Reverse Hydroxamate Inhibitors of Bone Morphogenetic Protein 1

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Supporting Information

ABSTRACT: Bone Morphogenetic Protein 1 (BMP1) inhibition is a potential method for treating fibrosis because BMP1, a member of the zinc metalloprotease family, is required to convert pro-collagen to collagen. A novel class of reverse hydroxamate BMP1 inhibitors was discovered, and cocrystal structures with BMP1 were obtained. The observed binding mode is unique in that the small molecule occupies the nonprime side of the metalloprotease pocket providing an opportunity to build in metalloprotease selectivity. Structure-guided modification of the initial hit led to the identification of an oral *in vivo* tool compound with selectivity over other metalloproteases. Due to irreversible inhibition of cytochrome P450 3A4 for this chemical class, the risk of potential drug–drug interactions was managed by optimizing the series for subcutaneous injection.

KEYWORDS: BMP-1, BMP1, PCP, TLL1, TLL2, MMP12, fibrosis

Bone Morphogenetic Protein 1 (BMP1, procollagen C-proteinase, PCP) is part of a family of tolloid metalloproteases (including TLL1 and TLL2) that cleave the C-terminal side of propeptides.¹ This extracellular cleavage is required as part of the cascade that generates mature collagen. Collagen is important for normal physiological functioning; however, excessive collagen or fibrosis occurs in certain disease states of the heart, liver, kidney, lung, and skin. Other researchers have reported that an antibody that inhibits BMP1 is advantageous for post myocardial infarction recovery in rats and that this could theoretically be expanded to humans.^{2,3} Myocardial infarction often results in significant fibrotic scarring of the heart with concomitant loss of cardiac function. The potential to improve cardiac function in patients after a myocardial infarction by small molecule inhibition is clearly compelling from a treatment perspective.

As an antifibrotic target, BMP1 has attracted interest for at least two decades.⁴ The most potent small molecule BMP1 inhibitors, exemplified by compounds 1–3, have been reported by Pfizer,⁵ Roche Biosciences,⁶ and Fibrogen,⁷ respectively, but without reported advancement to clinical study (Figure 1). These compounds share a common hydroxamic acid (C(O)-NHOH) zinc-binding motif. However, many clinical trials of hydroxamic acid-containing inhibitors have failed, aside from Vorinostat,⁸ largely a result of poor selectivity for matrix

Figure 1. Structure of published BMP1 inhibitors.

metalloproteinases (MMP) and/or poor metabolic stability of the hydroxamate.⁹ We sought BMP1 inhibitors with an alternative pharmacophore devoid of these liabilities. The goal of this effort was to discover potent and selective BMP1

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inhibitors with suitable systemic exposure to test for the potential treatment of fibrosis.

High-throughput screening for BMP1 inhibition revealed compound **4**,¹⁰ a reverse hydroxamate (NH(OH)C(O)), having submicromolar activity against BMP1 but lacking MMP selectivity. The X-ray crystal structure of the BMP1–**4** complex (Figure 2) revealed that the reverse hydroxamate

Figure 2. (A) Compound **4**. (B) Compound **4** and key interactions with BMP1. Compound **4** is shown in green carbon. Key residues of BMP1 are shown in cyan carbon. The zinc ion is in magenta. (C) X-ray crystal structure of the BMP1–**4** complex. Compound **4** and key interactions with BMP1 with the protein shown in cyan ribbon and light gray surface (RCSB 6BSM).

moiety engages in a bidentate zinc coordination, but unlike most MMP inhibitors, compound **4** exclusively occupies the nonprime side of the substrate binding pocket. Further, inhibitor binding is accommodated by a significant movement of a protein flap that contains a rare¹¹ vicinal disulfide bond formed between Cys65 and Cys66 and that closes over the S1 site in the published apo structure.¹²

The central methylene diamide of **4** is deeply buried and engages in two direct H-bond interactions with Ser67. Between this diamide and the reverse hydroxamate, the *n*-pentyl side chain lies in an extended conformation, tucked underneath the hydrophobic disulfide-containing flap. The benzofuran moiety occupies a rather large shallow pocket, presumably the S3 site, and makes a water-mediated interaction with the phenyl ring of Tyr68.

