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Discovery of chromenes as inhibitors of macrophage migration inhibitory factor

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is an essential signaling cytokine with a key role in the immune system. Binding of MIF to its molecular targets such as, among others, the cluster of differentiation 74 (CD74) receptor plays a key role in inflammatory diseases and cancer. Therefore, the identification of MIF binding compounds gained importance in drug discovery. In this study, we aim to discover novel MIF binding compounds by screening of a focused compound collection for inhibition of its tautomerase enzyme activity. Inspired by the known chromen-4-one inhibitor Orita-13, a focused collection of compounds with a chromene scaffold was screened for MIF binding. The library was synthesized using versatile cyanoacetamide chemistry to provide diversely substituted chromenes. The screening provided inhibitors with IC_{50}'s in the low micromolar range. Kinetic evaluation suggested that the inhibitors were reversible and did not bind in the binding pocket of the substrate. Thus, we discovered novel inhibitors of the MIF tautomerase activity, which may ultimately support the development of novel therapeutic agents against diseases in which MIF is involved.

1. Introduction

Macrophage migration inhibitory factor (MIF) is a central cytokine of the immune system. It is expressed in immune cells such as T-cells, macrophages, basophiles, eosinophils and B-cells.[1] Unlike other cytokines, MIF is constitutively expressed and stored in cytoplasmic pools and rapidly released in response to stimuli.[2] Upon release, MIF interacts with surface receptors on B-cells, T-cells, macrophages and some epithelial cells, which induce pro-inflammatory signal transduction. MIF has been shown to interact with the type II cluster of differentiation 74 (CD74) receptor, which is the invariant chain of the major histocompatibility complex II (MHCII). CD74 does not seem to have an intracellular signaling domain and is, therefore, expected to initiate intracellular signaling by recruiting other membrane receptors such as CD44, CXCR2 and CXCR4.[3–5] These interactions are important for the role of MIF in inflammatory signaling. In addition, MIF has also been suggested as a target in cancer due to its downregulation of p53 and its overexpression in several cancer cell types.[6–10] It was shown that neutralization of MIF through antibodies or genetic deletion was beneficial in several inflammatory disease models and a small molecule inhibitor of MIF was able to reduce tumor growth in mouse models.[11–15] Taken together these data indicate that development of MIF binding molecules has potential for drug discovery for inflammatory diseases and cancer.

MIF is a small protein of 115 amino acids, weighing approximately 12.4 kDa and exists predominantly in a homotrimeric form. One human homologue has been described, D-dopachrome Tautomerase (D-DT or MIF2), which shows a similar function to MIF.[16] MIF has structural similarity to two bacterial enzymes: 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase.[17] Inspired by these similarities, it was discovered that MIF not only functions as a cytokine, but has enzymatic activity as well. It has been shown to catalyze the interconversion of enol and keto isomers of D-Dopachrome and

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0968-0896/

phenylpyruvate.[18] One residue particularly important for this activity is the N-terminal proline which acts as a catalytic base in the tautomerase reaction.[19] Screening for inhibitors of MIF tautomerase activity has been recognized as an efficient way to identify MIF binding compounds that can be further investigated in more advanced disease models where MIF has been shown to play a role. A well-known inhibitor of the MIF tautomerase activity is the isoxazoline (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1, Fig. 1). ISO-1 is a competitive inhibitor of the MIF tautomerase activity and has beneficial effects in several disease models such as sepsis, chronic obstructive pulmonary disease (COPD) and cancer.[15,20–23] Based on ISO-1, several other MIF inhibitors have been developed, among which are the biaryltriazoles.[24–28] Using a structure-based virtual screening method, Orita-13 containing a chromen-4-one scaffold was identified as MIF inhibitor.[26,29] Additionally, covalent MIF inhibitors have been described, such as TP, as probes suitable for activity-based protein profiling.[30] Taken together, several small molecule binders of MIF have been developed (Fig. 1), but the identification of novel structural classes remains needed for a better understanding of the structural requirements for binding and to provide a broader basis for drug discovery.

Here, we describe the identification of novel MIF binders inspired by the chromen-4-one scaffold of Orita-13. A focused compound collection of 57 compounds was synthesized using cyanoacetamide-based chemistry. Screening of this library for inhibition of MIF tautomerase activity provided 6 inhibitors with potencies in the low micromolar range. The structural motif that was identified expands the number of scaffold available for further development of MIF inhibitors towards applications in disease models.

