Potent Triazolopyridine Myeloperoxidase Inhibitors

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S Supporting Information

ABSTRACT: Myeloperoxidase (MPO) generates reactive oxygen species that potentially contribute to many chronic inflammatory diseases. A recently reported triazolopyrimidine MPO inhibitor was optimized to improve acid stability and remove methyl guanine methyl transferase (MGMT) activity. Multiple synthetic routes were explored that allowed rapid optimization of a key benzyl ether side chain. Crystal structures of inhibitors bound to the MPO active site demonstrated alternate binding modes and guided rational design of MPO inhibitors. Thioether **36** showed significant inhibition of MPO activity in an acute mouse inflammation model after oral dosing.

KEYWORDS: Myeloperoxidase, peroxidase, methyl guanine methyl transferase, X-ray crystallography, lead optimization

Myeloperoxidase (MPO) is a member of the heme-containing peroxidase family which is predominantly expressed in neutrophils and at lower levels in monocytes and subpopulations of tissue macrophages. The enzyme catalyzes the oxidation of chloride to hypochlorous acid in the presence of hydrogen peroxide and also acts as a classic peroxidase, generating a wide range of free radicals and other reactive oxygen species. MPO plays a key role in host defense by generating reactive species that are key contributors to the antimicrobial activity of phagocytes.¹ However, excessive generation of reactive oxygen species by MPO has been linked to tissue injury in many chronic inflammatory diseases. A small molecule inhibitor of MPO might have the potential to treat inflammatory diseases such as atherosclerosis, multiple sclerosis, rheumatoid arthritis, multiply systems atrophy, inflammatory bowel syndrome, asthma, and cystic fibrosis.^{2–5}

Small molecules that inhibit the MPO via various mechanisms have been reported.⁶ Slow substrates, including tryptamines, nitroxides, and indoles, are converted in the active site to radical intermediates that react rapidly with physiological substrates and require high concentrations to be effective in vivo.^{7–10} Additionally, several irreversible inhibitors have been reported and entered clinical trials to treat various diseases.^{11,12} A reversible MPO inhibitor that blocks the active site has the potential advantage of not being expected to generate reactive intermediates that modify off-target proteins.^{13,14}

Recently, the discovery of triazolopyrimidine **1** was reported, which binds above the heme in the MPO active site and inhibits both oxidation of chloride to hypochlorous acid and single electron oxidations.¹⁵ Compound **1** (compound **6** in ref

15) is a moderately potent, reversible inhibitor with good oral bioavailability; however, the benzyl ether linkage is unstable in acidic conditions, and the compound is a known inhibitor of MGMT.¹⁶ Although MGMT inhibition may be desirable to enhance the effectiveness of chemotherapy agents, inhibition prevents the repair of alkylated DNA adducts, which could lead to mutations. Investigation of the core heterocycle identified that there was little tolerance for structural changes that maintained MPO inhibition. One exception was triazolopyridine **2**, which showed improved acid stability of the ether linkage; MGMT reactivity was still an issue (Figure 1). Optimization of the triazolopyridine ether series was undertaken, initially focusing on removal of the MGMT inhibition liability.

A published route allowed access to triazolopyridine **2**^{17,18} but involved generation of highly energetic intermediate **4** and required high temperatures with a large excess of benzyl alkoxides to access compounds in the final step, which limited efficiency and access to structural diversity (Scheme 1). A second-generation synthesis involved diversification by nucleophilic displacement of known arylhalide **7**¹⁹ at lower temperatures with benzyl alcohols and a weak base. Cleavage of the diazo bond to compound **8** followed by cyclization formed the desired compounds. While this route avoided the energetic intermediate and increased the yield of the synthetic sequence, introduction of the aryl ether still required conditions that were not compatible with many functional

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Figure 1. Initial leads for discovery of MPO inhibitors. The aminophenyl fluorescein (APF) assay measures inhibition of MPO chlorination activity in the presence of chloride, and the Amplex Red assay measures inhibition of MPO peroxidation activity in absence of chloride.

