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Teaser MIF and D-DT exert a broad range of functions, and their expression is often dysregulated in immune-inflammatory and chronic disorders. Pharmacological strategies targeting MIF and/or D-DT could represent a novel therapeutic avenue for human diseases.

Role of MIF and D-DT in immune-inflammatory, autoimmune, and chronic respiratory diseases: from pathogenic factors to therapeutic targets

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Macrophage migration inhibitory factor (MIF) is a protein that acts as a cytokine-, enzyme-, endocrine- and chaperon-like molecule. It binds to the cell-surface receptor CD74 in association with CD44, which activates the downstream signal transduction pathway. In addition, MIF acts also as a noncognate ligand for C-X-C chemokine receptor type 2 (CXCR2), type 4 (CXCR4), and type 7 (CXCR7). Recently, D-dopachrome tautomerase (D-DT), a second member of the MIF superfamily, was identified. From a pharmacological and clinical point of view, the nonredundant biological properties of MIF and D-DT anticipate potential synergisms from their simultaneous inhibition. Here, we focus on the role of MIF and D-DT in human immune-inflammatory, autoimmune, and chronic respiratory diseases, providing an update on the progress made in the identification of specific small-molecule inhibitors of these proteins.

Introduction

MIF was discovered at the end of the 1960s and acquired its name from its ability to inhibit the migration of macrophages. It is a pleiotropic protein sharing cytokine, endocrine molecular, chaperon-like protein, and enzyme-like properties [1,2] In addition to its role in the inflammatory and immune response, MIF also functions as a hormone, released by the anterior pituitary and adrenal gland in tandem with hypothalamic–pituitary–adrenal axis (HPA) activation. Furthermore, MIF serves as a cytosolic chaperon-like protein and exhibits multiple intrinsic enzymatic

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activities, such as D-dopachrome, phenylpyruvate tautomerase, and thiol-protein oxidoreductase activities.

Binding of MIF to its cell membrane high-affinity receptor CD74 (also known as the HLA class II histocompatibility antigen gamma chain) leads to recruitment of the cell-surface glycoprotein CD44 and subsequently mediates an intracellular signaling through activation of several signaling pathways, including, among others, the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK), Src, phosphoinositide 3-kinase (PI3K)/Akt, and nuclear factor (NF)-κB pathways [2,3]. In addition, MIF acts also as a noncognate ligand for the chemokine receptors, CXCR2, CXCR4, and CXCR7.

A second member of the MIF family, D-DT (also known as MIF-2), was recently characterized [4]. Both similarities and differences between MIF and D-DT have been reported. Whereas MIF catalyzes the keto-enol tautomerization of the non-naturally occurring compound D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid, D-DT produces 5,6-dihydroxyindole from keto-enol tautomerization and decarboxylation. In addition, D-DT is ten times less active than MIF in its tautomerase enzymatic activity and engages and activates the CD74 receptor, with a threefold higher acid dissociation constant and an 11-fold higher dissociation rate compared with MIF. As pointed out by Merck and collaborators, these differences could underlie the biological effects of the two proteins; for instance, D-DT binding to the receptor might not always trigger a signaling cascade, but could lead to its internalization [4].

In contrast to MIF, D-DT lacks the pseudo-(E)LR (Arg11, Asp44) motif that allows MIF to bind CXCR2 and CXCR4 [4,5]. Although our knowledge is still fragmentary, the structural differences between these two proteins could explain why, despite several synergistic actions of MIF and D-DT in multiple biological settings [4,6,7], these cytokines might also display opposite effects in certain settings, including adipose tissue and wound repair and myocardial ischemic-reperfusion injury [6,8]. Moreover, whereas plasma MIF and D-DT concentrations are similar, both under basal and septic conditions, lipopolysaccharide (LPS)-stimulated macrophages produce 20-fold more MIF than D-DT, suggesting that other *in vivo* sources of D-DT contribute to plasma D-DT levels [4]. However, better characterization of the biological activity of D-DT compared with that of MIF is required for the development of therapeutic strategies aimed at targeting the MIF superfamily signaling pathway.

In addition, anti-inflammatory properties of MIF and D-DT can be explained via activation of AMP kinase (AMPK), which is an endogenous downregulator of inflammatory responses [9]. In agreement with this concept, MIF can stimulate the secretion of both T helper type 1 and 2 (Th1 and Th2) cytokines by T cells, as well as interleukin (IL)-17 by lymph node cells, suggesting no single clear role in T cell polarization [2]. For example, MIF was protective in a model of acute kidney injury, despite the apparent paradox that this model is prevented by glucocorticoids (GC) and that MIF is a known GC antagonist [10]. Given that MIF and D-DT can also display anti-inflammatory effects, their tailored inhibition might not unequivocally improve all autoimmune and immunoinflammatory diseases. The possible dichotomous actions of cytokines in autoimmune diseases are clear, given the disease-worsening effects of antitumor necrosis factor (TNF)-α in patients with multiple sclerosis (MS), despite the clear beneficial effects associated with specific TNF inhibitors in multiple autoimmune diseases [11].

MIF and D-DT expression and regulation

MIF crystallizes as a homotrimer with a molecular mass of 37.5 kDa. The protein is ubiquitously expressed in all mammalian tissues and is detectable in the blood plasma at ng/ml concentrations [2,12]. The MIF protein is encoded by a single gene located on chromosome 22q11, which is in close proximity to three other genes, namely, *MMP11* (encodes stromelysin-3) and *GSTT1* and *GSTT2* (encode the glutathione *S*-transferase theta-1 and -2, respectively). Both the exonic structure and DNA sequence of MIF are highly conserved across phylogeny. MIF is present in fish, nematodes, and protozoa, but not in *Drosophila* and yeast. There is a 100% sequence identity between human and nonhuman primates and ~20% sequence identity between human and protozoa [5]. The human *Mif* gene comprises three exons (exon 1, 107 bp; exon 2, 172 bp; exon 3, 66 bp) and two introns (intron 1, 190 bp; intron 2, 96 bp). Importantly, two functional polymorphisms control MIF promoter activity and MIF expression levels, namely the −794 CATT 5–8 microsatellite repeat (rs5844572) and the −173 G > C SNP (rs755622) [2,13]. MIF expression correlates with the number of the CATT repeats at position −794, whereas the −173C allele is associated with higher MIF promoter activity, because of its linkage disequilibrium with the −794 CATT7 variant [2,13]. The transcription factor ICBP90 is crucial for MIF transcription in macrophages, lymphocytes, and synovial fibroblasts, and Toll-like receptor (TLR)-induced MIF expression is controlled in an ICBP90- and −794 CATT5–8 length-dependent manner [14]. MIF expression is influenced by both genetic and epigenetic mechanisms [15] and the activity of the MIF promoter is significantly upregulated in response to hypoxia [16].

