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Original article

Multiple binding modes of isothiocyanates that inhibit macrophage migration inhibitory factor

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that has roles in the innate immune response, and also contributes to inflammatory disease. While the biological properties of MIF are closely linked to protein–protein interactions, MIF also has tautomerase activity. Inhibition of this activity interferes with the interaction of MIF with protein partners e.g. the CD74 receptor, and tautomerase inhibitors show promise in disease models including multiple sclerosis and colitis. Isothiocyanates inhibit MIF tautomerase activity via covalent modification of the N-terminal proline. We systematically explored variants of benzyl and phenethyl isothiocyanates, to define determinants of inhibition. In particular, substitution with hydroxyl, chloro, fluoro and trifluoro moieties at the para and meta positions were evaluated. In assays on treated cells and recombinant protein, the IC_{50} varied from 250 nM to >100 μM. X-ray crystal structures of selected complexes revealed that two binding modes are accessed by some compounds, perhaps owing to strain in short linkers between the isothiocyanate and aromatic ring. The variety of binding modes confirms the existence of two subsites for inhibitors and establishes a platform for the development of potent inhibitors of MIF that only need to target one of these subsites.

1. Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that functions as a regulator of inflammation and a mediator of the innate immune response. MIF plays a critical role in chronic diseases such as atherosclerosis [1], rheumatoid arthritis [2] and has also been implicated in tumor growth [3–5]. Therefore MIF is a suitable target for the development of compounds for potential clinical use [6]. Following secretion, MIF binds to extracellular receptors, in particular the CD74 receptor, and is also internalized where it interacts with intracellular proteins. It has also been shown that MIF is a non-cognate ligand of the CXC chemokine receptors CXCR2 and CXCR4 [7,8]. MIF exists as a homotrimeric protein and possesses keto–enol tautomerase activity (against substrates L-dopachrome and phenylpyruvate), which is catalyzed by its N-terminal proline [9,10]. Whilst no physiologically relevant substrates have been identified, the catalytic site is in the region of the protein that is responsible for CD74 binding. Compounds that bind to the active site and inhibit MIF's tautomerase activity interfere with the interaction of MIF with CD74, and these inhibitors display activity in disease models of multiple sclerosis and colitis [11–13].

MIF has previously been identified as the major cellular target of isothiocyanates (ITCs) - phytochemicals which are commonly found in cruciferous vegetables including watercress, cauliflower, Brussels sprouts, mustard seeds and wasabi [6,14,15]. The isothiocyanate moiety reacts with sulfur- or nitrogen-containing biological nucleophiles as well as glutathione [16,17]. However, despite

Abbreviations: MIF, macrophage migration inhibitory factor; ITC, isothiocyanate; rhMIF, recombinant human MIF.

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the wealth of possible targets *in vivo*, phenethyl isothiocyanate reacts preferentially with the amino terminus of MIF [14]. This reflects the enhanced nucleophilic reactivity of the N-terminal prolyl amine of MIF, facilitated by neighboring hydrophobic residues in the active site that dramatically lower the pKa. This results in an unionized amino group compared to the ζN of a lysine residue which would normally be protonated under physiological conditions [18]. Using phenethyl isothiocyanate (**1**) as a starting point, we describe the preparation and biological assessment of a range of isothiocyanates against recombinant human MIF (rhMIF) and MIF within Jurkat T-lymphoma cells. Complementary structural analyses have also revealed the key requirements for ITC binding to MIF. This information will assist in the design of more potent MIF inhibitors.

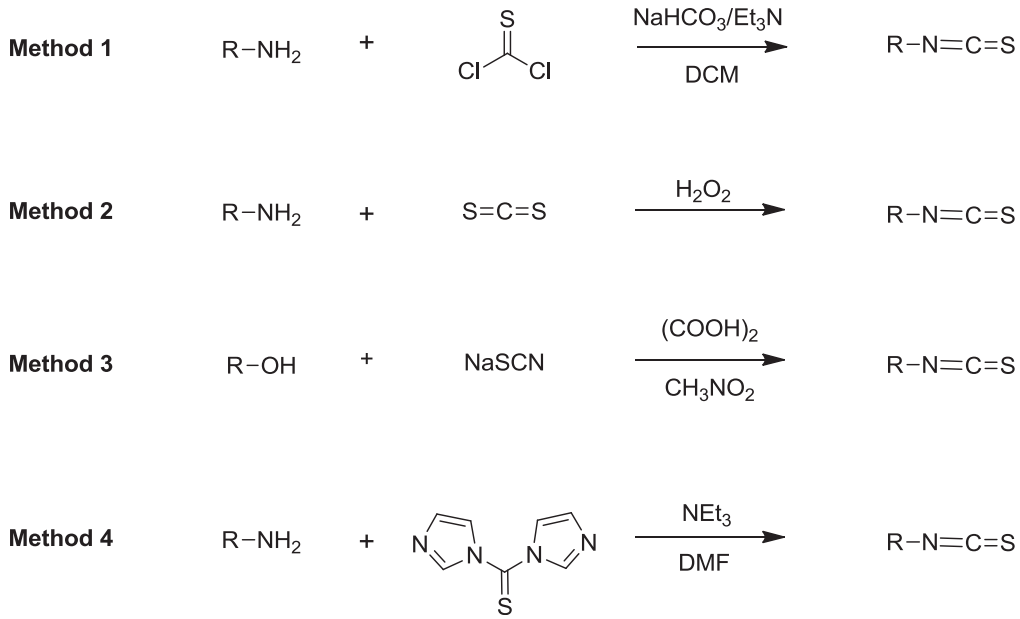
2. Chemistry

Isothiocyanates were synthesized via one of four methods (Scheme 1) starting from either the corresponding amine (methods 1, 2 and 4) or alcohol (method 3). The formation of the isothiocyanate was carried out with thiophosgene, carbon disulfide, sodium thiocyanate or 1,1′-thiocarbonyldiimidazole. See experimental section for details.