The reverse hydroxamate compounds were synthesized according to the conditions shown in Scheme 1.¹³ The

Scheme 1. Synthesis of Reverse Hydroxamate Inhibitors^a

^aReagents and conditions: (a) 2.5 M *n*-BuLi in hexanes, THF, −78 to −25 °C, 5 h (93%); (b) TiCl₄, DIEA, DCM, NMP, ice/acetone bath, 1.5 h (87%); (c) BnONH₂, 2 M Al(CH₃)₃ in toluene, THF, 0–5 °C, 1.5 h (70%); (d) MsCl, pyridine, 0–10 °C, 2 h (91%); (e) 40% tetrabutylammonium hydroxide in water, 2-MeTHF, 50 °C, 2 h; (f) CDI, formic acid, 2-MeTHF, 0–5 °C, 1 h (77% over two steps); (g) EDC, HOBt, DIEA, DMF, THF, 50 °C, 1 h (40–70%); (h) 10% Pd/C, H₂, EtOH, RT, 3 h (70%).

stereochemistry of the R¹ and R² side chains were set using a chiral Evans oxazolidinone. The reverse hydroxamate moiety was introduced by displacing the chiral auxiliary with benzyloxamine, Mitsunobu ring closure to the beta-lactam, and hydrolytic lactam opening followed by formylation to provide the protected reverse hydroxamate carboxylic acid intermediate. This was then coupled to an elaborated diamine via standard amide bond forming reaction and the final product was deprotected to reveal the reverse hydroxamate diamine product while maintaining the chiral integrity.

Initial explorations of the pentyl side chain SAR (Table 1) yielded no significant enhancement of potency. Reduction in

Table 1. BMP1 Potency and MMP12 Selectivity

compd	R1	R2	BMP1 ^a IC ₅₀ (nM)	MMP12 ^a IC ₅₀ (nM)
4	R-CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	H	160	400
5	R-CH ₂ CH ₂ CH ₃	H	1250	1250
6	H	H	6300	>10,000
7	R-CH ₂ Ph	H	160	4000
8	S-CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	H	4000	>10,000
9	R-CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	S-Et	>10,000	200
10	R-CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	R-Et	126	>10,000

^aAssay protocols are published.¹³ IC₅₀ values are the average of at least two determinations.

the chain length resulted in a loss of activity with compounds **5** and **6**, which is consistent with an efficient hydrophobic interaction. Increasing the size and restricting the conformational freedom had no measurable benefit on inhibition (**7**). It is clear from these compounds that the interaction of the lipophilic group with the vicinal disulfide accounts for almost two logs of potency at BMP1, which may be partially explained by preorganization into the bioactive conformation in solution. Matrix metalloprotease 12 (MMP12) was tested to represent general metalloprotease selectivity, and the SAR for this enzyme generally paralleled BMP1 (Table 1), including the stereochemical preference at R¹ (**8**). MMP9 activity (see Supporting Information) was very similar to MMP12.

Consideration of the X-ray crystal structures of related inhibitors in MMPs along with the docking of compound **4** into the MMP12 crystal structure (Supporting Information) suggested that **4** likely binds in the MMP12 prime site and that the *n*-pentyl side chain occupies a deep hydrophobic S1 pocket. Furthermore, and quite significantly, it appeared that the steric tolerance of a substituent adjacent to the reverse hydroxamate nitrogen (vicinal to the *n*-pentyl side chain) would have opposite stereochemical preferences by BMP1 and MMPs. In particular, an *R*-substituent alpha to the reverse hydroxamate might not be tolerated by MMP12 but was predicted to be tolerated by BMP1. Similarly, an *S*-substituent was predicted to have the opposite selectivity. Preparation of alpha ethyl analogs **9** and **10** resulted in one of each analog being preferred by each protease as predicted. Specifically, compound **10** has good activity at BMP1 and no measurable activity at MMP12.