2. Materials and methods

2.1. Chemistry general

All the reagents and solvents were purchased from Sigma-Aldrich, AK Scientific, Fluorochem, Abcr GmbH, or Acros and were used without further purification. All microwave irradiation reactions were carried out in a Biotage Initiator™ Microwave Synthesizer. Thin layer chromatography was performed on Millipore precoated silica gel plates (0.20 mm thick, particle size 25 μm). Nuclear magnetic resonance spectra were recorded on Bruker Avance 500 or 600 spectrometers ^{1}H NMR (500 MHz; 600 MHz), ^{13}C NMR (126 MHz; 151 MHz). Chemical shifts for ^{1}H NMR were reported as δ values and coupling constants were in hertz (Hz). The following abbreviations were used for spin multiplicity: s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, quin = quintet, dd = doublet of doublets, ddd = doublet of doublet of doublets, m = multiplet. Chemical shifts for ^{13}C NMR were reported in ppm relative to the solvent peak. Flash chromatography was performed on a Reveleris® X2 Flash Chromatography system, using Grace® Reveleris Silica flash cartridges (12 g). Mass spectra were measured on a Waters Investigator Supercritical Fluid Chromatograph with a 3100 MS Detector (ESI) using a solvent system of methanol and CO_2 on a Viridis silica gel column (4.6 x 250 mm, 5 μm particle size) or Viridis 2-ethyl pyridine column (4.6 × 250 mm, 5 μm particle size). High resolution mass spectra were recorded using a LTQ-Orbitrap-XL (Thermo) at a resolution of 60,000@m/z400.

2.2. General procedure for the synthesis of **1–57**

To a stirred solution of 2*H*-chromen-2-one (1.0 mmol) in dry ethanol (5 mL), the corresponding cyanoacetamide (1.0 mmol) and sodium ethoxide (0.2 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. The precipitate was filtered off and washed with cold ethanol (2 × 5 mL), yielding the final compounds without further purification in yields ranging from 35 to 81%. The characterization of all compounds can be found in the supporting information.

2.3. Single crystal x-ray structure determination

X-ray diffraction data for a single crystal of compound **7** was collected using a SuperNova (Rigaku-Oxford Diffraction) four circle diffractometer with a mirror monochromator and a microfocus MoKα radiation source (λ = 0.71073 Å). Additionally, the diffractometer was equipped with a CryoJet HT cryostat system (Oxford Instruments) allowing low temperature experiments, performed at 130 (2) K. The obtained data was processed with CrysAlisPro software (S1). The phase problem was solved by direct methods using SIR2004 (S2). Parameters of models were refined by full-matrix least-squares on F^2 using SHELXL-2014/6 (S3). Calculations were performed using WinGX integrated system (ver. 2014.1) (S4) Figure was prepared with Mercury 3.7 software (S5).

All non-hydrogen atoms were refined anisotropically. All hydrogen atoms attached to carbon atoms were positioned with the idealised geometry and refined using the riding model with the isotropic displacement parameter $U_{iso}[H] = 1.2$ (or 1.5 (methyl groups only)) $U_{eq}[C]$. Positions of hydrogen atoms linked to N2 were defined on the difference Fourier map and refined with no additional restraints. Crystal data and structure refinement results for presented crystal structure are shown in Table S1. The molecular geometry (asymmetric unit) observed in the crystal structure is shown in Fig. S1. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 1575884.

2.4. MIF tautomerase activity assay

Tautomerase activity inhibition of MIF by the synthesized chromene compounds was measured using recombinantly expressed His-tagged MIF, which was purified with cOmplete His-Trap purification resin (Roche, The Netherlands). The assay was done following the procedure of Dziedzic et al.[26] 4-hydrox-

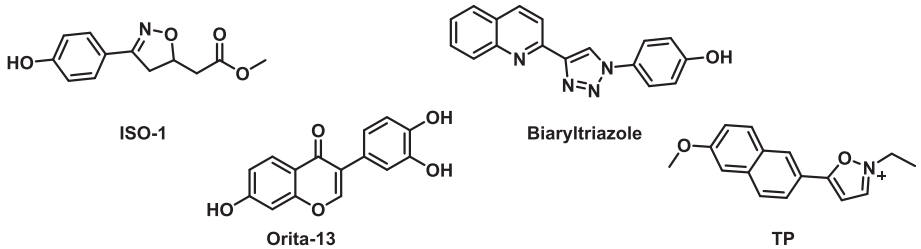


Fig. 1. Known MIF tautomerase activity inhibitors "ISO-1", a "biarylatriazole", "Orita-13" and activity-based probe "TP".

yphenyl pyruvate (4-HPP) was used as substrate to quantify tautomerase activity. Stock solutions of 10 mM 4-HPP were made in 50 mM ammonium acetate buffer pH 6.0, and incubated overnight at room temperature to allow equilibration between keto and enol form. Further dilutions of the substrate were made in the same acetate buffer. Inhibitor stock solutions had a concentration of 10 mM in DMSO. The inhibitor stock solutions were diluted in 0.4 M boric acid pH 6.2 to give final concentration in the screening assay of 25 and 50 μM. For the IC_{50} assay final concentrations of 250–0 μM or 100–0 μM or 25–0 μM in 5% DMSO, with 2 or 1.6-fold dilution series were applied. The control contained 5% DMSO as a vehicle control. This amount did not influence the MIF tautomerase activity. In the assays 50 μL of mixtures of MIF (dilution in 0.2 M boric acid pH 6.2, to give a final concentration of 340 nM) and the synthesized compounds were put in a UV-star F bottom 96-well plate. The enzymatic reaction was started by addition of 50 μL 4-HPP (to give a final concentration of 0.5 mM), and the increase of absorbance at 306 nm was followed over time using a Spectrostar Omega BMG Labtech plate reader. The positive control contained all the components excluding inhibitor (but including 5% DMSO), and the negative control was as the positive control without MIF. The data obtained were analyzed by firstly taking the slopes of the linear part of the increased absorbance over the time (that is the velocity of the enzymatic reaction), then normalizing them to the positive and negative control to give percentage of inhibition.

