Development of the First Potent and Selective Inhibitor of the Zinc Endopeptidase Neurolysin Using a Systematic Approach Based on Combinatorial Chemistry of Phosphinic Peptides*

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A new systematic approach, based on combinatorial chemistry of phosphinic peptides, is proposed for rapid development of highly potent and selective inhibitors of zinc metalloproteases. This strategy first evaluates the effects on the inhibitory potency and selectivity of the following parameters: 1) size of the phosphinic peptides, 2) position of the phosphinic bond in the sequence, and 3) the state (free or blocked) of the peptide extremities. After this selection step, the influence of the inhibitor sequence is analyzed in order to determine the identity of the residues that optimized both the potency and the selectivity. We demonstrate the efficiency of this novel approach in rapid identification of the first potent zinc metalloprotease inhibitor of the mammalian zinc endopeptidase neurolysin (24-16), able to discriminate between this enzyme and the related zinc metalloprotease thimet oligopeptidase (24-15). The most potent and selective inhibitor developed in this study, Pro-L-Phe(PO2CH2)Gly-Pro, displays a Kᵢ value of 4 nM for 24-16 and is 200 times less potent on 24-15. The specific recognition of such a free phosphinic tetrapeptide by 24-16, as well as the unique specificity of the 24-16 S₁ and S₂ subsites for proline, unveiled by this study, are discussed in terms of their possible significance for the function of this enzyme and its related zinc endopeptidase activities.

Potent enzyme inhibitors have long been recognized as powerful tools for assessing the physiological roles of enzymes and have sometimes led to therapeutic drugs able to modulate their activities in vivo (1). However, to be valuable tools, such inhibitors should also be as selective as possible and not interfere with all of the members of a particular enzyme family. This problem is of particular significance for the inhibitors designed based on enzyme mechanism considerations. This situation applies to the zinc metalloprotease family, for which a general strategy has been to synthesize short peptide sequences containing a particular functional group (thio, carboxyalkyl, hydroxamate) able to interact with the zinc atom of the active site, while the remaining peptide sequence fits within the enzyme cavity. This traditional approach has led to remarkable results in terms of potency (2), but taking into account the rapid expansion of the zinc metalloprotease family (3), we felt that it would be important to devise a more systematic approach for discovering highly potent and selective inhibitors of these enzymes. For this purpose, we elected to synthesize, by combinatorial synthesis methods, libraries of phosphinic peptides and then screened them with the proteases of interest to find potent and selective inhibitors. Such peptides have been chosen because they were proven to be highly potent inhibitors of several zinc metalloproteases, as expected for good transition state analogues of these enzymes (4–7). We recently tested this novel strategy for developing potent and specific inhibitors of two closely related mammalian zinc endopeptidases, the endopeptidases 24-15 and 24-16 (8). Indeed, these two peptides hydrolyze the peptide bonds of numerous natural (9–12) and synthetic peptides (13, 14) at the same location and are also both potently inhibited in an identical manner by several phosphonamide (15) or phosphinic (7) peptides developed previously. In addition, protein sequence comparison shows that these two enzymes possess 60% sequence homology (16). In this previous study, we demonstrated that the systematic substitution by 20 amino acids of the Yaa’ and Zaa’ positions in phosphinic peptides of the general formula Z-Phe(PO2CH2)Xaa’-Yaa’-Zaa’, with Xaa’ representing Gly or Ala, led to the identification of an extremely potent inhibitor of 24-15 (Z-Phe(PO2CH2)Asn-Arg-Met; Kᵢ = 15 pM), a molecule that is 3 orders of magnitude less potent toward 24-16 (8). Unfortunately, this approach did not allow the identification of specific inhibitors of 24-16. In fact, in these series of inhibitors, any combination of dipeptides in the Yaa’ and Zaa’ positions of the phosphinic peptides preferred by 24-16 was also well recognized by 24-15. This result led us to increase the molecular diversity of our phosphinic peptide libraries. We demonstrate that potent and specific inhibitors of 24-16 can be obtained through the use of this novel systematic strategy, in which the phosphinic peptide mixtures are generated not only by randomization of the peptide sequence, but also by taking into account the size of the peptide, the position of the phosphinic bond in the sequence, and the state (free or blocked) of their N- or C-terminal extremities.

