Bioorganic & Medicinal Chemistry Letters xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Irregularities in enzyme assays: The case of macrophage migration inhibitory factor

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ARTICLE INFO

Article history:
Received 9 April 2016
Revised 21 April 2016
Accepted 23 April 2016
Available online xxxx

Keywords:
MIF
Tautomerase assay
Protein crystallography
Reproducibility

ABSTRACT

Inhibitors of human macrophage migration inhibitory factor (MIF) previously reported in the literature have been reexamined by synthesis, assaying for tautomerase activity, and protein crystallography. Substantial inconsistencies between prior and current assay results are noted. They appear to arise from difficulties with the tautomerase substrates, solubility issues, and especially covalent inhibition. Incubation time variation shows that **3**, **4**, **6**, and **9** are covalent or slow-binding inhibitors. Two protein crystal structures are provided; one confirms that the twice-discovered **3** is a covalent inhibitor.

Macrophage migration inhibitory factor (MIF) is a homotrimeric toroidal protein, which has important functions as a cytokine in regulating inflammation, immune response, and aberrant cell growth.[1] Thus, there is much interest in development of therapeutic agents to interfere with the binding of MIF to its signal transduction partners including CD74. MIF also shows enzymatic activity as a keto-enol tautomerase. Though the activity may be vestigial in humans, the existence of the three catalytic sites at the interfaces of the monomer subunits provides an opportunity to discover small-molecule tautomerase inhibitors that may also interfere with the protein-protein interactions featured in MIF signaling.[2] Indeed, this strategy has been widely pursued and is supported by observed correlations between inhibition of tautomerase activity and biological function of MIF.[1–4]

Most MIF tautomerase inhibitors have arisen through screening efforts over the last fifteen years using D-dopachrome methyl ester (DOPA) and *p*-hydroxyphenyl pyruvic acid (HPP) as substrates.[2] Our laboratory has participated in such activities[4] as well as in de novo design.[5] Two series of compounds that came from the virtual screening also underwent lead optimization.[6,7] In a recent report,[5] we noted difficulties with the tautomerase assays and sizable variations in reports of inhibitory activity for the reference compounds (*R*)-ISO-1 (**1**) and Orita-13 (**2**). The present work expands this investigation to include the 11 previously reported MIF tautomerase inhibitors shown in Scheme 1. The compounds have been reassayed and two crystal structures for complexes with MIF are reported.

The HPP tautomerase assay was carried out as described before.[5] DOPA is a less preferable substrate; it is photosensitive, and it yields a shorter linear range for product formation than HPP, 25 versus 175 s. Inhibitory activity is monitored by measuring formation of the borate complex of the enol product of HPP at 305 nm using a Tecan Infinite F500 plate reader, after a 20-min incubation of the test compounds with MIF. Human MIF was only expressed on two occasions and consistency in activity was demonstrated using control compounds, especially MIF190 (**12**) and Orita-13 (**2**). We report K_i values by performing the assay with variation of the inhibitor's concentration for substrate concentrations of 1 and 2.5 mM using 200 nM MIF. Assay of **12** on 17 occasions has yielded 16 K_i values between 0.55 and 0.76 μM and one value of 0.85 μM. K_i results are much preferred to the more common IC_{50} reports that only require use of one substrate concentration. K_i is the binding constant for the inhibitor with the protein, while the IC_{50} is dependent on the substrate's concentration and Michaelis constant. Specifically, $IC_{50} = K_i \cdot (1 + [S]/K_m)$, so a K_i value should be smaller than an IC_{50}.[8]

In addition to **1**[9,10] and **2**,[11] recently reported MIF inhibitors with high potency were considered. **3** (RDR03785) was discovered in two independent high-throughput screens.[12,13] **4**–**6** are the three most potent MIF tautomerase inhibitors from the virtual screening effort of Xu et al.,[14] while **7** and **8** are the two new compounds reported by Youssef et al.[15] Most of the compounds were purchased from Alfa Aesar (**1**, **2**), Santa Cruz Biotechnology (**1**, **2**), Maybridge (**3**) ChemBridge (**4**, **6**), and Vitas-M Laboratory (**5**), though **7** and **8** had to be synthesized. We were unable to find a vendor for compounds DP00477 and ML00144 from the paper by Ouertatani-Sakouhi et al.[12] **9**–**11** were also reinvestigated; they

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http://dx.doi.org/10.1016/j.bmcl.2016.04.074
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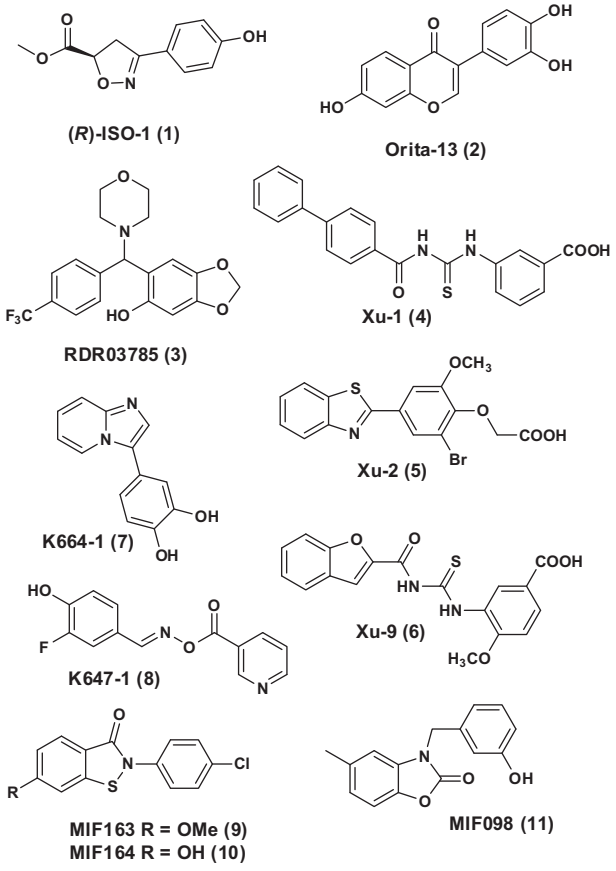
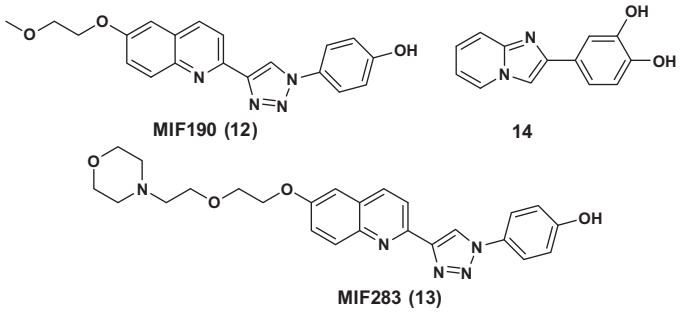


