Structure of malonic acid-based inhibitors bound to human neutrophil collagenase. A new binding mode explains apparently anomalous data

HANS BRANDSTETTER,^{1,5} RICHARD A. ENGH,^{1,2} ERICH GRAF VON ROEDERN,³
LUIS MORODER,³ ROBERT HUBER,¹ WOLFRAM BODE,¹ AND FRANK GRAMS⁴

¹Max-Planck-Institut für Biochemie, Abteilung Strukturforschung, 82152 Martinsried, Germany

²Boehringer Mannheim GmbH, Nonnenwald 2, 82377 Penzberg, Germany

³Max-Planck-Institut für Biochemie, Arbeitsgruppe Bioorganische Chemie, Am Klopferspitz 18a, 82152 Martinsried, Germany

⁴Boehringer Mannheim, Chemische Forschung, 68305 Mannheim, Germany

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Abstract

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases, which have been implicated in various disease processes. Various classes of MMP inhibitors, including hydroxamic acids, phosphinic acids, and thiols, have been previously described. Most of these mimic peptides, and most likely bind analogous to the corresponding peptide substrates. Among the hydroxamic acids, malonic acid derivatives have been used as MMP inhibitors, although optimization of their inhibition potency was not successful. Here we report the design of malonic acid-based inhibitors using the X-ray structure of a collagenase/inhibitor complex, which revealed a nonsubstrate-like binding mode. The proposed β -type turn-like conformation for the improved inhibitors was confirmed by X-ray crystallography. The observation of nonsubstrate-like binding confirms the original strategy for structure-based modeling of improved malonic acid inhibitors, and explains kinetic data that are inconsistent with substrate-like binding. Detailed interactions for the improved inhibitors seen in the crystal structure also suggest possibilities for further modifications in cycles of structure based drug design. Indeed, we have designed nonpeptidic inhibitors with approximately 500-fold improved inhibition based on these structures.

Keywords: binding mode; drug design; inhibitor; malonic acid; matrix metalloproteinase; MMP-8

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases involved in tissue remodeling (Woessner, 1991). They have also been implicated in various disease processes including tumor invasion and joint destruction. Therapeutic inhibition of one or several MMPs is, therefore, a promising approach for the treatment of, for example, arthritis and prevention of metastasis. Therefore, matrix metalloproteinases are attractive targets for inhibitor design, as demonstrated by the number and variety of MMP inhibitor research programs conducted by many pharmaceutical companies.

Various classes of MMP inhibitors, including hydroxamic acids, phosphinic acids, and thiols, have been previously described (Johnson et al., 1987; Beeley et al., 1994; Becker et al., 1995; Beckett

et al., 1996). Most of these mimic peptides and most likely bind analogous to the corresponding peptide substrates (Fig. 1), as has been shown in several X-ray crystallographic studies (Bode et al., 1994; Borkakoti et al., 1994; Lovejoy et al., 1994; Reinemer et al., 1994; Spurlino et al., 1994; Stams et al., 1994; Browner et al., 1995; Grams et al., 1995a, 1995b; Dhanaraj et al., 1996). The generalized structures of most of these inhibitors have been previously described (Beckett et al., 1996; Fig. 1). A zinc chelating group is followed by a methylene spacer, which may or may not be substituted with a residue [compare Batimastat (Campion et al., 1990; Davies et al., 1993) and GM6001 (Galaray, 1993), respectively; Fig. 1]. Following the spacer there is a hydrophobic residue R2 directed toward the S1' pocket that corresponds to a substrate L-amino acid, for example, leucine. It is followed by another L-amino acid or L-amino acid analog, which is usually hydrophobic (such as a C-terminally protected Trp, Phe, or similar unnatural amino acid). Earlier studies have shown tighter binding for these succinyl structures than for the corresponding malonic acid inhibitors that do not have the methylene spacer. Meanwhile, however, a crystal structure of a malonic acid inhibitor-MMP-8 complex was determined (Grams et al., 1995b), showing that the lack of a spacer

Reprint requests to: Frank Grams, Boehringer Mannheim GmbH, Chemical Research, Sandhofer Str. 116, 68305 Mannheim, Germany; e-mail: Frank_Grams@bm.bmg.boehringer-mannheim.com.