3. Results

The original identification of ITCs, in particular compound **1**, as inhibitors of MIF provided the starting point for this investigation [6,14,15,19]. A set (Fig. 1) of phenethyl and benzyl isothiocyanates and related compounds were either synthesized or purchased, and screened for inhibition of MIF against whole Jurkat cells as well as the recombinant human enzyme (Table 1). Cell based assay results were used as an initial indication of a positive hit. Inactive compounds were therefore not followed up with further recombinant tests. The naturally occurring compounds (**1**, **2**, **3**, **4**) all showed promising activity in both recombinant and cell based assays with improved activity compared to a literature standard, (*S*;*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1, **5**) which has an IC_{50} of 7 μM, assessed by inhibition of recombinant MIF tautomerase activity [20]. Conversely the inactivity observed with the isostructural isocyanate **6** and thiocyanate **7** confirmed that the isothiocyanate moiety is essential for activity. Previous crystallographic studies of MIF complexed with ITCs **1** or **4** [19,21] revealed two distinct binding modes for ITCs, described as (i) “downward”, occupying a deep, restricted, hydrophobic pocket, or (ii) “upward”, occupying a more open cleft exposed to the protein surface with both modes anchored by either a *cis* or *trans* thioamide bond, respectively, and influenced by the size of the side chain of the isothiocyanate inhibitor. In **8**, the presence of a hydroxyl group at the 4 position of the phenyl ring resulted in a five-fold increase in activity against the enzyme over the parent ITC **1**, while maintaining whole cell MIF activity (Table 1). The X-ray crystal structure of rhMIF covalently linked to **8** (Fig. 2) matches the “downward” binding mode previously seen for **1** with the distal end in a very restricted and mostly hydrophobic binding pocket, in this case with the additional phenolic hydroxyl forming a short hydrogen bond with Asn98. Assay results revealed that compounds with a substitution pattern different to **8** (e.g. **9** with a hydroxyl group at the 3 position and **10** with hydroxyls in the 3 and 4 positions, yielding IC_{50}'s 7.8 and 4.0 μM, respectively) or a larger substituent at these different positions (**11**, **12**, and **13**, showing trifluoromethyl or chloro substituents, IC_{50} 7.1, 10.4, and 1.6 μM, respectively) were still active in whole cell as well as enzyme based assays (Table 1). It was originally thought that ITC inhibitors could be developed that would span the deep binding pocket occupied by **1** and **8** as well as containing substituents that would project upwards towards Tyr37. However biological testing of **15** in a whole cell assay implied this would not work or would be difficult to optimize.

Given the encouraging activity observed with **2**, a range of benzyl isothiocyanate derivatives (Fig. 3) were also tested for their activity against whole cells as well as rhMIF. Two commercially available, fluoro-substituted benzyl derivatives, **16** and **17**, showed sub-micromolar and low-micromolar activity respectively in the cell based assay (Table 2). Subsequent biological testing of these two compounds at a later date revealed weaker biological data



Scheme 1. Synthesis of: isothiocyanates using thiophosgene in basic conditions (Method 1), hydroxyalkyl and aryl isothiocyanates using carbon disulfide (Method 2), isothiocyanates using sodium thiocyanate in acid (Method 3) and isothiocyanates using 1,1′-thiocarbonyldiimidazole and base (Method 4).

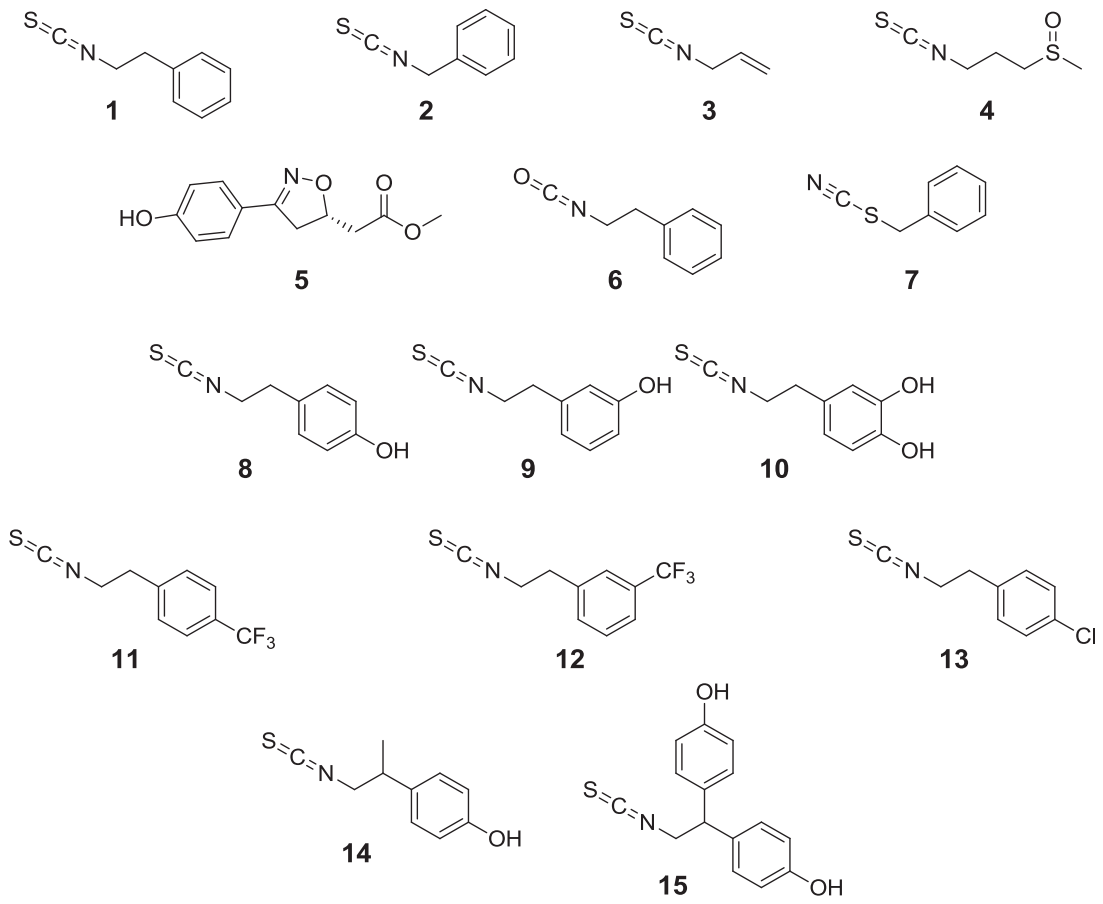


Fig. 1. Naturally occurring isothiocyanates (**1**–**4**), a non-covalent MIF inhibitor (ISO-1, **5**), isosteric analogs (**6**–**7**) and phenethyl isothiocyanate derivatives (**8**–**15**).

Table 1
IC_{50} of naturally occurring isothiocyanates, isostructural analogues and phenethyl isothiocyanate derivatives against recombinant human MIF (rhMIF) and Jurkat T-lymphoma cells.

ITC	rhMIF IC_{50} (μM, ± S.E.)[a]	Jurkat cell IC_{50} (μM, ± S.E.)[a,b]
1	16 ± 3	1.6 ± 0.2
2	0.53 ± 0.09	0.54 ± 0.01
3	1.1 ± 0.11	0.25 ± 0.03
4	11 ± 2	2.2 ± 0.1
5	~7[c]	11.3 ± 0.3
6	ND	>100[d]
7	ND	>100[d]
8	3.6 ± 1.1	1.2 ± 0.1
9	62 ± 18	7.8 ± 0.3
10	29 ± 3	4.0 ± 0.1
11	40 ± 6	7.1 ± 0.1
12	ND	10.4 ± 0.3
13	1.1 ± 0.1	1.6 ± 0.2
14	28 ± 3	4.8 ± 1.1
15	ND	>100[d]

[a] Concentration of ligand required to inhibit 50% MIF tautomerase activity.
[b] Activity of MIF determined from whole cell lysates following treatment with ITCs. All assays are the results of at least two replicates. ND – not determined.
[c] Literature value [20].
[d] Cell based assay results were used as an initial screen.

indicating some degradation. Mass spectral analyses on the degraded mixtures indicated the presence of dimerized thiourea adducts and among other peaks (HRMS for $C_{15}H_{14}F_2N_2S$ calculated 293.0846, found: 293.0889; Supplementary material). An attempt was also made to synthesize the benzylic derivative of **8**, 4-hydroxy