MIF secretion occurs via an unconventional export route, because it lacks an N-terminal signal sequence and inhibitors of the endoplasmic reticulum (ER)/Golgi transport, such as brefeldin A and monensin, do not influence MIF release from lipopolysaccharide (LPS)-challenged THP-1 monocytes [17].

MIF interacts with the Golgi complex-associated protein p115 in the cytoplasm, regulating its release from cells. Indeed, genetic depletion of p115 in monocytes and/or macrophages reduces the release of MIF, but does not affect the secretion of tumor necrosis factor (TNF)-α and IL-6 by endotoxin-stimulated monocytes. Interestingly, the MIF inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) inhibits MIF secretion by targeting the interaction between MIF and p115 [18].

In humans, D-DT is located ~80 kb from the *Mif* gene on chromosome 22. Similarly to MIF, D-DT comprises three exons and two introns and shows predicted binding sites for SP-1 and CREB. The human D-DT protein shares a 35% sequence similarity with MIF and catalyzes a similar enzymatic reaction. D-DT is ubiquitously expressed, with the highest levels detected in liver and testis. Furthermore, D-DT can be found in the cytoplasm and its release from necrotic cells suggests that it serves as a damage-associated molecular pattern (DAMP).

The multiple physiological functions of MIF and D-DT

Several studies identified MIF as a key modulator in promoting and modulating the magnitude of the inflammatory response. Differ-

ent cells of the innate and adaptive immune system and activated endothelial cells can release MIF and/or D-DT upon stimulation by stress, endotoxins, inflammatory, and immune stimuli. MIF has a pivotal upstream role in the inflammatory cascade by promoting the secretion of other inflammatory mediators, including TNF-α, IL-1, IL-6, IL-8, IL-12, interferon (IFN)-γ, cyclooxygenase-2 (COX-2), nitric oxide (NO), and matrix metalloproteinases (MMPs) [9,19]. MIF also supports macrophage survival and modulates their functions by inhibiting p53 [2,20]. In LPS-challenged mice, as well as in patients with sepsis, circulating levels of both D-DT and MIF increase with similar kinetics, although LPS-stimulated macrophages produce 20-fold more MIF than D-DT [4]. In addition, the inhibition of either D-DT or MIF in a murine model of endotoxemia showed improved survival rates compared with controls in a manner comparable with that achieved with anti-MIF antibody [4]. Likewise, MIF-knockout (KO) mice are resistant to high doses of LPS and have lower plasma TNF-α levels compared with wild-type mice [2,21]. In a model of cecal ligation and puncture, which leads to bacterial peritonitis, elevated MIF levels were found in the peritoneal fluid and in the systemic circulation of mice, and anti-MIF antibody treatment protected from lethality [22]. Consistent with these observations, staphylococcal toxic-shock syndrome toxin 1 (TSST1) or streptococcal pyrogenic exotoxin A (SPEA) induces MIF production, and mortality can be prevented by MIF immune -neutralization [23]. In addition, MIF upregulates TLR-4 expression [1] and counter-regulates the anti-inflammatory and immunosuppressive effects of GC [24].

In B cells, MIF binding to CD74 triggers a signaling pathway that involves the activation of the NF-κB p65/RelA homodimer and its co-activator, TAFII105, thus regulating the transcription of genes that control cell survival and proliferation. In particular, MIF has been shown to regulate cell entry into S-phase by increasing the levels of cyclin E and the expression of the antiapoptotic gene, *Bcl2* [25].

MIF is a major endogenous GC antagonist because it counteracts the action of GC at both the transcriptional and post-transcriptional level. The anti-GC effect of MIF depends on the activation by MIF of MAPK phosphatase-1 (MKP-1), a crucial MAPK signaling inhibitor, through which GCs signal to suppress proinflammatory cytokine secretion, as well as, on the prevention of cortisol-induced increase- in cytosolic IκBα, which in turn results in intensified NF-κB translocation to the nucleus [2,26]. Although further studies are required, recent investigations revealed that MIF is a nuclease that functions in poly-ADP-ribosylation (PARylation) PARP-1-dependent DNA fragmentation [27].

MIF and D-DT as promising therapeutic targets in several human diseases

Although the advent of several monoclonal antibodies (mAbs) directed against proinflammatory cytokines or their receptors has dramatically changed the course of several immunoinflammatory and autoimmune diseases, including inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and psoriasis, other autoimmune pathologies, such as MS, Guillain–Barré syndrome (GBS), and type 1 diabetes mellitus (T1DM), are not responsive to these treatments. In addition, several limitations dampen the use of mAbs in patients with autoimmune diseases, including the occurrence of primary nonresponders in approximately one-third of patients and the immunogenicity failure secondary to production of antibody in some 10% of treated patients per year [28].

In addition, these mAbs can be associated with significant adverse events, such as opportunistic infections and immune complications. Furthermore, they need parenteral administration and bear heavy healthcare costs [29,30].

The biological profile of MIF and D-DT has attracted attention because it might indicate the potential of these cytokines as novel therapeutic targets for autoimmune diseases, chronic respiratory diseases, cancer, and chronic inflammatory, cardiovascular, and neurological diseases. Thus, here, we review the emerging pathogenic contribution of MIF in immune-mediated inflammatory and autoimmune disease and chronic respiratory diseases, where solid evidence converging from clinical and preclinical data has been generated. These include autoimmune hepatitis (AIH), IBDs, RA, T1DM, MS, systemic lupus erythematosus (SLE), and GBS. The role of D-DT in these conditions is limited to pulmonary arterial hypertension (PAH) and MS.

