor of ISO-1 and inventor or co-inventor of several other small-molecule MIF inhibitors. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Executive summary

Macrophage migration inhibitory factor: main biological activities

- Induced by inflammatory stimuli, lipopolysaccharide, TNF-α and IFN-γ
- Inhibits macrophage migration (chemotaxis)
- Antagonizes glucocorticoids suppressive effects on inflammation
- Stimulates proinflammatory cytokine production
- Roles in cell growth/cell cycle checkpoint regulation (Jab-1), angiogenesis (cancer) and apoptosis.

MIF receptors/co-receptors/signaling

- $CD\text{-}74^{+/-}$ CD-44:
  - ERK ½ - PGE_2
  - Regulated intracellular proteolysis and subsequent NFκB/p65/Rel A–TAF2 activation
  - Serine PO_4
  - Co-receptors CXCR2, CXCR4 > $G\alpha_{\alpha l}$, integrins, calcium influx and Akt activation

Main MIF catalytic site inhibitors with potential for further development:

- SO-1 and derivative compounds 17 and 7 (Al-Abed Compounds) [25,28,29]
- OXIM-11 (Al-Abed) [30]
- 4-IPP (Mitchell) [33]
- AV411 (ibudilast), AV1013 (noncompetitive, allosteric inhibitors of macrophage migration inhibitory factor [MIF]) (Lolis) [36]
- Dagia Compounds (ISO-1 derivatives) [32]
- Jorgensen Compounds (from Zinc and Maybridge Libraries) [34]
- Lashuel Compounds (from NINDS Custom Collection II and Maybridge Libraries ; HCLP, Ebselen and lead compounds) [35]
- CPSI-1306/2705 [73]
- Avanir (Novartis Compounds) [31]

MIF elevations in disease states & models

- Rheumatologic:
  - Rheumatoid arthritis
  - Inflammatory bowel disease
  - Psoriasis
  - Endometriosis
  - Systemic lupus erythematosus
  - Multiple sclerosis
- Infectious:
  - Sepsis
  - Pneumonia
  - Viral arthritis
- Genetic/Environmental:
  - Cancer
  - Metabolic syndrome/type II diabetes
  - Polycystic ovary syndrome
  - Asthma
  - Atherosclerosis

ISO-1 studies

- Efficacy demonstrated in:
  - Murine colitis model (von-Hippel-Lindau factor knockout)
  - Murine type I diabetes Mellitus model (multiple low-dose streptozotocin)
  - Murine endotoxemia and sepsis (cecal ligation and puncture) models
  - Murine pneumonia models (reduction of cytokine levels and lung pathology, but no increase in overall survival)
  - *In vitro* murine viral arthritis studies
  - Prostate cancer cell line (DU-145) and murine prostate cancer xenograft models
  - Human lung adenocarcinoma cells and chronic lymphocytic leukemia B-cells
  - Human colorectal cancer cell line (CT26) and murine colon cancer model
  - Glioblastoma cell lines (LN229, LN18, U373 and HS683)
  - Human endometrial cells
  - Murine hyperalgesia (pain) model
  - Models of lung injury and repair (stem cells)
  - *In vitro* Alzheimer's model
  - Murine asthma model
  - Murine dengue-virus infection

Executive summary (cont.).

Past and future perspective

- MIF has intracellular, extracellular, local and systemic roles making it a complex drug target
- ISO-1 is the MIF inhibitor used most in initial disease models and for comparison in MIF inhibitor screens
- Other diseases and models where small-molecule MIF inhibitors may prove effective include rheumatoid arthritis, psoriasis, endometriosis, stem cell therapies, Alzheimer's disease and asthma
- Lead small-molecule MIF-inhibiting compounds will be screened for biological potency, bioavailability and toxicity and best candidates will be further developed
- As more is learned about MIF's biological functions and interacting proteins, compounds will likely be developed to specifically agonize or antagonize those interactions
- Small-molecule MIF inhibitors are attractive candidates for clinical testing in human diseases where MIF elevations are present

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