Structure-activity relationships for binding of 4-substituted triazole-phenols to macrophage migration inhibitory factor (MIF)

Zhangping Xiao, Marieke Fokkens, Deng Chen, Tjie Kok, Giordano Proietti, Ronald van Merkerk, Gerrit J. Poelarens, Frank J. Dekker

Abstract

Macrophage migration inhibitory factor (MIF) is a versatile protein that plays a role in inflammation, autoimmune diseases and cancers. Development of novel inhibitors will enable further exploration of MIF as a drug target. In this study, we investigated structure-activity relationships of MIF inhibitors using a MIF tautomerase activity assay to measure binding. Importantly, we notified that transition metals such as copper (II) and zinc (II) interfere with the MIF tautomerase activity under the assay conditions applied. EDTA was added to the assay buffer to avoid interference of residual heavy metals with tautomerase activity measurements. Using these assay conditions the structure-activity relationships for MIF binding of a series of triazole-phenols was explored. The most potent inhibitors in this series provided activities in the low micromolar range. Enzyme kinetic analysis indicates competitive binding that proved reversible. Binding to the enzyme was confirmed using a microscale thermophoresis (MST) assay. Molecular modelling was used to rationalize the observed structure-activity relationships. The most potent inhibitor inhibited proliferation of A549 cells in a clonogenic assay. In addition, attenuated MIF induced ERK phosphorylation in A549 cells. Altogether, this study provides insights in the structure-activity relationships for MIF binding of triazole-phenols and further validates this class of compounds as MIF binding agents in cell-based studies.

1. Introduction

Macrophage migration inhibitory factor (MIF) was discovered in 1966 by Bloom and Bennett as a cytokine that is implicated in the inhibition of macrophage motility [1]. Subsequently, MIF was also discovered to function as a hormone, a chemokine and as a molecular chaperone. Thus, MIF proved to be involved in various physiological and pathological processes [2–4]. Genetic deregulation of MIF (such as overexpression) has been implicated in many inflammatory and immune diseases in humans, such as diabetes, atherosclerosis and rheumatoid arthritis [5]. In addition, mounting evidence supports a role of MIF in tumorigenesis and progression [6]. Its key roles in health and disease raised interest in development of small molecule MIF modulators as potential therapeutic agents.

Many MIF functions are mediated by protein-protein interactions with membrane receptors. The cluster of differentiation 74 (CD74) receptor is the best characterized membrane receptor for MIF [7]. Recently, another membrane protein CD44 was reported as an integral component of the CD74 receptor complex that proved to be essential for MIF signal transduction [8]. By forming a complex with CD74 and CD44, MIF triggers activation of the mitogen activated protein kinase (MAPK) pathway. Activation of this pathway is associated with MIF mediated oncogenesis and inflammation. MIF functions in inflammation and immune cell chemotaxis are also mediated by interactions with chemokine receptors such as CXCR2 and CXCR4 [4]. Besides extracellular functions, MIF is known to bind to intracellular protein targets such as Jun-activated domain-binding protein 1 (JAB1), which results in slowing down JAB1 mediated cell growth [9]. Therefore, development of molecules to interfere with MIF protein-protein interaction has emerged as an attractive strategy to block MIF signaling.

Prior evidence indicates that treatment with anti-MIF antibodies or small molecule MIF modulators enables disruption of MIF mediated functions [10]. Besides discovering a statistically significant up-regulation of MIF concentration in the blood of septic
patients, Calandra et al. reported that an anti-MIF antibody can protect TNFα-deficient mice from fulminant septic shock [11]. Neutralization of MIF with anti-MIF antibodies provided to be beneficial in autoimmune encephalomyelitis [12], endotoxic shock [13] and even cancer [14]. From the perspective of drug discovery, development of small molecule MIF binders has advantages compared to the development of antibodies [15,16]. Many small molecule MIF binders have been discovered, but only a few of them have been tested for their biological activity [17–19]. Progression along this line is needed to shed light on the utility of MIF binders as a potential novel strategy for management of inflammatory diseases.

Normally, MIF exists in a homotrimeric form in which each monomer contains 115 amino acids and has a molecular mass of 12.4 kDa [20]. Apart from its functions in protein-protein interactions, the MIF trimmer also harbors keto-enol tautomerase activity. There are three tautomerase active sites in the MIF homotrimer, each located at the interface between two monomers, in which the residue Pro1 has a key role in catalysis [21]. Progression of the Jorgensen hybrid-triazole structure for which they confirmed reversibility of inhibition. Besides, we have explored alternatives for the quinoline functionality in the triazole 4-position in order to obtain novel triazole-phenols that reversibly inhibit MIF tautomerase activity and also provide activity on the cellular level.

2. Results and discussion

2.1. MIF tautomerase activity assay optimization

Recombinant human MIF was expressed and purified following methods published previously by our group [30] and others. [32] The MIF tautomerase activity assay using 4-HPP as a substrate (Fig. 2A) was established using previously described assay conditions [30]. To determine the IC50, the compounds were diluted from a 96 well plate. Subsequently, the dilutions were pre-incubated with the MIF enzyme solution (760 nM, 170 μL) for 15 min. The assay was started by mixing 50 μL of the inhibitor enzyme pre-incubation mixture with 50 μL of an aqueous 4-HPP (1.0 mM) solution. This provides a reaction mixture with 380 nM of the enzyme, 0.5 mM 4-HPP and various concentration of the inhibitor. In the positive control, MIF was incubated with a blanc DMSO dilution as a vehicle control before adding the 4-HPP substrate. In the negative control, the substrate was mixed with a blanc DMSO dilution in absence of MIF as an enzyme. In the negative control, the UV absorbance did not change over time, which was set to 0%, whereas the positive control was set to 100%.

To test the stability of MIF, 3 samples from the same batch of enzyme were stored at 4 °C for 1, 12 and 25 days, respectively, after being taken from the −80 °C freezer. Different storage times provide the same tautomerase activity levels (Fig. S1). In addition, unfolding temperatures of fresh enzyme and enzyme stored for 2 weeks in at 4 °C were determined by nanoDSF to be 77.9 °C and 78.9 °C (Fig. S1). These results together show that MIF is stable upon prolonged storage at −80 °C or 4 °C.