Autoimmune hepatitis

AIH is an immune-mediated inflammatory disease of the liver that occurs at all ages with a female predominance [31]. Although most patients with AIH respond to standard-of-care (SOC) treatment with prednisone and azathioprine, a significant proportion m exhibit partial or poor response and can progress to cirrhosis.

Regarding the role of MIF in AIH, it has been demonstrated that populations in the USA and Japan expressing the MIF-173 CC/GC genotypes correlated with more severe forms of AIH. Therefore, this polymorphism might represent a biomarker predictive of disease severity and therapeutic responses [32]. Patients with AIH from both the USA and Japan exhibited higher circulating levels of MIF [32]. Preclinical data demonstrated that MIF-KO mice were protected from the development of clinical and histological signs of hepatitis induced by concanavalin A compared with their wild-type counterparts [33]. Studies on the eventual contribution of D-DT to the development of AIH hepatitis in humans and rodent models are not yet available.

Inflammatory bowel diseases

IBDs are chronic immune-mediated disorders of the gastrointestinal tract that usually require lifelong medical therapy and eventually surgical intervention in severe disease. A meta-analysis showed that 1, 5-, and 10-year risk for surgical intervention for Crohn's disease was 16.3%, 33.3%, and 46.6%, respectively. The risk of surgery 1, 5, and 10 years after diagnosis of ulcerative colitis was lower, 4.9%, 11.6% and 15.6%, respectively [34].

Until the advent of biologic treatments with TNF-α inhibitors in 1997 [35], SOC treatment for IBD comprised steroids for the induction of remission and immunomodulatory drugs, such as azathioprine, mercaptopurine, or methotrexate to maintain remission. Subsequently, a variety of anti-TNF and other biological agents targeting TNF and other cytokines were introduced. However, many patients are still treated with various immunosuppressive medications because of a lack of efficacy, loss of response, or drug intolerance.

Several studies have evaluated the role of MIF in IBD. A meta-analysis indicated that MIF-173G/C polymorphism is associated with higher IBD risk, and increased circulating MIF levels are

reported in this context [36,37]. Preclinical studies showed that either anti-MIF antibody or genetic deletion of MIF by gene knockout conferred protection in a model of immune-inflammatory colitis induced by dextran-sulfate sodium (DSS) [38]. In addition, transgenic overexpression of MIF augmented the susceptibility of mice to DSS-induced colitis [38,39].

Systemic lupus erythematosus

SLE is a heterogeneous autoimmune disease that preferentially affects young women. Both Th1 and Th2-mediated events appear to be implicated in its pathogenesis [40]. Except for the recently approved anti-BAFF mAb belimumab, current SOC treatments, including corticosteroids, cyclophosphamide, cyclosporine A, chloroquine, methotrexate, and azathioprine, have limited efficacy and several adverse effects. These include: increased risk of infection and neoplasia; bladder toxicity for cyclophosphamide; hypertension and dyslipidemia for GCs and calcineurin inhibitors; and osteoporosis and hyperglycemia for GCs. The adverse effects associated with SLE SOC were recently reviewed elsewhere [41]. Although the prognosis has considerably improved over the past 20 years, novel therapeutic approaches are needed.

MIF has a complex role in SLE [2]. Initially, significant associations of genotypes carrying the −794 CATT7 and −173(*) C risk alleles with susceptibility to SLE and with a significant increase of TNF-α were found in a Mexican-Mestizo population. A meta-analysis demonstrated that, whereas MIF levels are increased in patients with SLE, there is no evidence of associations between MIF −173C/G and −794CATT5-8 polymorphisms and SLE susceptibility [2,42]. Another gene association study revealed that individuals with a high-expression MIF allele had reduced incidence of SLE. However, those patients with SLE and end-organ complications had increased frequency of high-expression MIF alleles. Plasma MIF levels and TLR-stimulated MIF production also reflect the underlying MIF genotype. Hence, MIF might exert a dual influence on the immunopathogenesis of SLE, with high-expression MIF alleles being protective, perhaps by stimulating the defective clearance of autoimmunogenic pathogens. However, once SLE develops, low-expression MIF alleles protect from ensuing inflammatory end-organ damage [2,43].

MIF levels are increased in patients with SLE, although this can be partly explained by increased GC use. However, GC-induced MIF levels have been positively associated with SLE disease damage (SLICC/ACR index) [2].

In patients with SLE, MIF is also involved in steroid resistance development by affecting the NF-κB/IκB signaling cascade [44]. The authors showed that the levels of MIF in serum and peripheral blood mononuclear cells (PBMCs) were higher in patients who were steroid resistant compared with those in patients who were steroid sensitive and healthy subjects. Also, in PBMCs from patients who were steroid resistant, NF-κB levels were significantly higher, whereas IκB levels lower, and were modulated upon MIF silencing.

Endogenous MIF is essential in murine SLE development (*Mif*$^{-/-}$*MRL/lpr* mice) because KO mice develop a milder disease than wild-type *Mif*$^{+/+}$*MRL/lpr-lpr* mice and ISO1 exerts beneficial effects both in SLE-prone MRL/*lpr* mice and NZB/NZW F1 mice. A Phase I study with anti-MIF mAbs has been carried out (NCT01541670) but no data have been released so far. In addition, a Phase Ib study is being carried out in patients with SLE using milatuzumab, a mAb directed against the MIF receptor, CD74 (NCT01845740).

Rheumatoid arthritis

RA is an autoimmune disease characterized by persistent synovitis in the joints and systemic inflammation. Nonsteroidal anti-inflammatory drugs (NSAIDs), oral corticosteroids, and biologic disease-modifying antirheumatic drugs (DMARDs) are widely used to treat RA. However, as discussed earlier, a significant number of patients still have inadequate responses to DMARDs and require novel therapeutic options.

The role of MIF in RA has been a focus of research [45]. A meta-analyses demonstrated increased circulating MIF levels in patients with RA, and associations between the MIF-173C/G and −794CATT5-8 polymorphisms and RA susceptibility were shown [46].

