

# Separating cytokine twins with a small molecule

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The cytokine macrophage migration inhibitory factor (MIF) has been characterized as a key immunomodulator and mediator of various diseases. Small molecule inhibitors based on the conserved enzymatic pocket of MIF have been valuable in elucidating MIF mechanisms and developing translational strategies. In contrast, our mechanistic understanding of the MIF homolog MIF-2/D-dopachrome tautomerase (D-DT) and its clinical translation has been hampered, partly because MIF-2-selective inhibitors have been elusive. Here, Tilstam *et al.* characterize a small-molecule inhibitor selective for MIF-2 that interferes with receptor binding and cell signaling. That could be a promising therapeutic lead and a valuable research tool.

Cytokines are protein mediators regulating intercellular communication. They are typically viewed as immunomodulators, orchestrating innate and adaptive immune responses, and are targets in inflammatory diseases and cancer. Many cytokine families are characterized by pleiotropy, and there are often homologs with overlapping activities and shared receptors. Cytokine homologs can also feature distinct, context- and disease-specific activity profiles, in which case tailoring of inhibitory strategies is mandated to achieve optimal therapeutic outcomes.

Macrophage migration inhibitory factor (MIF)<sup>2</sup> is the oldest cytokine to be discovered over a half-century ago (1, 2). In contrast to its name, MIF is a multifunctional chemokine-like inflammatory cytokine (1). MIF is a key regulator of innate immunity supporting host homeostasis, but ample evidence from preclinical and clinical studies demonstrates that dysregulated MIF critically contributes to inflammatory conditions, cardiovascular diseases, autoimmunity, and cancer (3, 4).

MIF is also of interest due to its extraordinary molecular properties. It does not belong to any of the known structural families for classical cytokines, and it signals both via its cognate type-II receptor CD74, and the CXC-type chemokine receptors

CXCR2, CXCR4, and CXCR7 (1, 3, 4). The MIF structure is evolutionarily conserved, and the MIF phylogenetic tree can be traced back by several hundred million years. Interestingly, the structure of the MIF trimer resembles that of bacterial tautomerase, including conservation of the proline-containing catalytic pocket (5). Human and murine MIF exhibit catalytic tautomerase activity *in vitro* with 4-hydroxyphenylpyruvate and D-dopachrome identified as substrates, suggesting that MIF could have a dual role as a cytokine and enzyme. However, a physiological role for the MIF tautomerase activity in mammals, if any, has yet to be unraveled. Mutagenesis studies have revealed that the residues of the catalytic pocket influence or overlap with the MIF receptor binding sites for CD74 and CXCR4 (4). The catalytic pocket of MIF has therefore attracted great attention as an intriguing opportunity for MIF-specific targeting. Specifically, the pocket offers the possibility to target MIF not only by antibody and peptide approaches but also by high-affinity small-molecule inhibitors (3, 4). This is unique among cytokines, as no other cytokine structures have a ligand pocket. Numerous anti-MIF inhibitors directed against the pocket, acting by a competitive or covalent mechanism, have been identified, and several are in preclinical and clinical testing for cancer and inflammatory diseases (4).

The MIF gene/protein copy number in various kingdoms and species ranges from zero to five (5), but in humans/mammals, MIF (now also called MIF-1), was long considered the only member of the family. A rat ortholog of MIF, termed D-dopachrome tautomerase (D-DT), was sequenced in 1995 (6) but not functionally characterized until 2011. Merk *et al.* (7), guided by the observation that antibody-based neutralization or genetic deletion of MIF-1 did not completely abrogate MIF-type responses and that deletion of CD74 produced a more pronounced phenotype than deficiency of the agonist, showed that D-DT (now also termed MIF-2) not only has a catalytic tautomerase pocket and activity similar to MIF-1, but is a functional homolog that shares MIF-like activity in certain disease models and binds to CD74 with equal affinity to MIF-1. Genetic models have also demonstrated overlapping activities between MIF-1 and MIF-2 (8). However, the specific role of MIF-2 remains unclear: sequence comparison indicates that MIF-2 may exhibit a differential MIF chemokine receptor activation profile, and recent studies in cancer cell models and cardiovascular and autoimmune patient cohorts support the notion that MIF-1 and MIF-2 have distinct functional profiles, depending on disease context (3, 4).