During our studies we noticed that irreproducible results were obtained from inhibitors synthesized using the CuAAC “click

Fig. 1. Reported inhibitors of MIF, ISO-1 is developed in 2002 by Al-Abed et al. and it is one of the most investigated inhibitors of MIF [28]. Orita-13 was found in 2001 by a structure-based computer-assisted search. Its Ks value was reported to be 38 nM [35]. Jorgensen-3bb is one of the most potent inhibitors of MIF reported to date with a Ks value of 57 nM [34].
reaction”. This raised the idea that transition metals such as copper could influence the MIF tautomerase activity assay that we employed in our studies. To our surprise, we found that copper(II) inhibits MIF tautomerase activity with an IC50 of 1.0 μM (Fig. 2B). Expanding on this finding we also found that zinc(II) inhibits MIF tautomerase activity with an IC50 of 1.0 μM (Fig. S3). This indicates that the observed inhibition does not depend on the reduct potential of the transition metal but rather suggests a role for the metal ion as Lewis acid. These observations imply that presence of (traces of) transition metals could contribute to irregularities in MIF tautomerase inhibition assays [36].

Subsequently, we investigated the inhibition of MIF tautomerase activity by copper(II) further. In the regular assays, we apply MIF with a C-terminal His-tag. To test the influence of the His-tag, we used MIF without His-tag and found that the tautomerase activity of His-tag free MIF was also completely blocked in presence of 20 μM copper(II) (Fig. S4). In addition, we found that copper(II) also blocked the activity of a related enzyme, 4-oxaloacryl tautomerase (4-OT), in a Michael addition reaction (Fig. S4) [37]. In the literature, it has been reported that the copper(II)-containing protein ceruloplasmin (CP) can suppress MIF enzymatic activity, [38], which is in line with our findings here. Altogether, these findings indicate that the transition metals copper(II) and zinc(II) can interfere with the 4-HPP tautomerization reaction catalyzed by MIF under the assay conditions applied in this study.

We adjusted the assay conditions to prevent interference of transition metals with MIF tautomerase enzyme activity in inhibitor binding studies. Including ethylenediaminetetraacetic acid (EDTA) in the assay buffer proved to be an effective strategy to prevent interference of copper(II) with the MIF tautomerase activity. Our results indicate that 125 μM copper(II) has no effect on 4-HPP tautomerization by MIF if 0.5 mM EDTA is added to the assay buffer (Fig. 2C). Addition of EDTA did not influence the Km value for 4-HPP conversion as both conditions provided a Km of 1.1 mM in the Michaelis-Menten enzyme kinetics (Fig. S2). Based on these findings we included 0.5 mM EDTA in the MIF tautomerase assay buffer in our studies. This is particularly important for activity measurements of 4-(1,2,3-triazole)phenol derivatives, as studied here, because their synthesis requires significant amounts of copper, which could result in copper pollution of the final products.

We note that inhibition of MIF tautomerase activity by transition metals is the first time reported here, but that EDTA has already been used before in the assay buffer in MIF inhibitor development [29,39]. However, other studies, including those using CuAAC for inhibitor synthesis, did not apply EDTA in the assay buffer [34,35]. Here we provide a rationale to include EDTA in the assay buffer in cases where the presence of traces of transition metals in the final products can be expected.

2.2. Synthesis

A focused compound collection of 4-(1,2,3-triazole)phenol derivatives was obtained by coupling 4-azidophenol to various terminal alkynes using CuAAC (Scheme 1). In this study, the CuAAC reaction was performed by addition of a catalytic amount of CuSO4 and sodium ascorbate in water to the alkyne andazole substrates dissolved in methanol. The reaction was allowed to proceed at room temperature or 60 °C for 12 h [40]. The terminal alkyne precursors for compounds of group A were prepared using propiolic acid as a key intermediate. Different amines were coupled to propiolic acid through a N,N'-dicyclohexylcarbodiimide (DCC) mediated amidation. The subsequent step provided the final products in overall isolated yields between 30% and 86%. Compounds of group B were synthesized by coupling various aliphatic terminal alkynes to 4-azidophenol at room temperature to provide a series of 4-(1,2,3-triazole)phenol derivatives. For this series the isolated yields varied between 12% and 96%. Compounds of group C were synthesized using 2-propargylamine as key intermediate. Different carboxylic acids were coupled to propargylamine using DCC mediated amidation to provide substituted terminal alkynes that were subjected to the “click” reaction. These two subsequent reactions were conducted at room temperature with isolated yields between 15% and 98%. The variability of the yields could be attributed to the low solubility of several products in combination with the filtration in the final step of the synthesis.

2.3. Enzyme inhibition study

The focused compound collection was screened for inhibition of MIF tautomerase activity by an assay employing 4-HPP as a substrate. The assay conditions, as described above, include 0.5 mM EDTA to avoid interference of residual copper(II) with the tautomerase activity. The inhibitors were screened for MIF binding by measurement of the residual MIF tautomerase activity in presence of 50 μM of the respective inhibitor. MIF tautomerase inhibitors ISO-1 and Jorgensen-2b were tested as a reference to literature values (Table 1). These inhibitors reduced the activity of MIF with percentages of 20% and 90% respectively at 50 μM, which is in line with their reported potency for MIF inhibition in literature [34].

Screening of the residual enzyme activity upon preincubation with 50 μM of the respective inhibitor provided clear structure-activity relationships for the three groups of 4-(1,2,3-triazole) phenol inhibitors as shown in Fig. 3. For compounds of group A the residual enzyme activity is around 75% of the positive control for all inhibitors, which indicates a potency similar to ISO-1 without a clear structure-activity dependence. For group B the residual enzyme activity varies between 90% and 10% of the positive control,
thus indicating a clear structure-activity dependence. Extending the 4-substitution of the 4-(1,2,3-triazole)phenol scaffold from propyl to pentyl and 5-hexynyl reduces the residual enzyme activity from 90% to 10% of the control, whereas octyl substitution provides increased residual enzyme activity. This indicates that an aliphatic tail with 5 or 6 carbon atoms is optimal in this series of inhibitors. The structure-activity relationships for the compounds in group C are less clear with residual enzyme activities between 40 and 10% of the control with compound 7c as the most active one in this series.

All the compounds that inhibited MIF tautomerase activity by more than 50% of the positive control were subjected for IC50 determination. The IC50 values are shown in Table 1. K_i values are calculated by the Cheng-Prusoff equation: 

\[
K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}
\]

in which K_M = 1.1 mM. The K_i value of ISO-1 was measured to be 44 ± 4.9 μM, which is in line with values reported in literature [30,36]. To further confirm the validity of our assays and to enable direct comparison, inhibitor Jorgensen-2b and 3b were synthesized according to literature [42]. Using Jorgensen-2b we investigated the effect of addition of EDTA to the assay buffer on the IC50 and K_i values in the MIF inhibition assay. For Jorgensen-2b, a K_i of 5.0 ± 0.6 μM was determined in presence of 0.5 mM EDTA, whereas a K_i value of 2.9 ± 0.3 μM was determined in absence of 0.5 mM EDTA. This demonstrates that EDTA can influence the Ki values to a certain extent but that the observed differences are limited to less than a two-fold change in potency. Ki value of Jorgensen-3b was determined to be 0.42 ± 0.03 μM with 0.5 mM EDTA present. The Ki values observed in our assay are in line with the Ki of 8.8 and 0.59 μM for Jorgensen-2b and 3b, respectively, reported before by Jorgensen [42].