2.5. Enzyme kinetic evaluation

To evaluate the reversibility of MIF tautomerase inhibition by the discovered chromene inhibitors, preincubation experiments were conducted using inhibitor **10** and **17**. The inhibitors (125–0 μM, 1.6-fold dilution series in 5% DMSO) were preincubated with the enzyme (340 nM) for 2 min (the time of preincubation in the regular IC_{50} assays) and 40 min prior to adding the substrate and starting the enzymatic reaction. Then the IC_{50} curves were made as described above.

Dilution experiments were performed using inhibitor **10**. To do this, an initial mixture with a relatively high concentration of MIF (34 μM) and the inhibitor (125 μM in 5% DMSO) was made. Subsequently, this mixture was diluted 100 times in a solution containing the substrate 4-HPP (0.5 mM) and boric acid. A control assay was done following the same procedure without inhibitor, but containing 5% DMSO. The enzyme activity was measured as described before. The absorbance was plotted against time.

To further investigate the mechanism of inhibition, kinetic experiments were conducted using inhibitor **10**. The velocity of the enzymatic reaction was measured at increasing concentrations of 4-HPP (0–2.56 mM, 1.25 × dilution) in the presence of MIF (340 nM) and inhibitor (0, 6.25 or 12.5 μM). The velocity of the reaction was plotted against the concentration of 4-HPP using GraphPad Prism 5.0. The curve was plotted using enzyme kinetics-allosteric sigmoidal, yielding the $V_{max\ app.}$, Hill slope and $K_{prime\ app}$. The concentration of 4-HPP that gives half of V_{max} (K_{half}) was calculated from the K_{prime} using the following equation:

$$Khalf = \sqrt[Hillslope]{Kprime}$$

3. Results and discussion

3.1. Chemistry

A library of approximately 60 fused amino-2*H*-chromenopyridine-diones was synthesized using methods as initially described by Rosati et al. (Fig. 2A).[31,32] The alignment of Orita-13 with the amino-2*H*-chromenopyridine-dione scaffold can be detected by checking the stereoscopic view of Orita-13, which indicates the potential of this library for MIF binding (Fig. 2B). These scaffolds combine a series of interesting features besides the chromene core, such as the amino group in 5-position and a fused piperidinodione ring. Moreover, the possibility to increase the diversity with two points of diversification and the rigid core structure attributed to the selection of this scaffold. It was possible to get the crystal structure of compound **7** revealing an intramolecular hydrogen bond between the exocyclic amine and the carbonyl group. This led to coplanarity between the fused rings, which provides interesting possibilities for the type of interactions under investigation (Fig. 2C, Fig. S1, Scheme S1).

Starting from our broad experience with cyanoacetamide chemistry in heterocycle synthesis,[33–36] we elaborated on the synthesis of Rosati et al.,[31] using a number of different cyanoacetamides and suitably substituted 2*H*-chromenes. Thus, we designed and synthesized a highly diverse medium-sized library in a medicinal chemistry frame utilizing aliphatic and aromatic substituents, heterocycles, hydrogen bond donors and acceptors. In addition, we enhanced the solubility of specific compounds with the introduction of morpholino substituents. The reactions proceeded under mild conditions with a plethora of different cyanoacetamides in good to very good yields in a parallel manner.

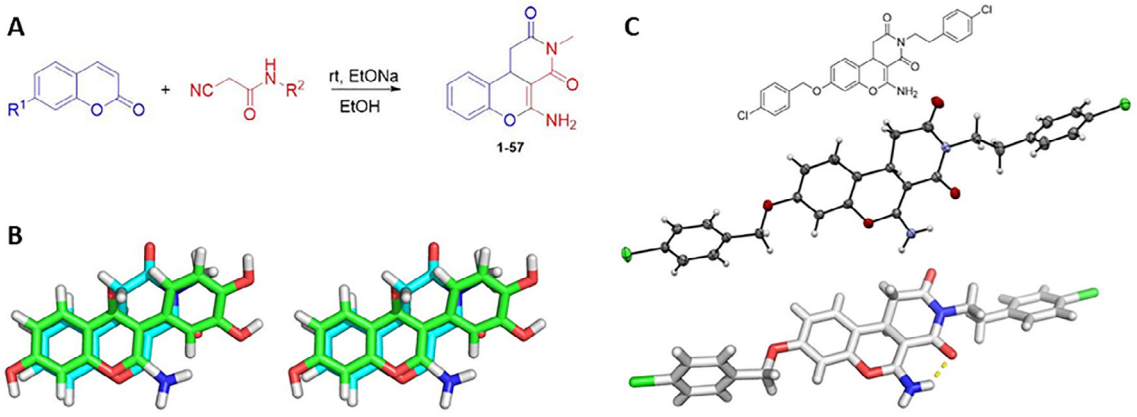
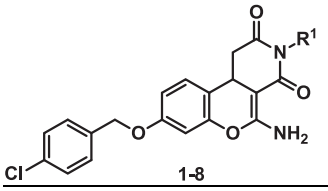
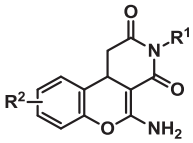


