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Model peptides to study the effects of P_2 and P_3 substitutions in statine-containing HIV proteinase inhibitors

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Through a series of synthetic model peptides, we have examined the structural requirements of the P_2 and P_3 residues in statine-based HIV protease (PR) inhibitors. Results agree with the general observations that, the more bulky the P_3 aromatic hydrophobic side chain, the more potent is the inhibitor. At P_2, an isopropyl side chain is critical in maintaining potency. Three-dimensional modeling demonstrates that the steric bulk of a leucyl residue or the unfavorable energy transfer, from water to enzyme, for a basic amino acid residue at P_2 markedly compromises activity. A naphthylalaninyl-valyl P_3–P_2 substituted analogue inhibits PR with an IC_{50} value of 6 nM, and was also effective as an antiviral agent.

Synthetic inhibitor; Modeling; Antiviral

1. INTRODUCTION

To date, reverse-transcriptase (RT) inhibitors (e.g. AZT) are the only antiviral agents available for treating AIDS (acquired immunodeficiency syndrome), a disease that remains to be lethal. An alternative target for rational antiviral agent design is the virally encoded aspartyl proteinase (PR) which has been shown to be essential for the posttranslational processing of viral $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ polyproteins of the pathogenic HIV. Recent reports suggest that the PR also plays a role in promoting the cellular mechanism of transcribing viral DNA [1] and in the early pre-integration phase of the replication cycle of HIV [2]. In addition, the cytopathic effect of HIV might be related to the proteinase's ability to specifically cleave non-viral substrates as observed in vitro [3,4]. Therefore, PR inhibition might provide very unique opportunities for treating AIDS.

Despite the fact that many synthetic PR inhibitors are reported to be highly potent in vitro ([5] and references therein), the design of PR inhibitors remains to be a challenging topic. We have reported a series of statine (or its variants)-containing hexa- to nonapeptides that are potent inhibitors against the PR as well as being antivirals [6]. We also demonstrated the antiviral efficacy of one mildly active statine-based protease inhibitor (SBPI) in an animal model of murine retroviral disease [7]. The reported results strongly support our and others' opinion that SBPIs represent an important approach in designing more effective antiviral agents either as potentially useful clinical agents or as pharmacological probes to delineate the mechanism of viral infection [8]. Drug resistance has emerged as a serious stumblingblock in antiviral therapy. HIV mutates rapidly, and new strains frequently evolve within the patient that are able to withstand the medicine, often within a few months after the start of the therapy. In designing protease inhibitors, incorporating the unusual amino acid statine at the P1P1′ position, because of its rather broad activity towards different aspartyl proteases, may offer an approach to study and verify the hypothesis that a broad-spectrum HIV protease inhibitor would be more effective in controlling HIV.

In the present work, we used the shortened analogue of a previously reported SBPI as a template to investigate systematically the structural requirements of the P_3 and P_2 residues in SBPIs. The relative potencies of the inhibitors were determined by measuring their IC_{50} values against PR. A computer model of the three-dimensional structure of HIV-1 proteinase and its inhibitors at the active site was then used to analyze the structure–activity relationships (SAR). The model predicts certain interactions between the inhibitors and the S_2 and S_3 subsites in the catalytic center of PR and explains some of the differences in inhibitory activity. Two active inhibitors were tested for their antiviral activity in a cell culture-based assay system. Details of the work are presented as follows.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

All commercial protected amino acids were obtained from Ad-

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vanced ChemTech, Inc. (Louisville, KY) and 2-quinolinecarboxylic acid was from Aldrich Chemical Co. (Milwaukee, WI). The side-chain protecting groups were tosyl for histidine and arginine, benzyl for tyrosine, and 2-chlorobenzyloxycarbonyl for lysine. N^{α}-Boc-*p*-(Fmoc)aminophenylalanine [9] and Boc-L-3(1′-naphthyl)alanine [10] were prepared as described. The synthetic peptides were prepared in a stepwise manner onto *p*-methylbenzhydrylamine resin using a standard manual solid-phase peptide synthesis method. For peptidyl resins carrying a Fmoc-*para*-aminophenylalanine residue, the removal of the Fmoc group was carried out as the last step by treating the peptidyl resin with 30% piperidine in DMF twice, for an hour each time. After HF cleavage, the peptides were purified by preparative HPLC, characterized by amino acid analysis and fast atom bombardment mass spectrometry.