Compounds in group A were not subjected to IC50 determinations due to a lack of inhibitory potency at concentrations of 50 μM. Group B provided two inhibitors with potencies in the low micromolar range. Compounds 5b and 7b had a K_i of 6.9 ± 0.3 μM and 6.5 ± 0.8 μM, respectively. The clear activity dependence on the length of the aliphatic tail indicates a role for lipophilic interactions in binding. For group C, inhibitors with variant monocyclic aromatic groups exhibit K_i values ranging from
### Table 1
Inhibition of MIF tautomerase activity by 4-substituted triazole phenols.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R₁, R₂, R₃ or R₄</th>
<th>%Residual activitya</th>
<th>IC₅₀ (µM)</th>
<th>Kᵢ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO-1</td>
<td>–</td>
<td>–</td>
<td>64 ± 7.1</td>
<td>44 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>Jorgensen</td>
<td>–</td>
<td>7.2 ± 0.9</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Jorgensen</td>
<td>–</td>
<td>–</td>
<td>0.64 ± 0.05</td>
<td>0.44 ± 0.03</td>
</tr>
</tbody>
</table>

**Group A**

| 1a    | 3,4-OMe-phenyl   | 77%                | –         | –       |
| 2a    | 4-F-phenyl       | 74%                | –         | –       |
| 3a    | 2-F-phenyl       | 80%                | –         | –       |
| 4a    | 2-amine-phenyl   | 77%                | –         | –       |
| 5a    | phenyl           | 71%                | –         | –       |
| 6a    | cyclopropyl      | 74%                | –         | –       |
| 7a    | 2-ethoxy-2-oxoethyl | 81%            | –         | –       |
| 1b    | hydroxymethyl    | 91%                | –         | –       |
| 2b    | 1-hydroxyethyl   | 73%                | –         | –       |
| 3b    | isopropyl        | 62%                | –         | –       |
| 4b    | propyl           | 50%                | –         | –       |
| 5b    | pentyl           | –                   | 10 ± 0.5  | 6.9 ± 0.3 |
| 6b    | cyclohexyl       | –                   | 26 ± 2.2  | 17 ± 1.5 |
| 7b    | 5-hexynyl        | –                   | 9.5 ± 1.1 | 6.5 ± 0.8 |
| 8b    | octyl            | 53%                | –         | –       |
| 9b    | 4-methoxy-4-oxobutyl | –              | 33 ± 2.0  | 23 ± 1.4 |
| 10b   | 3-oxo-3-(phenylamino)propyl | –                  | 47 ± 5.0  | 32 ± 3.4 |
| 1c    | phenyl           | –                   | 33 ± 1.4  | 23 ± 1.0 |
| 2c    | thiophen-2-yl    | –                   | 41 ± 4.0  | 28 ± 2.7 |
| 3c    | 1H-pyrrol-2-yl   | –                   | 43 ± 4.2  | 30 ± 2.9 |
| 4c    | 5-bromofuran-2-yl | –                  | 38 ± 4.1  | 26 ± 2.8 |
| 5c    | 5-methylthiophen-2-yl | –                  | 28 ± 2.0  | 19 ± 1.4 |
| 6c    | 4-F-phenyl       | –                   | 53 ± 3.0  | 36 ± 2.0 |
| 7c    | 1H-indol-2-yl    | –                   | 10 ± 0.7  | 6.9 ± 0.5 |
| 8c    | (1H-indol-3-yl)methyl | –                  | 62 ± 4.0  | 43 ± 2.8 |
| 1d    | H                | –                   | 4.8 ± 0.5 | 3.3 ± 0.3 |
| 2d    | Cl               | –                   | 1.4 ± 0.3 | 0.96 ± 0.2 |

**Group B**

**Group C**

**Group D**

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*a* Residual MIF tautomerase activity in presence of 50 µM inhibitor.

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**Fig. 3.** Screening of the inhibitory potency of the triazole-phenol collection at 50 µM inhibitor concentration. The enzyme activity in absence of inhibitor was set to 100%. Data are presented as mean ± standard deviation (n = 3).
19 to 35 μM. However, the bicyclic indole functionality provided stronger inhibition with a Ki value of 6.9 ± 0.5 μM for 7c. This could be due to both stacking and/or lipophilic interactions between the indole functionality of 7c and MIF. Extending the single carbon atom spacer between the triazole and the indole in 7c to a two-carbon atom spacer in 8c caused a loss of potency with a Ki of 43 ± 2.8 μM, which indicates a clear structure dependence of the activity.

2.4. Inhibitor optimization and enzyme kinetic study

To further improve the inhibitory potency of the inhibitors identified against MIF, we substituted the phenolic ortho-position with a fluorine [34]. Therefore, two new inhibitors were synthesized as shown in Scheme 1D. The resulting compound 1d provided a Ki of 3.2 ± 0.3 μM, which is two times enhanced compared to its equivalent without fluorine 7c (Fig. 4A). This proves again that an ortho-fluoro substitution is a favorable modification for triazole-phenol MIF inhibitors. By substitution of the 5-position of the indole in 1d with chlorine, inhibitor 2d was obtained, which proved to me more potent with a Ki of 0.96 ± 0.2 μM. The solubility of 1d and 2d were respectively 24 ± 0.19 μg/mL (41.41 ± 0.51 μM) and 10.8 ± 0.10 μg/mL (28.01 ± 0.26 μM) in pH 7.4 PBS buffer as measured by the shake-flask method (Fig. S1). Both 1d and 2d have significantly improved aqueous solubility compared to inhibitors Jorgensen-3b, which is 2.2 μg/mL [34]. This demonstrates that reducing the planar character of inhibitors indeed improved water solubility. We note, however, that 2d did not dissolve completely at concentrations of 50 μM and higher, which prohibits measuring full inhibition in the enzyme inhibition study.

To investigate the mechanism of inhibition and to avoid artefacts as described previously [25], the binding behavior of inhibitor 1d was characterized further. A pre-incubation and dilution assay was performed to study reversibility of binding for 1d to MIF. Towards this aim, MIF was pre-incubated with 83 μM of 1d for 10 min before 100-fold dilution and testing of the residual enzyme activity. Upon pre-incubation with 1d the MIF tautomerase activity could be fully recovered as compared to the positive control without inhibitor (Fig. 4B). This indicates reversible binding for 1d to MIF.