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1 The abbreviations used are: 24-15, thimet oligopeptidase (EC 3.4.24.15); 24-16, neurolysin (EC 3.4.24.16); Phe, phenylalanine; Pmc, N-(9-fluorenyl)methoxycarbonyl; Mcc, 7-methoxycoumarin-3-carboxylic acid; Lys(Dnp), Nᵢ–(2,4-dinitrophenyl)–L-lysine tBu; Boc, tert-butoxycarbonyl; Trt, triphenylmethyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Ad, adamantyl; rink amide resin, 4-(2,4-dimethoxyphenyl–Fmoc-aminomethyl)–phenoxy resin; Nle, 2-aminoheptan-4-ol; Tric, N-[2-hydroxy-1-bis(hydroxymethyl)ethyl]glycine.
The First Potent and Selective Inhibitor of 24-16

MATERIALS AND METHODS

All of the Fmoc amino acid derivatives, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 2-chlorotriyl resin, rink amide resin (4-2,4'-dimethoxyphenyl-Fmoc-amino-monomethyl)-phenoxy resin), and the Mcc-Pro-Leu-Gly-Pro-Loys(Dnp) were purchased from Novabiochem.

Purification of 24-15 and 24-16

The rat brain endopeptidases 24-15 and 24-16 were purified as described previously (17, 10).

Enzyme Assays and Inhibition Studies

24-15 and 24-16—Unless otherwise noted, all assays were performed at 25°C in 5 mM Tris/HCl buffer, pH 7.5, containing 0.1 mg/ml bovine serum albumin, in the absence (24-16) or presence (24-15) of 0.1 mM dithiothreitol. Under these conditions, 0.1-1 mM samples of the enzymes retain the same activity for 24 h. Endopeptidase activity assays were performed with Mcc-Pro-Leu-Gly-Pro-Loys(Dnp) substrate as described previously (14). Continuous assays were performed by recording the hydrolysis of the quenched fluorescence substrate with a fluorimeter, setting excitation and emission wavelengths at 347 and 405 nm, respectively. In a typical experiment, a cuvette containing 0.9 ml of buffer and 9 μM substrate was brought to thermal equilibrium in a jacketed holder in a compartment of a Perkin Elmer LS 50 luminescence spectrometer cell. The temperature was maintained by water circulating from Haake F3 bath. Injection of 3–30 μl of enzyme (final concentration, 0.1–1 μM) initiated the reaction.

Determination of K<sub>i</sub> Values—In most cases, the steady-state velocities observed with different inhibitor concentrations can be determined from the progress curves before there is significant substrate depletion. Under these conditions, the inhibition constants K<sub>i</sub> can be directly determined from Dixon plots. For slow binding inhibitors, progress curves can be fitted to the equation,

\[ P = V_0 (1 - \exp(-K_{app}t)) \]