⁵Present address: Department of Chemistry, MIT, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139.

Abbreviations: Bn, benzyl; iBm, iso-butyl malonyl; iPr, iso-propyl; iBu, iso-butyl; MAB inhibitor, malonic acid-based inhibitor; MMP, matrix metalloproteinase; NEP, neutral endopeptidase.

Fig. 1. Schematic comparison of substrate-like binding and nonsubstrate-like binding modes at the catalytic zinc ion (circle) and S1' pocket (arc). **A:** Substrate-like binding: general binding mode, Batimastat, GM6001 (from left to right). **B:** Nonsubstrate-like binding: general binding mode for malonic acid derived inhibitors, HONH-iBM-L-Asp-NHBn, HONH-iBM-L-Asn-NHBn(m-NH₂), and HONH-iBM-L-hPhe-NHhBn(p-Me) (from left to right).

leads to a different mode of binding. Structure-based drug design using binding features seen in this complex yielded compounds much more potent than the original malonic acid compounds. The increased potency arose mostly from the increased hydrophobic contacts in the S1' binding pocket with, however, a significant contribution from the introduction of a new hydrogen bond. Further reduction of molecule size while maintaining the hydrophobic character of the molecule yielded improved binding to about 500 times that of the original parent compound.

Results

Binding of HONH-iBM-L-Ala-Gly-NH₂ to MMP-8

The previously determined crystal structure of the HONH-iBM-L-Ala-Gly-NH₂/MMP-8 complex (Grams et al., 1995b) revealed several important binding features. In contrast to the substrate-analogue binding "primed-site inhibitors," the L-Ala-Gly-NH₂ peptide segment (Fig. 1B: R4-isobutyl, R5-methyl, R7-glycylamide) binds in a bent conformation rather than in the substrate-like extended geometry. Nevertheless, the carbonyl group succeeding the CH(iBu) group and the Ala carbonyl group still form hydrogen bonds with Leu160N of the active site edge strand and Tyr219N of the S1' wall-forming segment, respectively. The Ala-NH is hydrogen bonded to Pro217O; it adopts, however, a β -2-type turn con-

formation. As a consequence, the "inhibitor tail," i.e., Gly-NH₂, occupies the S1' pocket, while the iso-butyl group as a leucine analog interacts with the active site edge strand. We supposed that the strong fixation of the hydroxamate to the zinc ion in combination with the malonic acid-L-amino acid scaffold generally induces the unique conformation.

New inhibitors based on the HONH-iBM-L-Ala-Gly-NH₂ binding mode (see Table 1)

Optimization of interactions at the hydrophobic entrance of the S1' pocket suggested the addition of a larger and more hydrophobic group in the C-terminal portion of HONH-iBM-Ala-Gly-NH₂. In a first attempt, a furanyl residue was chosen (**2**); it showed a three-fold improved activity, confirming the concept at least from a thermodynamic point of view. However, due to the chemical instability of the resulting compound, the furanyl was replaced by a phenyl group (**3**), which yielded a stable inhibitor with approximately the same activity as seen with **2**. To provide more hydrogen bonding potential, an Asp or alternatively an Asn was introduced to replace the central Ala of the molecule (**4** and **5**). As shown by X-ray analysis (see below), the overall binding geometry occurs as predicted. No improvement in binding energy was obtained, however, with the addition of polar groups in place of Ala. Because the additional hydrogen bonds do contribute to binding, the failure to

Table 1. All compounds are racemic mixtures^a

<i>N'</i> -hydroxy-2-isobutyl-malonamide derivatives		
Formula	IC ₅₀ (μ M)	log P_{calc} ^a
HONH-iBM-Ala-Gly-NH ₂ (1)	139	−1.64
HONH-iBM-Ala-NHFur (2)	42	0.16
HONH-iBM-Ala-NHBn (3)	58	0.98
HONH-iBM-Asp-NHBn (4)	101	(0.49) ^b
HONH-iBM-Asn-NHBn (5)	63	−0.25
HONH-iBM-Asn-NHBn(<i>m</i> -NH ₂) (6)	30	−1.48
HONH-iBM-hPhe-NHhBn(<i>p</i> -Me) (7)	0.5–1.5	3.66
HONH-iBM-NH-(CH ₂) ₇ CH ₃ (8)	0.3	3.23

^aAll logPs (octanol/water coefficient) were calculated using the program PCModels clogp3 (Daylight Chemical Information Systems, Inc., 1993).

