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N-Hydroxyurea as zinc binding group in matrix metalloproteinase inhibition: Mode of binding in a complex with MMP-8

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Abstract—The first crystallographic structure of an *N*-hydroxyurea inhibitor bound into the active site of a matrix metalloproteinase is reported. The ligand and three other analogues were prepared and studied as inhibitors of MMP-2, MMP-3, and MMP-8. The crystal structure of the complex with MMP-8 shows that the *N*-hydroxyurea, contrary to the analogous hydroxamate, binds the catalytic zinc ion in a monodentate rather than bidentate mode and with high out-of-plane distortion of the amide bonds.

Matrix metalloproteinases are a class of proteolytic enzymes that use an electrophilic zinc ion to catalyze hydrolysis of the peptide bond.[1] They are involved in many important physiological processes. Over-regulation of their activity, however, results in uncontrolled degradation of the extracellular matrix in diseases such as cancer,[2] arthritis,[3] and multiple sclerosis.[4] Design and synthesis of low molecular weight MMP inhibitors have thus become an important target for many medicinal chemists and pharmaceutical companies.[5]

MMP inhibitors generally incorporate a substrate-like fragment that can be accommodated in at least one of the subsites of the enzyme active site and a functional group capable of binding the catalytic zinc ion. While a practically endless variability of the peptide or peptidomimetic fragment allows modulation of potency and selectivity against various MMPs, only a small set of zinc binding groups (ZBGs) have been identified and employed in the design of MMP inhibitors. They include, in an arbitrary, but generally accepted, order of efficacy,[6] hydroxamate > reversed hydroxamate > thiolate > phosphinate > carboxylate and phosphonate. An interesting series of eleven new heterocyclic zinc chelators, reminiscent of barbiturate ligands,[7] have been described,[8a] and the importance of novel ZBGs, together with elucidation of their mode of binding, has been further emphasized.[8b]

The hydroxamate[9] function appears to be ideally suited as ZBG for MMP inhibitors since it not only forms five-membered chelates with optimal zinc–oxygen distances, but even establishes two strong hydrogen bonds with the protein (Fig. 1a).[10] Hydroxamic acids, however, are frequently affected by rapid excretion, low oral bioavailability, and in vivo hydrolysis.[11] The *N*-hydroxyurea group, incorporating the CO–NH–OH determinant necessary for zinc chelation can, in principle, retain all the binding interactions of the hydroxamate. It has been therefore evaluated against enzymes containing a catalytic zinc ion such as carboxypeptidase A,[12] carbonic anhydrase,[13] and MMPs by Abbott researchers[14] and

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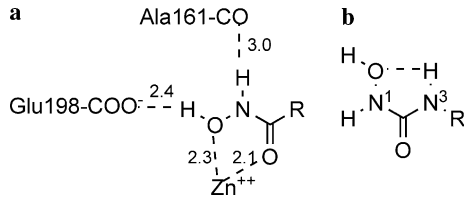


Figure 1. (a) Key bonding interactions of batimastat hydroxamate function as found in the complex with MMP-8 (distances in Å). (b) Preferred conformation of *N*-hydroxyureas bearing a hydrogen atom on N3 in the solid state.

by ourselves (unpublished results), providing rather weak inhibitors. This ZBG, however, may be interesting under the pharmacokinetic and toxicity profile, since several 5-lipoxygenase *N*-hydroxyurea inhibitors with improved oral bioavailability and slower in vitro metabolism, with respect to hydroxamates, were reported.[15]

To further investigate the behavior of this ZBG, we focused our attention on the described[16] nonpeptide hydroxamate **1** and the structurally related *N*-hydroxyurea **2a**[14] (Fig. 2). They were reported to bind in the MMP-3 active site, accommodating the lipophilic biaryl moiety into the large S1′ hydrophobic pocket, and ligating the catalytic zinc ion by the hydroxamate or *N*-hydroxyurea group, respectively. The reported IC_{50} of **2a** against MMP-3 was 80 μM,[14] more than 20-fold higher than that of the hydroxamate analogue **1**. The hydroxamate containing one less methylene in the linker between *N*-hydroxyurea and the biphenyl group[16] was some 100-fold more potent than **1** and would have been a much more attractive model for the present study, but the corresponding *N*-hydroxyurea presented serious problems of stability and synthesis.