described by Morrison and Walsh to determine V<sub>0</sub>, V<sub>0</sub>-P<sub>0</sub> and K<sub>app</sub>, where P is the product concentration, V<sub>0</sub> is the reaction velocity at t = 0, V<sub>0</sub>-P<sub>0</sub> is the final steady-state velocity, and K<sub>app</sub> is the observed first-order rate constant for the approach to steady state (18). However, as pointed out by Morrison and Walsh, relevant values for V<sub>0</sub> require that the portion of the progress curve used to determine V<sub>0</sub> corresponds to a time long enough to ensure that the equilibrium between the enzyme and the inhibitor has been reached. Some inhibitors in this study display K<sub>i</sub> values in the range of 10<sup>3</sup> M<sup>–1</sup> s<sup>–1</sup>. In these cases, even at 100 mM inhibitor concentration, the establishment of enzyme-inhibitor complex equilibrium takes about 3 h. Thus, for these inhibitors, to avoid the problem of substrate depletion, the only way to estimate their K<sub>i</sub> values is to preincubate them with the enzyme and, only after the equilibrium has been reached, to initiate the reaction by substrate addition. Measurements were made by equilibrating increasing concentrations of inhibitor in 2 ml of solution containing 0.1-1 mM of enzyme overnight or longer, and then initiating the reaction by the addition of 10 μl of substrate. The E<sub>i</sub> complex concentration can be easily determined by following the resultant enzyme activity. The E<sub>i</sub> complex dependence on inhibitor concentration makes it possible to determine the K<sub>i</sub> value, using the equation,

\[ K_i = \frac{1}{2}(k_{on} + K_i) - \left[ \frac{k_{off}(k_{on} + K_i)}{4E}[1 + k_{on}^2 - 4E]^{1/2} \right] \]

which takes into account depletion of both enzyme and inhibitor (19, 20). In this equation, E<sub>i</sub> and I<sub>i</sub> are known; thus, K<sub>i</sub> can be determined by fitting the data with the above equation. K<sub>i</sub> and K<sub>app</sub> values, in the range of, respectively, 10 nM and 10<sup>3</sup> M<sup>–1</sup> s<sup>–1</sup> suggest K<sub>0v</sub> values in the range of 10<sup>–5</sup> s<sup>–1</sup>, a very slow dissociation of the E<sub>i</sub> complex. In this case, the effect of the substrate addition on the equilibrium position of the E<sub>i</sub> complex can be neglected. The slow dissociation of such E<sub>i</sub> complexes has been confirmed by following the recovery of the activity of the E<sub>i</sub> complexes, which have been purified to remove free inhibitor excess and then diluted with buffer. These solutions were kept at 25°C and assayed periodically for return enzyme activity by combining a 2-ml sample with 5 μl of substrate solution. The return of activity was followed over an 8-h period, and the K<sub>0v</sub> value was estimated by fitting the data to a single exponential. For the determination of the K<sub>0v</sub> values, several progress curves at different inhibitor concentrations were recorded and fitted to Equation 1 by nonlinear least squares analysis. These procedures provide values for the three experimental parameters V<sub>0</sub>, V<sub>0</sub>-P<sub>0</sub> and K<sub>app</sub>. K<sub>0v</sub> values were determined by plotting K<sub>app</sub> values obtained at different inhibitor concentrations against the inhibitor concentration using the following equation.

\[ K_{app} = K_{off} + k_{on}(I/1 + 5/K_{0v}) \]

This equation assumes that the E<sub>i</sub> complex formation involved a single step (18).

Inhibitor Synthesis—Solid-phase synthesis was performed manually or in a model 357 Advanced ChemTech multiple peptide synthesizer. The side chain protecting groups used were Asp(tBu), Glu(tBu), Ser(tBu), Thr(tBu), Tyr(tBu), Lys(Boc), Trp(Boc), His(Trt), Asn(Trt), Gin(Trt), and Arg(Pmc). The phosphinic pseudopeptide Z-DL-Phe(PO2CH2)Gly-OEt was prepared as described previously (6). The protection of the hydroxynaphosphinyl group by the adamantyl group and the introduction of the Fmoc group will be described elsewhere. The protected phosphinic pseudopeptide Fmoc-DL-Phe(PO2CH2)Gly-OEt was incorporated as a block during the solid phase synthesis.

The C-terminal carboxylate peptides were synthesized as described previously, using the 2-chlorotriyl resin (8).

Peptides with a C-terminal amide group were synthesized on a rink amide resin. The conditions used for the synthesis of the peptides were those previously described (8). Cleavage of the peptides from the resin, together with the cleavage of the protecting groups, was performed by the action of trifluoroacetic acid containing 2.5% H<sub>2</sub>O, 2.5% thioanisole, 2.5% phenol, 1.25% ethanedithiol, and 1.25% trisopropylsilane.