^bCalculated as protonated acid.

improve the binding energy may be due to a correspondingly increased solvation of the unbound inhibitor. Introduction of a further potential hydrogen bond from the meta position of the P1' benzyl to either Tyr216O and Leu214O in a bidentate manner or to Leu193O yielded a twofold improvement in binding (**6**). Because the addition of hydrogen bonds improved binding only modestly, new strategies to increase the extent of hydrophobic bonding were used. One idea was to fill the entire volume of the S1' pocket by the C-terminal fragment NH-CH₂-CH₂-(C₆H₄)-*p*-CH₃; another was to occupy the hydrophobic binding area in the S2' site with the benzene moiety of a homo-Phe. The result was an inhibitor (**7**) with highly improved affinity (ca. 100-fold over HONH-iBM-Ala-Gly-NH₂). Similar P1' residues have been used in succinyl type inhibitors. They also display high activity. However, due to the different binding mode these residues are in "the front part" of the inhibitor, whereas in case of the malonic acid type inhibitors this moiety forms the "inhibitor tail."

The strategy of increased hydrophobicity (compound **7**) seemed, in general, more successful than, for example, specific hydrogen bonds, despite a likely unfavorable entropy effect due to the additional loss of flexibility upon binding. An extension of this strategy was to further increase both hydrophobicity and flexibility while removing the branching which, at least in compounds **4** and **6** leave one branch relatively exposed to solvent. This resulted in a set of "minimal" molecules with a simple linear aliphatic chain or an arylalkyl chain. We connected the malonic acid analog via an amide to the chain expected to fill the S1'. The best of these, HONH-iBM-NH-(CH₂)₇CH₃ (**8**), displayed an inhibition constant of 0.3 μ M toward MMP-8 (catalytic domain) (see Table 1).

The X-ray crystal structures of HONH-iBM-L-Asp-NHBn and HONH-iBM-L-Asn-NHBn(m-NH₂)

The inhibitors HONH-iBM-L-Asp-NHBn and HONH-iBM-L-Asn-NHBn(*m*-NH₂) bind with essentially the same interactions as the lead structure HONH-iBM-L-Ala-Gly-NH₂ (Grams et al., 1995b), with the additional interactions of the respective C-terminal groups (Fig. 2). Although racemic mixtures were used for crystallization, the electron density indicates that preferentially the *S,S* diastereomer of the inhibitor binds to MMP-8 (Fig. 3). In HONH-iBM-L-Asp-NHBn, however, the density disappears at the C α equivalent of iBM, so that binding of both stereoisomers could be possible.

Because the structure of the MMP-8/HONH-iBM-L-Ala-Gly-NH₂ complex has been described in detail (Grams et al., 1995b), we report here the distinguishing features of the new inhibitor structures (Fig. 4). The electron density (Fig. 2) shows that the aminobenzyl group of HONH-iBM-L-Asn-NHBn(*m*-NH₂) occupies the position of the Gly-NH₂ group of HONH-iBM-L-Ala-Gly-NH₂ (Fig. 3). Due to its greater length and bulk, it fills out the S1' pocket, which probably contributes to the tighter binding of the new inhibitors. The meta-amino group forms a hydrogen bond to Leu193CO (2.8 Å), while the CH group of the other meta position forms a close contact with Tyr216CO (2.9 Å). This latter type of CH-O contact has been observed often in proteins (Wahl & Sundaralingam, 1997); its contribution to binding energies is difficult to

Fig. 2. The $2f_o - f_c$ electron density for inhibitor HONH-iBM-L-Asn-NHBn(*m*-NH₂) at the zinc binding site. The density confirms binding of the *S,S* stereoisomer. Disorder of the isobutyl group is indicated by the branching of the density at C β . Red spheres indicate water molecules; a network of water is involved in binding.