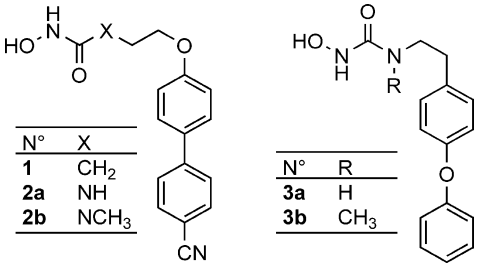


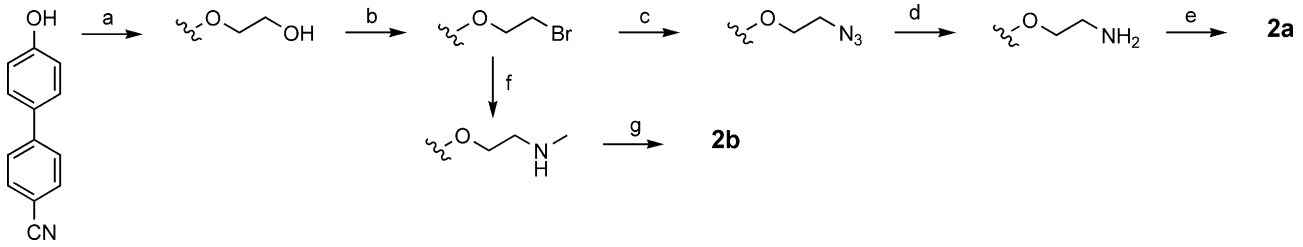
Figure 2. *N*-Hydroxyureas structurally related to the hydroxamate **1** studied as MMP inhibitors.

One reason for the decrease in binding affinity of *N*-hydroxyureas with respect to hydroxamate analogues is the difference in conformational preferences. In the solid state, *N*-hydroxyureas bearing at least one hydrogen atom on N3 (Fig. 1b) adopt a *trans* N1–CO amide bond conformation,[17] unproductive for zinc chelation. This *trans* conformation is stabilized by intramolecular hydrogen bonding in *N*-hydroxyureas,[17] while hydroxamic acids prefer, in general, the *cis* N–CO amide bond conformation. Furthermore, the partial sp^2 character of the *N*-hydroxyurea N3–CO bond, with its implications on the geometry and conformational preferences, strongly decreases the chain flexibility required for the optimal alignment of the ligand.

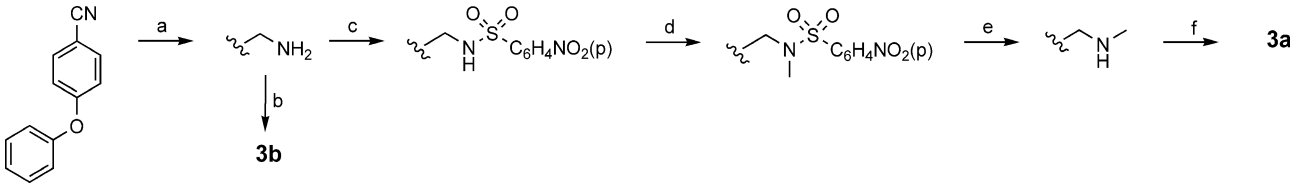
On the basis of these considerations, N3 methylation of *N*-hydroxyurea ligands was expected to improve binding affinity for MMPs by hampering the *trans* N1–CO amide bond conformation, balancing the stability of the *cis–trans* conformers of the N3–CO amide bond, and lowering the rotational barriers for their interconversion. The *N*3-methyl derivative **2b** of the *N*-hydroxyurea **2a**, and the new analogues **3a** and **3b**, based on a flexible biphenyl ether hydrophobic moiety and a shorter spacer, were synthesized (Schemes 1 and 2) and tested against MMP-2, MMP-3, and MMP-8 (Table 1). Only the *N*-methyl derivatives **2b** and **3b** allowed determination of their IC_{50}, owing to the increase of their solubility in the assay conditions. Available data show that methylation of **2a** and **3a** increased the binding affinity for MMP-2 and MMP-3, respectively. The IC_{50} against MMP-8, however, are in the range of millimolar and are too high to allow evaluation of the effect of methylation.

