Discovery of Human Macrophage Migration Inhibitory Factor (MIF)-CD74 Antagonists via Virtual Screening

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Macrophage migration inhibitory factor (MIF) is a cytokine that is involved in the regulation of inflammation as well as cell proliferation and differentiation. Deactivation of MIF by antibodies or inhibition of MIF binding to its receptor, CD74, attenuates tumor growth and angiogenesis. To discover small-molecule inhibitors of MIF's biological activity, virtual screening was performed by docking 2.1 million compounds into the MIF tautomerase active site. After visual inspection of 1200 top-ranked MIF-ligand complexes, 26 possible inhibitors were selected and purchased and 23 of them were assayed. The in vitro binding assay for MIF with CD74 revealed that 11 of the compounds have inhibitory activity in the micromolar regime, including four compounds with IC_{50} values below 5 μM. Inhibition of MIF tautomerase activity was also established for many of the compounds with IC_{50} values as low as 0.5 μM; Michaelis−Menten analysis was performed for two cases and confirmed the competitive inhibition.

Introduction

Macrophage migration inhibitory factor (MIF[a]) is an immunoregulatory and proinflammatory cytokine that is released by many cell types including macrophages and T-cells. Cytokines have been shown to be involved in the pathology of many human inflammatory diseases. As a cytokine that is detectable in circulation as well as in inflamed sites, MIF is implicated in several inflammatory and autoimmune diseases including rheumatoid arthritis, atherosclerosis, asthma, and lupus.[1−3] MIF also is involved in multiple aspects of tumor growth including control of cell proliferation and promotion of angiogenesis.[4,5] The central role of MIF in tumorigenesis has been further supported by genetic data showing that individuals with high expression alleles of the MIF gene are at greater risk for the development of invasive prostate cancer.[6]

The mechanism by which MIF acts as a proinflammatory mediator and thereby controls local and systemic immune responses is still unknown. An increasing body of evidence suggests that (a) MIF is indirectly promoting angiogenesis by stimulating tumor cells to produce angiogenic factors, such as IL-8 and VEGF,[5] (b) MIF directly down-regulates the expression and function of the tumor-suppressor protein p53,[7] (c) MIF is activating MAPKs,[8,9] thereby enhancing cellular responses,[10] and (d) MIF counter-regulates the expression of glucocorticoids,[11,12] which suppress the expression and release of many proinflammatory molecules. Recent studies have shown that MIF signal transduction is initiated by binding to a transmembrane protein, CD74.[13,14] Inhibition of MIF-CD74 binding has been shown to attenuate tumor growth and angiogenesis.[4]

Apart from its immunoregulatory role, MIF is also a phenylpyruvate tautomerase. Possible relationships between the tautomerase and immunological/inflammatory activities of MIF remain under investigation.[15,16] Though MIF may exert some biological function via an enzyme mechanism,[17] the catalytic activity of mammalian MIF is likely vestigial.[18] However, there is evidence that the interaction of MIF with its receptor, CD74, occurs in the vicinity of the active site and that MIF tautomerase inhibition is correlated with inhibition of MIF-CD74 binding.[16]

The emerging role of MIF in angiogenesis and tumorigenesis as well as in inflammatory diseases indicates that modulating the cytokine's activity can result in new therapies.[2,19,20] Specifically, inhibition of the biological activities of MIF by antibodies or genetic deletion leads to reduced cellular proliferation and inhibition of tumor growth and angiogenesis.[4,21,22] Moreover, as reviewed by Orita et al.,[2] immunoneutralization of MIF and deletion of the MIF gene have been shown to have therapeutic benefits toward inflammatory diseases and also to suppress tumor growth.

Although injectable biological agents such as anti-cytokine antibodies or soluble cytokine receptors have effectively inhibited MIF activities, these strategies have significant associated risks and limitations in addition to high cost and inconvenience of application.[19] Alternatively, MIF could be effectively targeted by oral formulation of small-molecule inhibitors. Biochemical and structure−function analysis of MIF has laid the basis for structure-guided drug design. The crystal structure for MIF revealed a new structural superfamily;[23,24] the 114-residue MIF monomer has a $\beta/\alpha/\beta$ motif, and three monomers associate to form a symmetrical trimer. The trimer is toroidal with a solvent-filled central channel. MIF also was found to show structural homology with two prokaryotic tautomerases, and phenylpyruvate and D-dopachrome were discovered to be MIF tautomerase substrates.[17,25] Site-directed mutagenesis and crystallography have defined the MIF catalytic site.[24] Each MIF trimer has three tautomerase active sites, which

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[a] Abbreviations: MIF, macrophage migration inhibitory factor; 4-HPP, 4-hydroxyphenylpyruvate; 4-IPP, 4-iodo-6-phenylpyrimidine; NAPQI, *N*-acetyl-*p*-benzoquinone; ISO-1, (*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; ISO-17, (*S*,*R*)-[3-(3-fluoro-4-hydroxyphenyl)-4,5-dihydroisoxazol-5-yl]acetic acid *tert*-butyl ester; MAPK, mitogen-activated protein kinase; IL-8, interleukin-8; PGE2, prostaglandin E2; VEGF, vascular epithelial growth factor; rhMIF, recombinant human MIF; TNF, tumor necrosis factor; PNPP, *p*-nitrophenyl phosphate; PNP, *p*-nitrophenol; AP, alkaline phosphatase; rmsd, root mean-squared deviation.

