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Discovery of covalent inhibitors for MIF tautomerase via cocrystal structures with phantom hits from virtual screening

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ABSTRACT

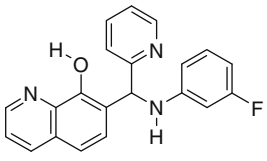
Biochemical and X-ray crystallographic studies confirmed that hydroxyquinoline derivatives identified by virtual screening were actually covalent inhibitors of the MIF tautomerase. Adducts were formed by N-alkylation of the Pro-1 at the catalytic site with a loss of an amino group of the inhibitor.

Macrophage migration inhibitory factor (MIF), first discovered in 1966 and cloned in 1989,[1] is a cytokine that plays a key role in the pathology of inflammatory diseases,[2] and has been implicated in cancer pathogenesis[3] and cardiovascular disease.[4] Although it is still poorly understood how MIF exerts its biological function, recent studies suggest that the MIF signaling pathway is initiated by its binding to the membrane-bound protein CD74.[5] MIF has tautomerase activity[6,7] and the catalytic active site of MIF tautomerase is likely linked to its cytokine activity.[8] MIF also exhibits thio-protein oxidoreductase activity.[9]

For more than a decade, effort has been directed towards searching for inhibitors targeting MIF tautomerase activity with the ultimate goal of antagonizing MIF biological function. A variety of inhibitors for MIF tautomerase has been reported and reviewed.[10,11] These inhibitors include indole derivatives,[12] dopachrome analogs,[13] ketones,[14,15] coumarin derivatives,[16] phenol derivatives,[17] ISO-1 and its analogs.[18] Several reports of covalent inhibitors such as 4-IPP,[19,20] 2-OBP,[21] NAPQI and phenylmethylsulfonyl fluoride,[22] show the potential for strong binding by a covalent bond.[23]

Biochemical and crystallographic studies[24] have demonstrated that Pro-1,[25] Lys-32,[26] and the C-terminal residues[27] are key residues involved in MIF tautomerase catalytic activity. The availability of X-ray crystal structures of MIF-inhibitor complexes[16,24] has provided data pivotal to the success of inhibitor discovery by rational design.

The X-ray crystal structures provided a template to perform in silico virtual screening, yielding several novel MIF tautomerase inhibitors.[19,28,29] For example, compound **1** has recently been identified from experimentally testing only a handful compounds selected by virtual screening.[28,30] The nearly identical compound **2** (de-fluoro *p*-methoxy)[31] was found to be active in inhibiting MIF tautomerase activity during the follow-up of the initial hits from a sanofi-aventis virtual screening campaign.[32]



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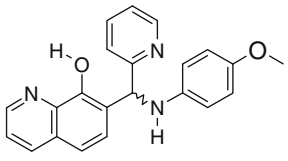

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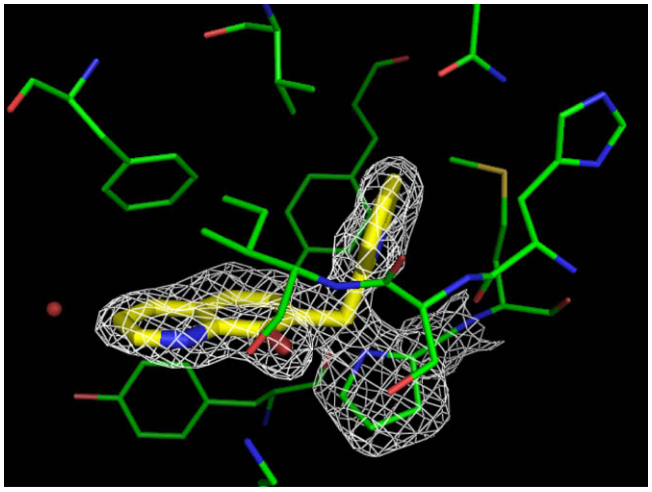

Figure 1. Superimposed are F_0–F_c electron densities of covalent adduct, contoured at 2σ.

To fully characterize the binding mode of compound **2**, we carried out X-ray crystallographic studies of its complex with MIF. The structure of human macrophage migration inhibitor factor crystals soaked with **2** was solved at a resolution of 1.86 Å as a trimer in the asymmetric unit.[33] The inhibitor has been refined in all three potential binding sites by C_3 symmetry. Each site has good electron density around the entire bound inhibitor, as the pyridinylmethyl quinoline fragment shown in black in Figure 1; no density is observed for the atoms (shown in red) of the *p*-methoxyaniline fragment of **2**.