Although none of the *N*-hydroxyureas **2a**–**3b** attains an IC_{50} lower than 58 μM, it is interesting to note that **2b** shows a 20-fold increase of potency by going from MMP-8 to MMP-2. Rationalization of these results would require some knowledge on the behavior of *N*-hydroxyurea as ZBG. To shed some light on this matter, lacking adequate amounts of MMP-2 for the cocrystallization, we determined the crystal structure[18] of the complex of **2b** with MMP-8.

The main binding interactions of **2b** in the active site of MMP-8 are reported in Figure 3. The lipophilic biphenyl substituent occupies the hydrophobic primary specificity pocket S1′. The *N*-methyl group is involved in favorable hydrophobic interactions with the Ile159 side



Scheme 1. Reagents and conditions: (a) $BrCH_2CH_2OH$, K_2CO_3, DMF, 90 °C, N_2; (b) CBr_4, PPh_3, THF, N_2; (c) NaN_3, Bu_4NI, CH_3CN, reflux; (d) PPh_3, *o*-dichlorobenzene, reflux; H_2O/acetic acid, reflux; (e) phenyl-*N*-hydroxycarbamate, pyridine, N_2; (f) KI, CH_3NH_2, THF, 40 °C; (g) *p*-nitrophenyl-*N*-hydroxycarbamate, CH_3CN, N_2.



Scheme 2. Reagents and conditions: (a) BH_3, THF, N_2; (b) phenyl-*N*-hydroxycarbamate **11a**, pyridine, N_2; (c) *p*-nitrobenzenesulfonyl chloride, pyridine, CH_2Cl_2; (d) CH_3I, K_2CO_3, DMF; (e) $HSCH_2COOH$, LiOH, DMF; (f) KI, CH_3NH_2, THF, 40 °C; (g) *p*-nitrophenyl-*N*-hydroxycarbamate, CH_3CN, N_2.

Table 1. IC_{50}[a] (μM), or percent inhibition, of *N*-hydroxyureas **2a**–**3b** against MMP-2, MMP-3, and MMP-8, at pH 7.5

Compound	MMP-2	MMP-3	MMP-8
1	ND[b]	3.4[c]	ND[b]
2a	>120[d]	22% at 120[e,f]	>120[d]
2b	58	200	1200
3a	>380[d]	>380[d]	>380[d]
3b	685	380	1000

[a] Replicate determinations indicate standard deviations less than 20%.
[b] Not determined.
[c] Ref. 16, pH 6.5.
[d] No inhibition observed at the reported concentration, upper solubility limit in the assay conditions.
[e] Percent inhibition at the reported concentration.
[f] IC_{50} 80 μM reported in Ref. 14.

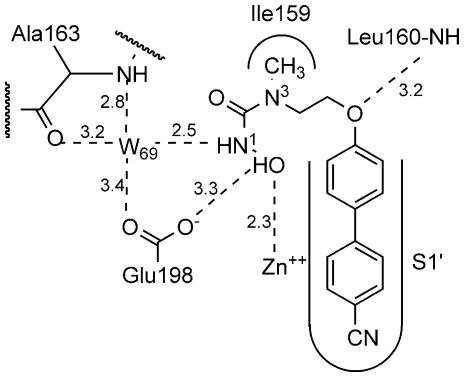


Figure 3. Schematic representation of the main bonding interactions of the *N*-hydroxyurea **2b** in the MMP-8 active site (distances in Å).

chain and the ethereal oxygen is H-bonded to Leu160 NH. The inhibitor hydroxyl is coordinated by the catalytic zinc ion (2.3 Å) and forms an H-bond with the Glu198 carboxylate, whereas the carbonyl oxygen is turned toward the solvent, 4.8 Å away from the zinc ion. The inhibitor NH forms a water mediated H-bond with the Ala163 CO group. The bridged water is further bonded to Ala163 NH and Glu198 carboxylate. Crystal structures of native MMP-2[19] and MMP-3[20] contain a water molecule coordinated by the catalytic zinc ion and H-bonded to the conserved Glu carboxylate, and all MMPs, presumably, bind water prior to substrate or inhibitor bonding. This water molecule may be completely displaced upon inhibitor binding,[6] or retained as bridged water, in some complexes with hydroxamates[21] and carboxylates.[22]

