Simple Pseudo-dipeptides with a P₂’ Glutamate
A NOVEL INHIBITOR FAMILY OF MATRIX METALLOPROTEASES AND OTHER METZINCINS

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Abstract

Background: MMP inhibitor design relies on the use of peptidic or non-peptidic scaffolds with a zinc-chelating function.

Results: Pseudo-dipeptides containing a L-glutamate motif are potent MMP inhibitors.

Conclusion: The L-glutamate scaffold is exploitable to develop potent inhibitors of metzincins.

Significance: A new family of zinc protease inhibitors has been discovered.

A series of pseudo-peptides with general formula X-L-Glu-NH₂ (with X corresponding to an acyl moiety with a long arylalkyl side chain) have been synthesized, evaluated as inhibitors of matrix metalloproteases (MMPs), and found to display remarkable nanomolar affinity. The loss in potency associated with a substitution of the P₂’ L-glutamate by an L-glutamine corroborates the importance of a carboxylate at this position. The binding mode of some of these inhibitors was characterized in solution and by x-ray crystallography in complex with various MMPs. The x-ray crystal structures reveal an unusual binding mode with the glutamate side chain chelating the active site zinc ion. Competition experiments between these inhibitors and acetohydroxamic acid, a small zinc-binding molecule, are in accord with the crystallographic results. One of these pseudo-dipeptides displays potency and selectivity toward MMP-12 similar to the best MMP-12 inhibitors reported to date. This novel family of pseudo-peptides opens new opportunities to develop potent and selective inhibitors for several metzincins.

The matrix metalloproteinases (MMPs) form a group of 23 proteins in humans, all of which contain a catalytic domain belonging to the zinc metalloproteinase family (1, 2). The association of these enzymes with a variety of pathological states (3–7) has stimulated impressive efforts to develop synthetic compounds able to effectively modulate the uncontrolled activity of these enzymes (8–15). Following the disappointment of clinical trials with early broad spectrum synthetic inhibitors of MMPs (16, 17), the field has refocused on the development of selective inhibitors better able to discriminate between different members of the family with the perspective of therapeutic applications (18, 19).

Retrospective analysis suggests that the strongly zinc-interacting hydroxamic acid moiety, incorporated in the structures of the first generation of synthetic MMP inhibitors, potentiates inhibition indiscriminately without specificity for members of this family (14) or even unrelated zinc proteinases (20). By using weaker zinc-binding groups such as phosphate or carboxylate functions, more selective compounds have been identified. The selective MMP-12 (macrophage metalloelastase) phosphinic inhibitor reported by our group (see Fig. 1, compound 1/RXP470 (21)) and compound 2 from Wyeth Research (22) belong to this group. A new generation of potent and highly selective inhibitors without any zinc-binding potential has also been developed. This latter group includes compounds 4 (23) and 5 (24), both highly selective for MMP-13. The crystallographic structures reveal these inhibitors occupy the S₁’ cavity, a hydrophobic pocket present in most MMPs, and do not interact with the zinc ion. They gain their selectivity by plunging deeply into this S₁’ cavity and exploiting an unusual conformation of the S₁’ loop characterized by an additional side pocket absent in other MMPs (19). In the past few years, several other highly selective MMP-13 inhibitors with different central cores but a similar binding mode have been reported (25–32). Following the same concept, the first non-zinc binding inhibitor that targets both MMP-8 and MMP-13 has been identified (compound 7) (33).

As a prelude to future clinical trials, the ability of such MMPs inhibitors to selectively interact with their target was highlighted in several animal models. Specifically, highly selective MMP-13 inhibitors were shown to be able to prevent cartilage lesion in mouse model of osteoarthritis (34, 35). More recently, compound 6, a selective MMP-13 inhibitor, was tested in a mouse model of atherosclerosis and shown to increase collagen content in evolving and established atherosclerotic plaques, improving their stability (36). The efficacy of compound 2, a selective MMP-12 inhibitor, in attenuating and reversing episodic airway narrowing was demonstrated in a

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*This article contains supplemental "Experimental Procedures," Tables S1–S3, and Figs. S1 and S2.
sheep asthma model (22). Phosphinic pseudo-peptide 1, a highly potent and selective MMP-12 inhibitor, was tested in mice with established atherosclerotic plaques, and its ability to block both their growth and rupture was demonstrated (37).