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are well-defined cavities located at the interfaces of the monomer subunits. The N-terminal proline of MIF resides in the tautomerase binding pocket and has an unusually low pK_a of 5.6−6;[26] this renders the proline nucleophilic and allows it to effect the tautomerization of substrates.[17]

Several small-molecule tautomerase inhibitors of MIF have been reported,[2,16,27−31] but none effectively inhibit MIF's cytokine activity. The first small-molecule inhibitor of MIF's biological activity was *N*-acetyl-*p*-benzoquinone (NAPQI), a metabolite of acetaminophen, which binds covalently to MIF's N-terminal proline.[16] NAPQI is a 40 μM tautomerase inhibitor, and it also decreases macrophage TNFα production as well as other biological actions of MIF.[16] Recently, another covalent inhibitor, 4-iodo-6-phenylpyrimidine (4-IPP), was reported including a crystal structure for its covalent complex with MIF.[30] Since covalent inhibitors are often not optimal as lead candidates, druglike molecules that bind reversibly to the MIF N-terminal region have also been sought. A tryptophan Schiff base that is a 1.65 μM MIF tautomerase inhibitor was found;[31] it decreases the number of THP-1 (human acute monocytic leukemia) cells that bind fluorescent MIF by 45% at 10 μM. Subsequently, a dihydroisoxazole derivative showed some inhibitory activity in assays of counter-regulation of glucocorticoid inhibition of TNFα, PGE_2, and COX-2 production,[29] but it shows low activity in a MIF-CD74 binding assay (vide infra). In view of this limited success with the discovery of small molecules that disrupt the biological activities MIF, we initiated an extensive virtual screening and assaying campaign to discover more potent and diverse lead compounds. As reported here, this effort resulted in the discovery of 11 novel compounds with IC_{50} values in the micromolar range including 4 with IC_{50} values below 5 μM. Such potent MIF-CD74 inhibitors not only provide leads for development of preclinical candidates for the treatment of cancer and inflammatory diseases, but also can be used as validation tools to further investigate the biology of MIF.

Methods

Virtual Screening. The crystal structure of MIF complexed with the substrate *p*-hydroxyphenylpyruvate (HPP) (PDB code 1CA7)[24] was employed in the docking calculations performed with Glide 4.0 (Schrödinger, LLC). Hydrogen atoms were added to the crystal structure, and the complex was submitted to a series of restrained, partial minimizations using the OPLS-AA force field[32] within the "Protein and Ligand Preparation" module of Glide. The key residue Pro1, which is postulated to act as a catalytic base to effect substrate tautomerization, was kept unprotonated, consistent with the observation that in the presence of no ligand or of an inhibitor, Pro1 has a pK_a of 5.6−6.[26] To compensate for the fixed protein structure, which is not expected to be optimal for a particular ligand, the van der Waals radii for nonpolar ligand atoms were scaled by a factor of 0.8, thereby decreasing penalties for close contacts. Receptor atoms were not scaled. For the protein preparation, grid generation, and ligand docking procedures, the default Glide settings were used.

Druglike subsets of the HitFinder collection from the Maybridge database [www.maybridge.com] and the ZINC database[33] were used for the virtual screening. The HitFinder collection was preprocessed with the Glide module "LigPrep", which prepared the ligands in multiple protonation and tautomerization states. This procedure resulted in ∼24 000 structures as compared to the initial 14 400 of the HitFinder collection. The ZINC "druglike" set had already been preprocessed and includes multiple protonation and tautomerization states.[33] The complete virtual library, prefiltered for properties based on Lipinski's rules,[34] totaled about 2.1 million compounds. All structures were docked and scored using the Glide standard-precision (SP) mode.[35] The 40 000 top-ranked structures from ZINC and the 1000 top-ranked ones from the Maybridge database resulting from the SP filter were redocked and rescored using the Glide extra-precision (XP) mode.[36]

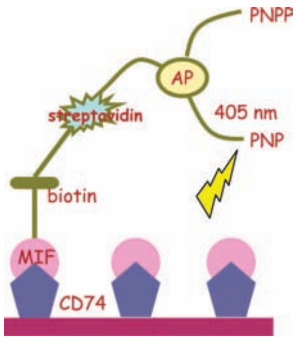


Figure 1. Schematic of the MIF-CD74 binding assay using biotinylated MIF and immoboilized CD74 ectodomain. For the bound complexes, alkaline phosphatase (AP) converts the substrate *p*-nitrophenylphosphate (PNPP) to *p*-nitrophenol (PNP), which absorbs light at 405 nm. In the presence of an inhibitor, MIF-CD74 binding and the product signal are reduced.

In essence, Glide performs a thorough conformational search for a ligand; then it determines all reasonable orientations ("poses") for each low-energy conformer in the designated binding site. In the process, torsional degrees of the ligand are relaxed, though the protein conformation is fixed. The SP "scoring function" is applied to judge the poses by considering, for example, hydrophobic and electrostatic interactions, hydrogen bonding, steric clashes, desolvation and internal energy of the ligand, and possible trapped or bridging water molecules in the binding site. In XP mode, the poses are further relaxed by complete energy minimizations. The resultant more accurate structures provide a basis for more detailed evaluation of contributions from explicit water molecules in the binding site and hydrophobic interactions.

Assayed Compounds. Compounds were purchased from commercial vendors. Their structures were confirmed by ^{1}H NMR and mass spectrometry. NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz), and ESI mass spectra were obtained on a Waters Micromass-ZQ at the Yale Chemical Instrumentation Center. Spectra are provided in the Supporting Information.

MIF-CD74 Binding Assay. The interaction between MIF and its receptor, CD74, was analyzed following methodologies previously described (Figure 1).[13] The 96-well plates were coated with recombinant MIF receptor ectodomain (sCD74 = $CD74^{73\text{-}232}$), washed 4 times, and blocked with Superblock (Pierce Biotechnology) at 4 °C overnight. Test compounds were preincubated with biotinylated human MIF (2 ng/μL) (Roche Applied Sciences) prepared recombinantly[37] for 30 min at room temperature. The mixtures then were added to wells for overnight incubation at 4 °C. The wells were washed 4 times, and strepavidin-conjugated alkaline phosphatase (R&D Systems) was added for 1 h of incubation at room temperature. After additional washes, 60 μL/well of *p*-nitrophenyl phosphate (Sigma) substrate was added. Absorbance at 405 nm was plotted as percent A_{405} relative to wells containing biotinylated human MIF alone. Each plot represented at least three independently performed assays, and each data point depicts a SEM ≤ 10%. Each test compound was analyzed for over 10−12 concentrations and over a ∼500-fold concentration range.