Construction of Peptide Libraries—The protocol used to synthesize the various mixtures of phosphinic peptides of the formula Yaa-DL-Phe(PO2CH2)Gly-Yaa', where Yaa and Yaa' represent 20 different natural amino acids (Cys replaced by Nle), was similar to that described previously (8).

For the tripeptide mixture of the formula Yaa-DL-Phe(PO2CH2)Gly, the protected phosphinic dipeptide (Fmoc-DL-Phe(PO2CH2)Gly-OEt) was coupled to the nonmodified 2-chlorotriyl or rink amide resin. This resin sample was then split into 20 identical samples to which a known amino acid (Yaa) was coupled. All 20 resin samples were then mixed to give a homogeneous mixture of 40 phosphinic tripeptides. In the case of the tripeptide mixture of the formula Yaa-DL-Phe(PO2CH2)Gly-Yaa', the protected phosphinic dipeptide (Fmoc-DL-Phe(PO2CH2)Gly-OEt) was coupled to a homogeneous mixture of 20 different amino acids (Yaa'), linked to the 2-chlorotriyl or rink amide resin, to give a mixture of 40 phosphinic tripeptides.

The (R configuration) or (S configuration) stereochemistry of the pseudophenylalanine residue was assigned as described previously (21).

RESULTS

Description of the Approach—The novel approach developed in this study was devised in an attempt to analyze the effects on the inhibitory potency and selectivity of the size of the phosphinic peptide, the position of the phosphinic bond in the peptide sequence, and the state of the N- and C-terminal extremities of the peptides. Thus, for a given peptide size, the development of different peptide mixtures could be envisaged (see Fig. 1). Due to the binding of the phosphoryl group with the catalytic zinc atom, the interaction of these peptide mixtures will differ according to the number and the nature of the subsites of the enzyme cavity involved in their binding. After this screening step, a further optimization of the potency and the selectivity of these inhibitors could be achieved by the identification of the amino acids improving these two parameters. In the present study, for chemical reasons, the nature of the phosphinic block Xaa(PO2CH2)Xaa has been kept unique. However, even with this restriction, the development of only tripeptide and tetrapeptide mixtures, as proposed by this strategy, could generate almost 5000 different phosphinic peptides, a molecular diversity expected to be sufficient for disclosing the existence of subtle differences between the active sites of related enzymes. In the present study, using the Phe(PO2CH2)Gly block, we show that specific inhibitors of

2 A. Yiotakis, S. Vassiliou, J. Jiracek & V. Dive, submitted for publication.
The data in Table I show that when the P2 position is substituted, only one mixture (1), with free N and C termini, displays both inhibitory potency and selectivity for 24-16. The protection of one of the two extremities affects both the affinity and the selectivity, with a more pronounced effect in the case of the N terminus modification. Interestingly, the effect of the protecting groups depends on the actual state of the N- or C-terminal extremity. For example, the acetylation of the N-terminal group reduces the potency of these mixtures for 24-16 by 2 orders of magnitude when the C terminus is free (mixtures 1 and 2), while the same modification has only a weak effect when the C terminus is already blocked (mixtures 3 and 4).

In the series of tripeptides substituted in the P2 position, mixtures 5, 6, and 7 exhibit significant inhibitory potency for both peptidases. In these series of tripeptides, the protection of the extremities produces less important changes in the affinity than in the series where the P2 position is substituted. In addition, the effects of the C- and N-terminal protection apparently depend on subtle rules. For example, the C-terminal protection of mixtures having a free N terminus does not change the affinity of the inhibitors for 24-16 (mixtures 5 and 7). On the other hand, when the N terminus is protected, the same modification of the C terminus gives inhibitors with highly reduced affinity for 24-16 (mixtures 6 and 8).