With the aim to develop a new generation of inhibitors of lower molecular weight than 1, compound 3, the most selective MMP-12 inhibitor to date, was designed (38, 39). The crystal structure of this inhibitor bound to MMP-12 in presence of acetohydroxamic acid (AHA) showed that this inhibitor adopts a "standard" binding mode with the two P2' and P3' glutamate side chains pointing toward the S2' and S3' subsites within the active site (39). Subsequent studies of this inhibitor and analogues possessing the general formula X-Glu-Glu-NH2, X-Gln-Glu-NH2, or X-Glu-NH2, where X corresponds to the long P1' aryl-alkyl side chain, have produced data that were not explained by MMP-12-AHA:3 complex crystal structure. Because the presence of AHA may affect inhibitor positioning in the crystal structure, experiments were carried out to evaluate this possibility. This included dual inhibition experiments and X-ray crystallography with new crystal manipulation methodology to obtain complexes with these inhibitors in the absence of the AHA molecule.

EXPERIMENTAL PROCEDURES

Chemical Synthesis—Pseudo-peptides 8 to 22 were synthesized on solid support from malonic building blocks or carboxylic acid derivatives as precursors. After cleavage, the resulting compounds were purified by preparative reverse-phase HPLC, and their purity was assessed by analytical HPLC and high resolution mass spectrometry analysis. All compounds were >95% pure. Further details on the synthesis and analysis are given in supplemental Table S2.

Enzyme Assays—MMP inhibition assays were carried out in 50 mM Tris/HCl buffer, pH 6.8, 10 mM CaCl2 at 25 °C as described previously (21). Assays were performed with a fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (13 mM) and human MMPs (nanomolar range concentration) from R&D Systems except for human MMP-12 produced and purified as described previously (40).

ADAMTS-4 inhibition assays were carried out in 50 mM Tris/HCl buffer, 100 mM NaCl, 10 mM CaCl2, pH 6.8, at 25 °C. Assays were performed using 5-FAM-Ala-Glu-Lys-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-TAMRA-NH2 as the substrate (0.18 mM) from Enzo and human ADAMTS-4 (1.05 nM) from R&D Systems. ADAMTS-5 inhibition assays were carried out in 50 mM Tris/HCl buffer, 100 mM NaCl, 10 mM CaCl2, pH 6.8, at 37 °C. Assays were performed using Abz-Threo-Glu-Ser-Glu-Ser-Arg-Gly-Ala-Ile-Tyr-Dap(Dnp)-Lys-Lys-NH2 as substrate (1.8 mM) from Enzo and human ADAMTS-5 (4.9 nM) from R&D Systems.

Substrate and enzyme concentrations were kept well below 10% substrate utilization to improve evaluation of initial rates. For each inhibitor, the percentage of inhibition was determined in triplicate at five inhibitor concentrations, chosen to target the 20–80% range of inhibition. Ki values were determined.
using the method proposed by Horovitz and Leviski (41) (supplemental Table S3). Continuous assays were performed by recording the increase in fluorescence induced by the cleavage of fluorogenic substrates. Black, flat-bottomed, 96-well non-binding surface plates (Corning-Costar, Schiphol-Rijkm, Netherlands) were used for this test. Fluorescence signals were monitored using a Fluoroskan Ascent photon counter spectrophotometer (Thermo-Labsystems, Courtaboeuf, France) equipped with a temperature control device and a plate shaker.

Dual inhibition studies on MMP-12 were conducted with fixed and varying concentrations of inhibitors and AHA. The experimental data were fit to provide a term $\alpha$ using Equation 1 (42),

$$V_{\text{ref}}/V = \frac{(1 + [\text{AHA}] / \alpha K_a)}{K_v(1 + [S]/K_m)} [I] + 1 + \left( \frac{[S]}{K_v(1 + [S]/K_m)} \right)$$

(Eq. 1)

where $V_{\text{ref}}$ is the initial velocity in absence of inhibitor and $V$ is the initial velocity in presence of both inhibitors (3, 8, 10, or 11) and AHA, $K_v$ and $K_m$ are the dissociation constants for inhibitors and AHA, respectively, and $\alpha$ is the interaction term defining the effects of the binding of one inhibitor on the affinity of the second inhibitor, in our case, AHA. All subsequent single or dual inhibitions studies incorporated the Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ substrate at a concentration as close to $K_v$ as possible (13 mM, $K_m = 8.5$ mM). For a fixed AHA concentration, $V_{\text{ref}}/V$ was reported in function of concentrations of inhibitors (supplemental Fig. S2). The slopes obtained were fit in function of AHA concentration to determine the $\alpha$ value for each inhibitor in competition with AHA (supplemental Fig. S2).