In Vitro Characterization of MIF Tautomerase Activity. Tautomerase activity was assessed using the substrate 4-hydroxyphenylpyruvate (4-HPP) following a previously reported procedure.[38] Briefly, 4-HPP was dissolved in 50 mM ammonium acetate at pH 6.0, allowed to equilibrate to room temperature, and stored at 4 °C. The substrate and buffer were mixed, followed by addition of purified rhMIF. Tautomerase activity was determined at 25 °C by adding 4-HPP to a quartz cuvette containing 0.435 M boric acid at pH 6.2 and the preincubated mixture of rhMIF and test compound, in triplicate, and measuring the increase in absorbance at 305 nm over 30−90 s. Compounds were tested over a 5000-fold concentration range. In the same manner, Michaelis−Menten

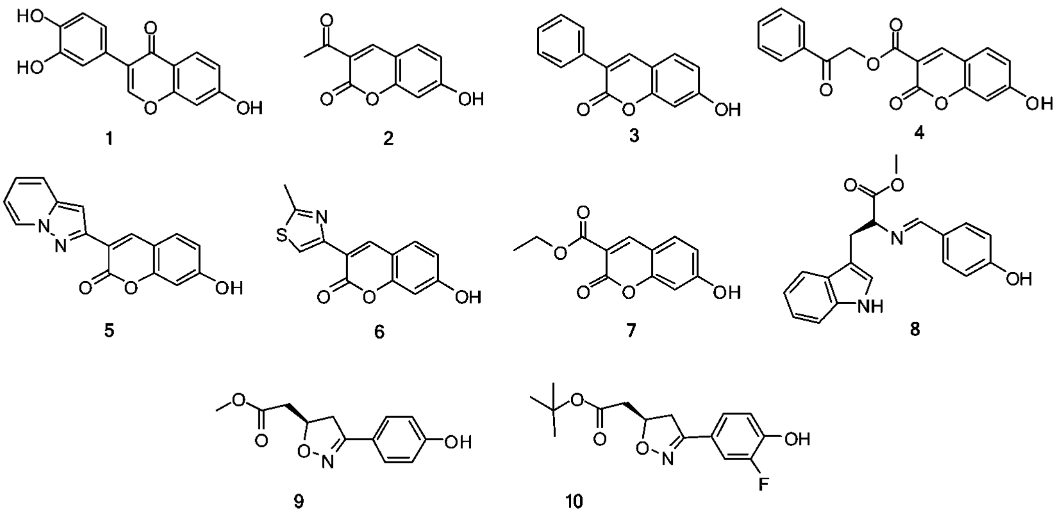


Figure 2. Structures of 10 reported MIF tautomerase inhibitors used to validate the docking procedure.

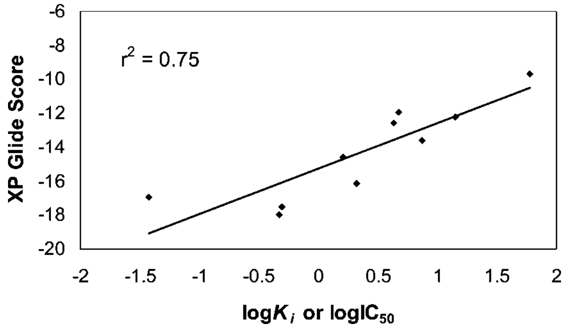


Figure 3. Glide XP score versus log K_i or log IC$_{50}$ for 10 known tautomerase inhibitors.

Table 1. Inhibition Constants, K_i, Half-Maximal Inhibitory Concentrations, IC$_{50}$, and Glide XP Gscores for Known MIF Tautomerase Inhibitors

compd	K_i (μM)	IC$_{50}$ (μM)	Glide XP Gscore
1	0.038[2]		−16.95
2	4.3[2]		−12.55
3	0.47[2]		−17.99
4	1.6[2]		−14.58
5	0.50[2]		−17.53
6	2.1[2]		−16.15
7	7.4[27]		−13.59
8		1.65[31]	−12.23
9		7[39]	−9.69
10		0.55[40]	−11.95

analysis was carried out for two of the inhibitors at concentrations of 0, 3, and 5 μM with 4-HPP as substrate; Lineweaver−Burk plots demonstrated the competitive inhibition.

Results and Discussion

Validation of the Docking Method. To test the performance of the Glide docking and scoring, 10 known MIF tautomerase inhibitors were processed with Glide as described above (Figure 2). The selected inhibitors were a chromene-4-one derivative (**1**),[2] coumarin derivatives (**2**−**7**),[2,27] an L-tryphophan Schiff base (**8**),[31] **9** (ISO-1),[39] and **10** (ISO-17).[40] The Glide XP scores showed significant correlation with the assay data for these known inhibitors (see Figure 3 and Table 1). Compounds **1**−**7** were assayed by Orita et al.,[2] who used D-dopachrome as the substrate of MIF, and K_i values were determined. Compounds **8**−**10** were assayed with the same protocol, but L-dopachrome methyl ester was the substrate and IC$_{50}$ values were determined. To accommodate the data from the two different assays, the regression analysis included an indicator variable (0 or 1) for the log IC$_{50}$ values of compounds **8**−**10**; this yielded an offset of 0.936 log unit for the three compounds.