The pyridinyl group points into the catalytic deep pocket of the active site. The quinoline ring points toward the surface of the protein, lying at the rim of the active site near the hydrophobic pocket

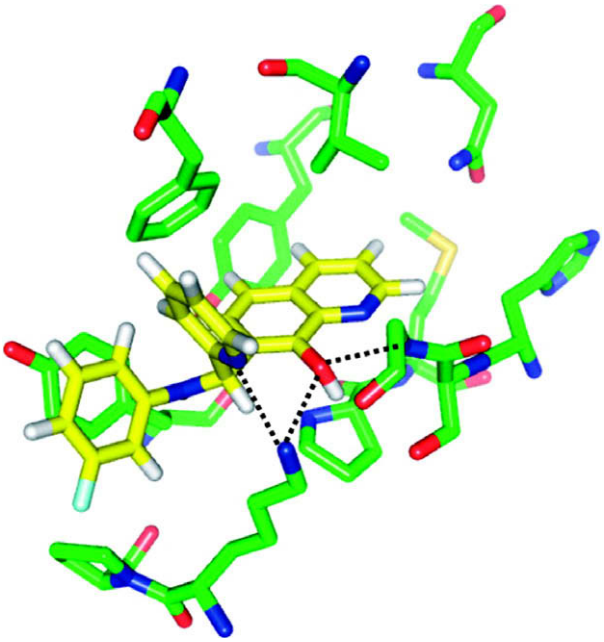

Figure 3. Predicted docking mode of **1** (Cournia et al. Ref. 28).

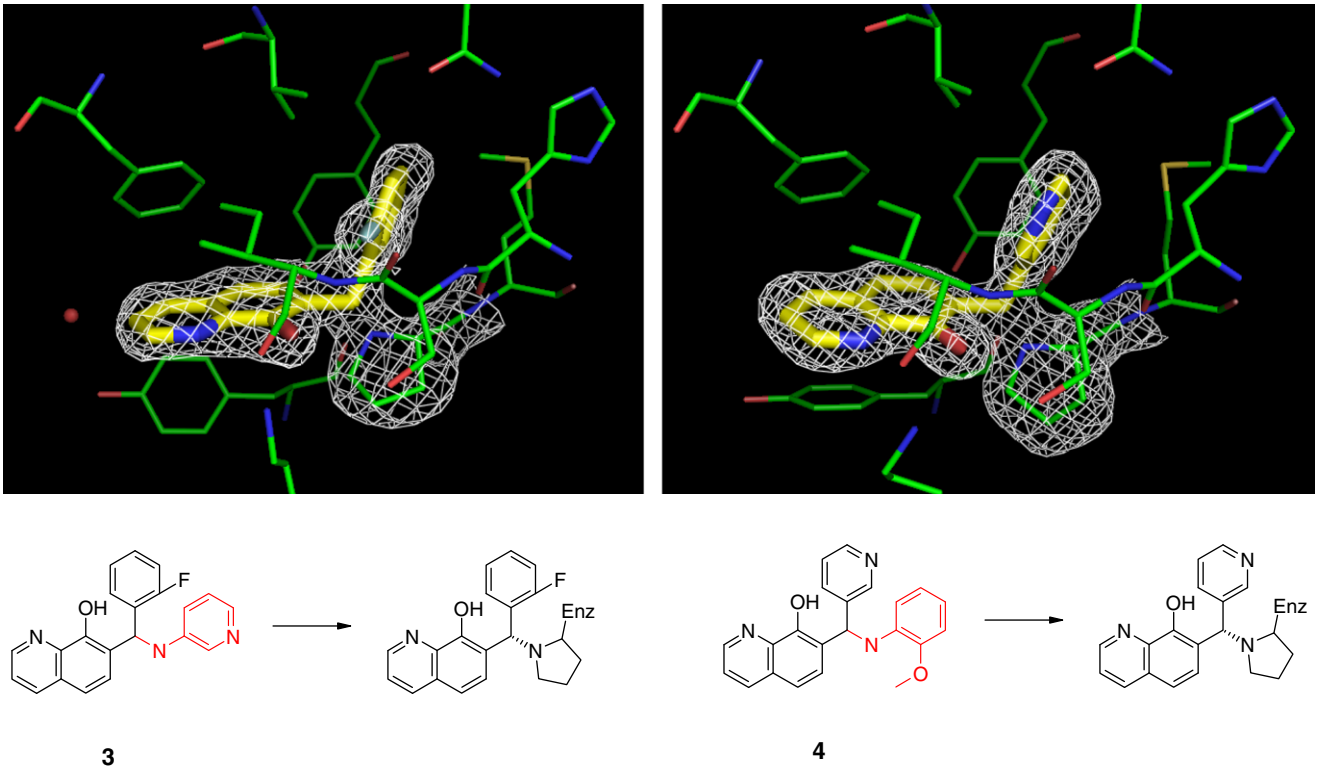

Figure 2. Superimposed are F_0–F_c electron densities of covalent adduct, contoured at 2σ.