2.2. *Assays*

In vitro PR inhibitory activities of the synthetic analogues were evaluated by an assay system consisting of site-specific proteolysis, at pH 5.5, of a fluorescent synthetic substrate by a recombinant HIV-1 proteinase we reported previously [6]. The antiviral potency assay was performed using human T-lymphoblastoid CEM and MT2 cells as reported [6].

2.3. *Molecular modeling*

The crystal structure of HIV-1 protease has been resolved independently by different laboratories [11–13]. In the present molecular modeling work, we have adopted the coordinates of the three-dimensional structure of HIV-1 protease reported by Wlodawer et al. [12] obtained from the Brookhaven Protein Databank. The molecular modeling method involved the minimization of the interaction energy between a statine-containing peptide inhibitor and the enzyme dimer. Energy calculations were performed using the AMBER 3.0 program. Initially, we utilized the atomic model of inhibitor MVT-101 and its orientation in the enzyme-inhibitor complex reported by Miller et al. [15] in positioning a statine residue in the catalytic site of HIV-1 protease. In doing so, a statine residue was docked in the catalytic site in a manner in which its backbone and isobutyl side chain superimposed with the backbone and the P_1 side chain of MVT-101. This orientation of statine resulted in close proximity between the residue's hydroxyl group and the catalytic Asp residues. All residues in the enzyme that were 8.0 Å away from the statine residue were held fixed. The rest of the enzyme molecule, including the active site water molecule, but not the Asp-25 and Asp-124 residues (the AMBER software numbered the enzyme dimer as if it were a single-chain polypeptide. Asp-124 would be Asp-25′ in a dimeric-enzyme numbering system), were allowed to move. The united atom option was invoked for the enzyme but an all atom inhibitor was used. The Asp-25 was modeled as the acid (protonated) residue and the Asp-124 was modeled as a base. After this preliminary model was constructed, the complex structure was refined by using the AMBER energy refinement program. With the statine residue assuming an optimal final position, other residues of the inhibitory peptide were incorporated as a β-sheet backbone. This preliminary enzyme complex was constructed in a manner that maximizes the hydrogen bonds between the peptidyl backbone of the inhibitor and the enzyme, and maximizes the hydrophobic interactions of the amino acid side chains, while minimizing any unfavorable steric interaction. Energy refinement was performed once again as usual. In the resulting model, the β-sheet conformation of the statine-containing inhibitors was preserved, in a manner similar to those of MVT-101 [15] and pepstatine [33]. The differences between our inhibitors and either one of the two known structures, pepstatin and MVT-101, is the significant area of concern. These differences are embodied in the fact that there are different side chains attached to the β-sheet backbone. The resulting minimized structure for one active inhibitor can serve as a starting position for another inhibitor, by changing the appropriate side chains. The graphics representation was achieved with the program INSIGHT and displayed on an Evans and Sutherland PS390.

3. RESULTS AND DISCUSSION

HIV-1 proteinase, a member of the aspartyl protease family, is known to hydrolyze a number of substrates each at a different rate. Therefore, the proteinase, even though it appears to be rather specific in regard to the scissile bonds that it cleaves, accommodates sequences of various structures [16,17]. These observations suggest that, unlike renin which is highly substrate specific, the catalytic subsites of PR can accommodate ligands that are quite different in structure. One may also translate, teleologically, these observations into more opportunities in designing potent PR inhibitors. An approach in rational inhibitor design starts with creating SAR data on subsite specificity of proteinase catalytic site ligands. A number of detailed studies on PR substrate subsite analysis have been reported [18–21] and the data generated has been effectively applied to reduced-bond inhibitor design [22].