It also was possible to make comparisons of the structures for three of the MIF−inhibitor complexes obtained from Glide XP with prior work. Specifically, Orita et al. reported a crystal structure for the complex with **7** as well as structures for the complexes of **1** and **3** from docking with the program DOCK 4.0.1.[2,27]

The crystal structure for the complex with **7** agrees very well with the conformation obtained by docking in the MIF binding site for the coumarin ring system (Figure 4). However, Glide predicts a syn geometry for the two carbonyl groups of compound **7**, likely due to the two hydrogen bonds that are formed with Lys32 of MIF in this configuration. The apparent discrepancy with the anti geometry in the crystal structure may be attributable to the fact that the ethyl ester moiety is disordered and showed poor electron density.[27] It also should be noted that the twisted ester moiety with a nearly 90° OCOC dihedral angle in both structures is highly unlikely; acyclic esters are predominantly *Z* with a dihedral angle near 0°. The rmsd between the Glide-calculated pose and the crystal structure of compound **7** is 0.59 Å when taking into account only the heavy atoms of the coumarin ring system and is 1.43 Å for all heavy atoms in the molecule. In general, the key features in the crystal structure are represented well by the Glide pose. The hydroxyl group at C7 of **7** forms a hydrogen bond with the side chain of Asn97. In fact, as drawn in Figure 2, the 10 known inhibitors all have a phenolic terminus, which is expected to participate in hydrogen bonds with the side chain amido group of Asn97. Furthermore, the backbone NH of Ile64 makes hydrogen bonds with both oxygens of the lactone fragment in the coumarin ring. The coumarin ring also appears to have favorable T-shaped aryl−aryl interactions with Tyr95 and Phe113. And again, the Glide pose features hydrogen bonds between the ammonium group of Lys32 and both carbonyl oxygens of **7**.

Significant similarities exist for the structures of the complexes with **7** and for those predicted by Glide for **1** and **3** (Figure 5). As in the previous models from DOCK,[2,27] the bicyclic ring systems are positioned in the same manner, and both inhibitors form hydrogen bonds between a hydroxyl group and the side chain of Asn97 and between the ether oxygen of the chromen-4-one or of the coumarin ring and the backbone

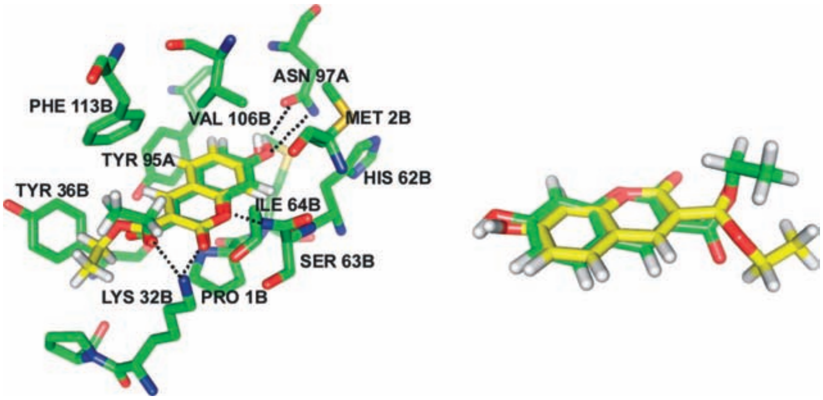


Figure 4. Comparison of the docked (yellow) and the observed crystal structure[27] (green) for the complex of MIF with **7**. The rmsd for heavy atoms in the coumarin ring is 0.56 Å, while for all heavy atoms it is 1.43 Å. The exocyclic ester group is disordered in the crystal structure.

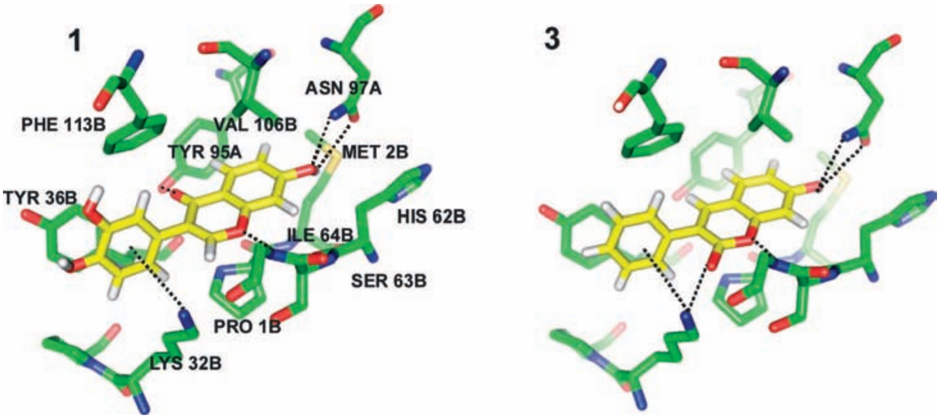


Figure 5. Docking poses from Glide XP for tautomerase inhibitors **1** and **3**. The side chain of Ile64B has been removed for clarity.

NH of Ile64. Aryl−aryl interactions in the binding pocket are notable between Tyr95 with the chromen-4-one or coumarin ring in a T-shaped geometry, while a parallel, stacked arrangement is predicted between the phenyl appendage and Tyr36. Also in accord with the prior docking results, **1** forms a hydrogen bond between its C4 carbonyl oxygen and the hydroxyl group of Tyr95; the O−O distance is 2.5 Å in the previous theoretical model[2] and 2.6 Å in the Glide pose. A cation−π interaction is indicated between the phenyl ring of both compounds and the ammonium group of Lys32, and compound **3** also features a hydrogen bond between its carbonyl oxygen and the ammonium group of Lys32. In all, the structural results and the activity correlation in Figure 3 provided optimism for the virtual screening exercise.