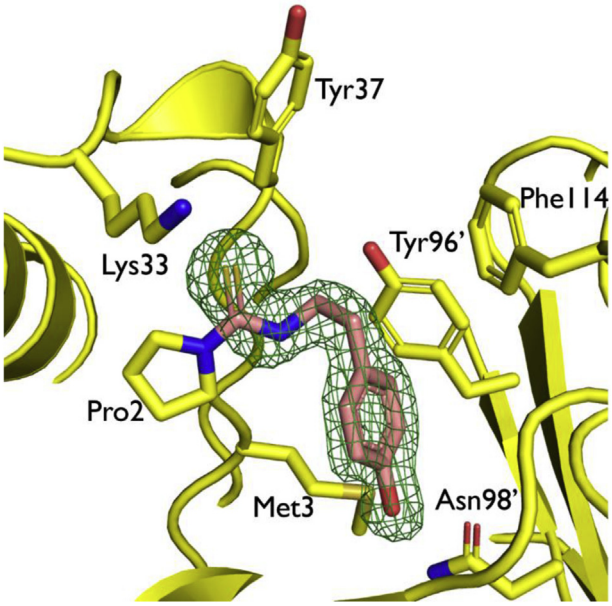


Fig. 2. The crystal structure of **8** covalently bound to rhMIF at 1.6 Å (PDB 4osf). Green mesh represents the *Fo* – *Fc* electron density omit map contoured at 3σ. The enzyme is shown as a yellow cartoon with the covalently bound inhibitor shown in salmon (oxygens, nitrogens and sulfur (thiourea) are colored red, blue and yellow respectively). Numbered residues are from chain A or the adjacent chain C identified with a "prime". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

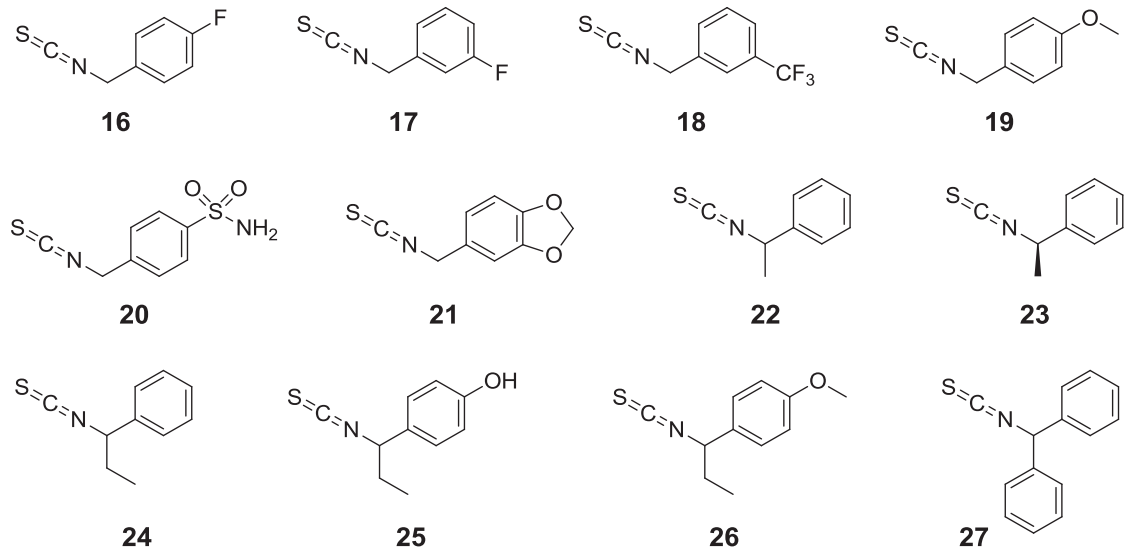


Fig. 3. Benzyl isothiocyanate derivatives tested as inhibitors of MIF.

Table 2
IC_{50} of benzyl isothiocyanate derivatives against recombinant human MIF and Jurkat T-lymphoma cells.

ITC	rhMIF IC_{50} (μM, ± S.E.)	Jurkat cell IC_{50} (μM, ± S.E.)[a,b]
16	ND 31 ± 3[c]	0.54 ± 0.04 9.6 ± 1.6[c]
17	ND 54 ± 3.3[c]	1.5 ± 0.1 11 ± 1[c]
18	ND	5.9 ± 0.1
19	0.7 ± 0.1	3.9 ± 0.8
20	0.75 ± 0.2	0.5 ± 0.1
21	2.9 ± 0.5	5.0 ± 1.6
22	1.0 ± 0.1	2.8 ± 0.02
23	ND[d]	1.1 ± 0.5
24	ND	21 ± 0.2
25	ND	>100
26	ND[d]	1.2 ± 0.3
27	ND	>100

[a] Concentration of ligand required to inhibit 50% MIF tautomerase activity.
[b] Activity of MIF determined from whole cell lysates following treatment with ITCs.
[c] IC_{50} data determined 24 months after initial values using the same sample stored in the laboratory. All assays are the results of at least two replicates. ND – not determined.
[d] Cell based assays give a good indication as to the MIF activity and therefore we did not examine these in any greater detail.

benzyl ITC, however subsequent analysis of this and a commercial sample revealed that both rapidly degraded to isothiocyanate and 4-methylenecyclohexa-2,5-dienone under alkaline conditions.

The presence of either a 4-methoxy or 4-sulfonamide substituent, **19** and **20** respectively, enhances the activity to sub micromolar with the latter compound also showing potent cell based activity (IC_{50} = 0.5 μM). Compound **22** shows promising activity as the racemic mixture with the cell based data of **23**, the *R* enantiomer, implying this to be the preferred stereochemistry.

It was initially assumed that simple benzylic ITC inhibitors would match the "downwards" binding mode of **1** and **8**. Crystallographic analysis of **2** complexed with rhMIF revealed unexpected results. In two separate crystallographic experiments using similar conditions (with the only difference being 1.9 M $(NH_4)_2SO_4$ compared with 2.0 M, see Experimental section), we observed different binding modes for **2**. In one instance we observed compound **2** binding in a "downwards" orientation in all three active sites of the trimer matching that seen for **1** and **8** (Fig. 4). The binding of **2** to MIF in this instance is effectively identical in all three active sites. Compound **2** forms a *cis* thioamide bond with the catalytic proline and projects the phenyl moiety down into the restricted, hydrophobic pocket where it makes an edge-to-face π interaction with Tyr96′. Val107 is close to the face of the phenyl ring of **2**, forcing a constrained conformation of the inhibitor where the angle of the benzylic carbon is more acute than that for an ideal tetrahedral carbon. The N-terminal proline of the "downwards" binding mode is shifted by up to 1.3 Å compared with the "upwards" orientation discussed below. The remainder of the pocket is lined by Met3, His63 and Met102. The crystal structure of **3** complexed with rhMIF at 1.7 Å revealed a similar downwards binding mode (PDB 3wns, Supplementary material).