**Crystallization**—The protein inhibitor solution for crystallization consisted of 0.53 mM of the catalytic domain of the F171D mutant of human MMP12 residues 106–263 with 100 mM AHA to prevent self-degradation of the protease prior to crystallization. The protein buffer was 3 mM CaCl$_2$, 200 mM NaCl with 20 mM Tris-HCl at pH 7.5. The inhibitors (compounds 3, 8, or 16) were added in a ratio 1:10 starting at 10 mM (water, NH$_3$aq 33% neutralization). This protein-inhibitor solution was mixed in a ratio 1:1 with reservoir solution containing the precipitant to give drops of 2 $\mu$l (initial volume). A set of working solutions was developed to co-crystallize the various MMP-12 inhibitor complexes. The drops were streak seeded (43) and equilibrated by sitting drop vapor diffusion for 1–4 days at 20 °C in a cooled incubator. The changes in precipitation propensity were counteracted by pH variations. The reservoir solutions consisted of 27% PEG 10,000, with 100 mM glycine, pH 9.0, for compound 3, 100 mM Tris-HCl, pH 9.5, for 8, and 200 mM imidazole maleate, pH 8.5, for 16.

Crystallization screening for catalytic domains of human MMP-8 and MMP-13 were carried out through the use of previously published conditions and of ab initio screening with the “Stura” screens (44), MD1–20 from Molecular Dimensions Ltd. Crystals of MMP-8 were obtained by co-crystallizing with a micromolar peptide-based inhibitor (gift of Dr. P. Cuniasse) in 17.5% PEG 20,000, 125 mM NaCl, 0.1 mM MES, pH 5.5. The peptide based inhibitor is slowly degraded by the enzyme and subsequent soaking for 24 h with 1 mM compound 17 in 25% PEG 4000, 100 mM MES, 125 mM NaCl, pH 5.5, preserves the integrity of the crystals and allows the establishment of 17 in the MMP-8 catalytic site. Crystal of the active catalytic domain MMP-13, were obtained from a sample of mixed pro-MMP-13 and catalytically active MMP-13 at 20 mg/ml. Only a few inhibitors could be co-crystallized with this procedure, among those compound 18 (supplemental Table S1).

For data collection, crystals were transferred into an appropriate cryoprotectant solution to which an amount of inhibitor to match that in the original protein-inhibitor drop was added. To remove excess AHA used to prevent degradation of the enzyme during preparation and crystallization a long soak in the cryoprotectant solution was deemed necessary. Because crystal can crack or dissolve during long soaks, a new kit CryoProtX (MDI-61 from Molecular Dimensions, Ltd.) was developed for this purpose (for further details, see supplemental Table S1). The length of the soak was determined on the basis of affinity of the inhibitor for the enzyme and, if known, its $\alpha$ value with respect to AHA. Given the lower affinity of compound 8 toward MMP-12, compare to that of compound 3, and considering its low $\alpha$ value, it was decided to carry out a 24-h soak to ensure the complete removal of AHA. For compounds 3, 16, 17, and 18, a 5-min soak was deemed sufficient to achieve AHA removal (supplemental Table S1). After the appropriate period in the cryo-protectant solutions, the crystals were then picked up with a loop and flash cooled in liquid nitrogen.