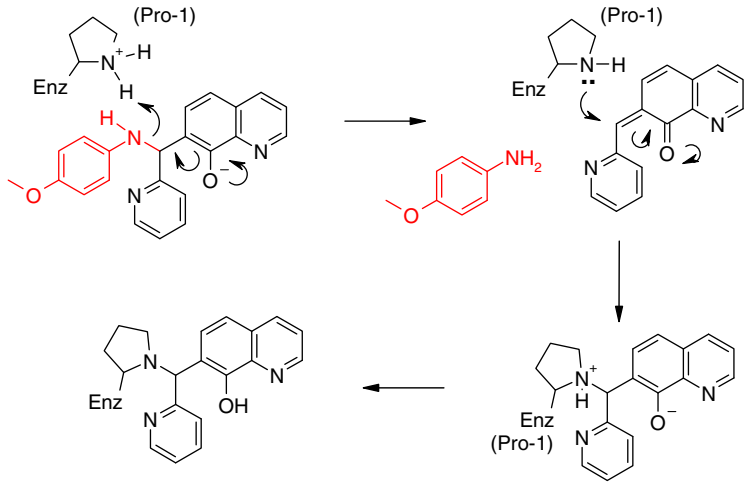

Figure 4. Proposed mechanism for the formation of covalent adduct.

formed by Tyr-36, Ile-64 and Phe-113. It forms aromatic/hydrophobic interactions with the side-chain of Tyr-36, Ile-64 and Phe-113. The amino group of Lys-32 forms a hydrogen-bond to the oxygen or the nitrogen of the quinoline ring, depending on the orientation of the Lys side-chain. The carbon bridging the two rings is at a distance from the nitrogen of Pro-1 (1.47 Å) that could only be explained by the formation of a covalent adduct with the *R* configuration around the introduced chiral center as shown in Figure 1.

Consistent with the formation of a covalent adduct with Pro-1, compound **2** has also been characterized as an irreversible inhibitor. Kinetic studies showed that the inhibitory potency of **2** increases as a function of pre-incubation time, similar to the time-dependent inhibition studies of known irreversible inhibitors, such as NAPQI.[22] In addition, crystal structures of two closely-related hydroxyquinolines (**3** and **4** in Fig. 2) also demonstrate adduct formation.[33]

Thus, compound **2** and analogs were opportunistically identified by virtual screening as MIF tautomerase inhibitors. It demonstrated that the structure-based in silico virtual screening is also capable of producing serendipitous hits, as its 'wet' counterpart of high-throughput screening. Although the predicted binding mode (as shown in Fig. 3) and the initial presumptions for the activities of these compounds were not confirmed by X-ray structures, the in silico virtual screening has led to the discovery of covalent inhibitors characterized by crystallographic and kinetic studies. Without selecting these compounds in the first place by virtual screening, none of these covalent inhibitors would have been discovered by experimentally testing only a limited number of compounds.

The formation of the covalent adduct was quite intriguing. That these compounds would act as an alkylating agent was unexpected. There was a possibility that the covalent adduct could have resulted from a small amount of impurity that reacted with the Pro-1 nitrogen, since these compounds (**2–4**) were purchased from external sources. The purities varied from 50% to 100% as shown by LC–UV–MS. One could also speculate that the basic amine of Pro-1 underwent nucleophilic substitution (S_N2) to displace the aniline in compound **2**. Mechanistically, that would be an extremely rare scenario since an amino or aniline group is a very poor leaving group,[34] unless it is stabilized by a proton sink. An alternative reaction pathway, shown in Figure 4, is proposed for the formation of covalent adducts from compound **2** and its analogs.[35] It involves a retro Michael addition reaction[36] leading to the in situ formation of the quinone methide intermediate, which is similar to the product of the de-amination reaction of phenolic benzylamine[37] via an activated amine as a leaving group.[38] A subsequent aza-Michael addition by the nucleophilic nitrogen of the Pro-1 to the quinone methide would give rise to the observed covalent inhibitor-MIF complex. This unusual reaction mechanism was not anticipated prior to virtual screening.

In summary, derivatives of hydroxyquinoline identified as 'hits' by virtual screening irreversibly inhibit MIF tautomerase via the formation of covalent adducts. While virtual screening played a pivotal role in the initial finding of these active inhibitors, structural biology and kinetic analysis revealed the irreversible nature of inhibition by a mechanism of N-alkylation of the Pro-1. The present work demonstrated a valuable strategy for lead seeking by coupling in silico virtual screening with prudent follow-up experimental studies.

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32. Virtual screen was performed by docking using Glide with the MIF crystal structure (1GCZ) and hydrogen bonding constraints to either Ile-64 backbone NH or Asn-97 side-chain.
33. Recombinant MIF was prepared as described by Orita et al. (Ref. 16). The protein was concentrated to 100 mg/mL in 0.1% beta-mercaptoethanol, 0.1 M sodium citrate, pH 5.0. Crystals were obtained by hanging drop vapor diffusion by mixing equal volumes of protein and reservoir solution (0.5–0.7 M ammonium sulfate, 0.1 M HEPES, pH 6.5) at 21 °C. Inhibitors (100 mM in DMSO) were added to the crystals to attain a concentration of 5 mM and incubated for 10 days before freezing in 50% sucrose. Data were collected either with a Rigaku *R*-Axis IV++ image plate and RU-H3R X-ray generator equipped with Xenocs Fox-2D optics or by Shamrock Structures LLC at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory (supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38). Data were processed with HKL2000 and CNX. Molecular replacement (MOLREP) used 1GCZ as starting structure. Coordinates have been deposited in the PDB (compd **2**: 3JTU; compd **3**: 3JSF; compd **4**: 3JSG).
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