A second crystal revealed **2** binding "upwards" towards Tyr37 in two of the three active sites (Fig. 4, lower panel). The inhibitor in the third site (Chain B) is seen binding "downwards" as described for the first instance. When bound in the "upwards" position, compound **2** forms a *trans* thioamide bond with Pro2. The phenyl ring, again makes an edge-to-face π interaction, but with Phe114 in this case. Two NH - π interactions occur between Gln'25 of a neighboring (symmetry related) molecule and the phenyl rings of **2** and of Tyr37 respectively. A "downwards" orientation is present in the third binding site where Glu'55, from a different symmetry related molecule is present between residues Lys33 and Tyr37, apparently competing with the phenyl ring of **2** for interaction with Tyr37. A recently deposited structure in the pdb (4p01) complexed with 4-cyanobenzyl ITC inhibitors in two of the active sites in the upwards orientation matching the binding orientation we see for **2**. However no inhibitor molecule has been built in the third active site and several amino acid residues are also disordered. The available crystallographic data surrounding the third catalytic proline indicates the presence of a thioamide moiety in a *trans* conformation suggesting substituted benzyl isothiocyanates preferentially bind in the upwards conformation. The "upwards" orientation in both sets of structures provides a clear explanation for the activity of compounds with multiple, as well as different aromatic, substitutions such as **17**, **18**, **20** and **21**.

Compounds that possess longer chains linking the isothiocyanate to the aromatic ring (Fig. 5) were tested for their biological activity and the majority showed IC_{50} ~2 μM (Table 3) with **29** giving an IC_{50} of 1.3 ± 0.9 against rhMIF. A crystal structure of compound **31** was determined in complex with rhMIF (Fig. 6). Whilst all three molecules of **31** are projected upwards, one adopts

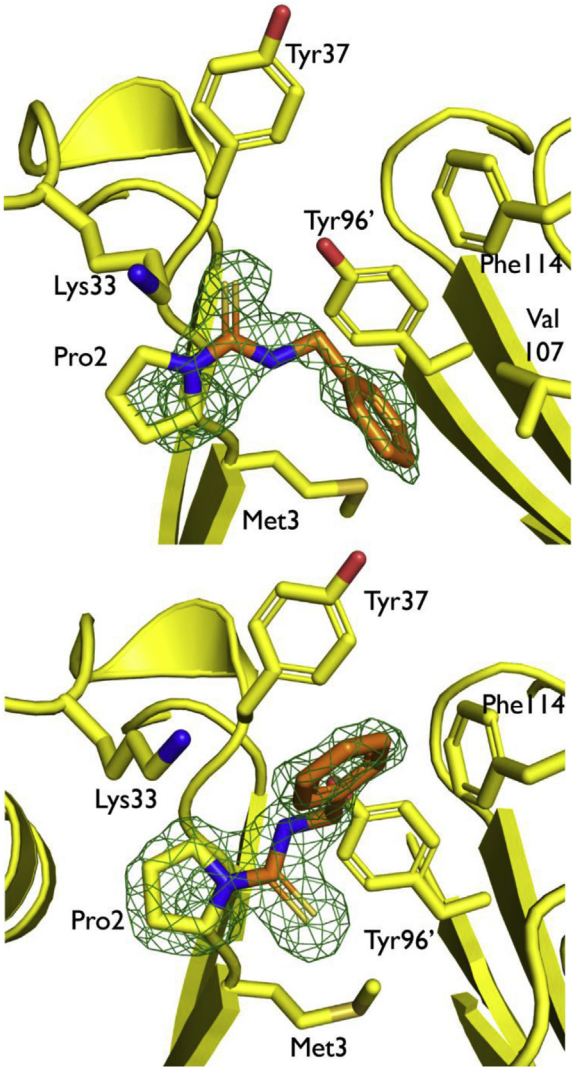


Fig. 4. The crystal structure of **2** covalently bound to rhMIF in two different binding modes. Upper panel: structure at 2.1 Å (PDB 3wnt) binding in the "downwards" orientation. Lower panel: crystal structure of rhMIF covalently modified with **2** binding in the "upwards" orientation at 2.0 Å (PDB 3wnr). Green mesh represents the *Fo* − *Fc* electron density omit map for the ligand and Pro2, contoured at 3σ. The enzyme is shown as a yellow cartoon with the covalently bound inhibitor shown in orange. All other colors are as described in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
IC_{50} of miscellaneous isothiocyanates against Jurkat T-lymphoma cells.

ITC	Jurkat cell IC_{50} (μM, ± S.E.)[a,b]
28	1.4 ± 0.1
29	1.8 ± 0.6
30	1.9 ± 0.1
31	4.2 ± 0.1
32	5.1 ± 0.2
33	11 ± 1

[a] Concentration of ligand required to inhibit 50% MIF tautomerase activity.
[b] Activity of MIF determined from whole cell lysates following treatment with ITCs. All assays are the results of at least two replicates.

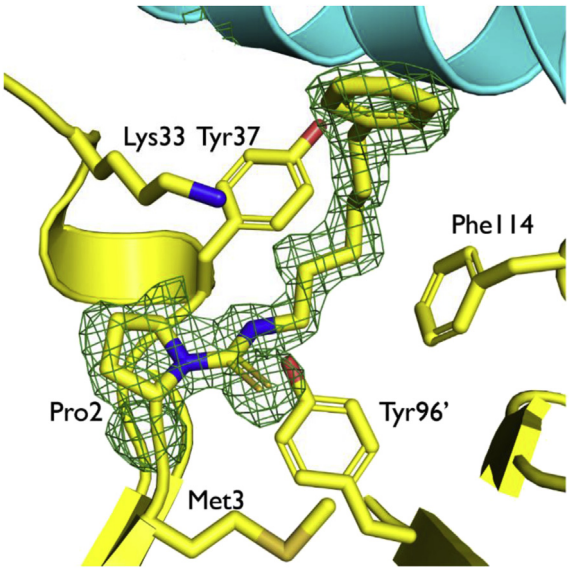


Fig. 6. The crystal structure of **31** covalently bound to rhMIF at 1.7 Å (PDB 4oyq). Green mesh represents the *Fo* − *Fc* electron density omit map for the ligand and Pro2, contoured at 3σ. The enzyme is shown as a yellow cartoon with the covalent inhibitor shown with yellow carbons within the mesh. A neighboring (symmetry related) protein molecule is shown in cyan (top of figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a different conformation (B chain) as a result of the crystal packing mentioned above and is relatively disordered compared to the other two positions. The disruption of one site by crystal packing requirements is also seen in a published MIF-L-sulforaphane structure [21].

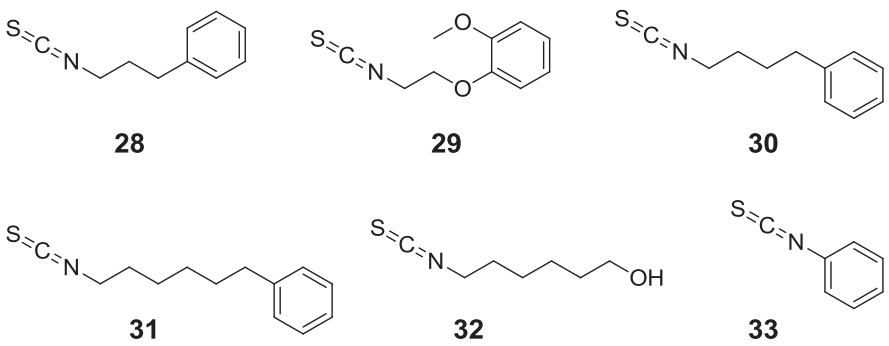


Fig. 5. Extended linker isothiocyanates, and phenyl isothiocyanate.