Fig. 2. A) Synthesis of fused amino-2*H*-chromenopyridine-diones. B) Stereoscopic view of the 3D-alignment of Orita-13 (green) with the amino-2*H*-chromenopyridine-dione scaffold (cyan). C) Structure of compound **7**, Molecular geometry observed in the crystal structures of compound **7**, showing the atom labelling scheme and an intramolecular hydrogen bond between the exocyclic amine and the carbonyl group is formed.

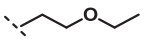
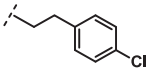
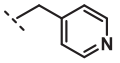
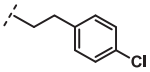
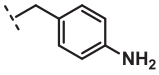
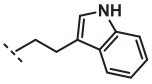
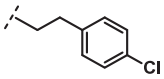
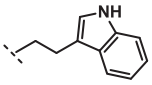
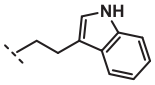
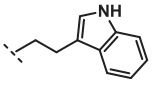
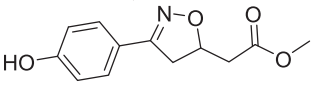
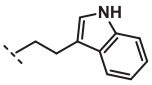
Table 1
Inhibition of MIF tautomerase activity by synthesized compounds of a chromene scaffold and reference compound ISO-1. IC_{50} values were given as mean ± standard deviation of at least 2 independent experiments. ND = not determined.



1-8



9-17

Compound	R^1	% inhibition at 25 μM	IC_{50} (μM)	Compound	R^1	R^2	% inhibition at 25 μM	IC_{50} (μM)
1		40%	ND	**9**		3-OEt	60%	21 ± 2.1
2		10%	ND	**10**		H	60%	18 ± 3.5
3		10%	ND	**11**		4-Me	50%	ND
4		80%	7.1 ± 1.0	**12**		H	15%	ND
5		15%	ND	**13**		3-Me	5%	ND
6		45%	ND	**14**		3-OEt	10%	ND
7		80%	13 ± 1.1	**15**		H	15%	ND
8		70%	8.0 ± 1.0	**16**		4-Me	25%	ND
ISO-1			79 ± 3.7	**17**		3-Me	55%	6.2 ± 0.6

3.2. Biological evaluation

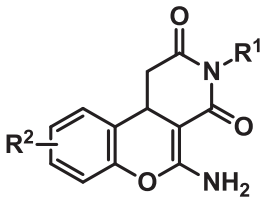
The compounds were tested for inhibition of the MIF tautomerase activity using a spectrophotometric assay based on the absorbance detection of the enzymatic enol product of 4-hydroxy phenylpyruvate (4-HPP) after reaction with boric acid.[26] First, a single point screening was done at a concentration of 25 μM and 50 μM and the compounds showing more than 50% inhibition of enzyme activity at 25 μM were tested for IC_{50} values (Figs. S2 and S3).