Fig. 3. Superposition of inhibitors HONH-iBM-L-Ala-Gly-NH₂ (green carbons), HONH-iBM-L-Asp-NHBn (white carbons), and HONH-iBM-L-Asn-NHBn(m-NH₂) (yellow carbons) at the binding site. The catalytic zinc is presented in magenta, whereas solvent molecules are indicated as red balls. Although the overall binding geometry is preserved, specific features are discernible at several positions. The two different conformations of the isobutyl group represents disorder and may occur simultaneously for the inhibitors. The electron density further indicated disorder at the Asp side chain of HONH-iBM-L-Asp-NHBn.

evaluate, however. In addition to the possibility of two stereoisomers binding, the electron density for HONH-iBM-L-Asp-NHBn indicated disorder at the S1' pocket; attempts to refine alternate orientations, however, typically restored the original geometry. It seems likely that the disorder arises from partial occupancy of HONH-iBM-L-Asp-NHBn in the crystal, and that the binding at S1' is identical for the two inhibitors. Alternatively, binding of two different stereoisomers throughout the crystal might also result in apparent disorder at the S1' pocket.

The hydrophilic replacements of Ala → Asp and Ala → Asn were made to increase the number of hydrogen bonds in the complex. This occurred, although the binding strengths were not improved. The amide group of HONH-iBM-L-Asn-NHBn(m-NH₂)

forms hydrogen bonds with 158GlyCO (2.9 Å) and 219TyrNH (2.9 Å). As in the case of S1' binding, the electron density for HONH-iBM-L-Asp-NHBn at this position also shows evidence of disorder and a solvent molecule interposed between the side chain and 158GlyCO, as depicted in Figure 4.

Attempts to crystallize the improved inhibitors with MMP-8 have failed thus far. This is consistent with our observation of inhibitor-dependent crystallization. Figure 5 suggests a rationale for this dependence, as a pair of hydrogen bonds at or adjacent to the inhibitor binding site may be critical for crystal growth. Small inhibitor-induced protein motions as well as direct inhibitor—protein contacts may, therefore, block crystallization with known crystallization conditions. However, crystallization of compound 7

Fig. 4. Schematic summary of binding interactions for HONH-iBM-L-Asp-NHBn, and HONH-iBM-L-Asn-NHBn(m-NH₂). CH-O type contacts are listed in parentheses. Although the overall set of interactions remains similar, specific interactions change with the meta-amino group of HONH-iBM-L-Asn-NHBn(m-NH₂) (hydrogen bond to Leu193CO), the Asp-Asn replacement (likely interposition of a water molecule), and a shift in the inhibitor position (weaker interactions to a water molecule).

Fig. 5. Crystal packing interaction between Asn 218 and Phe128C and Thr129OG of the neighboring molecule. The proximity of Asn218 to the binding site may explain the sensitivity of crystallization conditions to the inhibitor in co-crystallization experiments (unpubl. obs.).

was successful with a different MMP target (unpubl. obs.). The X-ray structure indeed verifies the assumed binding mode with the homophenyl group occupying the S2' site.

Discussion

In the absence of X-ray crystal structure information, collagenase inhibitor optimization has focussed on similarities to the peptidic substrates. This meant that Phe, Leu, or analogs are typically found in P1' and analogs of Phe, Leu, or Trp are found in P2' (Netzel-Arnett et al., 1991, 1993; Niedzwiecki et al., 1992). The crystal structures reported here show that such optimization was successful only with the spacer containing inhibitors, simply due to the nonsubstrate-like binding of the malonic acid-type inhibitors. As opposed to the succinyllic acid type and thiolpropionic acid type inhibitors, which presumably bind analogous to substrates (at least when the substrate analogue residues are used), the malonic acid-based inhibitor binds to the zinc with its hydroxamic acid function slightly twisted, in comparison with hydroxamates of the succinyllic acid type. The C α of the MAB inhibitor occupies the position of the C β of a substituent on the spacer of the succinyllic compounds. The C α of the second amino acid of the MAB inhibitors