4. Discussion and conclusions

Macrophage migration inhibitory factor has been associated with numerous disease states including inflammatory bowel disease [4], rheumatoid arthritis [2], pain [22] and multiple sclerosis [13]. It has also been found to be overexpressed in numerous tumor types including breast cancer [23], colon cancer [24,25], non-small cell lung cancer [26], prostate cancer [27] as well as melanoma [28] and glioblastoma multiforme [29]. In the majority of these disease states, MIF has been up-regulated or over-expressed and subsequent inhibition of MIF has impeded its action. Several reports have also shown efficacy of isoxazoline inhibitors (e.g. ISO-1, **5**) *in vivo* including murine models of multiple sclerosis and colitis [12,13]. There are numerous inhibitors in the literature including the noncovalent competitive inhibitor **5** [20], allosteric inhibitors including the anti-inflammatory drug ibudilast (a phosphodiesterase inhibitor) [30] and a small set of covalent inhibitors including 4-iodo-6-phenylpyrimidine [31] and several isothiocyanates including **1**, **2** and **4** [6,14]. Prior to the identification of MIF as the target of isothiocyanates, ITCs have been associated with anti-cancer and anti-inflammatory activity [6,14,15]. Allyl ITC **3**, in combination with celecoxib, was shown to inhibit growth of bladder cancer [32] and to trigger apoptosis in MDA-MB-468 breast cancer cells [33]. The anti-tumorigenic activity of **3** can be attributed to MIF inhibition in the most part.

Here we report ITC inhibitors of MIF which adopt one of two distinct binding modes described as (i) “downward”, occupying a deep, restricted, hydrophobic pocket, or (ii) “upward”, occupying a more open cleft exposed to solvent. The deep binding pocket is optimal for only a select few inhibitors, those being isothiocyanates with phenyl side chains separated by either a methyl (benzyl isothiocyanate, **2**) or ethyl group with substituents limited to a hydroxyl in the 4-position (**1** and **8**). Crichlow et al. commented that the energetic disadvantage of the formation of a *cis* bond would be compensated for by the affinity of the phenethyl side chain of **1** being buried deep within the hydrophobic pocket in the crystal structure [21]. The “upwards” binding mode shows that MIF is far more accommodating with ITCs that can bind to the active site, with the results implying that compounds binding in this mode can do so with a higher affinity than similar compounds in the downward orientation, e.g. **2** and **20** compared to **1** and **8**. While the “downward” binding mode imposes tight constraints on the size of the inhibitor, the “upward” mode allows a much wider scope for design of inhibitors. In contrast, classical competitive inhibitors are seen to generally occupy both downward and upward regions. However a covalent thioamide linkage in the *trans* orientation removes the necessity to bind deep within the hydrophobic cavity but still maintain potent activity. This provides for the ability to develop a lower molecular weight inhibitor.

Several fluoro-substituted compounds in this study have been shown to degrade over time. Whilst the mechanism of degradation is not known, it may be possible to stabilize these compounds in the form of a prodrug as glucosinolates (β-thioglucoside-*N*-hydroxysulfates). ITCs exist in nature in the form of glucosinolates and the active ITCs are formed *in situ* by the plant enzyme myrosinase, a glucoglutahydrolase, as a natural defense mechanism. Intestinal bacteria also produce myrosinase which provides humans with the ability to process dietary glucosinolates. Hydrolyzed glucosinolates as prodrugs for ITCs have been previously tested in a number of cancer cell lines with reasonable activity (low micromolar IC_{50}) [34]. This strategy may protect susceptible compounds from degradation.

Isothiocyanates are known to react with biological nucleophiles, including the formation of reversible conjugates with glutathione. Alterations in the efficiency of these off-target reactions will contribute to the amount of free intracellular isothiocyanate available to react with MIF during the period of the cellular assays. This could be the major factor for the differing results in cell-based and recombinant MIF assays. However, in order for them to react with nitrogen-containing amino acids the amine must be uncharged, which therefore precludes the majority of nitrogen-containing amino acids on the surface of proteins e.g. Lys67 and Lys78 of MIF. We have previously shown that MIF is the major molecular target within cells and that Pro2 is the only residue which interacts with isothiocyanates [14]. Non-reacting moieties include surface residues as well as the three cysteine residues of each MIF monomer. Further stability of ITCs against adverse interactions could also be aided via the use of glucosinolate prodrugs.

In conclusion, our results show that MIF is inhibited by numerous different ITCs possessing an aromatic moiety. Expansion of this set is focused on the optimization and expansion of benzyl substituents as well as optimization of the alkyl chain. Given a number of reports on allosteric binding [30,35], it is clear that there is further optimization available to enhance binding of ITCs via extended interactions in this upward orientation. This would also assist in blocking interactions with the target receptor CD74. We have shown it is possible to develop small, sub-micromolar inhibitors of MIF which are active on both the enzyme and cells. We are currently investigating additional isothiocyanates which exploit the covalent binding and the upwards orientation. There is significant potential to further develop multiple isothiocyanates for different indications including tumors or inflammatory diseases of the gastrointestinal tract.

5. Experimental

5.1. General experimental details

All commercial reagents and anhydrous solvents were obtained from commercial sources and were used without further purification, unless otherwise specified.

High resolution electrospray ionization (ESI) mass spectra were recorded on a microTOF$_Q$ mass spectrometer. ^{1}H and ^{13}C NMR spectra (δ, ppm) were recorded at 400 MHz using a Varian MR spectrometer. The purity of the final compounds was assessed on the basis of analytical HPLC, and the results were greater than 95% unless specified otherwise. HPLC analysis was carried out using a Shimadzu Prominence system on a C18 column (Phenomenex Prodigy ODS(3) 5 μm 100 Å, 250 × 3 mm) with a 2 × 4 mm C18 guard column, peaks detected at 210 and 254 nm, solvents acetonitrile in aqueous 0.05% TFA: $t_0 = 10\%$, $t_{12.5} = 100$, $t_{15} = 100$, $t_{17} = 10$, $t_{20} = 10\%$.

(2-isothiocyanatoethyl)benzene (**1**), 3-Isothiocyanato-1-propene (**3**), (2-isocyanatoethyl)benzene, (**6**), 1-chloro-4-(2-isothiocyanatoethyl)benzene (**13**), 1-fluoro-4-(isothiocyanatomethyl)-benzene (**16**), 1-(isothiocyanatomethyl)-4-methoxybenzene (**19**), 1,1′-(isothiocyanatomethylene)bisbenzene (**27**) and isothiocyanatobenzene (**33**) were purchased from Sigma Aldrich. (Isothiocyanatomethyl)benzene (**2**), (3-isothiocyanatopropyl)benzene (**28**), (4-isothiocyanatobutyl)benzene (**30**), (6-isothiocyanatohexyl)benzene (**31**), 1-isothiocyanato-4-(methylsulfinyl)butane (*R,S*-Sulforaphane, **4**), (thiocyanatomethyl)benzene (**7**), (*R*)-(1-isothiocyanatoethyl)benzene [*R*(−)-a-Methylbenzyl isothiocyanate, (**23**) were purchased from LKT laboratories, Inc (St Paul, MN, USA). ISO-1 (*S,R*)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid, methyl ester (**5**) was purchased from Calbiochem. 1-fluoro-3-(isothiocyanatomethyl)-benzene (**17**) was purchased from Oakwood Chemicals.