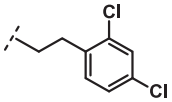
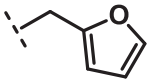
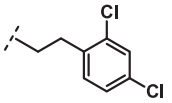
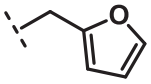
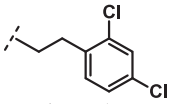
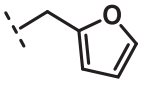
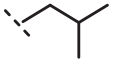
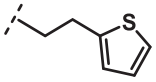
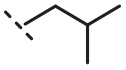
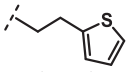
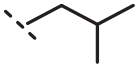
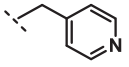
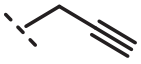
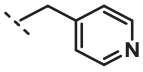
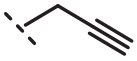
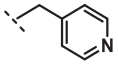
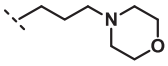
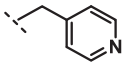
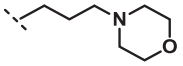
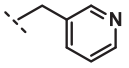
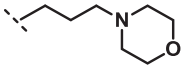
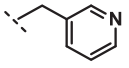
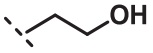
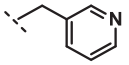
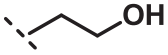
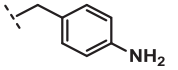
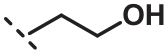
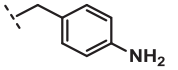
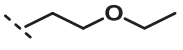
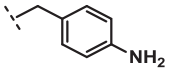
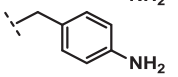
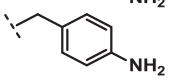
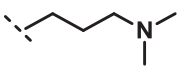
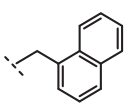
The investigation started with 4-chlorobenzyloxy chromene derivatives, bearing various aliphatic or aromatic substituents on the R^1 position (compounds **1**–**8**, Table 2). Short aliphatic substituents (**1**–**3**) showed less than 50% inhibition at 25 μM, whereas compound **4** carrying a longer aliphatic substituent provided an IC_{50} of 7.1 ± 1.0 μM. The compounds with aromatic substituent (**5**–**8**) also showed inhibition, of which a 4-chlorophenethyl substituent (**7**, IC_{50} = 13 ± 1.1μM) and an indole with ethyl spacer (**8**, IC_{50} = 8.0 ± 1.0 μM) gave the best results. This suggests that lipophilic interactions are important for the inhibition of MIF. Next, these active derivatives (**4, 7** and **8**) were further investigated. To investigate whether the bulky 4-chlorobenzyloxy was necessary, it was removed (R^2 = H) or replaced with several smaller substituents such as 3-Me, 4-Me or 3-OEt on the R^2 position (Table 1). In case of the long dodecane substituent (**9**–**11**), when smaller substitutions on position R^2 were introduced, activity did not improve. In contrast, introducing smaller substitutions on R^2 in case of compounds with a 4-chlorophenethyl on position R^1 (**12**–**13**) caused a loss of activity. Concerning the indole substituted compounds (**14**–**17**), a methyl substituent at R^2 improved slightly the activity, but others were not active. Several other compounds were synthesized combining different types of substituents at the R^1 position, such as morpholines, naphthalenes, furans, thiophenes or aliphatic chains with different heteroatoms (Table 2), but these did not lead to an improved inhibition. The IC_{50} value of reference MIF inhibitor ISO-1 was determined under the conditions used for the chromene compounds. The IC_{50} value of ISO-1 was within the range reported in literature.[37] The activity of Orita-13 has been reported to be similar to ISO-1.[27] The most potent chromene compounds were active at lower concentrations compared to the reference compound ISO-1. Therefore, compounds **10** and **17** were taken for further investigation.

3.3. Kinetic evaluation

To investigate the reversibility of the inhibition of MIF by the discovered inhibitors, a preincubation assay was performed with **10** and **17**. The inhibitors were preincubated with MIF for 2 or 40 min before initiating the enzymatic reaction. Then, the IC_{50} curve was made as described before. No difference in IC_{50} was observed between incubation times, suggesting that the inhibition was not time-dependent on the investigated time scale (Fig. 3A, Fig. S4). To further investigate reversibility we performed dilution experiments with compound **10** in which the inhibitor and enzyme were preincubated at a high concentration (10 × IC_{50}) before dilution in a substrate solution to 10× below the IC_{50} of the inhibitor. In combination with an irreversible inhibitor, the enzyme will show no activity after dilution. With a reversible inhibitor, however, the activity of the enzyme can be recovered.[38] The dilution assay with compound **10** showed that the activity of MIF could be recovered after dilution (Fig. 3B), which is consistent with reversible inhibition as observed in the preincubation assay.

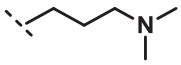
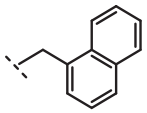
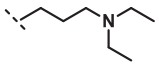
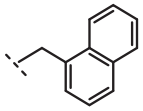
Table 2
Additional chromene compounds tested for inhibition of MIF. Percentage inhibition at 25 μM is given as mean of at least 2 independent experiments.



Compound	R^1	R^2	% inhibition at 25 μM	Compound	R^1	R^2	% inhibition at 25 μM
8		3-OEt	30%	**38**		3-OMe	0%
19		4-Me	20%	**39**		H	0%
20		3-Me	20%	**40**		4-Me	15%
21		3-OEt	10%	**41**		3-OMe	15%
22		3-OMe	15%	**42**		4-Me	10%
23		4-Me	0%	**43**		3-OEt	0%
24		3-OMe	0%	**44**		3-OMe	0%
25		3-Me	0%	**45**		4-Me	0%
26		3-OEt	0%	**46**		3-Me	0%
27		3-OMe	0%	**47**		3-OEt	10%
28		4-Me	0%	**48**		3-OMe	0%
29		3-OMe	0%	**49**		4-Me	0%
30		H	0%	**50**		3-OEt	0%
31		4-Me	0%	**51**		3-OMe	0%
32		3-OEt	0%	**52**		H	0%
33		3-OMe	0%	**53**		4-Me	10%
34		3-Me	0%	**54**		3-Me	0%
35		3-OMe	0%	**55**		3-OEt	50%

(*continued on next page*)

Table 2 (*continued*)

Compound	R^1	R^2	% inhibition at 25 μM	Compound	R^1	R^2	% inhibition at 25 μM
36		3-Me	0%	**56**		3-OMe	25%
37		4-Me	0%	**57**		3-Me	35%