Inhibition of Zinc Endopeptidases 24-16 and 24-15 by Phosphinic Tetrapeptide Inhibitors of the General Formula Yaa-DL-Phe(PO2CH2)Gly-Yaa and Preference for the P2 and P2 positions. —The development of tetrapeptide mixtures was then considered to improve both the inhibitory potency and selectivity of the inhibitors. According to our proposed strategy three different tetrapeptide-type mixtures would have to be developed: 1) Zaa-Yaa-Phe(PO2CH2)Gly, 2) Yaa-Phe(PO2CH2)Gly-Yaa, and 3) Phe(PO2CH2)Gly-Yaa-Zaa. The third type of mixture was not retained at this stage, because we hypothesized that it would give similar results to those obtained with the Z-Phe(PO2CH2)Gly-Yaa-Zaa tetrapeptides developed previously (8). On the other hand, according to the results reported in Table I, it appears that the presence of a residue in the Yaa position is important both for inhibitory potency and selectivity of the peptides toward 24-16. Thus, the second tetrapeptide mixture was first developed. The results in Table I also led us to investigate phosphinic peptides with both free N- and C-terminal extremities. Twenty different peptide mixtures, having the general formula Yaa-DL-Phe(PO2CH2)Gly-Yaa, each containing a single amino acid in the Yaa position, while a mixture of 20 different amino acids is present in the Yaa position, were synthesized, and their activities were determined both on 24-15 and 24-16. It should be emphasized that the concentration used to test these mixtures was 1 µM and 10 µM for 24-15 and 24-16, respectively, pointing out the better recognition of these mixtures by 24-16. Furthermore, the data in Fig. 2 indicate that the 24-16 S2 subsite has a clear preference for the inhibitor with a proline residue in the P2 position. The preferred amino acids for 24-15 in this position are histidine and arginine.

Based on these results, the synthesis of 20 different phosphinic peptides of general formula Pro-DL-Phe(PO2CH2)Gly-Yaa was performed, with Yaa representing 20 different amino acids. The influence of the P2 position on the potency of these inhibitors toward these two endopeptidases is reported in Fig. 3. The concentration of each peptide was 100 nM for 24-16 and 40 µM for 24-15, respectively. Here again, the inhibitor containing a proline in the P2 position appears the most potent toward 24-16, while those with an arginine or a lysine in this position are preferred by 24-15. The K, values determined on both peptidases for the Pro-DL-Phe(PO2CH2)Gly-Pro compound in-

![Diagram](image)
Inhibitory potency and selectivity of phosphorus-containing tripeptide mixtures of the general formula Yaa-L-D-Phe(PO₂CH₂)Gly-Yaa for endopeptidases 24–16 and 24–15.

Values for Kᵢ were determined as described under “Materials and Methods.” Assays were carried out in 5 mM TricineNaOH, 0.1 mg/ml bovine serum albumin in the presence (endopeptidase 24–15) or absence (endopeptidase 24–16) of 0.1 mM dithiothreitol. Selectivity corresponds to the ratio IC₅₀(24–16)/IC₅₀(24–15).

| Inhibitors                  | IC₅₀ (24–16) | IC₅₀ (24–15) | Selectivity |
|-----------------------------|--------------|--------------|-------------|
| Pro-L-Phe(PO₂CH₂)Gly-Yaa'   | 5            | 880          | 176.0       |
| N-Ac-Pro-L-Phe(PO₂CH₂)Gly-Yaa' | 600         | 1020         | 1.7         |
| Pro-L-Phe(PO₂CH₂)Gly-Yaa-‘NH₂ | 150         | 215          | 1.4         |
| N-Ac-Pro-L-Phe(PO₂CH₂)Gly-Yaa-‘NH₂ | 585         | 880          | 1.5         |
| Yaa-Pro-L-Phe(PO₂CH₂)Gly     | 18           | 60           | 3.3         |
| N-Ac-Yaa-Pro-L-Phe(PO₂CH₂)Gly | 6           | 15           | 1.9         |
| Yaa-Pro-L-Phe(PO₂CH₂)Gly-‘NH₂ | 14           | 380          | 27.1        |
| N-Ac-Yaa-Pro-L-Phe(PO₂CH₂)Gly-‘NH₂ | 167         | 300          | 1.8         |