Scheme 1. Reported MIF tautomerase inhibitors.

were previously synthesized in our laboratory and assayed by a collaborator.[6,7] The identities of all assayed compounds were confirmed by NMR and mass spectrometry, and the purity was >95% as judged by HPLC.



The current K_i results are compared with the data from the literature in Table 1. In general, less consistency may be expected for weaker inhibitors than stronger ones, reflecting solubility and aggregation issues as well as spectral interference at higher concentrations. For (*R*)-ISO-1 (**1**) the current assay protocol has been executed three times yielding K_i values of 21, 24 and 28 μM, while a wide range of IC_{50} values is found in the literature.[4,9,10,13,15] As previously discussed,[5] we also find **2** to be much less active than the original report of an HPP IC_{50} of 0.038 μM.[11] The compound has been assayed several times by us with all results in the 13–22 μM range.

For new results, it should be stated from the outset that we find **3**, **4**, **6**, and **9** to be covalent inhibitors or possibly slow, tight-binding inhibitors. This was established by observing that their activity varied with the incubation time for the inhibitor with MIF prior to addition of HPP (Fig. 1).[16] The bound/unbound equilibrium is rapidly established for reversible inhibitors, while bond formation is a slower process. The Pro1 nitrogen of MIF with a pK_a of 5–6 is well known to act as the catalytic base for the tautomerase reaction and as a nucleophile forming covalent adducts.[1,2] We have previously reported crystal structures for two biaryl triazoles including **12** that confirm formation of the expected non-covalent complexes.[5] Consistently, for the closely related **13**, there is negligible effect of increasing the incubation time, while for **3**, **4**, **6**, and **9** the initial velocities decrease markedly with increasing time. **4** and **6** both contain an acylthiourea functionality, which is prone to nucleophilic attack and has led to covalent modification of MIF for related compounds.[12,17]

For **3**, we also obtained an 1.85-Å X-ray crystal structure clearly showing a covalent bond between Pro1 and the benzylic carbon atom of **3** (r(N–C) = 1.48 Å) with the morpholine ring missing (Fig. 2).[18] The remaining fragment is oriented with the CF_3 group inward near Asn97; there is a hydrogen bond between the carbonyl group of the fragment and the NH of Ile64, and the benzodioxole ring is π-stacked between Tyr36 and Phe113. Several mechanisms are possible; the simplest is that the morpholine nitrogen is protonated and attack by the Pro1 nitrogen atom displaces neutral morpholine. Alternatively, the phenol assists E1 elimination of the morpholine, and Pro1 adds to the resultant

Table 1
Results for inhibition of the tautomerase activity of MIF (μM), *Clog P*, and aqueous solubility

Cmpd	Lit.[a] IC_{50}	Refs.	Present[b] K_i	*Clog P*	S (μg/mL)
1	7, 18, 25, >100	9,15,13,10	24	2.10	
2	0.038[b]	11	15	1.49	
3	(0.57, 0.36)[b,c]	12,13	(0.052)[c]	3.49	27.6
4	(0.348)[c,d]	14	(4.27)[c]	4.54	<1
5	4.521[d]	14	10.9	3.86	6.0
6	(5.141)[c,d]	14	(26.79)[c]	3.26	<5
7	0.11	15	45.2	2.62	
8	0.41	15	3.30	1.26	
9	(1.9)[b,c]	7	(5.37)[c]	3.99	
10	1.0[b]	7	9.20	2.49	
11	0.010[b]	6	NA	3.35	21.9
12			0.55–0.85[e]	3.98	3.6
13			0.41	4.24	48.5
14			26.6	2.62	

[a] Dopachrome substrate.
[b] HPP substrate.
[c] Covalent inhibitor.
[d] K_i.
[e] Range for 17 measurements.

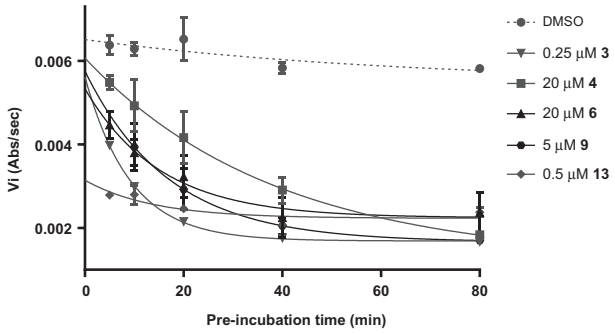


Figure 1. Effect of incubation time on the initial velocity of the MIF tautomerase reaction in the presence of inhibitors. Data shown are from triplicate experiments; mean ± SEM are plotted.