While substrate subsite specificity studies have led to general conclucions on certain chemical preference at different subsites within a series of synthetic ligands, such SAR may not always extrapolate to inhibitor binding. Since subsite specificity of PR may also be strongly influenced by the sequence of the substrate [16,21,23,24], it becomes apparent that a systematic subsite specificity search on catalytic site inhibitors would generate more relevant SAR data in designing antiviral agents. In designing PR inhibitors, the incorporation of a hydroxyl-containing transition-state residue in place of the P_1–$P_{1'}$ scissile-bond dipeptide has been proven to be a very effective approach in producing potent inhibitors ([5] and references therein). In analogy to the proteinase's substrates, the affinity of each series of transition-state inhibitors is remarkably influenced by the structures of the side chains at the P_2, P_3, $P_{2'}$ and $P_{3'}$

Table I

Optimal structure at the P_3 position

Code	Sequence (P_3 P_2 P_1 $P_{1'}$ $P_{2'}$ $P_{3'}$)	IC_{50} (nM)
A	Ac-Phe-Val-Statine-Leu-Phe-NH_2	90
B	Ac-paF-Val-Statine-Leu-Phe-NH_2	80
C	Ac-YOM-Val-Statine-Leu-Phe-NH_2	16
D	Ac-Tyr-Val-Statine-Leu-Phe-NH_2	43
E	Ac-Trp-Val-Statine-Leu-Phe-NH_2	13
F	Ac-Pro-Val-Statine-Leu-Phe-NH_2	1,062
G	Ac-Nal-Val-Statine-Leu-Phe-NH_2	6
H	QC-Val-Statine-Leu-Phe-NH_2	3,650

statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; Nal, 3-(1′-naphthyl)alanine; YOM, *O*-methyltyrosine; paF, *p*-aminophenylalanine; QC, quinoline-2-carbonyl. The synthetic peptides were characterized by amino acid analysis and fast atom bombardment mass spectrometry. HIV proteinase inhibition was assayed as outlined in section 2. Each IC_{50} value is the average result of three experiments.

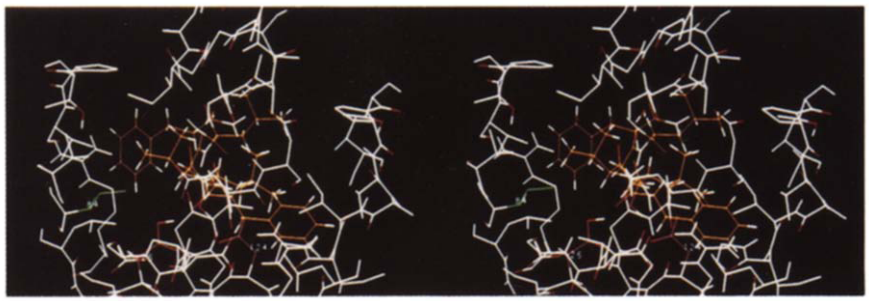

Fig. 1. Stereoscopic computer model of inhibitor A Ac-Phe-Val-Statine-Leu-Phe-NH_2 (orange color) in the HIV-1 active site. Amino acid residues of the proteinase shown are within 8 Å away from the P_2 position in the inhibitor. The catalytic aspartate side chains (numbered 25 and 124) and the statyl-OH group are in red color. The Ile-84 side chain of the S_2 hydrophobic pocket is in green.

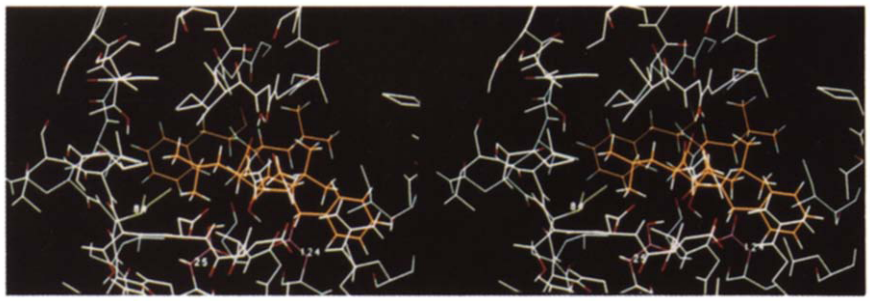

Fig. 2. Stereoscopic computer model of inhibitor I Ac-Phe-Leu-Statine-Leu-Phe-NH_2 (orange color) in the HIV-1 active site. The model shows the occurrence of steric hindrance between the P_2-Leu side chain and that of Ile-84. See legend to Fig. 1 for other description.