occupies the position of the nitrogen of the second amino acid in the succinyllic acid-based inhibitors, and the C α of the third amino acid occupies the position of the P1' C γ of a succinyllic acid-based inhibitor. Examples of inhibitors with a substrate analog sequence include: HONHCO-(*RS*) CH(iBu)CO-Leu-Gly-OEt and HONHCO-CH₂-(*RS*) CH(iBu)CO-Leu-Gly-OEt. Whereas the first of these, a malonic acid derivative, shows only a 29- μ M inhibition constant against MMP1, an inhibition constant of 40 nM is observed for the succinyllic acid derivative. Further elongation of the spacer reduces inhibition (>10 μ M for HONHCO-CH₂-CH₂-(*RS*) CH(iBu)CO-Leu-Gly-OEt) (see Table 2). It can be assumed that of the three only HONHCO-CH₂-(*RS*) CH(iBu)CO-Leu-Gly-OEt binds in a substrate analog manner. A similar but less dramatic result is observed with the thiol-containing inhibitors HS-(*S*)CH(iBu)CO-Leu-Gly-OEt (8.5 μ M) and HS-CH₂-(*RS*)CH(iBu)CO-Leu-Gly-OEt (1.3 μ M). It is possible that these thiol inhibitors also bind in two modes, analogous to the hydroxamates: the first via a β -turn in, and the second with typical substrate geometry. At least for a similar succinyllic inhibitor, substrate-like binding has been verified (Grams et al., 1995b).

These discrete binding modes might also be distinguished among metzincins in general (Bode et al., 1993; Stöcker et al., 1995) but

Table 2. Influence of a spacer between chelating group and following residue

Inhibitor	K_i Thermolysin (nM)	K_i NEP (nM)	IC ₅₀ MMP1 (μ M)	Reference
HONHCOCH ₂ CH(iPr)CO-Trp-OH	2,000	5		Bihovsky et al. (1995)
HONHCOCH(iPr)CO-Trp-OH	5	0.4		Bihovsky et al. (1995)
HS-(<i>S</i>)CH(iBu)CO-Leu-Gly-OEt			8.5	Johnson et al. (1987)
HS-CH ₂ -(<i>RS</i>)CH(iBu)CO-Leu-Gly-OEt			1.3	Johnson et al. (1987)
HONHCO-(<i>RS</i>) CH(iBu)CO-Leu-Gly-OEt			29	Johnson et al. (1987)
HONHCO-CH ₂ -(<i>RS</i>) CH(iBu)CO-Leu-Gly-OEt			0.040	Johnson et al. (1987)
HONHCO-CH ₂ -CH ₂ -(<i>RS</i>) CH(iBu)CO-Leu-Gly-OEt			>10	Johnson et al. (1987)

not presumably among all metalloproteinases with a thermolysin-like fold. As shown by Grams et al. (1995b), the binding of substrate-analogue inhibitors in thermolysin is different from inhibitor binding in the MMPs. (Notably, thermolysin was used as a model for the MMPs prior to their X-ray determination!) Thermolysin preferentially binds the D-isomer of, for example, HONHCOCH₂CH(Bn)Ala-Gly-OH (with respect to the benzyl α -carbon) (Kortylewicz & Galarzy, 1990), with an altered direction of the C-terminal chain proceeding the C α of CH(Bn). Comparison of the malonic acid and succinyl type inhibitors also reveals a difference to the MMPs. Although the succinyl type is preferred by the MMPs when using a substrate analogue inhibitor sequence, this is not the case with thermolysin. HONHCOCH₂CH(iPr)CO-Trp-OH binds with an affinity of 2,000 nM for thermolysin, but the malonic acid type inhibitor HONHCOCH(iPr)CO-Trp-OH binds with a K_i of 5 nM (Table 2). This selectivity is the opposite of than seen for the MMPs. A similar effect can be observed with the closely related NEP (5 nM for the succinyl, 0.4 nM for the malonic acid type inhibitor; see Table 2).