Conformational changes required to accommodate the *N*-hydroxyurea ZBG in its planar conformation, with retention of the binding interactions proper of hydroxamate, are beyond the extensive flexibility of the MMP active site loop.[23] Thus, the *N*-hydroxyurea is forced to bend and to ligate as monodentate ZBG. The O=C–N1–O torsion angle (−122°) highly deviates from planarity, and the two oxygen atoms are closer to the *trans* rather than *cis* amide bond conformation, adopted by hydroxamate in zinc chelation. The amide N3–CO bond is also significantly distorted from planarity as shown by the high value of the O=C–N3–Me torsion angle (−43°). The presence of three non-H substituents on N3 allows evaluation of the valence angles of this atom. Their sum (325°) denotes a substantial sp^3 character of the nitrogen, which is in accordance with the high value of the out-of-plane distortion (43°) of its amide bond. The high value (58°) of the out-of-plane distortion of the N1–CO amide bond indicates negligible amide resonance and suggests substantial sp^3 character also for the N1 nitrogen.

Two hypothetical monodentate *N*-hydroxyurea zinc interactions in the active site of carboxypeptidase A[12] and carbonic anhydrase[13] were previously reported and the *N*-hydroxyurea group is proposed to bind in the deprotonated form, in accordance with the assumption of a value of 6.16 for its pK_a. An experimental pK_a value of 9.7 has been however reported[24] for H_2N–CO–NHOH, and calculated pK_a values, in the 10–11 range, are easily available[25] for several *N*3-alkyl-*N*1-hydroxyureas. In accordance with its weak acidity, the *N*-hydroxyurea group of the MMP-8:**2b** complex binds in the protonated form (Fig. 2, N1–O ... $^-$OOC–Glu198 distance 3.3 Å).

It was also interesting to compare the mode of binding of the *N*-hydroxyurea **2b** and the reference hydroxamate **1** containing an sp^3 methylene group in place of the N3-CH_3 group of **2b**. To this purpose, hydroxamate **1** was docked into the MMP-8 binding site (Fig. 4) using the program FLO+/QXP.[26] The simulated docking conformation of **1** (yellow carbon atoms) retains both zinc chelation and the two H-bonds typical of the hydroxamate ZBG, although the bisaryloxy group is more deeply buried into the S1′ pocket and the H bond between the ethereal oxygen and Leu160 NH is lost. In the MMP-8:**2b** complex, both the high out-of-plane distortions, involving N1 and N3 amide bonds, and the pyramidal character of N3 show that the presumably planar preferred conformation of the *N*-hydroxyurea[17] is forced to bend, in the active site of MMP-8, in order to coordinate the catalytic zinc

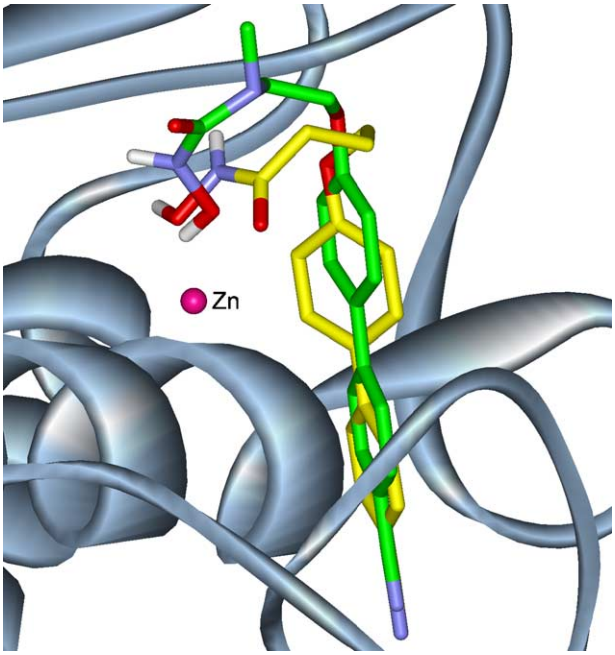


Figure 4. Superimposition of the crystallographic pose of **2b** (green carbon atoms) with the hydroxamate **1** (yellow carbon atoms) as docked into the MMP-8 binding site. MMP-8 is represented as a solid ribbon.

ion by the hydroxyl oxygen and simultaneously insert the biphenyl group into the S1′ pocket.