Analysis of the −173G > C and CATT(5-8) MIF polymorphisms in patients with RA and healthy controls showed a similar frequency of genotypes and haplotypes in the two groups. By contrast, joint damage was significantly higher in patients carrying the MIF −173G > C or the MIF CATT(5-8) variants, but no synergism between these genetic variants was found. The MIF −173C and MIF CATT7 alleles were associated with significantly higher circulating MIF levels compared with individuals carrying none of these alleles, and increased plasma concentrations of MIF correlated with more severe joint damage. Therefore, MIF polymorphisms are not associated with RA susceptibility but are associated with high levels of joint damage [13,47]. More recently, the functional regulation of CD44 by high-expression MIF alleles has been described [48].

In RA, synovial MIF concentrations correlate with disease activity [49]. In patients positively responding to anti-IL-6 receptor blockade with tocilizumab, serum MIF levels are significantly reduced, in contrast to other proinflammatory mediators [50]. The pathogenic role of MIF in RA is also supported by studies on preclinical models of RA [1,51–53]. For example, synovial inflammation induced by anti-CII Ab/LPS was reduced in MIF-KO mice compared with wild-type mice. In addition, MIF-KO mice exhibited reduced proliferation of synoviocytes and an increase in p53 expression and apoptosis [52]. In a rodent model of RA, administration of recombinant MIF reversed the inhibition of antigen-induced arthritis exerted by dexamethasone to a severity score superimposable to that of mice treated with recombinant MIF alone [54].

Type 1 diabetes mellitus

T1DM is an autoimmune disease characterized by selective loss of pancreatic β cells and an inability to maintain glucose homeostasis in absence of exogenous insulin. Clinical studies indicate that immune-modulatory approaches administered during the early stages of the disease improve the disease course by preserving residual β cell function, which might allow better glucose homeostasis and, therefore, reduce long-lasting diabetic complications [55].

Over the past decades, MIF has been shown to have a key pathogenic role in autoimmune diabetogenesis [1]. A study of pediatric patients with T1DM with duration of disease of 5.36

years (range 1–15 years) demonstrated that these patients had significantly higher circulating MIF levels compared with healthy children. MIF levels correlated with serum CCL2 levels, but not with glycosylated hemoglobin or duration of diabetes [56]. Accordingly, a more recent study reported that, in adult T1DM, MIF levels and its receptors CD74 in monocytes were elevated during the established phase of the disease and that MIF levels positively correlated with disease duration. A significant increase was also observed in the cognate receptor of MIF, the CD74 antigen [57].

Similar findings were reported in the nonobese-diabetic (NOD) mouse model of T1DM, in which a significant increase in circulating MIF levels was observed only in longstanding diabetic NOD mice compared with nondiabetes-prone control C57BL/6 animals, and NOD mice in prediabetic stages (8-week-old NOD animals) as well as acute diabetics [57].

However, increased *MIF* gene expression and CD74+ F4/80+ macrophages were found in the pancreas of 12-week-old NOD mice with ongoing diabetogenesis. In addition, MIF antagonism of NOD macrophages with ISO-1 prevented their activation-induced cytokine production and delayed the onset of autoimmune diabetes induced by transfer with diabetogenic T cells [57].

MIF transcriptional levels are increased in splenic lymphocytes of spontaneously diabetic NOD mice as well as in 8-week-old NOD mice treated with cyclophosphamide compared with 2-week-old nondiabetic NOD and healthy C57BL/6 control mice. In addition, administration of recombinant MIF to NOD mice increased disease incidence by 31% [58]. Consistent with these findings, it was demonstrated that treatment with anti-MIF antibodies reduced the incidence of diabetes in both adoptive spleen cell-transferred and cyclophosphamide-challenged NOD mice [19].

Similarly, MIF-KO mice were less susceptible to the induction of diabetes by multiple low doses of streptozocin (MLD-STZ) than were wild-type mice [19]. MIF deficiency also resulted in decreased production of IL-18, TNF-α, IL-1β, and inducible NO synthase in the islets of Langerhans [19]. MIF immunostaining was significantly increased in islet cells of MLD-STZ diabetic mice and anti-MIF antibody or ISO-1 treatment improved clinical, biochemical, and anatomopathological signs of the disease and reduced *ex vivo* the islet antigen-specific proliferative responses by splenic mononuclear cells [59].

It was also demonstrated that, whereas in pancreatic islets from wild-type mice, an increase in MIF release anticipated pancreatic islet death induced by IFN-γ + TNF-α + IL-1β, the islets from MIF-KO mice exhibited significant resistance to this pathway of cytokine-induced death. In addition, when exposed to IFN-γ + TNF-α + IL-1β, the expression of insulin was unaltered in pancreatic islets from MIF-KO mice and these islets produced less COX-2 than those from wild-type mice. Finally, the activation of mitochondrial membrane pore-forming protein Bcl-2-associated X protein and effector caspase 3, which is the last step in cytokine-induced apoptosis in pancreatic islets from wild-type mice, was not observed in islets from MIF-KO mice. Additionally, MIF-KO islets exhibited upregulation of prosurvival kinase ERK1/2 [60].

Guillain–Barre syndrome

GBS is a prototypic acute inflammatory neuropathy that is most often characterized by a rapidly progressive, symmetrical, and often ascending weakness of extremities accompanied by variable sensory deficits. The annual incidence of GBS ranges between 0.8 and 1.9 per 100 000 worldwide. SOC treatments for GBS comprise intravenous immunoglobulins and plasma exchange. However, nonresponders to SOC have a poor prognosis, with severe disability or death in 9–17% of all patients with GBS [61].

Patients with GBS have higher circulating MIF levels compared with control subjects [62] and these MIF levels increase progressively with the level of disability. Interestingly, higher MIF levels were not found in the cerebrospinal fluid (CSF) of patients with GBS [63].

Silencing of MIF attenuated the upregulation of TLR4 and translocation of NF-κB into the nucleus in response to lipo-oligosaccharide (LOS) from *Campylobacter jejuni*, which is a major etiological factor of the disease. Upregulation of IL-12p40, TNF-α, IL-6, CXCL8, and CCL5 in GBS monocytes was also induced by LOS, and small interfering (si)RNA-MIF overrides the effects of LOS on the production of TNF-α, IL-6, and CXCL8 [64].