6. Experimental procedures

Isothiocyanates were synthesized via one of four methods (Scheme 1) starting from either the corresponding amine or alcohol.

Compounds **8**, **11**, **12**, **18** were synthesized from commercially available amines according to method 1 published by Ryu et al. (Scheme 1, method 1) [36].

6.1. Method 1

The amine (e.g. tyramine.HCl 200 mg, 1.15 mmol, 1 equiv.) in dichloromethane (6 mL) with saturated sodium hydrogen carbonate (3 mL) or triethylamine (4 equiv.) was stirred at 0 °C for 5 min. Thiophosgene (95 μL, 1.2 mmol, 1.05 equiv.) was added and the mixture stirred at 0 °C for 20 min then brought to RT and stirred for 2 h. Dichloromethane was separated, the aqueous phase was then acidified and extracted with chloroform. The combined organic phases were dried (magnesium sulfate), filtered and concentrated to give the crude product as an orange gum. The crude material was purified using silica gel chromatography 0–5% ethyl acetate in dichloromethane which gave the pure product.

Hydroxyalkyl and aryl isothiocyanates **9**, **10**, **32**, were synthesized from commercially available amines according to method 2 published by Tajima et al. (Scheme 1, method 2) [37].

6.2. Method 2

Carbon disulfide (5 equiv.) in a tetrahydrofuran solution with the amine (1 equiv.), a catalytic amount of triethylamine (0.1 equiv.), and the mixture was stirred for 30 min at 30–35 °C. After cooling to 0–5 °C, hydrogen peroxide 30% aq. (2.8 equiv.) was added dropwise to this solution at 0–10 °C. As soon as the hydrogen peroxide was added, the solution was acidified with concentrated hydrochloric acid, concentrated, and filtered to remove free sulfur. The filtrate was extracted with ethyl acetate. The organic phase was dried (magnesium sulfate) and concentrated to give a yellow oil. Purification was performed by silica-gel chromatography (ethyl acetate:chloroform 1:3).

Compounds **22**, **24**, **25**, **26** and **27** were synthesized from commercially available alcohol according to method 3 published by Miyake et al. (Scheme 1, method 3) [38,39].

6.3. Method 3

Oxalic acid (2.0 mmol, 1 equiv.) and sodium thiocyanate (2.4 mmol, 1.2 equiv.) were added to a nitromethane (5 mL) solution of 4-(1-hydroxypropyl)phenol (2.0 mmol, 1 equiv.). The mixture was stirred for 2–3 h at 60 °C and was then poured into water and extracted with ethyl acetate. The combined organic phases were washed with water, dried (magnesium sulfate) and concentrated. The crude material was purified by silica-gel chromatography with dichloromethane-ethyl acetate mixtures.

Compounds **20**, **21** and **29** were synthesized from commercially available amines according to the method published by Lee et al. (Scheme 1, method 4) [40].

Triethylamine (3 or 6.25 equiv.) was added to a solution of the amine (1 equiv. in dimethylformamide at RT under N_2). To the resultant solution, 1,1′-thiocarbonyldiimidazole (2 equiv. in DMF at RT under N_2) was added in a dropwise fashion over 15 min, with continued stirring at RT for 18 h. The solution was quenched with water and extracted with ethyl acetate. The combined organic phases were washed with water and brine, dried (magnesium sulfate) and concentrated. The crude material was purified using silica gel chromatography (ethyl acetate-hexanes) to afford the desired isothiocyanate.

4-(2-isothiocyanatoethyl)phenol (**8**) [41] was prepared from tyramine.HCl and thiophosgene according to method 1 (yellow oil, yield 24%) [36]. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.08 (d, 2H, J = 8.0 Hz, ArH), 6.80 (d, 2H, J = 8.0 Hz, ArH), 4.76 (bs, 1H, OH), 3.68 (t, 2H, J = 7.0 Hz, CH_2NCS), 2.92 (t, 2H, J = 7.0 Hz, $ArCH_2$). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.6, 130.0, 129.2, 115.6, 46.6 and 35.7. HRMS $[M-H]^-$ for C_9H_9NOS calculated 178.0332, found 178.0369.

3-(2-Isothiocyanatoethyl)-phenol (**9**) [41] was prepared from 3-hydroxyphenethylamine, carbon disulphide and hydrogen peroxide according to method 2 [37]. IR (neat) 3383, 3039, 2932, 2179, 2089, 1588, 1491 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.25–7.15 (m, 1H), 6.83–6.71 (m, 3H), 4.92 (s, 1H), 3.71 (t, 2H, J = 6.9 Hz), 2.94 (t, 2H, J = 6.9 Hz) ppm. ^{13}C NMR: 36.3, 46.2, 114.2, 115.8, 130.0, 130.9, 138.8, 155.7 ppm. Spectra in agreement with literature values.

4-(2-Isothiocyanatoethyl)-1,2-benzenediol (**10**) [37] was prepared from dopamine, carbon disulphide and hydrogen peroxide according to method 2 [37]. IR (neat) 3297, 3033, 2965, 2936, 2187, 2114, 1602, 1517 cm^{-1}. ^{1}H NMR data matched the reported data [37]. ^{1}H NMR ($CDCl_3$): 6.85–6.63 (m, 3H), 5.55 (s, 1H), 5.40 (s, 1H), 3.66 (t, 2H, J = 6.8 Hz), 2.86 (t, 2H, J = 6.8 Hz) ppm. ^{13}C NMR: 35.8, 46.5, 115.7, 115.9, 121.4, 130.1, 130.5, 142.6, 143.7 ppm. Spectra in agreement with literature values.

(2-isothiocyanatoethyl)-4-(trifluoromethyl)-benzene (**11**) [42] was obtained from (2-aminoethyl)-4-(trifluoromethyl)-benzene and thiophosgene according to method 1 [36]. IR (neat) 2932, 2186, 2083 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.62 (d, 2H, J = 8.0 Hz), 7.36 (d, 2H, J = 8.0 Hz), 3.78 (t, 2H, J = 6.8 Hz), 3.08 (t, 2H, J = 6.8 Hz) ppm. ^{13}C NMR: 36.2, 46.0, 124.1 (J = 272 Hz), 125.8 (J = 3.8 Hz), 129.2, 129.6 (J = 32.5 Hz), 131.8, 141.0 (J = 8.8 Hz) ppm. ^{19}F NMR (CF_3CO_2H) −63.0 ppm.