Using the knowledge of this new binding mode, we designed inhibitors to increase the number of hydrogen bonds, especially in the S2' region, but also in the S1' pocket. Although these hydrogen bonds were indeed formed, only a very small improvement was observed for the S1' hydrogen bond-forming compound. The presumably favorable binding enthalpy of these hydrogen bonds, especially for that in S2', is apparently counterbalanced by other unfavorable enthalpic or entropic changes. Indeed, there is even a net loss of binding free energy with the addition of a carboxyl group to the alanine (compare 3 and 4). One likely explanation is a solvation effect for these much more hydrophilic compounds (for calculated logPs see Table 1). An alternate approach for increasing binding strengths was to increase interactions in hydrophobic binding regions; introduction of a phenyl ring in P2', as seen in Batimastat and a large hydrophobic group occupying S1', which is also beneficial in succinyl type inhibitors, led to success. Further, introduction of both hydrophobicity and flexibility via the construction of an inhibitor with an optimal "tail" residue and elimination of a residue pointing to S2' improved binding 500-fold over that of the original parent compound.

Conclusions

Previous data with malonic acid-type inhibitors showed anomalous results when analyzed assuming substrate-like binding. Therefore, only very few data were published with these inhibitors, which have been ideal as thermolysin inhibitors. Chain elongation studies quickly showed the superiority of succinyl inhibitors with structures analogous to ideal substrate sequences. Therefore, all the potent MMP inhibitors published to date are of this latter type. The reason that the inhibition of malonic acid-based inhibitors does not correlate well with ideal substrate sequences has been shown by X-ray structures of three different examples crystallized in complexes with MMP-8. This new interpretation of old data points toward new strategies of drug design for these pharmaceutically interesting enzymes. The different interactions with the protein also might facilitate the design of compounds with a different pharmacological profile.

Materials and methods

Synthesis of the inhibitors

The synthesis of the inhibitors will be published elsewhere.

Modeling

Modeling studies have been performed with the program package InsightII/Discover (MSI, San Diego, California).

Crystallization and data collection

Crystals of the MMP-8-inhibitor complexes were grown with the vapor-diffusion technique at room temperature. A 10- μ L hanging drop consisting of 2 μ L of protein solution (5 mg/mL Met80-Gly²⁴²MMP-8 buffered at pH 6 with 0.2 M MES/NaOH), 3 μ L of aqueous 50 mM inhibitor solution, and 5 μ L of PEG solution (10% m/v PEG 6000 buffered at pH 6 with 0.2 M MES/NaOH) was equilibrated against a reservoir solution of 1 M Na₂HPO₄/NaH₂PO₄ at pH 6. Diffraction data to 1.9 Å (HONH-

Table 3. Data and refinement statistics

Inhibitor	HONH-iBM-Asn-NHBn(m-NH ₂)	HONH-iBM-Asp-NHBn
Data evaluation		
R_{sym} (resolution range)	4.0% (6.5–1.8 Å)	5.6% (8.0–1.9 Å)
R_{sym} (final shell)	21.5% (1.9–1.8 Å)	27.2% (2.0–1.9 Å)
Measured/unique reflections	25,225/14,805	23,574/10,905
Refinement		
Resolution	6.0–2.0 Å	6.0–2.0 Å
Completeness after $sF_{obs} > 2$ cutoff	86%	75%
Completeness, final shell	73% (2.07–2.00 Å)	55% (2.07–2.00 Å)
Final R -factor	18.8%	18.3%
Model		
Number of nonhydrogen atoms	1,365	1,357
Number of solvent molecules	83	83
Bond deviations from ideal values	0.015 Å	0.014 Å
Angle deviations from ideal values	1.9°	1.8°

iBM-Asp-NHBn) and 1.8 Å (HONH-iBM-Asn-NHBn(m-NH₂)) were collected on the X1000 Siemens multi-wire area detector using Siemens FRAMBO/SADIE/ASTRO data collection software and processed with Siemens SAINT data reduction software.

Refinement

The structures were refined with XPLOR (Brünger, 1989) (X-PLOR: A System for X-ray Crystallography and NMR, Version 3.1, Yale University Press, New Haven, CT) beginning with the crystal structure of the HONH-iBM-Ala-Gly-NH₂/MMP-8 complex (Grams et al., 1995b). Data and refinement statistics are summarized in Table 3. The atomic coordinates of the X-ray structures have been deposited at the Brookhaven Data Bank with the accession codes 1A85 and 1A86.

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