Virtual Screening. The docking was then extended to the 2.1 million structures obtained from the ZINC and Maybridge databases. The distribution of the Glide XP scores for the top-ranked 1000 compounds from the ZINC database, the top-ranked 1000 compounds from the Maybridge set, and the 10 known tautomerase inhibitors is shown in Figure 6. It is evident that the ZINC collection delivers many significantly lower XP Gscores that are likely to result in more potent inhibitors. This fact undoubtedly arises from the far larger size and diversity of the ZINC library (~2.1 M compounds from 10 vendors) in comparison to the Maybridge Hitfinder collection (14 400 compounds). The average molecular weight for the top-scoring 1000 compounds from the ZINC database is 321.7, while it is 306.1 for the top 1000 Maybridge compounds. Thus, although the top ZINC compounds have on average one additional non-hydrogen atom, the greater structural variety of the larger database is undoubtedly an important asset.

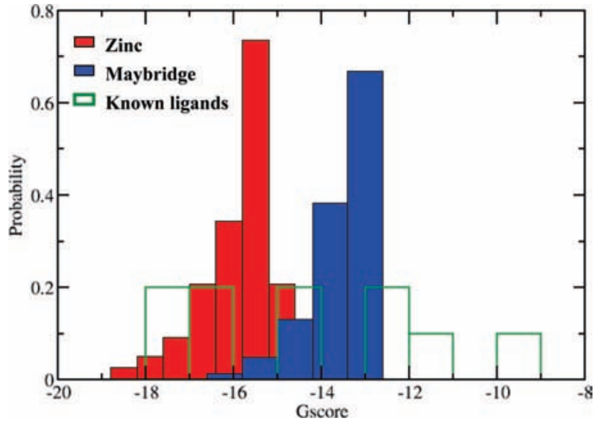
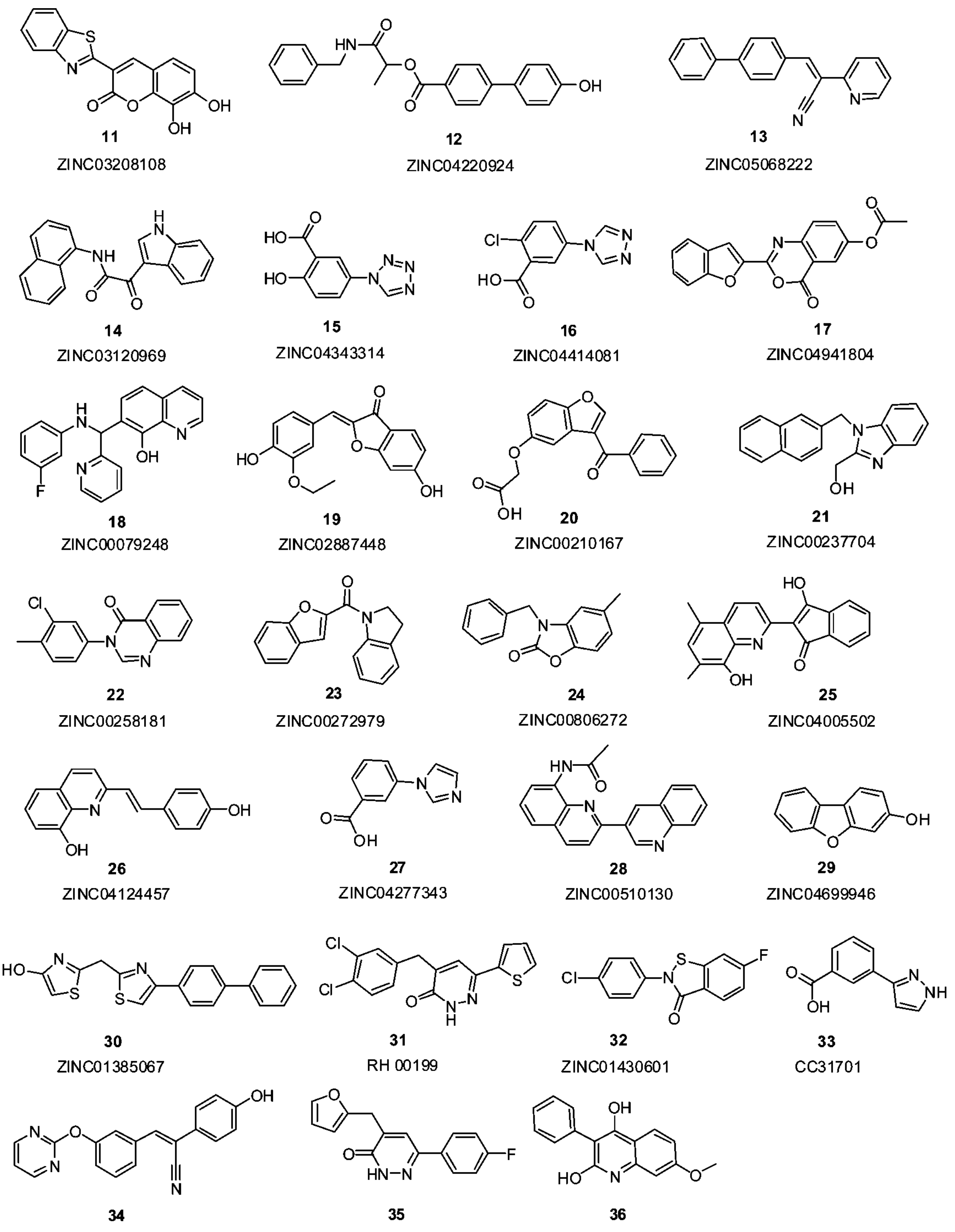


Figure 6. Distributions of the Glide XP scores for the top-ranked 1000 ZINC compounds, the top-ranked 1000 Maybridge compounds, and the 10 known tautomerase inhibitors.