(2-isothiocyanatoethyl)-3-(trifluoromethyl)-benzene (**12**) was obtained from (2-aminoethyl)-3-(trifluoromethyl)-benzene and thiophosgene according to method 1 [36]. IR (neat) 2936.2, 2875.3, 2186.6, 2083.8, 1596.8, 1492.3 cm^{-1} ^{1}H NMR ($CDCl_3$): 7.40–7.60 (m, 4H), 3.76 (t, 2H, J = 6.8), 3.05 (t, 2H, J = 6.8 Hz) ppm. ^{13}C NMR: 36.2, 46.0, 124.0 (J = 272.4 Hz), 124.1 (J = 3.8 Hz), 125.5 (J = 3.8 Hz), 129.3, 131.2 (J = 32.3 Hz), 132.3, 132.3, 137.9 ppm. ^{19}F NMR (CF_3CO_2H): −63.1 ppm.

4-(2-Isothiocyanato-1-methylethyl)phenol (**14**) [43] was obtained from 4-(2-amino-1-methylethyl)phenol by Boc protection of the alcohol [44] followed by O-benzylation (benzyl bromide, potassium carbonate), N-deprotection (hydrochloric acid) and reaction with thiophosgene. Finally, debenzylation by reaction with triphenylphosphine hydrobromide in acetonitrile under microwave conditions [45], gave the required compound (**14**). ^{1}H NMR ($CDCl_3$): 7.09 (d, 2H), 6.82 (d, 2H), 4.90 (bs, 1H), 3.56 (m, 2H), 3.08 (sextet, 1H), 1.35 (d, 3H) ppm. Spectra in agreement with literature values.

4,4′-(2-isothiocyanatoethylidene)bis-phenol (**15**) [46] was produced from 4,4′(2-aminoethylidene)bisphenol by Boc protection of the amine [47] followed by bis O-benzylation (benzyl bromide, potassium carbonate), N-deprotection (hydrochloric acid) and reaction with thiophosgene [36] to give the bis O-benzyl derivative of **15**. The free phenol (**15**) was then obtained by reaction with triphenylphosphine hydrobromide in acetonitrile under microwave conditions [45]. ^{1}H NMR (CD_3OD): 7.67 (d, 2H), 7.14 (d, 2H), 6.83 (d, 2H), 6.72 (d, 2H), 5.04 (dd, 1H), 4.59 (dd, 1H), 4.40 (dd, 1H) ppm. ^{13}C NMR: 171.1, 162.1, 158.2, 133.9, 131.2, 129.2, 125.6, 116.5, 116.4, 73.3, 55.0 ppm.

1-(isothiocyanatomethyl)-3-(trifluoromethyl)-benzene (**18**) [48] was obtained from 3-(trifluoromethyl)benzylamine and thiophosgene according to method 1 (colorless oil, yield 45%) [36]. IR (neat) 2929.2, 2171.9, 2078.3, 1599.0, 1494.4 cm^{-1} ^{1}H NMR ($CDCl_3$): 7.50–7.64 (m, 4H), 4.80 (s, 2H) ppm. ^{13}C NMR: 48.3, 123.7 (J = 272.4 Hz), 123.7 (J = 3.8 Hz), 125.3 (J = 3.8 Hz), 129.6, 130.1, 131.4

(J = 32.5 Hz), 134.0, 135.3 ppm. ^{19}F NMR (CF_3CO_2H): −63.2 ppm. Spectra in agreement with literature values.

4-(isothiocyanatomethyl)benzenesulfonamide (**20**) [49] was obtained from 4-aminomethyl benzenesulfonamide hydrochloride and 1,1′-thiocarbonyldiimidazole according to method 4 (white solid, yield 37.5%) [50]. ^{1}H NMR data matched the reported data [49]. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.87 (d, 2H, J = 8.4 Hz); 7.56 (d, 2H, J = 8.4 Hz); 7.40 (s, 2H, NH_2); 5.05 (s, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 143.9, 138.5, 129.9, 127.6, 126.2, 47.6. HRMS for $C_8H_8N_2NaO_2S_2$ calculated 250.9919, found: 250.9912.

5-(isothiocyanatomethyl)benzo[*d*][1,3]dioxole (**21**) [34] was obtained from 1,3-benzodioxol-5-methylamine and 1,1′-thiocarbonyldiimidazole according to method 4 (yellow oil, 68.1%) [50]. ^{1}H NMR data matched the reported data [34]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.81–6.75 (m, 3H); 5.98 (s, 2H, CH_2); 4.60 (s, 2H, OCH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 148.2, 147.7, 127.9, 120.6, 108.5, 107.6, 101.4, 48.6). HRMS [M-H]$^-$ for $C_9H_6NO_2S$ calculated 192.0125, found: 192.0120.

(1-isothiocyanatoethyl)-benzene (**22**) [51] was obtained by reacting 1-phenylethanol with oxalic acid and sodium thiocyanate according to method 3 [38,39]. IR (neat) 3062, 3029, 2975, 2169, 2928, 2870, 2081, 1603, 1492 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.37–7.26 (m, 5H), 4.92 (q, 1H, J = 6.8 Hz), 1.68 (d, 3H, J = 6.79) ppm. ^{13}C NMR: 25.0, 57.0, 125.4, 128.2, 128.9, 132.3, 140.2 ppm. Spectra in agreement with literature values.

(1-isothiocyanatopropyl)-benzene (**24**) [52] was obtained by reacting 1-phenyl-1-propanol with oxalic acid and sodium thiocyanate according to method 3 [38,39]. IR (neat) 3063, 3029, 2967, 2932, 2874, 2075 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.15–7.35 (m, 5H), 4.62 (t, 1H, J = 8.0 Hz), 1.88 (m, 2H), 0.94 (t, 3H, J = 8.0 Hz) ppm. ^{13}C NMR: 10.5, 32.3, 63.2, 125.9, 128.1, 128.8, 132.0, 138.8 ppm. Spectra in agreement with literature values.

4-(1-isothiocyanatopropyl)phenol (**25**) [38] was obtained by reacting 4-(1-hydroxypropyl)phenol (AKos) with oxalic acid and sodium thiocyanate according to method 3 [38,39]. IR (neat) 2115, 2078, 1721, 1512 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.26 (m, 2H), 6.84 (m, 2H), 4.62 (dd, 1H, 4.0 Hz, 4.0 Hz), 1.92 (m, 2H), 0.99 (t, 3H, 4.0 Hz) ppm. ^{13}C NMR: 10.6, 32.3, 55.4, 62.9, 114.2, 127.2, 131.1, 131.7, 159.4 ppm. Spectra in agreement with literature values.

1-(1-Isothiocyanato-2-methylethyl)-4-methoxybenzene (**26**) was obtained by reacting 1-(4-methoxyphenyl)-1-propanol (Aldrich) with oxalic acid and sodium thiocyanate according to method 3 [38,39]. IR (neat) 2049, 1510, 1244 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.20 (d, 2H, J = 7.2 Hz), 6.90 (d, 2H, J = 6.8 Hz), 4.62 (m, 1H), 3.81 (s, 3H), 1.95 (m, 2H), 1.01 (t, 3H, J = 6.0 Hz) ppm. ^{13}C NMR: 10.6, 32.3, 55.4, 62.9, 114.2, 127.2, 131.1, 131.7, 159.4 ppm.