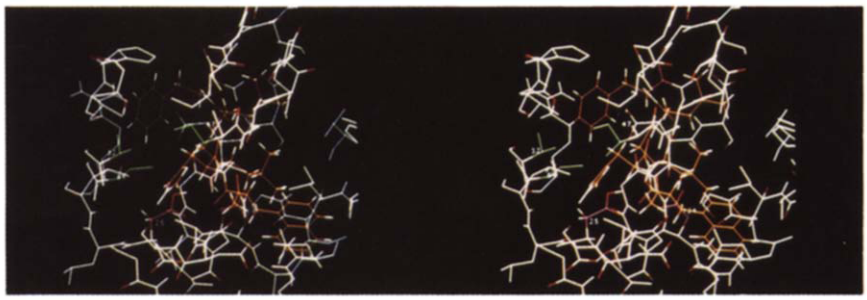

Fig. 3. Stereoscopic computer model of inhibitor L Ac-Nal-His-Statine-Leu-Phe-NH_2 (orange color) in the HIV-1 active site. The side chains of Ile-84, Val-32 and Ile-47 of the S_2 hydrophobic pocket surrounding the P_2-histidyl imidazole group are in green. See legend to Fig. 1 for other description.

positions [25–27]. Therefore, knowledge of the interaction of each of these positions to their corresponding enzyme subsites would help in designing optimal inhibitors. Thus the mode of binding of the P_1 or $P_{1'}$ subsite of a series of hydroxyethylene isostere protease inhibitors was delineated recently through in vitro enzyme inhibition studies and in combination with X-ray crystallography [30–32].

We reported previously a series of potent seven to nine amino acids statine-containing proteinase inhibitors [6]. Based on X-ray crystallography evidence that the PR ligand binding cleft extends from S_3 to $S_{3'}$ subsites [15,31], a series of SBPIs spanning from P_3 to $P_{3'}$, with systematic replacement of either the P_2 or P_3 amino acid residue, were synthesized in attempt to arrive at principles that could be used to design potent SBPIs. Crystallographic analysis of complexes between HIV PR and active-site inhibitors revealed that the S_3 subsite binds the inhibitor's P_3 side chains through hydrophobic interactions [15,32,33]. Billich et al. studied the effect of variations of P_3 structures on the relative cleavage efficiency of a series of synthetic substrates [16]. They reported that aromatic residues, especially Phe and Tyr are preferred over aliphatic (except methionine) residues. In our study, a series of analogues containing aromatic ring structures were synthesized in order to examine the optimal structure. The α-amino termini were acetylated since it was shown that acetyl-pepstatine is significantly more potent than isovaleryl-pepstatine [34] probably due to difference in the solvation effect of the capping structures [33].

Table I summarizes our results on the optimal structure at the P_3 position in binding to the enzyme. The aromatic amino acids tested and their relative affinity towards the proteinase were 1′-naphthylalanine > tryptophan > *o*-methyltyrosine > tyrosine > *p*-aminophen-

ylalanine > phenylalanine. The most potent inhibitor was **G** which exhibited an anti-proteinase IC_{50} value of 6 nM. It appears that the more bulky the aromatic side chain, the more potent the inhibitor is. Our study agrees with those of Prasad et al. who reported the best inhibition activity occurred when either 2′-naphthylalanine or *m*- biphenylalanine was incorporated at the P_3 position of a series of hydroxyethylamine-containing substrate inhibitors [35]. In another report describing molecular modeling of a potent dihydroxyethylene(DHE)-containing inhibitor (1-naphthoxyacetyl-His-DHE-Ile-AMP) complexed with HIV-1 protease, the naphthoxy group is part of a terminal group which allows a large degree of flexibility. The naphthyl ring is not buried in the enzyme model and is lying partially outside the active site with no specific orientation but apparently interacting non-specifically with a number of large hydrophobic regions on the enzyme surface [36]. In contrast, molecular modeling of compound **A** (Fig. 1) and **G** (not shown), when docked in the active cleft of PR, demonstrates, in the case of the statine-containing inhibitors, that both the phenyl and naphthyl rings are buried within the enzyme. They are stacked parallel to the Asp-Arg ion pair in the enzyme, and against the Val of the inhibitor in position P_2. Since the inhibitor backbone maintains a β-sheet conformation it limits the position and orientation of the α-methylene unit (corresponding to the ether oxygen of the naphthoxy group above). These restraints force the naphthyl group in the statine-containing inhibitors into the position shown in Fig. 3. In addition, the conformation of this naphthyl group is fixed and cannot rotate 180 degrees to be more exposed to solvent, since to do so would result in a steric interaction between the methylene group at the naphthylene-1-position and the hydrogen at the naphthylene-8- position (peri interaction).