The influence of inhibitor 1d on the enzyme kinetics of MIF-catalyzed conversion of 4-HPP was analyzed (Fig. 4C, 4D). In presence of 2 or 5 μM 1d the Km increases from 1.14 to 1.60 and 1.95 μM respectively, whereas the Vmax remains constant between 0.24 and 0.29 (absorbance/min) compared to 0.27 for the control (Table 2). Thus, the enzyme kinetics demonstrate that 1d is a competitive inhibitor, which is consistent with previous reports for MIF inhibition by inhibitors with a triazole-phenol core [34]. Similar results were obtained for 2d as shown in the supporting information.

2.5. Binding affinity study

To confirm binding of 1d to MIF, we performed a microscale thermophoresis (MST) assay. MST is a newly emerging technology for analysis of binding to proteins. This technology exploits the ligand-induced changes in the molecular movement of fluorescently labeled proteins in a temperature gradient [43]. We determined the thermophoresis shift upon titration of different concentration of 1d to 50 nM MIF. The Kd value was determined from the changes in thermal shifts upon titration of 1d, which provides an sigmoidal curve from which the binding affinity (Kd) was calculated to be 3.63 μM. Thus, the Kd observed in the MST assay is comparable to the Ki value of 3.2 μM calculated from the enzyme kinetic experiments (Fig. 5). Altogether, we conclude that 4-(1,2,3-triazole)phenol inhibitor 1d binds reversibly to MIF with a potency in the low micromolar range.

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor (μM)</th>
<th>Kd (mM) ± SD</th>
<th>Vmax (absorbance/min) ± SD</th>
</tr>
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<tbody>
<tr>
<td>1d</td>
<td>1.14 ± 0.17</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>1.60 ± 0.24</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>2.5</td>
<td>1.95 ± 0.33</td>
<td>0.24 ± 0.02</td>
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The p-values show that the slopes are significantly non-zero (n = 3, p-value < 0.05).

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Fig. 4. Inhibition of MIF tautomerase activity by 1d. A) Dose-response curve for inhibition of MIF tautomerase activity by 1d. B) Pre-incubation and dilution assay of 1d and MIF. After 10 min pre-incubation at 83 μM 1d for 10 min the MIF solution was diluted 100-fold and employed for 4-HHP conversion. In the control group no enzyme was added. C) Michaelis-Menten plots of MIF activity at concentration of 0, 1.0 and 2.5 μM of inhibitor 1d. D) Lineweaver-Burk plots of MIF at concentration of 0, 1.0 and 2.5 μM of 1d (n is 3 for all results shown in this figure.).
2.6. Molecular modeling

Docking studies were performed to rationalize the structure-activity relationships observed for MIF inhibition. Structural information for the MIF interaction of the analogous biaryltriazoles was used as a basis for molecular modeling (PDB code: 4wrb and 5hvs) [34]. Modelling was performed using the software Discovery Studio 3.0. The compounds were docked into the crystal structure of MIF and energy minimized. The highest scoring poses were analyzed and compared with reference inhibitor Jorgensen-3bb [34]. The 2-fluoro-4-(1,2,3-triazole)phenol part of 1d occupies the same position as observed in Jorgensen-3bb (Fig. 6A, 6B, 6C). A main difference is that the quinolone in Jorgensen-3bb occupies a position different from the indole 2-carboxamide functionality in 1d. Instead, 1d has a hydrogen bond with Lys32 and a hydrophobic interaction with Ile64. Although 1d does not reach the same potency as reported for Jorgensen-3bb, the interactions observed in the modelling appear to be useful, because they enable targeting of a different part of the binding pocket.

The structure-activity relationship was further rationalized by investigating details from docking poses of 1d, 7c and 3c. A main difference between 1d and 7c is formation of a hydrogen bond between the fluoro and residue Asn97 in 1d, which is not present in 7c. The extra hydrogen bond can contribute to the two-times enhanced potency of 1d compared to 7c. Comparison was also made between 7c with the indole 2-carboxamide that provided a Ki of 6.7 μM and 3c with a pyrrole 2-carboxamide that provided a Ki of 29 μM (Fig. 6D, 6E). The 4-fold change in potency between 3c and 7c could be attributed to additional hydrophobic interaction with Ile64 in 7c.

2.7. A549 cell colony formation assay and inhibition of ERK phosphorylation

MIF inhibitors have been shown to inhibit the growth of A549 non-small cell lung cancer cells, in which MIF plays an essential role for anchorage-independent growth and invasive behavior [44,45]. The growth inhibitory potency of 2d against A549 cells was measured using a colony formation assay. A549 cells were seeded in 6-well plates with 200 cells per well. The cells were incubated for 10 days in medium containing various concentrations of the respective inhibitor. ISO-1 was applied as a positive control. The results are shown in Fig. 7A. Inhibitor ISO-1 inhibits colony formation at 20 and 100 μM. Also treatment of A549 cell with inhibitor 2d at 2.5, 10 and 20 μM decreased the number of colonies in a concentration-dependent manner. This demonstrates that compound 2d inhibits cell proliferation in the clonogenic assay at 10-fold lower concentrations compared to MIF inhibitor ISO-1.

To gain more insight into the effect of MIF inhibitors to tumor cell proliferation, phosphorylation of ERK is investigated in A549 cells. Towards this aim the cells are incubated with 2d or ISO-1 before treatment with MIF. Both 2d, as well as ISO-1, attenuate MIF induced ERK phosphorylation in A549 cell (Fig. 7B). Thus inhibitor 2d is able to inhibit ERK phosphorylation and cell
proliferation in A549 cells.

3. Conclusion

In this study, we investigated the structure-activity relationships for inhibition of MIF tautomerase activity by inhibitors with a 4-(1,2,3-triazole)-phenol core. During our studies we discovered that the transition metals copper(II) and zinc(II) inhibit MIF tautomerase activity with potencies in the low micromolar range. Addition of 0.5 mM EDTA to the assay buffer proved to be effective to avoid potential interferences from residual metal ions in inhibitor preparations. We aimed to replace the previously employed quinolone functionality by screening of a focused compound collection of 4-(1,2,3-triazole)-phenols. Several novel inhibitors were identified from which 7c with an indole 2-carboxamide functionality proved to be the most potent one. Further improvement of potency was achieved by ortho-fluorination of the phenol functionality to provide 1d. Compound 1d proved to be a reversible and competitive inhibitor of MIF tautomerase activity with a Ki value of 3.2 μM. Binding was confirmed using an MST assay, which provided a KD of 3.6 μM. In addition, we demonstrated that addition of a chlorine in the indole 5-position of 1d provided compounds 2d with further enhanced potency. This compound proved also effective in the inhibition of colony formation and attenuation of ERK signaling in A549 cells. Altogether, we present novel insights in the MIF tautomerase activity assay and we provide a novel triazole-phenol inhibitor 2d with cellular activity, which provides a basis for further development of MIF inhibitors.