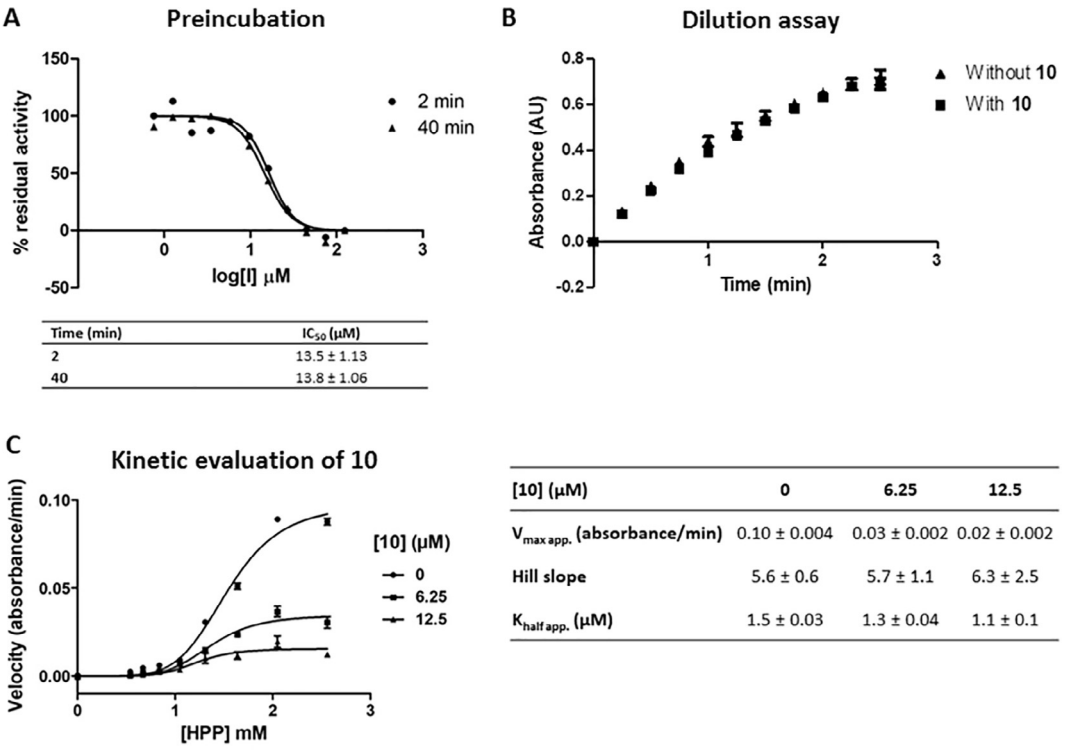


Fig. 3. A) MIF (340 nM) was preincubated with compounds **10** (125–0 μM) for 2 or 40 min prior to starting the enzyme reaction by adding the substrate. No significant change in IC_{50} value was observed. B) MIF (34 μM) was incubated with a concentration of 125 μM of compound **10**. Subsequently, this mixture was diluted 100x with the substrate and the enzyme activity was monitored. Diluting the inhibitor recovered the enzyme activity. C) The velocity of the enzyme reaction was measured at increasing concentrations of the substrate (4-HPP) in the presence of different concentrations of inhibitor **10**. The $V_{max\ app.}$, Hill slope and $K_{half\ app.}$ were determined for each inhibitor concentration.

To further investigate the mechanism of inhibition of the inhibitors, a kinetic evaluation of compound **10** was done (Fig. 3C). The velocity of the enzyme reaction was measured at increasing concentrations of the substrate (4-HPP) in the presence of different concentrations of inhibitor **10**. From this curve, the apparent maximum velocity ($V_{max\ app.}$), the Hill slope and the concentration of 4-HPP that gave half of $V_{max\ app.}$ ($K_{half\ app.}$) were determined. The experiment showed a sigmoidal curve with a Hill slope larger than 1, not following Michaelis-Menten kinetics, which is in line with observations from Lubetsky et al.[39] The K_{half} values were consistent with the values reported by Lubetsky et al. (denoted as $[S]_{0.5}$). An increasing concentration of compound **10** gave a decrease in $V_{max\ app.}$ The change in $K_{half\ app.}$ is less pronounced. This indicates that there is no direct competition between the substrate 4-HPP and the inhibitor **10**. This observation is in contrast to the binding mode described for Orita-13 that has been shown to bind the MIF active site.[29]

4. Conclusions and future perspectives

MIF binding to its molecular targets plays a key role in inflammatory processes and cancer. Therefore, MIF binders are considered to be potential therapeutics. In this study, we employed the MIF tautomerase enzymatic activity to identify MIF binding compounds that could potentially interfere with MIF functions. Using cyanoacetamide chemistry a focused compound collection with a chromene scaffold was synthesized and subsequently screened for inhibition of MIF tautomerase activity. This enabled identification of several novel MIF inhibitors with IC_{50}'s in the low micromolar range. Kinetic evaluation suggested that compound **10** and **17** were reversible inhibitors and that inhibitor **10** does not bind in direct competition with the substrate 4-HPP. Taken together, a novel structural class of MIF inhibitors has been identified that could be used to further investigate the tautomerase activity of MIF and may ultimately lead to the development of novel therapeutic agents.