**Data Structure Determination and Refinement**—Data for the various MMP-inhibitor complexes were collected at the European Synchrotron Radiation Facility, beam lines ID23-1 and ID23-2 (Grenoble, France) at 100 K from single crystals. Crystals of MMP-12 and MMP-8 diffracted to high resolution (1.9 and 1.6 Å, respectively). Those from MMP13 crystallized from mixed pro-MMP-13/active-MMP-13 diffracted to only 2.5 Å. The automated data processing system at the European Synchrotron Radiation Facility based on XDS (46) was used for the data collected from MMP-8 and MMP-13. Data reduction for MMP-12 with compound 8 was carried out with MOSFLM (47) and scaled with SCALA from the CCP4 suite of programs (48). Data reduction for MMP-12 with compound 3 made use of the script “xdsfree” and structure solution by molecular replacement using MOLREP (50) with model coordinates from PDB code 3LIK (39). Rigid body refinement with REFMAC (51) was sufficient for MMP-12 with compound 8 as the lattice parameters did not vary substantially from those of compound 3 (supplemental Table S1). The constraint files for the inhibitors were generated with Monomer Library Sketcher from the CCP4 suite of programs (48) and for its use in COOT (52) recalculated with phenix.elbow (53). In all cases, a single conformation was seen for the inhibitor. The original MMP-8 model for molecular replacement of the MMP-8 inhibitor complexes was PDB entry 1OY2 (54). The structure of the MMP-8 complex with compound 17 was solved by molecular replacement using as a starting model another

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3 L. Vera and E. A. Stura, manuscript in preparation.
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MMP-8 inhibitor complex not yet published. Similarly, the model used for molecular replacement of the MMP-13 catalytic domain consisted of residues 104–272 of a structure of the full form not yet published. Molecular replacement was carried out using PHASER (55) and after rigid body refinement with REFMAC (51), compound 18 was added to the structure. Final fitting and stereochemical analysis of the refined model was carried out with COOT (52). The figures were made with PyMOL from Schrödinger, LLC (56).

Protein Data Bank Accession Number—The coordinates and structure factors for the catalytic subunit of human MMP-12, MMP-8, and MMP-13 inhibitor complexes have been deposited in the RCSB Protein Data Bank with the following codes: MMP-12 with compounds 3 (code 3TS4), 8 (code 3TSK) and 16 (code 4EFS); MMP-8 with compound 17 (code 3TT4), and MMP-13 with compound 18 (code 3TVC) (supplemental Table S1).

RESULTS

P2′ Position and Inhibitor Potency—The replacement of the P2′ glutamate in compound 3 by a glutamine (compound 8) resulted in a marked loss of potency (Fig. 2A), whereas the same replacement performed at the P3′ position (compound 9, Fig. 2A) had almost no effect on the potency nor on the selectivity profile. This led us to synthesize compound 10, lacking a substituent in position P3′ and measure its Kᵢ values on various MMPs (Fig. 2A). Compounds 3 and 10 have similar values showing that a P3′ residue plays a minor role in determining potency and selectivity profiles toward MMP-12 (Table 1). The P2′ glutamine analogue of 10 (compound 11) shows a loss in affinity (14-fold for MMP-12; supplemental Table S3). The importance of the P3′ t-glutamate was further investigated using conservative substitutions, either an aspartate or a homoglutamate in a t-configuration (compounds 12 and 13, respectively, Fig. 2B), or in a d-configuration (compound 14, d-Glu; and 15, d-Asp; Fig. 2B). All modifications produced a marked loss in potency toward MMP-12, with the exception of the t-aspartate derivative 12 where the potency loss was less substantial.

These latter results point to a critical role for a P2′ t-glutamate and the glutamine substitutions suggest that the side chain carboxylate at this position makes an interaction within the active site. The crystal structure of the complex between MMP-12 and compound 3 in presence of AHA does not support this suggestion (39). AHA is a small zinc-binding group that acts as a weak MMP inhibitor, classically used to prevent enzyme autolysis during protein preparation and crystallization (40) (57). However, the presence of AHA may affect inhibitor binding and efforts were made to obtain crystals of MMP complexes without AHA to remove this possible source of bias.