Scheme 1. Initial Synthetic Routes to Benzyl Triazolopyridines^a

1st Generation:

2nd Generation:

^aReagents and conditions: (a) MeOCOC₂Cl, DIEA; (b) HNO₃, H₂SO₄, 13% over 2 steps; (c) TEA, H₂O; (d) H₂, Pd/C; (e) NaNO₂, AcOH, 16% over 3 steps; (f) NaH, ROH (excess), 20%; (g) (i) 4-chloroaniline, NaNO₂, HCl, (ii) NaOAc, H₂O, 99%; (h) Cs₂CO₃, ROH, DMSO, 100 °C, 20–90%; (i) Zn, AcOH, EtOH, 10–85%; (j) NaNO₂, AcOH, 25–75%.

groups and low yields were observed with electron withdrawing substituents on the phenyl ring.

Limitations with functional group tolerance and late-stage diversification drove the development of a third synthetic route (Scheme 2). Known diamine **10**²⁰ was reacted with 4-chlorobenzenediazonium chloride to form diazene **11**. Cleavage of the diazene bond with zinc to afford the diamine, followed by cyclization with isoamyl nitrite, formed compound **2** in high yield. Bis-trityl protection followed by debenzoylation via transfer hydrogenation provided key intermediate **13**. Other protecting groups were investigated, but trityl provided

Scheme 2. Triazolopyridine Synthesis Supporting Late-Stage Diversification^a

^aReagents and conditions: (a) (i) 4-chloroaniline, NaNO₂, HCl, (ii) NaOAc, H₂O, >90%; (b) Zn, AcOH, EtOH; (c) isoamyl nitrite, AcOH, >80% over 2 steps; (d) TrtCl, TEA, DCM, 50%; (e) Et₃SiH, Pd(OAc)₂, TEA, MeOH, 80%; (f) alkylation or Mitsunobu; (g) TFA/TES, 15–90% over 2 steps.

the best stability to a variety of chemical transformations combined with ease of deprotection in the final step. Alkylation of **13** with alkyl bromides or a Mitsunobu reaction with alcohols allowed introduction of ethers, followed by acidic deprotection provided the desired compounds (**9**). This optimized route allowed rapid exploration of a diverse set of substituents.

The Amplex Red (AR) assay was used to measure the inhibition of MPO peroxidase activity. In the presence of hydrogen peroxide, MPO oxidizes Amplex Red, 10-acetyl-2,7-dihydroxyphenoxazine, to produce a red-fluorescent product. The reported compounds were also active in the aminophenyl fluorescein (APF) assay,¹⁵ which measures the MPO chlorination activity. Because changes to the APF assay procedure were made over the course of this research, only the AR assay is reported for most compounds to allow a consistent comparison.

Because previous work indicated that there was little tolerance for changes to the heterocyclic core,¹⁵ efforts focused on exploring SAR by changing the benzyl ether. Determining if MGMT activity could be removed as a liability was the highest priority. Literature studies demonstrated that inactivation of MGMT occurs via transfer of the benzyl group onto the active site cysteine in the protein.²¹ We hypothesized that ortho substitution of the phenyl ring could hinder access of the cysteine to the benzylic carbon, preventing reaction with MGMT. In fact, compounds studied with an ortho substituent, such as halobenzyl ethers **14** and **15**, did not inactivate MGMT, whereas substitution at the meta position with compounds **17** and **18** inactivated the protein. Because ortho substitution provided a path forward to remove MGMT inhibition, the focus switched to improving potency and other molecular properties. Subsequent compounds were not tested for MGMT activity due to limited availability of the assay.

Exploration of substitution around the benzyl ring revealed similar potency with small groups such as fluoro and chloro at the ortho position. However, MPO potency was decreased with polar groups such as a methylsulfone (Table 1). A diverse set of small and large substitutions were tolerated at the meta position of the benzylic ring, which is not surprising considering the large binding pocket shown in the crystal structure. Figure 2 shows the initial HTS hit **1** overlaid with the crystal structure of compound **20** in the MPO active site. The heterocyclic core stacks on the heme and both

Table 1. Activity of Monosubstituted Triazolopyridine Benzyl Ethers

Compound	R	AR IC ₅₀ (nM)
14		30
15		26
16		720
17		26
18		21
19		21
20		21
21		78
22		26
23		160

Figure 2. Crystal structure of compounds 1 (light blue)¹⁵ and 20 (orange, 5QJ2) bound in the active site of MPO. Heme is shown in green. Only key residues in the active site are shown.