In the murine experimental allergic neuritis model of GBS, administration of either anti-MIF antibodies or ISO-1 significantly reduced the cumulative score and disease duration compared with control vehicle-treated mice [62]. However, older data that were recently discussed in a review of the role of MIF in GBS suggested that MIF also represents a neurotrophic factor released from macrophages invading the peripheral nervous system (PNS) and that it might help Schwann cell survival and regeneration [65]. If so, MIF might have a dichotomic role during the course of GBS and experimental allergic neuritis (EAN), entailing pathogenic effects at disease onset and beneficial actions later on affording survival of myelin and its regeneration in the PNS. Therefore, specific MIF inhibitors should be given early during the course of the disease.

Multiple sclerosis

MS is a chronic immune-inflammatory disease that is probably autoimmune in origin in most cases, It affects central nervous system (CNS). MS affects 2.3 million people worldwide, with most patients developing disease between 20 and 40 years of age. There are different subtypes of MS based on their clinical phenotype: primary-progressive MS (PPMS); relapsing-remitting MS (RRMS); and secondary-progressive MS, which appears ~10–15 years after RRMS in up to 50% of patients [66].

First-line SOC treatment for MS comprises injectable treatments, such as IFN-β and glatiramer, as well as oral therapies, such as teriflunomide and dimethyl-fumarate. Second-line therapies include fingolimod and the intravenous natalizumab, which present higher levels of efficacy in reducing the relapse rate [67]. Moreover, alemtuzumab, cladribine, and ocrelizumab were recently added as approved therapies [67]. By contrast, the only treatment approved for the treatment of MS relapses comprises high doses of steroids that appear to act also by inhibiting the action of proinflammatory cytokines, such as IL-1 [68]. All these treatments are systemic immune-modulatory or immunosuppressive treatments, with risks of adverse events; therefore, additional treatments are being evaluated.

Both Th1 and Th17-mediated events have a major role in the pathogenesis of MS. Emerging evidence also point to a key role of MIF in the pathogenesis of MS. Data on MIF genetic polymorphisms are discordant, with one paper pointing to a lack of association between the *MIF* gene −173G > C polymorphism with

MS in a Turkish population [69]. Recently, it was shown that *MIF* functional polymorphisms (−794 CATT5-8 and −173 G > C) are associated with circulating MIF serum levels, severity, and progression in male patients with MS from a western Mexican population [70].

Other data demonstrate that MIF levels in the CSF of patients with RRMS are increased compared with those in patients with other neurological diseases. No significant differences were observed in MIF levels in the CSF from patients in remission [71]. It was also shown that circulating MIF levels were significantly higher in patients with disability progression compared with patients with stable disease, and that MIF serum levels correlates with the expanded disability status scale (EDSS) score [72]. Recently, it was reported that, in males with secondary progressive and primary progressive MS, MIF levels were higher than in patients with clinically isolated syndrome or relapsing-remitting course MS, but not significantly higher compared with healthy controls [73]. Furthermore, MIF and D-DT levels in male patients but not in female patients were correlated with the EDSS score. Male patients presenting with progressive disease showed also increased MIF and D-DT levels, which were significantly correlated with the presence of two high-expression promoter polymorphisms located in the *MIF* gene: a $-794CATT_{5\text{-}8}$ microsatellite repeat and a −173 G/C SNP [73]. Accordingly, MIF is highly expressed in active MS lesions at immunohistochemistry analysis [74]. However, in partial contrast with these studies, a recent *in silico* analysis of the expression of MIF and D-DT and the receptors CD74, CD44, CXCR2, and CXCR4 in PBMCs from patients with MS and encephalitogenic T cells from mice with experimental autoimmune encephalomyelitis (EAE) mice demonstrated a variable but consistent upregulation of these receptors, but not of MIF and D-DT [76]. Also, a significant increase in MIF receptors was found in CNS lesions associated with MS. However, the authors were not able to fully discriminate between male and female patients and forms of the disease, because relatively limited information was available from the data sets used in the analysis [75].

Preclinical studies using rodent models of MS (EAE) support a pathogenetic role of MIF in immunoinflammatory demyelination, entailing both increased intralesional levels of MIF during active disease [77] and beneficial effects obtained with different approaches aiming at genetic deletion or selective inhibition of endogenous MIF on the outcome of the disease [78,79]. Interestingly, induction and progression of EAE is reduced in a similar manner in MIF-KO and D-DT-KO mice, respectively,which suggests a nonredundant roles for MIF and D-DT in EAE and perhaps MS [73].

Given that MIF inhibits GC activity [2,80], both MIF and eventually D-DT-inhibitors could be used for those patients with MS who develop resistance to steroid treatment during relapses. Recent preclinical studies demonstrate that MIF promotes resistance to GC treatment in EAE [24].

Chronic respiratory diseases

Persistent unresolved inflammation within the respiratory tract is one of the underlying mechanisms responsible for the pathogenesis of chronic respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF), and PAH. It is generally accepted that the complex interplay of multiple sets of mediators and specific receptors in the lung and various environmental factors (e.g., cigarette smoking, pollutants, environmental particles, irritants, microbes, allergens, and toxic molecules) can cause impaired resolution and sustained inflammation of the respiratory tract.

Increasing evidence supports the potential of MIF to be used as an effective biomarker and/or therapeutic target candidate in these chronic respiratory diseases.

MIF levels are increased in patients with asthma [81], COPD [82], CF [83], IPF [84], and pulmonary hypertension (PH) [85]. Higher circulating MIF levels were found in patients with asthma compared with controls, and patients with symptomatic asthma had higher MIF levels compared with patients with asymptomatic asthma [81]. Similar observations were made in patients with COPD, who exhibited higher circulating MIF levels compared with controls. An additional increase in MIF levels was observed in patients presenting with acute COPD exacerbation [82]. Higher circulating MIF levels were also found in patients with IPF, were particularly abundant in bronchoalveolar lavage (BAL) from these patients, and were mainly expressed in areas of active pulmonary fibrosis [84].