4. Experimental section

4.1. Chemistry

4.1.1. General

All the reagents and solvents were purchased from Sigma-Aldrich, AK Scientific, Fluorochem or Acros and were used without further purification. Reactions were monitored by thin layer chromatography (TLC). Merck silica gel 60 F254 plates were used and spots were detected with UV light. MP Ecochrom silica 32–63, 60 Å was used for column chromatography. Nuclear magnetic resonance spectra, 1H NMR (500 MHz) and 13C NMR (126 MHz), were recorded on a Bruker Avance silica. Chemical shifts were reported in ppm relative to the solvent. High-resolution mass spectra were recorded using Fourier Transform Mass Spectrometry (FTMS) and electrospray ionization (ESI) on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. Melting points were measured by Electrothermal IA9100 Melting Point Apparatus.

4.1.2. Azidophenol synthesis

Concentrated HCl (3.5 mL) was added dropwise to a solution of 4-aminophenol (1.5 g, 13.7 mmol) in water (20 mL) over a period of 5 min. After cooling the resulting solution to 0 °C, NaNO2 (1.9 g, 27.5 mmol) was added portion-wise. The mixture was left stirring for 1 h at room temperature. A freshly made solution of NaN3 (1.8 g, 27.5 mmol) in a few mL of demi-water was added dropwise to the reaction mixture and left stirring for 1 h at room temperature. The reaction mixture was extracted with ethyl acetate (3x50 mL). The combined organic layers were extracted with brine and dried with MgSO4, filtered and concentrated under reduced pressure. The product was obtained as a dark liquid and used without further purification.

4-azido-2-fluorophenol was prepared analogously to 4-azidophenol using 4-amino-2-fluorophenol as starting material. The product was obtained as a dark liquid and used without further purification.

4.1.3. Synthetic procedure of compounds in group A

Compounds of group A were synthesized by coupling of propiolic acid to the corresponding amines followed by CuAAC.

![Fig. 7. MIF inhibition in A549 cells. A) A549 cells were treated with varying concentration of ISO-1 or 2d and stained with crystal violet. Bar chart showing the decreased number of colonies after incubation with ISO-1 or 2d. Colonies were counted by by ImageJ and confirmed by manually counting. One colony was defined to be an aggregate of >50 cells. Data was shown as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 vs control group. B) Effect of MIF inhibitors on activated ERK signaling pathway. The cells were treated with the respective MIF inhibitors for 10 min, followed by the stimulation of 50 ng/μl MIF for 15 min at 37 °C. pERK:GAPDH ratio was applied to quantify pERK level (n = 1, duplicates will be provided). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)](image-url)
coupling to azidophenol. Towards this aim, propionic acid (1 eq.) and N,N'-dicyclohexylcarbodiimide (DCC, 1.0 eq.) were dissolved in dry acetonitrile (MeCN) and cooled in an ice bath for 15 min. The corresponding amines were added into the respective mixture and stirred at room temperature for 2 h. The resulting precipitate was removed by filtration. The solvent was removed under reduced pressure and the products were directly used for the next step. 4-azidophenol (1.0 eq) and the appropriate alkyne (1.0 eq) were dissolved in MeOH (4 mL) in a round bottom flask equipped with stirring bar. Fresh prepared solutions of CuSO4 (0.1 eq) in demi-water (0.20 mL) and sodium ascorbate (0.20 eq) in demi-water (0.20 mL) were added sequentially to the reaction mixture. The reaction mixture was left stirring overnight at 60 °C. The reaction mixture was diluted with AcOEt and then filtered. The residue was washed with a small amount of methanol. The filtrate was extracted with brine and then concentrated under reduced pressure. The residue was purified using column chromatography with CH2Cl2: MeOH 10:1 to yield the desired products.

4.1.3.1. N-(3,4-dimethoxyphenyl)-1-(4-hydroxyphenyl)-1H-1,2,3-triazole-4-carboxamide (1a, ZP023). Yield 77%. m.p. 250.4–253.0 °C. 1H NMR (500 MHz, DMSO-d6) δ 10.36 (s, 1H), 10.03 (s, 1H), 9.20 (s, 1H), 7.47 (d, J = 8.2 Hz, 1H), 7.33 (dd, J = 8.7, 2.4 Hz, 1H), 6.96 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 8.8 Hz, 1H), 3.75 (s, J = 3.2 Hz, 1H), 3.74 (s, J = 3.2 Hz). 13C NMR (126 MHz, DMSO-d6) δ 158.19, 157.84, 148.44, 145.26, 143.63, 132.05, 128.34, 125.01, 122.42, 116.09, 112.34, 111.84, 105.61, 55.74, 55.43. HRMS, calculated for C17H17O2N4 [M+H]+: 281.1034, found 281.1031.

4.1.3.6. N-cyclopentyl-1-(4-hydroxyphenyl)-1H-1,2,3-triazole-4-carboxamide (6a, ZP049). Yield 42%. m.p. 233.2–233.5 °C. 1H NMR (500 MHz, DMSO-d6) δ 10.02 (s, 1H), 9.07 (s, 1H), 8.65 (d, J = 4.3 Hz, 1H), 7.73 (d, J = 8.6 Hz, 2H), 6.94 (d, J = 8.6 Hz, 2H), 2.88 (dt, J = 10.1, 4.9 Hz), 0.72–0.63 (m, 4H). 13C NMR (126 MHz, DMSO-d6) δ 161.18, 158.54, 143.90, 128.84, 124.68, 122.73, 116.51, 23.01, 6.18. HRMS, calculated for C13H13O2N4 [M+H]+: 245.1033, found 245.1031.

4.1.3.7. Ethyl (1-(4-hydroxyphenyl)-1H-1,2,3-triazole-4-carbonyl) glycinate (7a, ZP025). Yield 78%. Decomposed at 220 °C. 1H NMR (500 MHz, DMSO-d6) δ 10.04 (s, 1H), 9.14 (s, 1H), 8.95 (t, J = 6.0 Hz, 1H), 7.75 (d, J = 8.8 Hz, 2H), 6.95 (d, J = 8.9 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H), 4.02 (d, J = 6.1 Hz, 2H), 1.22 (t, J = 7.1 Hz, 3H). 13C NMR (126 MHz, DMSO-d6) δ 170.09, 160.42, 158.62, 143.32, 128.80, 125.11, 122.74, 116.51, 60.99, 41.19, 14.54. HRMS, calculated for C13H17O2Na [M+Na]+: 291.1088, found 291.1086.