1,1′-(isothiocyanatomethylene)bisbenzene (**27**) was obtained from Aldrich or from reacting diphenylmethanol with oxalic acid and sodium thiocyanate according to method 3 [38,39]. IR (neat) 3055, 3031, 2925, 2162, 2139, 2090 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.48–7.23 (m, 10H), 5.99 (s, 1H) ppm.

1-(2-isothiocyanatoethoxy)-2-methoxybenzene (**29**) was obtained from 2-(2'-methoxy)phenoxyethyl amine and 1,1′-thiocarbonyldiimidazole according to method 4 (white solid, yield 64.2%) [50]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.01–6.91 (m, 4H); 4.22 (t, 2H, J = 5.6 Hz); 3.90 (t, 2H, J = 4.4 Hz); 3.88 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 150.3, 147.3, 122.9, 120.9, 116.0, 112.5, 67.9, 56.0, 44.7. HRMS for $C_{10}H_{11}NNaO_2S$ calculated 232.0403, found: 232.0407.

6-Isothiocyanato-1-hexanol (**32**) [53] was prepared from 6-amino-1-hexanol, carbon disulphide and hydrogen peroxide according to method 2 [37]. ^{1}H NMR data matched the reported data [53]. IR (neat) 3367, 2934, 2859, 2099 cm^{-1}. ^{1}H NMR ($CDCl_3$): 3.65 (t, 2H, J = 6.5 Hz), 3.52 (t, 2H, J = 6.6 Hz), 1.72 (m, 2H), 1.59 (m, 2H) 1.43 (m, 4H) ppm. ^{13}C NMR: 25.0, 26.4, 29.9, 32.4, 45.0, 62.0 ppm.

7. Biological assays

7.1. Isothiocyanates and related compounds

Stock solutions (1 M) of isothiocyanates and related compounds were prepared in sterile DMSO and stored at 4 °C. Isothiocyanates were diluted further with DMSO and added to cells so that the final concentration of DMSO in the media was kept constant at 0.1%.

7.2. Recombinant MIF; cloning, expression and purification

Human MIF cDNA was obtained from HeLa cells, ligated into a pET28α expression vector (Novagen), and transformed into *E. coli* DH5α (Invitrogen). Resultant clones were selected in the presence of kanamycin (50 μg/mL).

MIF expression was induced with isopropyl-D-1-thiogalactopyranoside (IPTG) and the cells were harvested by centrifugation. Cells were lyzed by freeze thawing, dounce homogenization, and vortex mixing with an equal volume of glass beads (106 pm; Sigma G-8893). The lysate was clarified by centrifugation at 38,000 g for 30 min, followed by filtration with 0.45 μM and then 0.22 μM membrane filters.

The clarified lysate was fractionated through a DEAE anion exchange column using fast protein liquid chromatography. MIF-containing fractions were pooled and loaded onto a MONO-Q HR5/5 column (GE Healthcare). The bound protein was eluted using Tris-buffered saline (50 mM Tris-Cl, 150 mM NaCl, pH 7.5), and the fractions containing purified rhMIF were concentrated using a centrifugal filter device with a 5 kDa molecular-weight cutoff (Corning Lifesciences). The mass of full length MIF (12,345 Da) was confirmed by mass spectrometry using a Velos Pro ion trap mass spectrometer (Thermo Fisher Scientific).

rhMIF was diluted to 0.8 μM with sterile Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4) and 19 μL aliquots were transferred to a 96 well plate. Appropriate dilutions of ITCs (1 μL) were added to the rhMIF and incubated at room temperature for 5 min before measurement of tautomerase activity.

7.3. Cell culture

The Jurkat T-lymphocyte cell line was obtained from American Type Culture Collection (#TIB-152, Rockville, MD, USA) and maintained in RPMI 1640 containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells were resuspended in fresh antibiotic-free medium 1 h before treatment at a density of 1 × 10^6 cells/mL. Following treatment with ITCs for 30 min, 1.5 × 10^6 Jurkat cells were collected and resuspended in 60 μL of lysis buffer (40 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.6 mg/mL Complete™ protease inhibitors and 1% CHAPS). Insoluble material was removed by centrifugation at 15,000 g for 4 min. Protein extracts (20 μL) were transferred to a 96 well plate in duplicate for measurement of tautomerase activity.

7.4. MIF tautomerase assay

L-dopachrome methyl ester was prepared by combining 500 μL of a 20 mM sodium periodate stock with 1.5 mL of 20 mM L-3,4-dihydroxyphenylalanine methyl ester (in ultrapure H_2O) respectively in 8 mL of Bis Tris buffer (50 mM Bis Tris, pH 6.2, 1 mM EDTA). Components were reacted for 5 min before immediate use in the tautomerase assay. 180 μL of dopachrome solution was added to each well and the change in absorbance at 475 nm due to dopachrome tautomerization was monitored for 2 min using a Softmax Pro 190 spectrophotometer (Molecular Devices, Sunnyvale, CA,

USA). A blank containing TBS or lysis buffer was included in all experiments.

8. X-ray crystallography

The isothiocyanate-MIF complexes were prepared using methods described elsewhere [19]. Crystallization was carried out using either 1.9 or 2.0 M ammonium sulfate, 100 mM Tris pH 8.0, 200 mM NaCl and 4% (v/v) 2-propanol. Diffraction data was obtained from single crystals using either the Cu Kα radiation on the University of Otago's Rigaku MicroMax-007 HF X-ray generator with Osmic VariMax mirrors and an R-AXIS IV++ image plate detector or the MX1 beamline at the Australian synchrotron using an ADSC Quantum 210r detector. Diffraction data were processed with iMosflm [54] and scaled using SCALA [55]. For phasing, molecular replacement was implemented using Phaser (using PDB entry 4F2K as a search model) [19]. Modeling and refinement of the structures was carried out using Coot [56] and REFMAC [57] from the CCP4 suite [58] and Phenix.refine [59]. Restraints for BITC were taken from Cambridge Structural Database (BASXAW, YAXZEE and XEWFIF) [60]. Statistics for data collection, reduction and refinement are listed in Supplementary material.

9. Accession codes

Coordinates and crystallographic structure factors have been deposited with the Protein Data Bank [accession code 3WNR for **2** − MIF complex (upwards), accession code 3WNT for **2** − MIF complex (downwards), accession code 3WNS for **3** − MIF, accession code 4OSF for **8** − MIF complex and accession code 4OYQ for **31** − MIF].

Author information

Author contributions

M.B.H and J.D.A.T conceived the experiments, analyzed results and wrote the paper. E.S.S. carried out all the biological activity testing. E.J.D. synthesized and characterized most of the compounds. A.L.G. determined a crystal structure and C.T.V. synthesized and characterized several compounds and determined a crystal structure. J.D.A.T. determined the remainder of the crystal structures. M.T.R. purified and derivatized rhMIF with ITCs and set up crystallization experiments. Y.N. assisted with small molecule parametrization. A.B.G, R.A.J.S and S.M.W. help to design the experiments, analyzed results and provided constructive comments on the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.02.012.

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