Accumulating evidence indicates that pharmacological antagonism of MIF is beneficial in animal models of asthma [86,87]. ISO-1 attenuated corticosteroid-insensitive inflammation and airways hyperresponsiveness in an ozone-induced model of COPD [88] and reduced inflammatory responses associated with allergen-induced lung inflammation and fibrosis in a murine model of chronic asthma [86].

Pulmonary hypertension

A substantial increase in circulating MIF protein levels in the serum of patients with idiopathic and heritable PAH compared with controls without changes in D-DT has been reported [85]. T lymphocytes represent a source of this upregulated MIF in idiopathic PAH [85]. CD74 is overexpress in the endothelium of muscularized pulmonary arterioles and in cultured pulmonary endothelial cells (EC) from patients with idiopathic PAH, contributing to an exaggerated adhesion and recruitment of PBMCs. The MIF antagonist ISO-1 or anti-CD74 neutralizing antibodies partially attenuate PH development in rats and substantially reduced inflammatory cell infiltration [85]. Interestingly, MIF stimulates angiogenic factor expression [89] and induces vascular leakage [90]. Therefore, functional interdependence between MIF and hypoxia-inducible factor (HIF)-1α protein stabilization and transactivation activity has been reported [16]. In addition, nonlaminar shear stress induces MIF through a Krüppel-like factor 2 (KLF2)-dependent mechanism [91]. Similarly, MIF is involved in the recruitment and migration of endothelial progenitor cells [16].

Approaches for the identification of small-molecule inhibitors of MIF and D-DT

Significant effort is being invested in the search for inhibitors of MIF or D-DT signaling, with a strong focus on small molecules, which have many advantages over biologics, such as lack of immunogenicity, possibility for oral administration, and lower manufacturing cost [3].

MIF contains several sites of interest that influence its biological functions, such as the tautomerase active site, the thiol-protein oxidoreductase (TPOR) active site, or the pseudo-(E)LR motif [3]. The TPOR active site, which contains a CXXC motif around cysteines located at positions 56 and 59, is able to reduce insulin and 2-hydroxyethyldisulfide, and has a potential role in cellular redox homeostasis, apoptosis inhibition, defense against redox stress, and monocyte and/or macrophage activation. Ebselen, a small molecule that acts as a MIF trimer-disrupting inhibitor, was shown to irreversibly bind to all three MIF cysteines, including Cys-56 and Cys-59 of the CXXC motif [92]. The pseudo-(E)LR motif, comprising Asp45-X-Arg12, was found to be involved in MIF binding to chemokine receptor CXCR2, because the MIF alanine mutant of Asp-45 or Arg-12 showed almost complete inhibition of MIF/CXCR2-mediated chemotactic and arrest function. However, to date, no MIF inhibitors are known to target the pseudo-(E)LR motif [93].

Among these sites of interest, the tautomerase active site, which is extensively studied, constitutes an attractive entry point for the design and testing of small-molecule inhibitors of MIF. Indeed, it has been demonstrated that small molecules binding to the MIF tautomerase pocket are able to inhibit MIF biological functions, although the biological relevance of this site is controversial [3].

D-DT and MIF have a highly conserved tertiary and quaternary structure, but show only 34% amino acid sequence homology in humans [4]. Although D-DT lacks the CXXC and pseudo-(E)LR motifs, it does have, similar to MIF, a tautomerase active site in which three of the five key tautomerase active residues of MIF are conserved (N-terminal Pro-1, Lys-32, and Ile-64) [6,7,89]. Thus, MIF and D-DT have selective and reversible small-molecule inhibitors that bind to their tautomerase pocket, such as ISO-1 and 4-CPPC [7]. These differences could also influence the biological activities of MIF and D-DT resulting from their binding to the MIF receptor CD74 [5]. For instance, mutation of the Pro-1 residue of MIF to glycine suppresses the catalytic activity and reduces the binding affinity to its receptors CD74 and CXCR2 [94,95]. By contrast, Pro-1 mutant of MIF does not activate CXCR4, suggesting that the tautomerase active site is essential for binding or inducing CXCR4 signaling. In addition, the tautomerase-null, Pro1 → Gly1 MIF protein (P1G-MIF), despite being catalytically inactive, maintains the ability to bind CD74 and the intracellular binding protein JAB1/CSN5, although to a reduced extent. Moreover, cells derived from P1G-MIF knock-in mice showed growth and tumorigenic characteristics that are intermediate between those of the wild type and complete MIF deficiency [96].

Several classes of small-molecule MIF tautomerase inhibitors have been reported, including competitive, covalent, and allosteric inhibitors [97,98]. Here, we focus exclusively on the recent identification of small molecules that competitively bind the MIF tautomerase active site with high affinity.

Targeting the MIF tautomerase active site

MIF catalyzes the tautomerization of the non-naturally occurring D-dopachrome compound and that of phenylpyruvate and *p*-phenylpyruvate (4-HPP) [99]. Testing the MIF tautomerase inhibitory activity of small molecules using 4-HPP as substrate is usually the first assay performed when targeting the MIF tautomerase pocket. The tautomerase active site has the advantage of being amenable to high-throughput screening [3]. However, to confirm a MIF antagonism effect, tautomerase inhibitors generally undergo a second-pass screening using a MIF-dependent cell-based assay, where GC override, cellular proliferation, or cytokine release can serve as a readout [3].

The X-ray crystal structure of MIF complexed with 4-HPP [Protein Data Bank (PDB) 1CA7] revealed that the tautomerase cavity is located at the monomer–monomer interfaces within the MIF trimer, and requires an N-terminal Pro-1 residue to act as a catalytic base. Other interactions between 4-HPP and the tautomerase pocket residues Lys-32 and Ile-64 from one subunit of the trimer, and Tyr-95 and Asn-97 from an adjacent subunit were also highlighted [100]. Structure-based and computer-aided designs of MIF tautomerase inhibitors have greatly benefited from this X-ray crystal structure. As an example, several MIF tautomerase inhibitors incorporate a phenolic moiety that interacts trough hydrogen bonding with Asn-97 at the backbone of the tautomerase pocket, as also reported for 4-HPP [101].