**DISCUSSION**

The development of a systematic approach based on combinatorial chemistry of phosphinic peptides led us to identify a potent and selective inhibitor of 24-16. The ability of 24-16 to interact specifically with Yaa-Phe(PO₂CH₂)Gly-Yaa’ peptides is intriguing, since the importance of the presence of a C-terminal tripeptide from the scissile bond of natural substrates seemed to indicate that a residue in the P₃ position was a strong requirement for 24-16 recognition (10). In agreement with this, the addition of norleucine in the P₃ position of the inhibitor produces a more potent inhibitor of 24-16 (Table II, compounds 1–6). But as is also shown in this table, the residue in the P₃ position plays a much more crucial role in inhibitor binding for 24-16 than for 24-15. Based on this important result, analysis of the role played by the Yaa and Yaa’ positions in the rather selective Yaa-Phe(PO₂CH₂)Gly-Yaa’ mixture rapidly results in the identification of the preference of the 24-16 S₂ and S₃ subsites for proline, as compared with 24-15, leading to the first potent inhibitor able to discriminate between 24-16 and 24-15.

This study also provides evidence for the essential role played by the free amino acid group in the P₃ position of the inhibitors in their binding to 24-16. For example, the acetylation of the proline in compound 1 promotes a decrease in affinity of almost 3 orders of magnitude. It should be emphasized that in most of the literature reports, peptide substrates or inhibitors are screened with blocked extremities, because...
charged extremities are believed to play a repulsive role in the binding of these molecules to the active site of endopeptidases (22, 23).

Another important reason to use a systematic approach is related to the possibility of gaining deeper insight into the specificity of these peptidases. The analysis of the effects caused by systematic substitution of a particular position, both in the present and previous study, indicates that among 20 amino acids tested, only a few residues, one or two at most, were greatly preferred over the others. Thus, this restricted number justifies the use of a systematic approach to achieve a proper inhibitor potency and selectivity for a particular target. The specificity of the 24-16 S2 subsite, as revealed by this study, could also explain, a posteriori, why the dipeptides Pro-Ile and Pro-Phe are specific blockers of 24-16 (24). In fact, based on our results, one may hypothesize that these peptides interact with the S2 and S3 subsites of 24-16, the specificity of this interaction being ensured by the binding of the free proline to the S2 subsite. This mode of binding will place the C-terminal interaction being ensured by the binding of the free proline to Leu-Gly-Pro-D-Lys(Dnp)OH (14). In fact, the cleavage by 24-16 is able to hydrolyze the synthetic substrate Mcc-Pro-Phe-Gly-Pro-Nle (6) (22, 23). This peptide has also been used for many years to study the respective contribution of these two peptidases in the catabolism of neurotensin in the central nervous system (7) and particularly to examine the influence of these inhibitors on neurotensin-induced hypothermic and analgesic effects after their central administration.

Recently, rat brain 24-16 was sequenced (16) and was found to share 90% homology with a zinc peptidase of rabbit liver. According to Kawabata et al., this peptidase may be involved in the processing of vitamin K-dependent proproteins (33–34). The rat brain peptidase also presents 90% homology with a zinc peptidase able to bind, under particular conditions, angiotensin II and which was later shown to also display peptidase activity (35). Serizawa et al. (36) recently published a study on a 24-16-like peptidase, which is supposed to play an important role at the level of the inner membrane of mitochondria. Without any doubt, the potent and specific inhibitor of 24-16 developed in this study will permit investigation of all these putative functional roles for these 24-16-like peptidases.

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