4.1.4. Synthetic procedure of compounds in group B

For compounds of group B, 0.5 mmol of 4-azidophenol (1.0 eq) and 0.5 mmol appropriate alkyne (1.0 eq) were dissolved in MeOH (4 mL) in a round bottom flask. Fresh prepared solutions of CuSO4 (0.1 eq) in demi-water (0.20 mL) and sodium ascorbate (0.20 eq) in demi-water (0.20 mL) were added sequentially to the reaction mixture. The reaction mixture was left stirring overnight at room temperature. The reaction mixture was diluted with AcOEt and precipitate was removed by filtration. The residue was washed with methanol. The filtrate was extracted with brine and then concentrated under reduced pressure. The residue was purified using column chromatography with CH2Cl2: MeOH 50:1 to yield the desired products.

4.1.4.1. 4-(4-(hydroxymethyl)-1H-1,2,3-triazol-1-yl)phenol (1b, ZM028). Yield 21%. m.p. 212.5–213.8 °C. 1H NMR (500 MHz, DMSO-d6) δ 9.92 (s, 1H), 8.49 (s, 1H), 7.66 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 8.9 Hz, 2H), 5.29 (t, J = 5.6 Hz, 1H), 4.59 (d, J = 5.5 Hz, 2H). 13C NMR (126 MHz, DMSO-d6) δ 158.04, 149.19, 129.41, 122.26, 121.34, 116.47, 55.44. HRMS, calculated for C13H13O2N3 [M+H]+: 219.0768, found 219.0769.

4.1.4.2. 4-(4-(1-hydroxyethyl)-1H-1,2,3-triazol-1-yl)phenol (2b, ZM043). Yield 96%. m.p. 89.9–90.3 °C. 1H NMR (500 MHz, DMSO-d6) δ 9.91 (s, 1H), 8.44 (s, 1H), 7.66 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 5.34 (d, J = 4.0 Hz, 1H), 4.93–4.85 (m, 1H), 1.47 (d, J = 6.3 Hz, 3H). 13C NMR (126 MHz, DMSO-d6) δ 157.99, 129.47, 119.94, 116.45, 62.02. HRMS, calculated for C13H16O2N3 [M+H]+: 206.0924, found 206.0925.

4.1.4.3. 4-(4-isopropyl-1H-1,2,3-triazol-1-yl)phenol (3b, ZP180). Yield 52%. 1H NMR (500 MHz, Methanol-d4) δ 8.15 (s, 1H), 7.62 (d, J = 8.9 Hz, 2H), 6.96 (d, J = 8.9 Hz, 2H), 3.14 (m, 1H), 1.38 (d, J = 7.0 Hz, 6H), 1.3C NMR (126 MHz, Methanol-d4) δ 158.03, 154.60, 129.51, 121.90, 118.74, 115.70, 25.66, 21.49. HRMS, calculated for C15H16O2N3 [M+H]+: 240.1131, found 240.1131.

4.1.4.4. 4-(4-Propyl-1H-1,2,3-triazol-1-yl)phenol (4b, ZM018). Yield 12%. 1H NMR (500 MHz, Chloroform-d) δ 8.89 (s, 1H), 8.37 (s, 1H), 7.63 (d, J = 8.9 Hz, 2H), 6.91 (d, J = 8.9 Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H), 1.66 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, DMSO-d6) δ 157.45, 147.58, 129.05, 121.63, 120.03, 115.98, 27.10, 22.19, 13.67. HRMS, calculated for C17H21O2N3 [M+H]+: 281.1033, found 281.1031.
2.67 (t, J = 7.6 Hz, 2H), 1.66 (m, 2H), 1.33 (m, 4H), 0.89 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, DMSO) δ 157.90, 124.88, 129.51, 122.11, 120.42, 116.42, 31.31, 29.05, 25.46, 22.36, 14.38. HRMS, calculated for C13H13ON3 [M+H]+: 232.1444, found 232.1442.

4.4.1.6. 4-(4-Cyclohexyl-1H-1,2,3-triazol-1-yl)phenol (6b, ZP179). Yield 46%. 1H NMR (500 MHz, DMSO-d6) δ 9.89 (s, 1H), 8.36 (s, 1H), 7.64 (d, J = 8.8 Hz, 2H), 6.92 (d, J = 8.8 Hz, 2H), 2.67 (t, J = 7.6 Hz, 2H), 1.66 (m, 2H), 1.33 (m, 4H), 0.89 (t, J = 7.4 Hz, 3H). 13C NMR (126 MHz, DMSO) δ 157.98, 153.42, 129.57, 122.12, 119.29, 116.42, 35.13, 33.03, 25.95. HRMS, calculated for C14H12ON [M+H]+: 244.1444, found 244.1443.

4.4.1.7. 4-(4-(hex-5-yn-1-yl)-1H-1,2,3-triazol-1-yl)phenol (7b, MZ024). Yield 21%. Decomposed at 220 °C. 1H NMR (500 MHz, DMSO-d6) δ 9.88 (s, 1H), 8.38 (s, 1H), 7.63 (d, J = 8.9 Hz, 2H), 6.91 (d, J = 8.9 Hz, 2H), 2.77 (t, J = 2.6 Hz, 2H), 2.69 (t, J = 7.6 Hz, 2H), 2.21 (td, J = 7.1, 2.6 Hz, 2H), 1.73 (m, 2H), 1.52 (m, 2H). 13C NMR (126 MHz, DMSO) δ 157.92, 147.92, 129.49, 122.12, 120.48, 116.44, 84.87, 71.80, 28.41, 27.93, 24.93, 17.91. HRMS, calculated for C14H12ON3 [M+H]+: 242.1288, found 242.1298.

4.4.1.8. 4-(4-Octyl-1H-1,2,3-triazol-1-yl)phenol (8b, ZP087). Yield 30%. 1H NMR (500 MHz, DMSO-d6) δ 9.91 (s, 1H), 8.37 (s, 1H), 7.63 (d, J = 8.4 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 2.66 (t, J = 7.6 Hz, 2H), 1.64 (m, 2H), 1.35–122 (m, 10H), 0.86 (t, J = 6.7 Hz, 3H). 13C NMR (126 MHz, DMSO) δ 157.89, 148.27, 129.51, 122.04, 120.39, 114.63, 51.36, 29.35, 29.24, 29.10, 25.49, 25.46, 22.57, 14.43. HRMS, calculated for C14H14O3N3 [M+H]+: 274.1914, found 274.1911.