triazolopyrimidine and triazolopyridine cores hydrogen bond with Arg-239 and a heme carboxylate. The triazoles make close interactions with Gln-91 and His-95 and bind in proximity to the heme iron. The methyl pyrazole extends into a hydrophobic pocket and makes a hydrogen bond with Thr-238. A 4× loss of potency by the methyl pyrazole isomer **21** could be due to the absence of this hydrogen bond. Smaller substituents such as chloro at the para position maintain potency, whereas steric bulk resulted in lower potency, as shown by biphenyl **23**. The diverse SAR in the benzyl ether binding pocket allowed assessment of a wider variety of substitutions in order to find a balance of properties.

Once monosubstitution of the benzyl ether was investigated, the best substituents were combined to optimize the potency of the chemotype. Combination of ortho substitution to prevent MGMT inhibition with meta aryl groups produced compounds such as **24** and **25**. When these biaryl compounds were docked in the binding site, it was unclear how a molecule such as **24** could fit in the active site with the phenyl group in a similar region as the compounds shown in Figure 2 due to a clash of the ortho chloro with Phe-366. It became clear when a new crystal structure was solved that ortho substitution tips the benzylic group into a different vector, which allows the aryl group to access a different binding pocket (Figure 3). The new crystal structure helped explain the broad tolerability of large groups in this region and influenced design of subsequent molecules.

Figure 3. Crystal structure of compounds 20 and 24 (5QJ3) bound in the active site of MPO. Only key residues in the active site are shown.

In attempt to increase the potency of the series, ortho substituted aryl rings were explored to reach into the new binding pocket that could now be accessed with ortho substitution. Biphenyls such as compound **24** provided vectors to extend into the region, but these compounds had high protein binding and were potent inhibitors of CYP 3A4 (compound **24**: >99.5% protein bound and CYP 3A4 IC₅₀ = 60 nM). Investigation to replace either of the phenyl rings with heterocycles to improve these properties and increase potency was pursued (Table 2). Replacement of the distal phenyl ring with a methylpyrazole (compound **26**) maintained potency while reducing free fraction and improving the CYP inhibitor profile (93.2% protein bound and CYP 3A4 IC₅₀ = 1500 nM).

Table 2. Activity of Disubstituted Benzyl Ethers and Heterocyclic Ethers

Compound	R	AR IC ₅₀ (nM)
24		24
25		23
26		26
27		290
28		400
29		120
30		27
31		12
32		250

Replacement of the other phenyl with an oxazole (27), triazole (28), or isoxazole (29) resulted in a significant loss in potency. However, methyltriazole 30 retained the potency observed with substituted phenyl compounds such as 24 and not surprisingly had a similar binding mode (not shown). Difluoromethyl analogue 31 moderately improved potency, while hydroxylation to compound 32 resulted in a loss in potency, consistent with tolerance of only small, nonpolar groups at the ortho position. The heterocyclic replacement of the second phenyl ring did moderately improve free fraction and CYP 3A4 inhibition, as demonstrated by compound 30 (98.0% protein bound, CYP 3A4 IC₅₀ = 370 nM). While nanomolar potency was not achieved in the series with reduced CYP3A4 activity, the crystal structures show multiple binding modes could be accessed by small changes in the biaryl structures.

Thioethers were also investigated in order to see if the larger sulfur atom may offer any advantages. Compounds were synthesized using intermediate 33, which was obtained from 2,6-diamino-4-bromopyridine using a similar synthetic sequence as shown in Scheme 2. Introduction of a protected thiol could be achieved using palladium coupling conditions to obtain intermediate 34 (Scheme 3). A one-pot deprotection with *tert*-butoxide, followed by alkylation and acid depro-

tection, provided the targeted compounds and allowed for rapid SAR exploration.

Scheme 3. Synthesis of Thioether Triazolopyridines^a

^aReagents and conditions: (a) DPEPhos, Pd₂(dba)₃, K₂CO₃, RSH, toluene, 75%; (b) (i) *t*-BuOK, (ii) alkyl halide; (c) TFA/TES, >75% over 2 steps.