Crystal Structures of Compounds 3 and 8 Bound to MMP-12 in Absence of AHA—To allow AHA to diffuse out of the MMP-12-3 complex crystals, the time in the cryoprotectant solution was increased from typically just 30 s to 5 min. This required a cryoprotectant in which the crystals remain stable and maintained their ability to diffract to high resolution (1.6 Å, Fig. 3A). In this MMP-12-3 complex without AHA, the P1′ and P2′ residues adopt very well defined positions (Fig. 3A) but with different binding modes compared with that observed in the previous complex with AHA (Fig. 3B). A complete reorganization of the upper part of the inhibitor is observed, with the P3′ glutamate folding back allowing a direct interaction between its distal carboxylate function and the zinc ion (Fig. 3, A and B). In addition to the reorientation of the P3′ glutamate side chain, several other differences characterize this new binding. First, the P3′ glutamate points toward the solvent, and only one hydrogen bond remains between the carbonyl of the first glutamate and the NH of Leu1851 (d = 2.9 Å, Fig. 3A), whereas in the previous complex, five hydrogen bonds were formed between the inhibitor backbone and the enzyme. Furthermore, due to a significant difference in the positioning of this H-bonded carbonyl (1.3 Å, Fig. 3B), the P1′ side chain of compound 3 plunges less deeply in the S1′ cavity. The S1′ loops in the two complexes in the presence or absence of AHA are unperturbed by the repositioning of the inhibitor.

By contrast, compound 8, even in absence of AHA, with a P2′ glutamate adopts a canonical binding mode with the two P2′ and P3′ side chains pointing toward the enzyme S1′ and S3′ subsites (Fig. 3C). Thus, the conformation of 8 within the MMP-12 active site superposes well with the one adopted by compound 3 in presence of AHA. The presence or absence of a P3′ zinc-chelating group, capable of establishing interactions with the catalytic zinc ion, appears to be critical in influencing the inhibitor binding mode.

Characterization of Binding Mode of Compounds 3, 8, 10, and 11 in Solution—Because differences may exist between the inhibitor-binding mode observed in the crystal and that occurring in solution, competition experiments between AHA and inhibitors possessing or not a P2′ zinc-chelating group were carried out. In the case that the distal carboxylate function of the P2′ glutamate interacts with the zinc ion, the presence of a second zinc-binding compound such as AHA should interfere with inhibitor binding. Alternatively, if the inhibitor adopts a “substrate-like” canonical binding mode exemplified by a P2′ glutamine (Fig. 3B), a non-competitive situation would exist allowing the formation of a ternary inhibitor-MMP-12-AHA complex in solution. The Yonetani-Theorell graphical method can be used to determine whether ternary complexes are formed. In this approach, an α parameter is determined (supplemental Fig. S1 and Table S5); with an α value close to 1, there is no reciprocal influence between the two inhibitors, and both can bind in the enzyme active site, forming a ternary complex. In contrast, with value of α << 1 or >> 1, there is mutual interference between inhibitors. An α << 1 indicates that the affinity of one molecule is increased by the presence of the other, corresponding to a cooperative behavior enhancing the formation of a ternary complex. With α ≫ 1, there is a full antagonism between inhibitors, and only a binary complex will be formed in solution. Compounds 8 and 11 with a P2′ glutamine fit the graph with a low α value (α = 10 and 9.7 respectively, supplemental Table S5), suggesting that these inhibitors and AHA bind to enzyme active site independently of each other and form a ternary complex in solution. This result is in agreement with the binding mode observed in the crystal structure of the complex between compound 8 and MMP-12 (Fig. 3C). High α
FIGURE 2. Comparison of affinity/selectivity profiles for inhibitor 3 (A) and its analogues varying in their $P_2'$ and $P_3'$ positions inhibitor 10 (B) and its $P_2'$ analogues on a set of MMPs. For each MMP, the $1/K_i$ (M) values are reported.
values were obtained with inhibitors 3 and 10 harboring a carboxylate function in their P$_2^\prime$ positions ($\alpha = 230$ and $\alpha \to \infty$ respectively; supplemental Table S5), a situation where the carboxylate function of the P$_2^\prime$ glutamate and AHA compete in binding to the catalytic zinc ion, in accordance with the crystal structure of the complex between MMP-12 and compound 3 in the absence of AHA.