Exploration of SAR around the benzofuran moiety revealed that replacement of the fused bicycle with the more elongated phenyl furan led to a 6-fold boost in potency. When combined with the *R*-ethyl group alpha to the reverse hydroxamate, this led to the discovery of compound **11** (Figure 3), which

Figure 3. Structure of oral BMP1 inhibitor **11**.

exhibited excellent inhibitory activity against BMP1 (32 nM), TLL1 (100 nM), and TLL2 (160 nM) and was selective against 15 metalloproteases (>30-fold relative to BMP1).¹⁴ Additionally, **11** had good oral exposure in a rat¹⁵ (dose normalized area under the curve (DNAUC) = 1.6 h·kg/L) and thus represents a selective *in vivo* tool compound.

Although compound **11** is a suitable oral tool for *in vivo* experimentation, unfortunately, it demonstrates cytochrome P450 3A4 time-dependent inhibition (4.3-fold decrease in enzyme activity over 15 min @ 33 μM). For a medication, this is concerning because it could potentially cause drug–drug interactions for patients on other medications. The reverse hydroxamate functionality appears to be responsible for this effect, which has also been reported previously.¹⁶ Unfortunately, BMP1 activity is dramatically reduced without the reverse hydroxamate metal chelator, similar to a published report that replacements for a hydroxamic acid metal chelator

reduced BMP1 activity.¹⁷ To address this issue, we decided to pursue considerably more potent and soluble molecules that could be dosed subcutaneously and thus avoid the possibility of inactivating gut 3A4 that could occur with oral delivery.

To improve the potency, substitution on the aryl ring (Table 2, R³) was explored. Potency improvements were obtained

Table 2. BMP1 Potency with Substituents

compd	R2	R3	BMP1 IC ₅₀ (nM)
12	H	H	25
13	H	3-CO ₂ H	8.0
14	H	3-OCH ₃	20
15	H	3-OCH ₂ CH ₃	1.0
16	H	4-CO ₂ H	1.0
17	<i>R</i> -Et	3-OCH ₂ CH ₃ , 5-CO ₂ H	<1 ^b
18	<i>R</i> -Et	3-OCH ₂ CH ₃ , 4-CO ₂ H	<1 ^b
19	<i>R</i> -Et	3-OCH ₂ CH ₃ , 5-PO ₃ H ₂	<1 ^b
20	<i>R</i> -Et	3-OCH ₂ CH ₃ , 4-PO ₃ H ₂	<1 ^b
21	<i>R</i> -Et	3-OCH ₂ CH ₃ , 5-C(O)-S-NCH ₂ (CO ₂ H) (CH ₂ CO ₂ H)	<1 ^b
22	<i>R</i> -Et	3-OCH ₂ CH ₃ , 4-C(O)-S-NCH ₂ (CO ₂ H) (CH ₂ CO ₂ H)	<1 ^b

^bLower limit of assay sensitivity is approximately 250 pM.

with both para and meta substitution (compounds **13**–**16**), although 3-ethoxy and 4-carboxy showed the most dramatic improvements (>10-fold). The ethoxy group on **15** may be making a positive van der Waals interaction with the upper pocket defined by Ala78 and Gln77, whereas **16** likely makes a positive interaction with the flexible Arg72.

An even larger boost in potency was obtained when two substituents were added. An entire series of compounds was identified, which had acidic groups to facilitate solubility and subnanomolar potency (compounds **17**–**22**).