Although *N*-hydroxyurea group was reported[27] to form chelation complexes with Fe(III) and Cu(II) metal ions, it seems to be less effective than hydroxamate for zinc chelation in MMP inhibitors. The monodentate zinc coordination observed in the MMP-8:**2b** complex is probably also conserved in the active site of MMP-2 and MMP-3. When **2b** was minimized[26b] into the active site of these enzymes, maintaining the experimentally observed zinc coordination geometry, the simulated complexes could explain the increased affinity for MMP-2 and MMP-3. The MMP-3:**2b** complex (Fig. 5) is stabilized by a further H-bond of the ethereal oxygen with Ala165 NH, in addition to the usual H-bond with

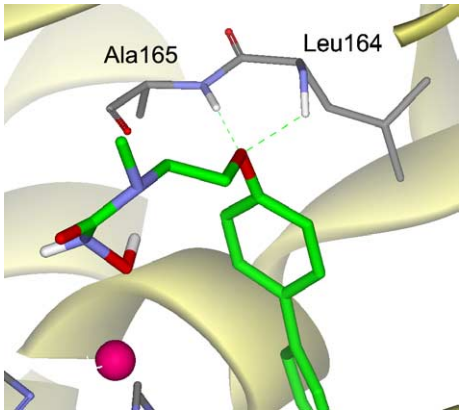


Figure 5. Calculated geometry of **2b** (green carbon atoms) minimized in the MMP-3 active site. Two H-bonds connecting **2b** ethereal oxygen with Ala165 and Leu164 are shown as green dotted lines. The rest of the enzyme is represented as a solid ribbon.

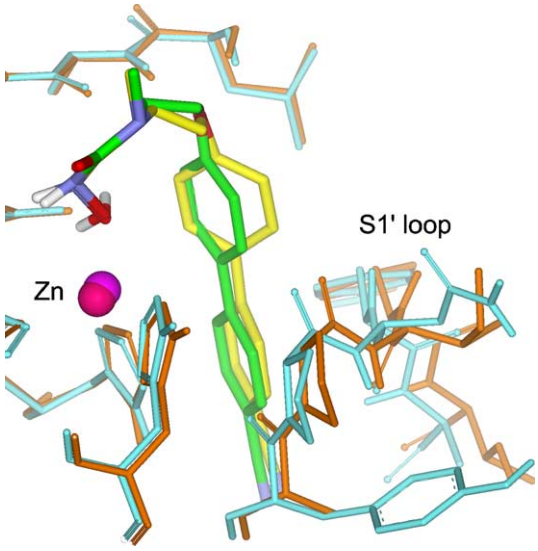


Figure 6. Superimposition between the calculated geometry of the adduct formed by MMP-2 (orange) with the inhibitor **2b** (light blue carbon atoms) and the crystallographic pose of the same inhibitor (green carbon atoms) in the MMP-8 (yellow). Only the relevant section of the active site is displayed.

Leu164 NH. In the MMP-2:**2b** complex (Fig. 6), the loop defining the S1′ pocket is moved away about 1 Å from the biphenyl internal ring, with respect to MMP-8:**2b**. While the two rings of the biphenyl in the MMP-8:**2b** complex are forced in a practically eclipsed conformation (dihedral angle 9°), they can be better accommodated, in the wider MMP-2 active site, with a dihedral angle of 47°. The ensuing release of the torsional strain probably contributes to the observed increase in affinity for MMP-2. To further improve *N*-hydroxyureas as MMP inhibitors, work is in progress to design and test ligands that may favor zinc chelation by the *N*-hydroxyurea group in its preferred planar conformation.

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Supplementary data

Chemistry; crystallographic details; MMP inhibition; molecular modeling. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.057.

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