One of the first reported reversible small-molecule MIF tautomerase inhibitors, Orita-13, is a chromene-4-one derivative that displayed a submicromolar IC_{50} value that was found later in the micromolar range by another group [102,103]. Among other earlier reversible small-molecule tautomerase inhibitors reported, the isoxazole derivative ISO-1, identified in 2002 [104], represents an important prototypical tool for investigation of MIF-mediated signaling [105], although it exhibits a moderate *K*i value of 24 μM in the 4-HPP tautomerase assay. ISO-1 antagonizes GC-mediated inhibition of LPS-stimulated cytokine production in monocytes, and NF-κB activation from LPS-treated macrophages [104]. Its efficacy has been demonstrated in numerous animal models of immunoinflammatory and autoimmune diseases and chronic respiratory diseases, such as asthma [86]. The X-ray crystal of ISO-1 complexed with MIF (PDB: 1LJT) showed similar hydrogen-bonding interactions with Lys-32, Ile-64, and Asn-97, and cation-π interaction with Tyr-95, as also reported for 4-HPP. However, the low potency and off-target effects of ISO-1 have precluded its development. Several ISO-1 derivatives displaying better IC_{50} values in the MIF dopachrome assay were further generated, such as ISO-66 and ISO-92 (1,5 and 1,07 μM respectively versus 18 μM) [106].

In 2009, virtual screening performed by molecular docking of a large library of molecules into the MIF tautomerase active site led to the identification of 11 MIF inhibitors displaying inhibitory activity in the low micromolar range, including four compounds with IC_{50} values <5 μM, when tested in an *in vitro* binding assay for MIF with CD74 [107].

More recently, it was shown that small molecules binding the MIF tautomerase pocket need to have a solvent-exposed moiety to effectively inhibit CD74 biological activation [108]. This finding was supported by the fact that the PAnM mutants and covalent inhibitors of MIF used in this study that inactivate or partially activate MIF binding to CD74 were those showing higher solvent-accessible areas. The authors also demonstrated, using site-directed mutagenesis, that the catalytic pocket of MIF is not involved in interactions with CD74, whereas key surface-exposed residues (Tyr-36, Lys-66 Trp-108, Ile-64, and Asn-109) are.

Using molecular dynamics and sited-directed mutagenesis experiments, the same team also highlighted the important role of Tyr-99 located at the end of the solvent channel. Indeed, based on atomistic molecular dynamics simulations of wild-type MIF over 1 μs, Tyr99 residue was found to dynamically influence MIF activation of CD74. This was confirmed by the fact that the alanine mutant of Tyr-99 had no CD74-dependent neutrophil recruitment activity. Interestingly, a dynamic signal communication with Tyr-99 and Asn-109 occurring via the β-strand system of MIF was brought to light [109].

Recent advances in the discovery of novel MIF tautomerase inhibitors

Other competitive inhibitors of MIF tautomerase activity exhibiting high binding affinity with *K*i values in the submicromolar range have been reported, such as biaryltriazole (**1**-**2**), pyrazole **3**, and benzoxazol-2-thione **4** derivatives (Fig. 1). The design, especially the determination of the binding mode of these molecules, has benefited from X-ray crystallography studies, to the point that many MIF tautomerase inhibitors claimed as competitive inhibitors have turned out to be covalent inhibitors on the basis of their crystal structure complexed with MIF.

Starting from a 37-μM biaryltriazole docking hit, Dziedzic and coworkers generated several more potent analogs, including compound **1**, which has a *K*i value of 0.65 μM [110]. The three distinct hydrogen bonds formed in the X-ray crystal structure of the MIF–**1** complex (PDB: 4WRB) between the triazolylquinoline moiety and active site residues Ly32A and Ile64A contributed to the 57-fold gain in affinity compared with the starting hit. Further optimization of **1** afforded biaryltriazole derivative **2**, which exhibits a low *K*i value of 0.057 μM and an aqueous solubility of 47 μg/ml.

The same group also successfully replaced the phenolic moiety found in several MIF tautomerase inhibitors with a pyrazole bioisostere that retains hydrogen bonding with Asn-97 [111]. Optimization of a 113-μM pyrazole docking hit led to the identification of pyrazole derivative **3**, which exhibits a low *K*i value of 0.067 μM and an aqueous solubility of 67 μM.

An *N*-benzyl benzoxazol-2-thione derivative **4** was recently identified as a potent MIF tautomerase inhibitor, exhibiting a *K*i value ranging from 0.3 to 1 μM [112]. Interestingly, compound **4** dose-dependently inhibited MIF-induced AKT phosphorylation in pulmonary endothelial cells derived from patients with PAH, and reversed established PH induced by monocrotaline in rats after oral administration [112]. In addition, compound **4** significantly decreased right ventricle fibrosis in the monocrotaline model in contrast to ISO-1, which promoted it [85,112].

Targeting the D-DT tautomerase active site

Until recently, the suicide inhibitor 4-IPP, which covalently binds with Pro-1 of both MIF and D-DT, was the only known tautomerase inhibitor of D-DT [113]. The resulting 6-phenylpyrimidine (6-PP) adducts of MIF and D-DT reduced the recruitment of neutrophils to the lung by nearly 50% compared with apo proteins [7]. However, the lack of selectivity of 4-IPP makes it difficult to discriminate the effects of D-DT versus MIF inhibition.

4-CPPC was found to competitively inhibit D-DT tautomerase activity with a *K*i value of 33 ± 0.7 μM versus 431 ± 37 μM for MIF, which represents a 13-fold selectivity for D-DT [101]. Interestingly, the X-ray crystal structures of MIF complexed with 4-CPPC revealed an induced-fit mechanism that was not observed in the numerous MIF–inhibitor complexes. Thus, 4-CPPC should make it possible to discriminate the effects of D-DT versus MIF inhibition.

ISO-1 had no effect on D-DT tautomerase activity even at 1000-fold molar excess, which suggests that the design of a dual competitive tautomerase inhibitor of MIF and D-DT would be challenging [6,7,114].

Drug repositioning

Repositioning of existing drugs that could treat novel indications through the identification of a new target offers a faster, less risky, and cheaper development process compared with the traditional drug discovery and development model. In this context, repositioning of ibudilast and iguratimod (T-614) as MIF inhibitors is being considered.