Biochemical BMP1 potency was measured using small peptide substrates that included the BMP1 cleavage sequences of physiological protein substrates. The lower limit of the BMP1 profiling assay in Table 2 was ca. 250 pM (half the enzyme concentration), so tight-binding analysis was used for more potent inhibitors **19** and **22**, incorporating an enzyme–inhibitor preincubation step to ensure equilibrium binding. As would be predicted from the crystal structure of **22**, compounds **19** and **22** are substrate-competitive inhibitors. Binding of **19** is single step, reversible with an on-rate constant ($k_{on} = 3.6 \mu\text{M}^{-1} \text{s}^{-1}$) measured by stopped flow and an off-rate constant $k_{off} = 0.93 \text{ h}^{-1}$ ($t_{1/2} = 45 \text{ min}$) measured by jump dilution. Increased potency of **22** compared to **19** appears to be driven by a faster on-rate ($k_{on} = 55 \mu\text{M}^{-1} \text{s}^{-1}$) accompanied by a similar off-rate constant $k_{off} = 2.3 \text{ h}^{-1}$ ($t_{1/2} = 18 \text{ min}$). Inhibitor binding affinities (K_i) measured by tight binding were comparable to those calculated from stopped flow and jump dilution rate constants ($K_i = k_{off}/k_{on}$). Both **19** and **22** have BMP1 K_i values that are low picomolar (40 and 6.8 pM, respectively). Inhibition constants (K_i) of TLL1 for **19** and **22** (240 and 31 pM, respectively) and TLL2 for **19** and **22** (260 pM, and 39 pM, respectively) were obtained using similar methods.

To help explain the exceptional potency of compound **22**, an X-ray crystal structure bound to BMP1 was solved (Figure 4).

Figure 4. Cocrystal structure of **22** bound to BMP1. Compound **22** is shown in green carbon, BMP1 in cyan carbon and a light gray surface, and the zinc is in magenta (RCSB 6BSL).

Several advantageous interactions were noted compared to compound **4** (Figure 2) that are consistent with the SAR. Introduction of the phenylfuran as a benzofuran replacement produced an edge-to-face π - π interaction with Tyr68, and substitution on the phenylfuran with a 3-ethoxy group led to improved hydrophobic contact in the upper pocket. Additionally, there appear to be key interactions between both acidic groups of the aspartic acid substituent and Arg61 and Arg72, all of which appear to contribute to the >10,000-fold increase in BMP1 activity of **22** over **4**.

Further considerations for the quality of compounds **19** and **22** are metalloprotease selectivity and multiple species exposure. Both compounds were found to be >100-fold selective against 14 metalloproteases.¹⁴ Also, compounds **19** and **22** had reasonable exposure, low to moderate Cl, and a MRT > 2 h after subcutaneous injection in both rat¹⁵ and dog¹⁵ (Figure 5). Due to the overall profile, both **19** and **22** were progressed for further development consideration.

Figure 5. Structure of subcutaneous (SQ) BMP1 inhibitors.

In summary, high throughput screening identified a reverse hydroxamate pharmacophore that binds BMP1. Cocrystal structures revealed a binding mode on the nonprime side of the BMP1 active site accommodated by a shift in the vicinal disulfide loop. The chemical series was optimized for selectivity and exposure to discover an oral *in vivo* tool, compound **11**. This class of compounds demonstrates irreversible cytochrome P450 3A4 inhibition, so the series was further optimized to picomolar potency for subcutaneous injection to avoid gut 3A4, which could potentially cause drug–drug interactions. Two compounds were identified that were suitable for profiling as precandidate compounds, **19** and **22**. Pharmacology data for these compounds will be reported in subsequent publications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00173.

Synthetic procedures and characterization data for preparing BMP1 inhibitors, selectivity information, protein crystallization, binding kinetics, and a docking file (PDF)

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All authors have given approval to the final version of the manuscript, aside from Marlys Hammond, our friend and former colleague who passed away in October 2017.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DNAUC, dose normalized area under the curve; SAR, structure–activity relationship; MMP12, matrix metalloproteinase-12; SQ, subcutaneous

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