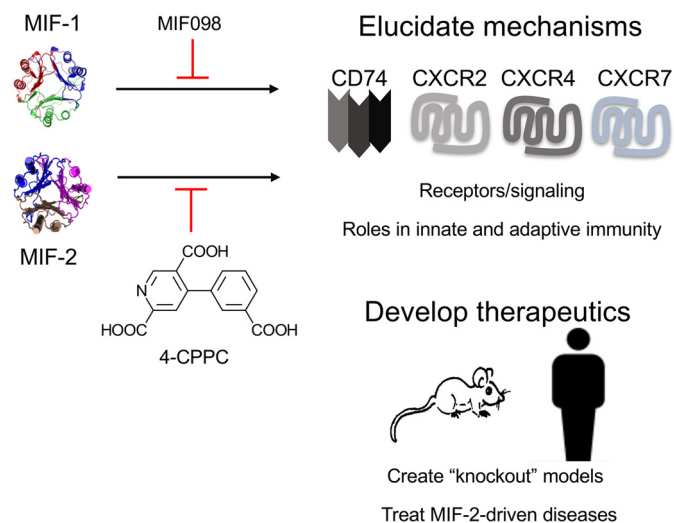
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<sup>2</sup> The abbreviations used are: MIF, macrophage migration inhibitory factor; D-DT, D-dopachrome tautomerase; 4-CPAC, 4-(3-carboxyphenyl)-2,5-pyridinedicarboxylic acid; 4-IPP, 4-iodo-6-phenylpyrimidine; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; DKO, double knockout; PDB, Protein Data Bank.

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**Figure 1. Utility and applications of MIF-2-selective small-molecule inhibitors.** The potential utility and applications of 4-CPPC and MIF-2-selective small-molecule inhibitors in general are depicted. Together with MIF-1-selective small molecules, such as MIF098 or antibodies (not shown), such compounds could be useful in studying the role of both MIF-1 and MIF-2 in homeostasis and host defense. Similarly, the compounds could be valuable in elucidating distinct MIF-1- versus MIF-2-driven mechanisms, driving distinct receptor and signaling pathways. Moreover, MIF-2-selective inhibitory strategies may become valuable in developing future therapeutics for MIF-2-dominated diseases and in depleting MIF-2 in mouse disease models. MIF-2 and MIF-1 are represented as *three-dimensional ribbon structures* of the trimeric proteins as indicated (MIF-2, PDB entry 1DPT; MIF-1, PDB entry 1MIF).

This is where the current study by Tilstam *et al.* (9) comes in. The authors begin their search for MIF-2 inhibitors with an *in silico* screen of 1.6 million compounds docked into the MIF-2 catalytic pocket (9), leading to 1821 hits that bound to two conformational states. They tested 176 of these candidates in the tautomerase assay, finding one that showed activity. This compound, 4-(3-carboxyphenyl)-2,5-pyridinedicarboxylic acid (4-CPPC), exhibits an enzymatic  $IC_{50}$  of 27  $\mu\text{M}$  for MIF-2 versus 450  $\mu\text{M}$  for MIF-1, corresponding to a 17-fold selectivity for MIF-2 over MIF-1. Of note, among numerous MIF tautomerase inhibitors, the only “useful” compound also targeting MIF-2 had been 4-iodo-6-phenylpyrimidine (4-IPP). However, 4-IPP is nonselective for MIF-2 and is a covalent inhibitor, precluding translational development. Thus, 4-CPPC is the first selective and reversible MIF-2 inhibitor.

A previous study demonstrated that 4-CPPC binds to the active site of MIF-2 and induces a major conformational change of the C-terminal region (10), a behavior that principally differs from that of other MIF tautomerase inhibitors and could potentially impact cellular function. Tilstam *et al.* now provide evidence for this critical feature. They demonstrate that 4-CPPC dose-dependently and selectively blocks the binding between MIF-2 and the CD74 ectodomain and attenuates MIF-2/CD74-mediated ERK-MAPK signaling in human fibroblasts, but does not impact MIF-1 signaling. In contrast, the MIF-1-selective inhibitor MIF098 blocks MIF-1 but not MIF-2 function.

This work elegantly exploits the unique MIF structure, identifying only the second known class of an anti-cytokine small molecule. As illustrated in Fig. 1, compounds such as 4-CPPC could have an important utility as leads for the development of

therapeutics for diseases/disease stages driven or dominated by MIF-2 (*i.e.* as cytokine-selective or cytokine subclass-selective tailored strategies). They can also help us to better interpret ongoing clinical studies employing MIF-1 antagonists. Moreover, they can be tools to disentangle the pathways and mechanisms of the MIF family ligand/receptor network, which necessitates discerning between MIF-1- and MIF-2-skewed responses. These compounds can also be applied as a “pharmacological knockout” of MIF-2, enabling researchers to circumvent lengthy breeding periods for *Mif-1/Mif-2*-double knockout (DKO) mice, with a *Mif-1-KO*/MIF-2 inhibitor setting representing a functional DKO mouse.

In summary, whereas 4-CPPC is the first identified MIF-2-specific small molecule, it certainly will require extensive optimization by medicinal chemistry approaches to improve selectivity, affinity, and potency. The study by Tilstam *et al.* (9) lays out a novel translational avenue for MIF protein family-directed therapies in particular and cytokine homolog-specific strategies in general and provides the scientific community with a long-awaited research tool to elucidate the functions of the MIF ligand/receptor network in physiology and pathophysiology.

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