The complexes for the top-ranked 1000 compounds from the ZINC database resulting from the XP processing, the top-ranked 100 compounds from ZINC with just the SP filter, and the top-ranked 100 XP hits from the Maybridge database were submitted to display and visual inspection. Many well-ranked structures were ruled unlikely owing to poor conformations including twisted amide and ester groups or overly short nonbonded contacts. Final selection of compounds for assaying also took into account chemical diversity, properties predicted by the QikProp program,[41] and QikProp alerts concerning potentially undesirable chemical substructures. Structural diversity in compound selection is desired to provide alternative lead series for optimization. Previously reported cores, substituted phenols



11 ZINC03208108
12 ZINC04220924
13 ZINC05068222
14 ZINC03120969
15 ZINC04343314
16 ZINC04414081
17 ZINC04941804
18 ZINC00079248
19 ZINC02887448
20 ZINC00210167
21 ZINC00237704
22 ZINC00258181
23 ZINC00272979
24 ZINC00806272
25 ZINC04005502
26 ZINC04124457
27 ZINC04277343
28 ZINC00510130
29 ZINC04699946
30 ZINC01385067
31 RH 00199
32 ZINC01430601
33 CC31701
34 ZINC01384384
35 ZINC00119581
36 NRB 02373

Figure 7. Chemical structures of the purchased compounds and their database codes.

and especially coumarin derivatives,[2] were avoided in the selection with some exceptions for interesting substituents.

Finally, 34 potential inhibitors were designated for purchase; however, only 24 turned out to be commercially available. Compounds **11**−**30**, **32**, **34**, **35** were chosen from the ZINC database, and compound **31** was chosen from the Maybridge collection (Figure 7). For the unavailable compounds, a similarity search was performed using SciFinder Scholar [www.cas.org]. Commercially available compounds that showed 97% or greater similarity to the desired ones were redocked using Glide, as described above. If the Gscore of a compound was within the range of previously selected XP or SP compounds, it was purchased. Compounds **33** and **36** were chosen in this manner. Thus, 26 compounds were finally acquired including five (**14**, **15**, **16**, **27**, **33**) that were selected on the basis of their SP performance and visualization, though they were not in the top 1000 from the XP scoring. Ultimately 23 compounds were assayed. The chemical structures and purity of the compounds were verified with ^{1}H NMR and mass spectrometry at the Yale Chemical Instrumentation Center. Compound **34** was impure and gave incorrect NMR and mass spectra, while **35** and **36** were delivered in insufficient quantities for spectral analysis and were thus not assayed.

In Vitro Assays. The 23 compounds were first assayed in vitro for disruption of human MIF binding to its receptor, CD74. Eleven of the 23 compounds were discovered to be active with

Table 2. Experimental Activities from the Tautomerase and MIF-CD74 Inhibition Assays and Glide Scores and Rankings

	IC_{50} (μM)					
compd	MIF-CD74 binding assay[a]	4-HPP tautomerase assay[a]	XP Gscore	SP Gscore	XP rank	SP rank
11	NA		−17.89		32	
12	NA		−15.77		443	
13	NA		−15.90		389	
14	NA			−9.71		21
15	550	NA		−9.50		52
16	250	NA		−9.86		8
17	2.5	730	−16.21		285	
18	2.5	530	−16.57		182	
19	NA		−15.74		458	
20	1500	980	−15.06		977	
21	NA		−16.41		223	
22	NA		−15.92		379	
23	4.0	3.0	−15.39		696	
24	1.5	0.5	−15.89		394	
25	NA		−16.24		278	
26	8	max 40%	−15.13		938	
27	NA			−9.63		31
28	NA		−17.99		26	
29	900	max 38%	−15.8		430	
30	65	300	−16.73		150	
31	250	430	−18.15		1	
32	NA	4.2	−15.89		393	
33	15	max 30%		−9.67		27
9	max 40%	NA				
4-IPP	NA	4.5				
anti-MIF antibody	0.4					

[a] NA indicates measured but not active. Blank cells indicate not measured.

half-maximal inhibitory concentrations (IC_{50}) in the micromolar range including four below 5 μM (Table 2). The IC_{50} of the most potent compound, **24**, was 1.5 μM. Thirteen of the compounds were also assayed for MIF tautomerase inhibition, and **23**, **24**, and **32** were found to have IC_{50} values of 3, 0.5, and 4 μM, respectively. It should be noted that **9**, a previously reported tautomerase inhibitor in the dopachrome-based assay,[39] was inactive in the 4-HPP tautomerase assay and marginally active in the MIF-CD74 binding assay. It shows maximal inhibition of 40% at 10 μM, so an IC_{50} could not be obtained (Table 2). As other references, a biologically neutralizing anti-MIF IgG1 monoclonal antibody (clone12302, R&D Systems) was found to be a 0.4 μM inhibitor in the binding assay, while 4-IPP is inactive in the binding assay, but it is a 4.5 μM inhibitor in the 4-HPP tautomerization assay.[30] Notably, the dihydroindole and benzooxazolone derivatives **23** and **24** are potent in both assays.

In order to check the expected competitive inhibition of the tautomerase activity, Michaelis−Menten analysis was performed for inhibitors **23** and **24** with 4-HPP as the substrate. Linear Lineweaver−Burk plots were generated using seven substrate concentrations and the inhibitors at concentrations of 0, 3, and 5 μM. For **23** the corresponding three K_m values are 222 ± 20, 322 ± 35, and 533 ± 50 μM, and for **24** they are 235 ± 20, 322 ± 40, and 444 ± 50 μM. The values in the absence of the inhibitors are consistent with a previously reported K_m of 170 ± 40 μM.[42] In all cases, the *y*-intercept was the same, indicating no change in the maximal rate for the catalyzed reaction, V_{max}. In conjunction with the increasing K_m values with increasing inhibitor concentrations, competitive inhibition is indicated.