**Pseudo-peptides X-L-Glu-NH$_2$ as Potent MMP Inhibitors**—A small library of pseudo-peptides with the general formula X-L-Glu-NH$_2$ has been generated using the template inspired by compound 10. The components consist of an L-glutamate residue functionalized at its N position with different acyl moieties X, where X designates the variable aryl-alkyl side chain designed to fit within the P$_1^\prime$ cavity. This library was screened against various MMPs (supplemental Fig. S2). Among the tested molecules, compounds 16, 17, and 18 were identified as potent MMP inhibitors (Table 1 and Fig. 4A). Compound 16 is a potent and selective MMP-12 inhibitor ($K_i = 2.5$ nM, Table 1); compound 17 is a mixed MMP-8/MMP-12 inhibitor, ($K_i = 5.3$ nM for MMP-8 and $K_i = 3.3$ nM for MMP-12, Table 1) and compound 18 exhibits high affinity toward MMP-2, MMP-10, MMP-12, and MMP-13, with $K_i$ values of 47, 54, 3, and 18 nM, respectively (Table 1). X-ray structures have been obtained for each of the three compounds 16, 17, and 18 in complex with MMP-12, MMP-8, and MMP-13, respectively (Fig. 4). Inhibitors 16, 17, and 18 adopt a binding mode within the MMP active sites that is similar to that observed for compound 3 in complex with MMP-12. More specifically the carboxylate function of P$_2^\prime$ glutamate chelates the catalytic zinc ion and the carbonyl adjacent to the P$_1^\prime$ methylene is hydrogen bonded to the amide of MMP-12 Leu$^{181}$, MMP-8 Leu$^{160}$, or MMP-13 Leu$^{185}$. These anchor points allow the P$_2^\prime$ side chain to insert deeply into the S$_3^\prime$ pocket. The P$_2^\prime$ glutamine analogues (compounds 20 and 21, supplemental Table S3) of inhibitors 17 and 18 were evaluated, and as expected, a drop in potency was observed for all MMPs tested (3- to 275-fold; supplemental Table S3).

**Pseudo-peptides X-Glu-NH$_2$ as Inhibitor of Other Metzincins**—Finally, the same library of pseudo-peptides (X-Glu-NH$_2$) was evaluated on ADAMTS-4 and -5, two aggrecanases whose active sites share close structural features with MMPs (58). Compound 19 (Table 1 and Fig. 4A) was the most potent inhibitor, with $K_i$ value of 92 and 585 nM for ADAMTS-4 and ADAMTS-5, respectively. Again, the glutamine analogue (compound 22, supplemental Table S3) showed a drastic drop in affinity ($K_i > 10 \mu M$), confirming the key role of a P$_2^\prime$ carboxylate function in their P$_2^\prime$ values were determined in 50 mM Tris/HCl buffer, pH 6.8, 10 mM CaCl$_2$ at 25 °C. ND, not determined.

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ylate, in coherence with a binding in aggrecanases comparable with that observed for MMPs.

**DISCUSSION**

The crystal structure for compound 3 in complex with MMP-12 without AHA shows a shift away from the peptidic substrate-like binding mode toward a new mode where the distal carboxylate of the P2′ l-glutamate side chain of the inhibitor, now acts as a zinc-binding group. The marked loss in potency observed for compound 8, the glutamine analogue of 3, and the competition experiments performed in solution against AHA lead to the conclusion that the crystallographic results are pertinent beyond the solid state. Thus, the binding mode seen in the crystal structure exists in solution. Structural and inhibition data reveal that further simplification is possible by removing the P3′ substituent. Such simplification is performed without any loss in inhibitor potency (compare 3 and 10; Table 1) and leads to the identification of a minimal pseudo-peptide scaffold of the type X-l-Glu-NH2. The crystal structure of compound 16 in complex with MMP-12 (Fig. 4B) shows that this simple scaffold engages two interactions within the active site: the zinc chelation and a canonical hydrogen bond at the entrance of the S1′ cavity. The same binding mode characterizes the inhibitors 17 and 18 in complex with MMP-8 and MMP-13, respectively (Fig. 4, C and D). However, the zinc chelation by the P2′ carboxylate group is likely submitted to structural and conformational constraints because a loss of potency is observed in certain analogues of compound 10 (Fig. 2B, analogues 12, 13, 14, and 15). The binding of the P2′ carboxylate group to the zinc ion might be conditioned by the nature of the P1′ side chain. This interdependence between the P1′ and P2′ side chains may account for the selectivity profiles observed for compounds 10, 16, 17, and 18. Thus, it will be interesting to understand whether the lower potency exhibited by these inhibitors toward certain MMPs might be caused by a suboptimal interaction between the P2′ carboxylate and the zinc ion and/or the P1′ side chain and the S1′ cavity, with the inhibitors adopting in some cases alternative binding modes in solution.