Acknowledgements

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.12.032.

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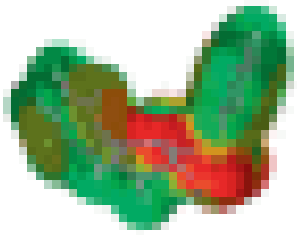

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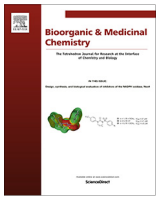

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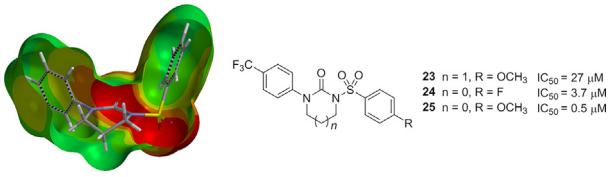

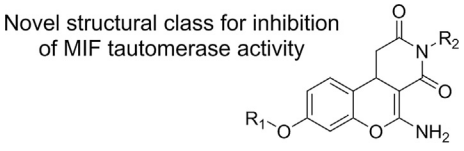

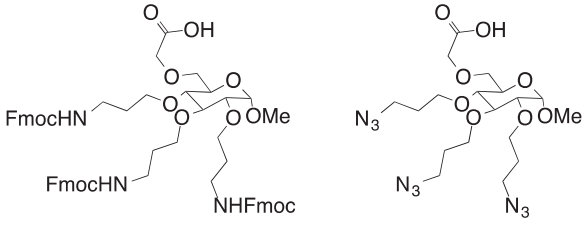

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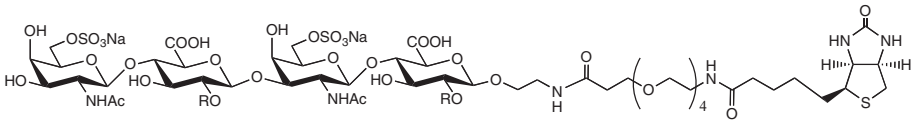
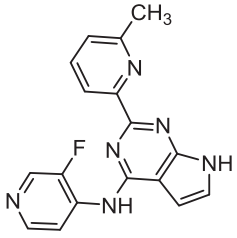
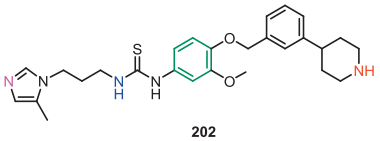


CS-DD R=SO_3Na, **CS-CC** R=H



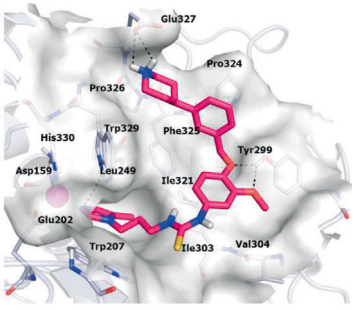
Compound **19**
TGFβRI Ki apparent = 0.14 nM
PSMAD cellular IC_{50} = 24 nM

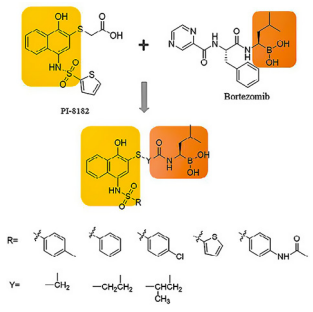


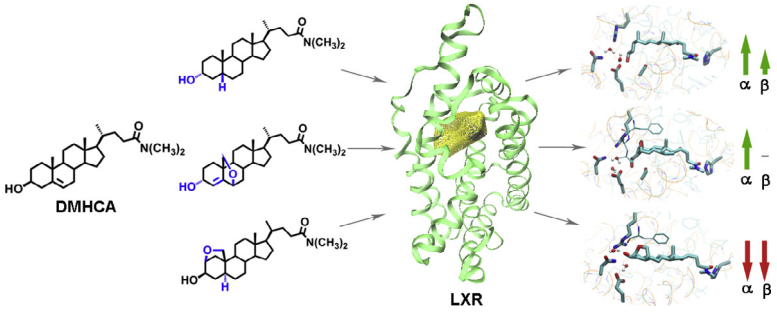
202

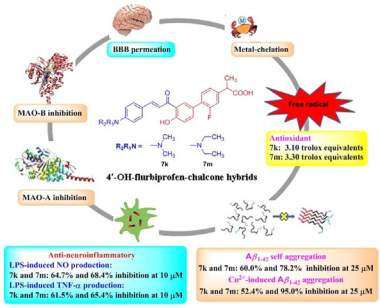
A : zinc binding motif
B : hydrogen bonding donor
C : aromatic group
D : amino group

- Glu-Phe-Arg mimetic of $A\beta_{42}$
- hQC IC_{50} = 6.2 nM
- in vivo (icv) : 30% inh. of $hA\beta_{N3pE-42}$