There is not a straightforward correspondence between activity in the MIF-CD74 and tautomerase assays, and it would not be expected. In the simplest model for MIF-CD74 binding occurring near the tautomerase active site, the structures of the inhibitors affect how much they protrude from the active site. The leftmost residues in Figure 5 are on the surface of MIF. The extent, if any, to which the inhibitors protrude and the concomitant variability in how surface residues such as Lys32 rearrange would be expected to modulate the MIF-CD74 interaction. Small, potent tautomerase inhibitors like 4-IPP might have little effect on CD74 binding, while larger, weaker tautomerase inhibitors like **17** and **18** could be more disruptive of the interaction with CD74, as observed. Conversely, molecules that show no or little tautomerase inhibitory activity could still interfere with the binding of MIF to CD74, e.g., by binding in the same vicinity but having little effect on the binding of tautomerase substrates. In general, the MIF-CD74 antagonists do provide some tautomerase inhibition; the exceptions are **15**, **16**, and **9**, which are very weak MIF-CD74 antagonists. This can be interpreted as additional support for the notion that the interaction of MIF with its receptor, CD74, occurs in the vicinity of the active site.[16] However, there is no question that detailed understanding of the inhibitory effects of specific compounds awaits experimental structures for MIF-CD74-inhibitor complexes. The fact that MIF is trimeric with three catalytic sites also adds potential complexity for interpretation of the tautomerase results, which may depend on the effects on activity at one site upon binding at another site.

What is absolutely clear from the results in Table 2 is that through virtual screening, which targeted the tautomerase active site of MIF, there has been substantial success in finding both promising inhibitors of MIF's tautomerase activity and of its binding to CD74. The reported leads also provide opportunities for pursuing analogue series that can be expected to yield valuable insights on the relationship of activity and structure.

As can be seen in Table 2, most compounds that were chosen for purchase and assaying did not receive the highest XP rankings for the reasons noted above. However, the no. 1 XP-ranked compound from the Maybridge database (**31**) was purchased and found to be a 250 μM inhibitor in the receptor assay, while **28** and **11**, which were ranked 26th and 32nd from the ZINC database, were inactive. The particularly promising compounds **23** and **24** were no. 696 and no. 394 in the ZINC XP evaluation. In addition, three of the five compounds selected on the basis of the SP scoring, which were not in the top 1000 for XP, did show some activity. Overall, the docking results were provocative and essential, but skilled human filters are still necessary in our experience.

Table 3. Predicted Properties from QikProp for the Most Active Compounds

compd	MW[a]	QP log *P*[b]	QP log *S*[c]	QP P_{Caco}[d]
16	223.618	1.55	−2.50	61.3
17	321.289	2.09	−3.74	584.3
18	345.375	4.61	−5.46	2362.6
23	263.295	3.33	−3.76	3998.2
24	239.273	2.97	−3.17	2660.7
26	263.295	3.22	−4.01	840.4
30	350.452	5.07	−6.46	1605.7
31	337.223	4.75	−6.10	1594.1
33	188.185	1.31	−2.18	69.0

[a] Molecular weight. [b] Predicted octanol/water log *P*. [c] Predicted aqueous solubility; *S* in mol/L. [d] Predicted Caco-2 cell permeability in nm/s.

Calculated Properties and Structural Basis for Inhibition. Some predicted properties from QikProp for the most active MIF-CD74 inhibitors are summarized in Table 3.[41] The rms errors for QikProp predictions are 0.5−0.6 log unit. When QikProp is run on 1700 known oral drugs, 90% have MW < 470, QP log *S* > −5.7, QP log *P* < 5.0, and QP P_{Caco} > 22