Inhibitors with a simple scaffold and a carboxylate group that chelates the zinc ion have been reported, and the crystal structures for some of these in complex with MMP-12 have been determined (45, 49). Similarly, all of these inhibitors utilize a carboxylate zinc-binding group, an oxygen atom to form a hydrogen bond with the amide of Leu181 and possess a long side chain that fills the S1′ cavity (Fig. 5). Two important differences can be discerned between these inhibitors: the number of atoms linking the carboxylate chelating function to the H-bond acceptor (n, Fig. 5A) and the geometry around the H-bonded heteroatom. In this respect, superimposition of the inhibitors reveals small differences in the position adopted by the carboxylate and the heteroatom that may affect the enthalpy of interaction (Fig. 5E). Compared with inhibitors with a carbonyl group that functions as H-bond acceptor, the inhibitor with a hydroxyl group has lower affinity (Fig. 5, B–D). A different orientation of the oxygen atom and suboptimal hydrogen bonding with the amide of Leu181, as illustrated in Fig. 5E, might in part explain its lower

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**FIGURE 4.** A, schematic representation of inhibitors 16, 17, 18, and 19. B, complex of inhibitor 16 in interaction with MMP-12. Carbon atoms of 16 are shown as sticks colored in yellow. The electron density map of the inhibitor-binding region is in gray, and hydrogen bonds are represented in red dotted lines. C, complex of inhibitor 17 in interaction with MMP-8. Carbon atoms of 17 are shown as sticks colored pink. Electron density map of the inhibitor-binding region is in gray, and hydrogen bonds are represented as red dotted lines. D, complex of inhibitor 18 in interaction with MMP-13. Carbon atoms of 18 are shown as sticks colored green. Electron density map of the inhibitor-binding region is colored gray, and hydrogen bonds are represented in red dotted lines.
affinity. Regarding the linker, increasing its length (variable \( n \); Fig. 5A) is likely to reduce inhibitor potency. Indeed, longer linkers should induce higher flexibility in the inhibitor structure and thus incur a higher entropic penalty when bound to the enzyme, modulated by other constraints. Data reported in Fig. 5 showed that this is not the case. Compound 16 (\( n = 4 \), Fig. 5D) displays a similar affinity to an inhibitor with a shorter linker (\( n = 2 \), Fig. 5C). This might be explained by the better ability of compound 16 through higher flexibility to optimize key interactions within the enzyme active site. Potency therefore depends on achieving a subtle balance between the geometry around the H-bonded heteroatom and the length of the linker separating the chelating carboxylate function and the H-bond acceptor. In the series of inhibitors developed in these studies, the L-glutamate scaffold with linker length comprising four atoms appears to be optimal. Compare with compound 10, a shorter linker \( n = 3 \) (compound 12), a longer one \( n = 5 \) (compound 13), as well as changes in chirality (\( \alpha \)-glutamate derivative 14) decreases the affinity for MMP-12. It is worth to mention that, as dipeptide structures, the \textit{in vivo} stability of such compounds can be a potential hurdle. However the presence in their structure of a \( \text{P}_{1} \)’ side chain different to those of natural amino acids, should prevent from hydrolyzing by dipeptidas.

Despite significant chemical efforts over the past twenty years, only a few highly potent and selective inhibitors of MMPs have been developed. Exploiting the phosphinic peptide chemistry, we have reported one of the most selective MMP-12 inhibitors. Even if phosphinic peptides have been demonstrated in several examples of zinc proteases to behave as highly potent and selective inhibitors, this chemistry involves several difficult steps, making the production of inhibitors a hard task. In contrast, the synthesis of pseudo-peptide exploiting the L-glutamate scaffold involves a simple coupling step between a large set of commercially available carboxylic acids and an appropriately protected L-glutamate. Thus, libraries based on this motif will combine high chemical diversity and lower costs. Subsequent screening, as achieved here, should yield new potent and selective inhibitors for MMPs and other proteases similar to ADAM and ADAM-TS whose active sites possess structural features similar to those in MMPs.

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REFERENCES

MMP Inhibitors


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