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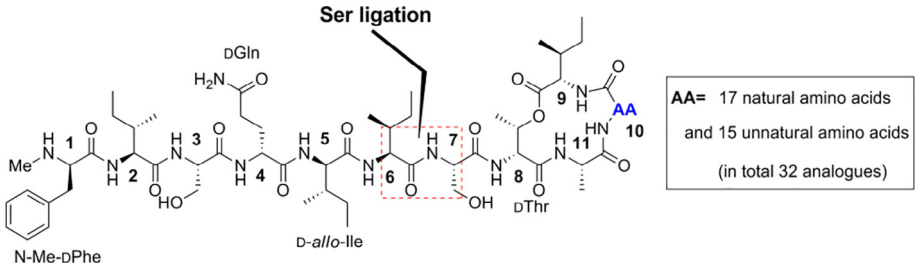

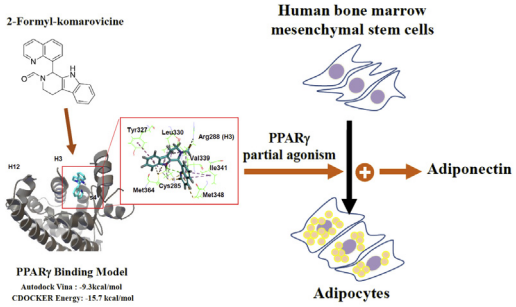

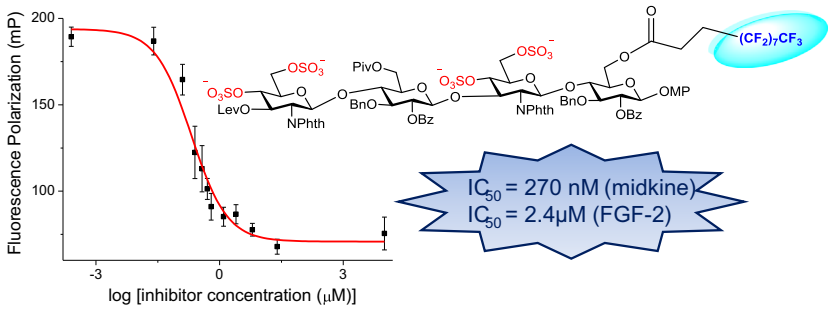

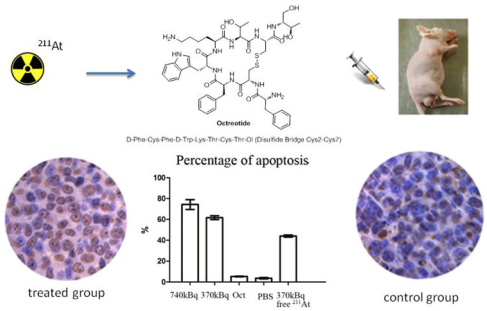

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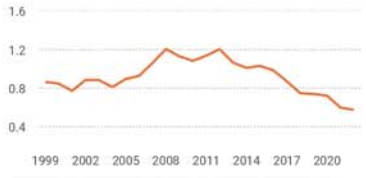

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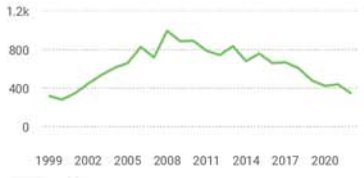

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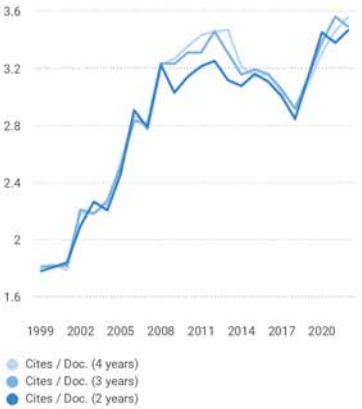

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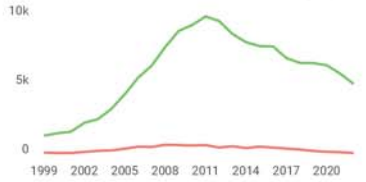

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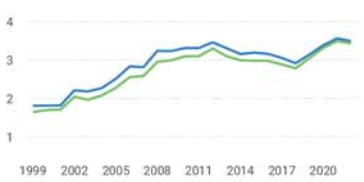

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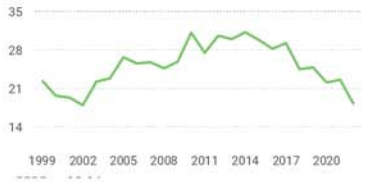

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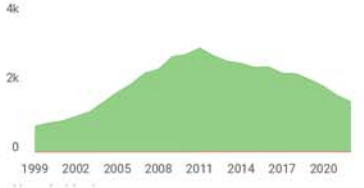

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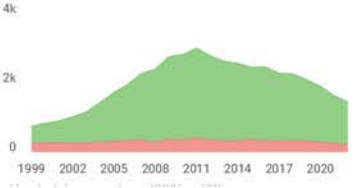

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