Ibudilast is a nonselective phosphodiesterase (PDE) inhibitor exhibiting anti-inflammatory and neuroprotective properties that was approved nearly 30 years ago in Japan for the treatment of asthma and poststroke complications. Ibudilast suppresses activated microglia-induced neuronal cell death *in vitro* via inhibiting production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α [115]. It also inhibits tautomerase activity of MIF with an IC_{50} value of 30.9 μM, and blocks MIF-induced PBMC chemotaxis [116]. A phase IIb clinical trial evaluating Ibudilast in patients with primary progressive MS has been completed (NCT01982942).

Iguratimod (T-614) is a NSAID approved for the treatment of RA in Japan and China [117]. It inhibits the production of various inflammatory cytokines and NF-κB activation in several cell types [97,118]. Iguratimod also exhibits an IC_{50} value of 6.81 μM in the MIF dopachrome assay [106]. However, its mode of MIF tautomerase inhibition remains to be elucidated. Iguratimod potentiates the anti-inflammatory activity of GCs in murine RAW 264.7 macrophages and human THP-1 monocytes and in the EAE model of MS, suggesting potential clinical usefulness as a steroid-sparing compound [106].

Concluding remarks

In conclusion, MIF and D-DT are multifunctional proteins with a range of functions, including immunomodulatory properties, and their expressions are often upregulated in several diseases. In addition, these two proteins could be effective biomarkers or promising therapeutic target candidates in several human disorders. However, whether high levels of these cytokines represent a cause or an effect of the inflammatory milieu associated with disease pathogenesis remains unknown. Current efforts aim to develop specific strategies to restore the expression of MIF and/or D-DT and a better understanding of the overall risk:benefit ratio of these different approaches.

Despite the report of different classes of potent and selective small-molecule MIF inhibitor directed against the MIF tautomerase active site, none have been approved for clinical use. One of the current challenges is to design such inhibitors with optimized drug-like properties for clinical trials. To date, the most advanced anti-MIF therapy is imalumab, an anti-MIF antibody currently in a clinical trial for cancer treatment (NCT01765790).

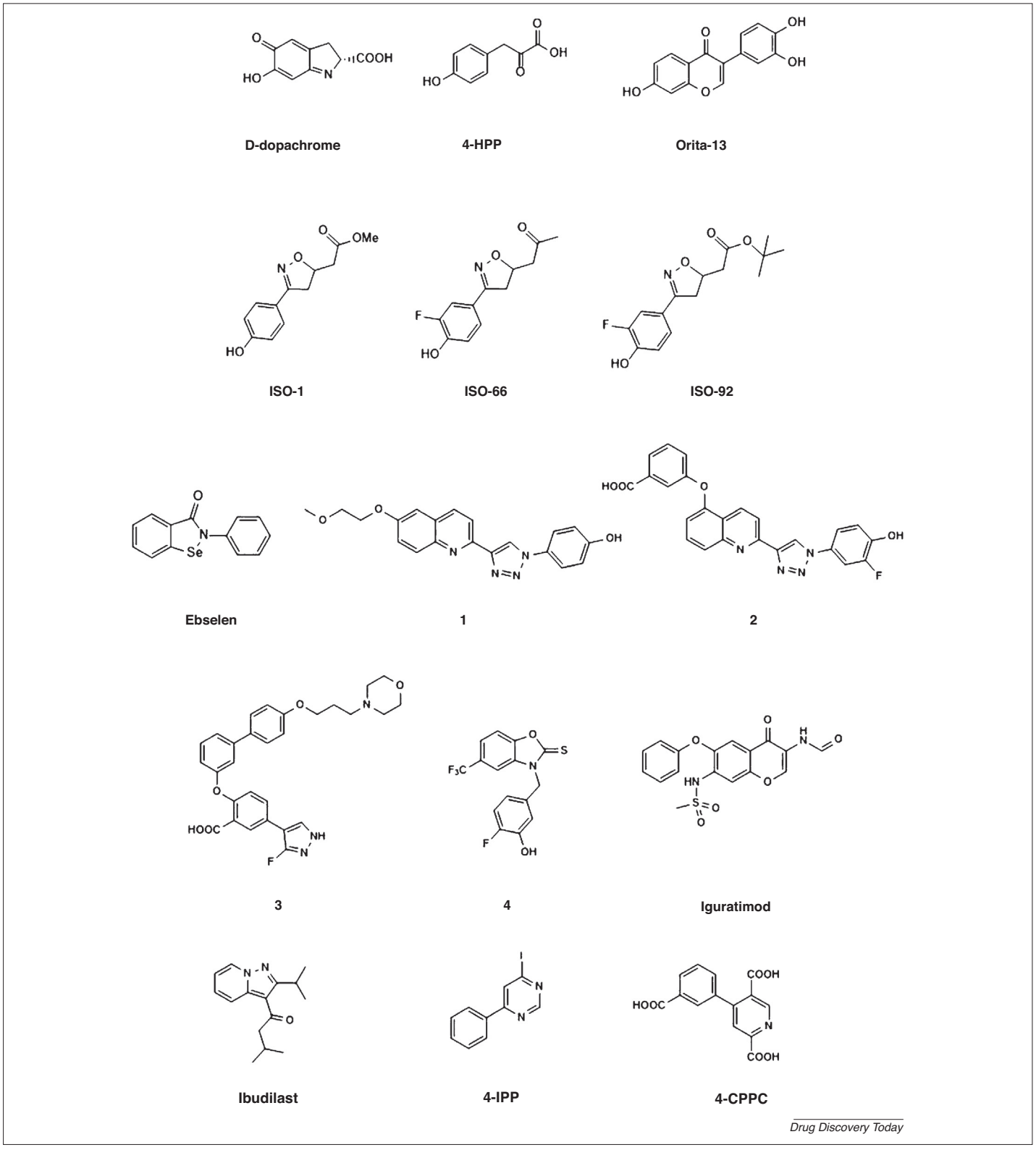

FIGURE 1

Structures of the non-physiological substrates for the macrophage migration inhibitory factor (MIF) tautomerase activity, D-dopachrome and 4-HPP, and of a selection of MIF tautomerase inhibitors.

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