Thioethers have similar potency when compared to the corresponding ether compounds and most matched pairs were within ~3-fold potency when comparing the Amplex Red Assay data (Table 3). To see if activity of thioethers was a

Table 3. A Comparison of Thioether and Ether Linked Triazolopyridines

Compound	R	X	AR IC ₅₀ (nM)
14 36		O S	30 8.7
37 38		O S	6.7 4.6
31 39		O S	12 8.5

result of oxidation under the assay conditions, representative sulfoxide and sulfone analogues were synthesized and had only weak activity (not shown). A more detailed profile of thioether 36 (Table 4) compared to the initial HTS lead 1 shows good potency for MPO in both the chlorination assay (APF) and the peroxidase assay in absence of chloride (AR), as well as related family member, eosinophil peroxidase (EPX). Inhibition of

Table 4. Comparison of HTS Lead and Compound 36

compd	1	36
MPO Amplex Red (nM)	110	8.7
MPO APF (nM)	110	6.8
EPX (nM)	17	22
TPO (nM)	42000	4000
LPO (nM)	>100000	>8000
% bound, human	99.4	98.0
% bound, mouse	87.3	80.8
mouse PK, PO 3 mpk		
C _{max} (nM)	31000	17000
AUC (nM × h)	22000	18000

EPX may prove beneficial for treating inflammatory conditions as well.²¹ The compound is inactive for lactoperoxidase (LPO) at the concentrations tested and shows some selectivity over thyroid peroxidase (TPO), which is a significant off-target liability. The selectivity for the peroxidase family is characteristic for the series. Because other reported thioethers have been reported as irreversible inhibitors, the reversibility of inhibition by compound **36** was investigated (see data in [Supporting Information](#)). In the absence of chloride, inhibition of MPO in the Amplex Red assay was observed, even after dilution, indicating irreversible activity. However, in the presence of chloride, inhibition was diminished after dilution, signifying reversible inhibition. Because chloride is present in biological systems, it is possible that reversible inhibition of MPO is the mechanism in vivo, but additional studies would be needed to prove this assertion. Stop flow analysis of a closely related compound demonstrated that the series likely does not act as a substrate for MPO (see data in [Supporting Information](#)).

Because compound **36** was a potent MPO inhibitor with good oral bioavailability, it was evaluated in an acute mouse model of inflammation. Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) allows detection of reactive oxidizing species (ROS) in biological systems by emission of blue luminescence and is used clinically to screen for defective oxidative burst in neutrophils. Although luminol can detect many ROS, studies have shown that the luminol signal is MPO dependent in blood, and in a previous report no luminescence was observed in MPO knockout mice treated in a similar manner.^{15,22–26}

Mice were treated with compound **36** and 20 min later were dosed with phorbol 12-myristate 13-acetate (PMA), which stimulates MPO activity via degranulation of neutrophils. After 2 min, the blood samples from the mice were treated with luminol to measure MPO-dependent hypochlorous acid production. Compound **36** decreased MPO activity in a dose proportional manner at 10 and 30 mpk ([Figure 4](#)).

Figure 4. PMA acute inflammation model with compound **36**. The experimental procedures can be found in the [Supporting Information](#). Plasma levels for compound **36** were 21 μ M (10 mpk, $n = 10$) and 70 μ M (30 mpk, $n = 9$).

A triazolopyrimidine HTS lead was optimized to improve potency and remove MGMT activity. Thioether **36** showed significant inhibition of MPO activity in an acute mouse inflammation model after oral dosing. The compound was not progressed into chronic models due to inhibition of TPO at the concentrations where MPO inhibition was observed. Efforts on the ether and thioether linked triazolopyrimidines were stopped due to difficulty in identifying orally bioavailable, potent inhibitors without CYP and TPO activity. Crystal structures of inhibitors bound to the MPO active site

demonstrated alternate binding modes and have guided rational design of MPO inhibitors, including alternate chemotypes that will be reported in future communications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](#) at DOI: [10.1021/acsmmedchemlett.8b00308](https://doi.org/10.1021/acsmmedchemlett.8b00308).

Biology methods, syntheses, and characterization data for new compounds ([PDF](#))

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CYP, cytochrome P450 enzymes; mpk, milligrams per kilogram; MPO, myeloperoxidase; MGMT, methyl guanine methyl transferase; AR, Amplex Red; APF, aminophenyl fluorescein; po, orally dosed; SAR, structure–activity relationship

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