4.4.1.9. Methyl 4-((4-(4-hydroxyphenyl)-1H-1,2,3-triazol-4-yl)butanoylcarbamoyl (9b, MZ056). Yield 17%. 1H NMR (500 MHz, DMSO-d6) δ 9.90 (s, 1H), 8.40 (s, 1H), 7.63 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.9 Hz, 2H), 2.39 (s, J = 3.9 Hz, 2H), 2.70 (t, J = 7.6 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 1.91 (m, 2H). 13C NMR (126 MHz, DMSO) δ 173.75, 157.91, 129.42, 122.20, 120.78, 116.48, 51.76, 33.08, 24.69, 24.58. HRMS, calculated for C14H13O4N3 [M+H]+: 262.1186, found 262.1184.

4.4.1.10. 3-(4-(4-Hydroxyphenyl)-1H-1,2,3-triazol-4-yl)-N-phenylpropanamide (10b, ZP078). Yield 27%. 1H NMR (500 MHz, DMSO-d6) δ 9.99 (s, 1H), 9.90 (s, 1H), 8.38 (s, 1H), 7.60 (m, 4H), 7.28 (t, J = 7.9 Hz, 2H), 7.02 (t, J = 7.4 Hz, 1H), 6.91 (d, J = 8.9 Hz, 2H), 3.01 (t, J = 7.5 Hz, 2H), 2.75 (t, J = 7.6 Hz, 2H). 13C NMR (126 MHz, DMSO) δ 170.62, 157.99, 147.18, 139.67, 129.41, 124.19, 123.52, 122.16, 120.68, 119.52, 116.48, 36.09, 21.47. HRMS, calculated for C17H17O2N4 [M+H]+: 309.1346, found 309.1343.

4.5. Synthetic procedure of compounds in group C

For compounds of group C, 0.5 mmol 2-propynylamine was used to form amides with corresponding carboxylic acid derivatives mediated by N,N'-dicyclopentylcarbodiimide (DCC, 1.0 eq.) in dry acetonitrile (MeCN). Conditions and procedures used here were same as those in group A. Afterwards, different amides were mixed with 4-azidophenol (1.0 eq.), fresh prepared solutions of CuSO4 (0.1 eq) in demi-water (0.20 mL), sodium ascorbate (0.20 eq) in demi-water (0.20 mL) in MeOH (4 mL). The reaction mixture was left stirring overnight for at 60 °C. The CuAAC reaction and purification were also done in the same way as for group A described above.
4.1.5.8. N-((1-(4-hydroxyphenyl)-1H-1,2,3-triazol-4-yl)methyl)-2-(1H-indol-2-yl)acetamide (8C, ZP075). Yield 47%. m.p. 225.9–226.8 °C. 1H NMR (500 MHz, DMSO-d6) δ 8.88 (s, 1H), 9.93 (s, 1H), 8.46 (t, J = 5.6 Hz, 1H), 8.23 (s, 1H), 7.55 (m, 3H), 7.34 (d, J = 8.1 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 7.06 (s, 1H), 6.96 (d, J = 7.6 Hz, 1H), 6.91 (d, J = 8.9 Hz, 2H), 4.36 (d, J = 5.6 Hz, 2H), 3.56 (s, 2H). 13C NMR (126 MHz, DMSO) δ 170.87, 156.77, 145.88, 136.14, 128.80, 127.22, 123.94, 121.76, 120.98, 120.80, 118.77, 118.35, 116.07, 111.38, 108.80, 34.40, 32.66. HRMS, calculated for C22H26O5N6F [M+H]+: 334.1299, found 334.1297.

4.1.6. Synthetic procedure of compounds in group D

2-fluoro-4-nitrophenol (1.15 g, 7.3 mmol) was dissolved in ethanol (10 mL) and added to Pd/C (60 mg). Subsequently the flask was charged with hydrogen (H2) gas and the black suspension was stirred at room temperature for 4 h. After 4 h the starting material was evaporated under reduced pressure to provide the crude product that was used directly for the next reaction step. The crude product was dissolve in water (10 mL) with concentrated HCl (2 M). Subsequently, NaN3 (1.0 g, 15 mmol) and NaN2 (0.95 g, 15 mmol) were added sequentially to yield 4-azido-2-fluorophenol. Yield 35%. 1H NMR (500 MHz, DMSO) δ 7.37 (m, 2H), 6.79 (d, J = 6.3 Hz, 1H), 7.06–6.90 (m, 2H), 6.79 (d, J = 11.9 Hz), 136.92, 131.90, 128.99 (d, J = 8.7 Hz), 127.53, 123.82, 122.00 (d, J = 19.1 Hz), 121.67 (d, J = 6.1 Hz), 120.00, 118.69, 117.04 (d, J = 16.3 Hz), 117.22, 109.50, 103.32, 34.82. 19F NMR (376 MHz, DMSO-d6) δ −133.75 to −133.81 (m). HRMS, calculated for C12H13F2N5ClF [M+H]+: 410.0207, found 386.0823.

4.2. Enzyme activity study

4.2.1. Protein expression and purification

C-terminal His-tagged recombinant human MIF was expressed with pET-20b (+) plasmid and Escherichia coli BL21 according to literature procedures [46]. After culturing Escherichia coli cells were pelleted by centrifugation at 4000 rpm for 20 min. Cell pellets were resuspended in a lysis buffer containing 20 mM Tris–HCl (titrated to pH 7.5 with an aqueous concentrated NaOH solution), 20 mM sodium chloride, 10% glycerol, 2 mM magnesium chloride, and 0.2 × complete EDTA-free protease inhibitor cocktail (Roche). Subsequently, the cells were lysed by sonication and centrifuged at 17,000 g for 20 min. The supernatant was purified by high pressure chromatography system (Biologic DuoFlow) equipped with a His trap HP (5 mL) column with detection at 280 nm for the eluent. The binding buffer contained 50 mM Tris and 10% glycerol that was titrated to pH 7.4 using 1 M NaOH or 1 M HCl. The elution buffer contained 500 mM imidazole, 50 mM Tris, 10% glycerol that is also titrated to pH 7.4 using 1 M NaOH or 1 M HCl. The collected protein was purified again by PD-10 column (GE healthcare) to remove the high concentration of imidazole. The resulting MIF was assessed by SDS gel electrophoresis and no impurities were observed (>95%). The concentration of MIF was determined by Bradford protein assay to be 1.83 mg/mL (135 μM). The purified protein was aliquoted and stored at −80 °C. The stability of the protein was tested by the tautomerase assays (Synergy H1 Hybrid Reader, BioTek) and thermal stability assays (nanoDSF, Prometheus NT.48).