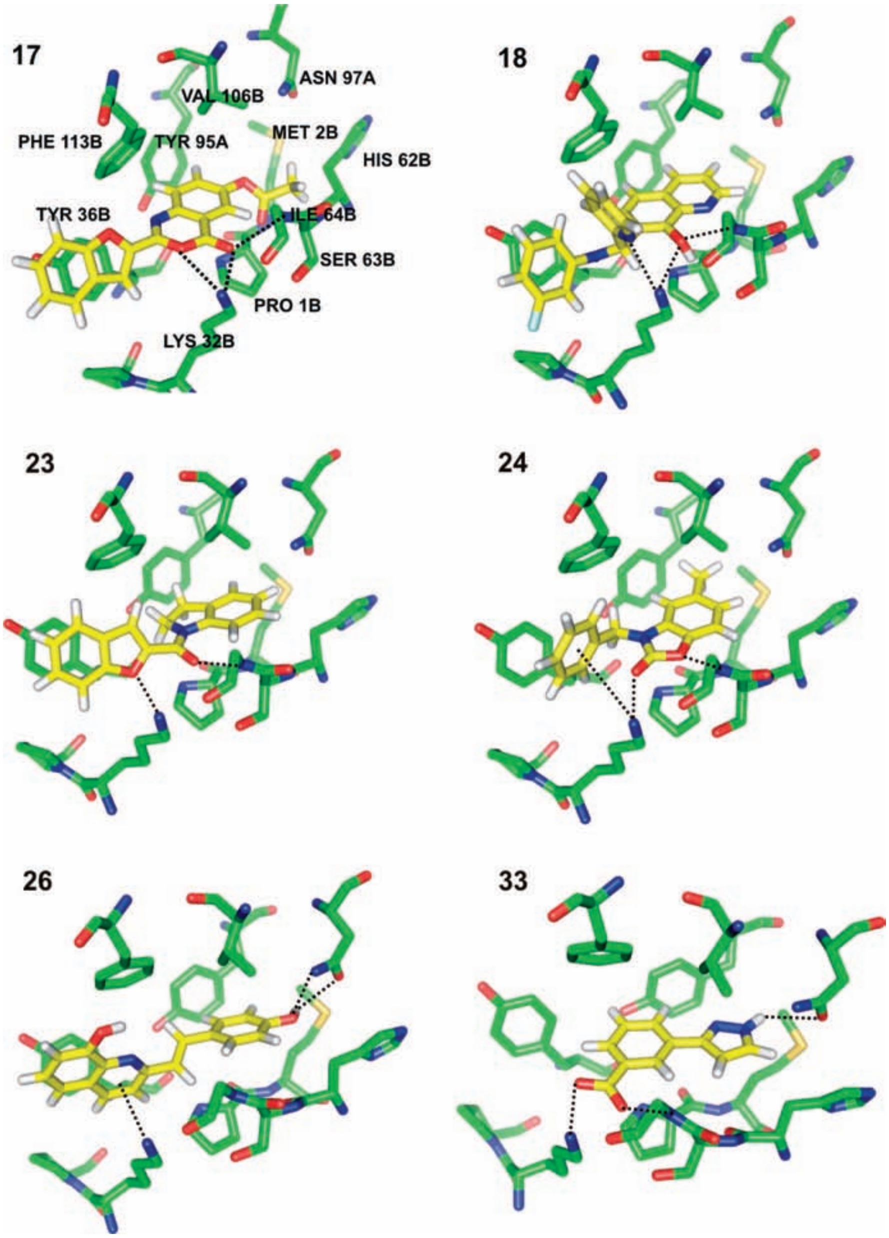


Figure 8. Docking poses for six of the most potent MIF-CD74 inhibitors: **17**, **18**, **23**, **24**, **26**, **33**. The side chain of Ile64 has been removed for clarity.

nm/s.[43,44] The results in Table 3 compare favorably with these limits, so optimism can be expressed for the utility of the compounds as drug leads. Development starting from **30** and **31** would need to take into account their low predicted solubilities.

Figure 8 illustrates the computed poses for complexes between MIF and the six most potent MIF-CD74 inhibitors, **17**, **18**, **23**, **24**, **26**, and **33**. The compounds represent six unique chemotypes for potential lead optimization. Aryl−aryl interactions, hydrogen bonds, and seemingly favorable van der Waals contacts provide the binding energy. The compounds are predicted to bind in the MIF catalytic pocket in a similar manner to the known tautomerase inhibitors in Figures 4 and 5. **17**, **18**, **23**, **24**, and **33** form a hydrogen bond with the backbone nitrogen of Ile64 as well as with the ammonium group of Lys32. Of the nine compounds with a MIF-CD74 inhibitory activity at or below 250 μM, only **26** is predicted to form the two hydrogen bonds with Asn97 that are expected for the known inhibitors **1**−**10**. However, the pyrazolyl NH of **33** participates in a hydrogen bond with the carbonyl oxygen of the Asn97 side chain. The imidazole analogue **27** was purchased thinking that it was likely zwitterionic and that the protonated nitrogen might participate in an analogous hydrogen bond with Asn97; **15** and **16** provided additional variations of the theme and show some activity. Though the remaining most-active compounds lack a hydrogen-bonding group in the proximity of Asn97, introduction of such a group could be explored during lead optimization. Incidentally, it seems possible that alternative poses are viable in some cases by flipping the ligand end-to-end in the binding site, e.g., the benzofuran unit could be proximal to Asn97 in the complexes for **17** and **23**.

Notably, all of the purchased compounds have conjugated ring systems that were expected to be well buried in the active site. As shown in the examples in Figure 8, Tyr95 is involved in an edge-to-face aryl−aryl interaction in all cases, while Tyr36 and Phe113 are also providing stacking and edge-to-face interactions with all inhibitors except **33**. The latter compound is the only charged inhibitor depicted in Figure 8; its carboxylate group is predicted to participate in a salt bridge with Lys32 and to accept an additional hydrogen bond from the backbone NH of Ile64. Though **33** has a molecular weight of only 188, it appears to have excellent hydrogen-bonding and aryl−aryl compatibility with the MIF active site. Another useful design element is potential cation−π interactions with Lys32, as with **24** and **26**.

Conclusions

Virtual screening of druglike subsets of the publicly available ZINC and Maybridge databases was performed to seek inhibitors of the biological activity of the cytokine MIF. The docking procedure was validated on three known MIF tautomerase inhibitors by comparing the Glide binding poses with previously calculated models for two of these compounds as well as a crystal structure. The structures from Glide reproduced well the expected geometries of the complexes. Furthermore, the Glide XP scores showed significant correlation with tautomerase assay data for 10 known inhibitors. Following the virtual screening, the complexes for the top-ranked 1200 compounds were visually inspected and 26 compounds were purchased. One compound was impure and gave incorrect spectra, and two others were delivered in inadequate quantities. Finally, 23 compounds were assayed in vitro for disruption of MIF-CD74 binding and 13 for MIF tautomerase inhibition. Notably, 11 of the 23 compounds were found to inhibit the binding of MIF to its receptor in the micromolar range including four with IC_{50} values below 5 μM.

Visualization of the computed structures shows potentially favorable aryl−aryl interactions, hydrogen bonding, and van der Waals contacts between the inhibitors and the active site of MIF. However, a tautomerase inhibitor is not necessarily a MIF-CD74 antagonist. While compounds **32** and 4-IPP inhibit the enzymatic activity, they were inactive in the MIF-CD74 binding assay. On the other hand, compounds **17**, **18**, and **26**, while being MIF-CD74 inhibitors in the low micromolar realm, are weak tautomerase inhibitors. A deeper understanding of the structure−activity relationships would greatly benefit from crystallographic investigations of MIF-CD74-inhibitor complexes. Nevertheless, judging by the success of the present research, it appears likely that the MIF-CD74 inhibitors do bind in the MIF tautomerase active site.

The present work has led to discovery of small-molecule inhibitors of MIF-CD74 binding, which are the most potent that have been reported to date. The structural diversity of the inhibitors has provided valuable alternative series for ongoing lead optimization. Potent, selective MIF-CD74 inhibitors are anticipated to provide promising candidates for the treatment of cancer and inflammatory diseases. Moreover, they can be used as validation tools to further explore the biology of MIF.

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Supporting Information Available: NMR and mass spectral data for the assayed compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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