4.2.2. Tautomerase assay

Inhibition of the tautomerase activity and kinetics of MIF was measured using 4-hydroxyphenyl pyruvic acid (4-HPP) as substrate. A stock solution was prepared by dissolution of 4-HPP in 50 mM ammonium acetate buffer that was titrated to pH 6.0 using 1.0 M NaOH or 1.0 M HCl. 4-HPP was dissolved to provide a concentration of 10 mM and this solution was incubated overnight at room temperature to allow equilibration of the keto and enol forms. Subsequently this 4-HPP stock solution was stored at 4 °C. The MIF stock solution (80 μL, 135 μM MIF) was diluted in the boric acid buffer to provide a MIF solution (12 mL, 0.9 μM) in boric acid buffer (435 mM, pH 6.2). The enzyme activity was determined by pre-incubating 170 μL of the MIF dilution with 10 μL EDTA (20 mM in demin.water) and DMSO (20 μL). This mixture was pre-incubated for 15 min. Next, 50 μL of this mixture was mixed with 50 μL of 1 mM 4-HPP solution in 50 mM pH 6.0 ammonium acetate buffer. Subsequently, MIF tautomerase activity was monitored by formation of the borate–enol complex, which was measured by the increase in UV absorbance at 305 nm. The increase in UV absorbance was monitored over the first 10 min of incubation using a BioTek Synergy H1 Hybrid plate reader. In experiments where EDTA was excluded the 10 μL of the 20 mM EDTA solution is replaced for demi water. Inhibitors were added to the experiment as DMSO solutions by replacing the blank DMSO for a DMSO solution with a corresponding inhibitors concentration that provides the final inhibitors concentration after dilution. Initially, the inhibitors were dissolved in DMSO at 10 mM, which was diluted further in DMSO to provide 1 mM from which 20 μL was added to the enzyme activity assay to provide a final concentration of 50 μM in the screening. For compounds that showed ca. 50% or greater inhibition at 50 μM, an IC50 was measured. Towards this aim the compounds were stepwise diluted in DMSO and subsequently the MIF tautomerase activity was measured using the same protocol. The DMSO concentration in all assays was kept constant at 5% and control experiments
demonstrated that this DMSO concentration did not influence the tautomerase activity. MIF tautomerase activity in the presence of blank DMSO was set to 100% enzyme activity. In the negative control the enzyme was excluded to monitor non-catalyzed conversion of the substrate, which did not show a change in absorbance at 305 nm. Data from the first 3 min were used to calculate the initial velocities and the nonlinear regression analyses for the enzyme kinetics were repeated three times with the program Prism6 (GraphPad).

4.3. Docking study

Docking studies were performed to gain insight in the structure-activity relationships. All molecular modelings were done with the program Discovery studio (Dassault systems) version 2018 and the crystal structures of human recombinant MIF (PDB-code:4wrb, [32], 5hvs [32]) were used. The CDOCKER protocol was used for docking which is a CHARMM based algorithm. Docking was verified by use of the ligand 3-(6-((3-fluoro-4-hydroxyphenyl)-1H,1,2,3-triazol-4-yl)naphthalen-1-yl)oxy)benzoic acid (Jorgensen-[3bb] from the crystal structure 5hvs. This ligand contains the 4-(1,2,3-triazole)phenol functionality, which is also present in our molecules. First, the ligand was removed from the protein and subsequently docked back in the crystal structure. All 10 highest ranked poses show a comparable position compared to the original pose from the crystal structure in the 4-(1,2,3-triazole)phenol functionality (Fig. S8). Poses with the lowest CDOCKER energies were chosen for representation.

4.4. MST

MST experiments were performed on a Monolith NT.115 system (NanoTemper Technologies) using 100% LED and 20% IR-laser power. Laser on and off times were set at 30s and 5s, respectively. Recombinant His-tagged MIF was labeled with RED-tris-NTA for 30 min (NanoTemper Technologies) and applied at a final concentration of 50 nM. A two-fold dilution series was prepared for compound 1d in PBS-T with 5% DMSO. Subsequently, 10 µL of labeled MIF was mixed with 10 µL samples with different concentration of compound 1d. Samples were filled into hydrophilic capillaries (Monolith NT.115 capillary, standard treated) for measurement.

4.5. Colony formation assay

A549 cells were seeded in 6-well plates, each well contained 2 mL medium with 200 A549 cells and incubated for 24 h. 10 mM stock solutions of ISO-1 or 2d were prepared by dissolution in DMSO. Subsequently, the inhibitors were diluted to different concentration in fresh medium before addition into the corresponding well upon which the cells were treated continuously for 10 days. Finally, the cells were fixed with paraformaldehyde for 20 min and stained with crystal violet for 20 min. After washing, the image of each well was photographed and analyzed with ImageJ. We defined one colony as an aggregate of >50 cells. The numbers of colonies was analyzed as the ratio of the numbers found in inhibitor treated samples compared to untreated samples.

4.6. ERK signaling pathway study

Cells were seeded into a 6-well plate at a density of 2 × 10^5 cells per well with RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Costar Europe, Badhoevedorp, The Netherlands), and 1% penicillin/streptomycin. After overnight culturing, the cells were treated with the respective MIF inhibitors for 10 min, followed by the stimulation of 50 ng/µL MIF for 15 min. After that, cells were lysed by RIPA buffer. The BCA Protein Assay Kit (Pierce, Rockford IL, USA) was used to determine the protein concentration according to the manufacturing instruction. Thirty-microgram protein was separated by a pre-cast 10% NuPAGE Bis-Tris gel (Invitrogen, USA). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Five percent skimmed milk was used to block the membrane for 1 h at RT. The blocked membrane was incubated with appropriate primary antibody (phospho-ERK, pERK, #9101, 1:1000, Cell Signalling; GAPDH, #97166, 1:10000, Cell Signalling) overnight at 4 °C, followed by the treatment of an HRP-conjugated secondary goat anti-rabbit antibody (#P0448, 1:2000) or rabbit anti-mouse antibody (#PO260, 1:2000) (Dako Cytomation, Glostrup, Denmark) at RT for 1 h. The protein bands were visualized with enhanced chemiluminescence (ECL) solution (GE Healthcare, Amersham, UK). The figures were quantified with imageJ software (National Institutes of Health, USA) based on greyscale.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References