In agreement with a substrate subsite specificity study [16], Pro at P_3 was not well tolerated in our inhibitor. Compound **F** having a Pro at P_3 was three orders of magnitude less potent than its naphthylalanyl analogue **G**. Through a systematic SAR search, Roberts et al. discovered a quinoline-2-carbonyl (QC) moiety at P_3 is a highly preferred structure in designing potent proteinase inhibitors that contain an (*R*)-hydroxyethylamine $P_1P_{1'}$ replacement [25]. However, in contrast to what had been observed, the substitution at P_3 with a QC group in statine-containing inhibitors did not produce a potent inhibitor. Compund **H** was mildly active (IC_{50} = 3.65 μM) when compared with other P_3-aromatic members of the series.

We previously reported that for statine-containing octapeptide inhibitors, PR discriminates remarkably between a valine and a histidine at the P_2 position. The virus-specific proteinase accommodated inhibitors with valine at P_2 but apparently rejected the histidyl analogues. In the present study, we further characterized the structural requirements of the P_2 residue in a series of pentapeptides. Table II lists the potency values of the peptides substituted at P_2 position with aliphatic, polar (neutral), and basic residues. Although it is believed, like the S_3 subsite, that the S_2 subsite of PR is hydrophobic in nature, limited crystallographic detail is available in analysing the interaction between the subsite and its ligand side chain structure. However, empirical replacements of the P_2 residue have been based on hydrophobic (e.g. [31]) and polar characteristics (e.g. [25]) of the substituting residue in comparison to those occurring in the natural substrates [16]. In the present series of statine-containing peptides, there is a dramatic difference in preference of valine over leucine at P_2 even though both residues are hydrophobic aliphatic homologues. Changing from an isopropyl to an isobutyl side chain resulted essentially in an inactive peptide (**A** vs. **I**). This result shows a stringent steric requirement at the enzyme S_2 pocket. It was reported that synthetic chromogenic substrates consisting of β-branched residues, Val or Ile, at the P_2 position were hydrolyzed by PR at a faster rate than that of a Leu-containing analogue [19]. The polar amino acid Asn occurs repeatedly as a P_2 residue in a number of known substrates for HIV PR. This observation led to a popular approach of incorporating Asn immediately adjacent to the amino side of a transition-state $P_1P_{1'}$ replacement when designing potent proteinase inhibitors (see [5]). However, in the statine-containing series (Table II) Asn, as well as Gln, remarkably lowered potency. Both Asn- or Gln-substituted peptides (**K** and **J**) were three orders of magnitude less potent than the valyl analogue (**G**). A similar result was observed by Richards et al. in three pseudo-palindromic sequences substituted by an aromatic variant of statine at $P_1P_{1'}$ carrying an Asn or Gln at the P_2 position, the peptides were either weak inhibitors or inactive [17].

In our experience with statine-containing analogues that span from $P_{3'}$ to P_5 or P_6, a histidine at P_2 produced ineffective PR inhibitors [6]. In contrast to our observation, a highly potent hydroxyethylene-containing proteinase inhibitor that spans from $P_{3'}$ to P_3 with a histidine at the P_2 position has been reported [36]. We extended our study to the effect of other basic amino

Table II

Comparison of P_2 structures[a]

Code	Sequence	IC_{50} (nM)
A	Ac-Phe-Val-Statine-Leu-Phe-NH_2	90
I	Ac-Phe-Leu-Statine-Leu-Phe-NH_2	~10,000
G	Ac-Nal-Val-Statine-Leu-Phe-NH_2	6
J	Ac-Nal-Gln-Statine-Leu-Phe-NH_2	3,300
K	Ac-Nal-Asn-Statine-Leu-Phe-NH_2	4,600
L	Ac-Nal-His-Statine-Leu-Phe-NH_2	7,200
M	Ac-Nal-Lys-Statine-Leu-Phe-NH_2	na
N	Ac-Nal-Arg-Statine-Leu-Phe-NH_2	na

[a] See footnote to Table I. The symbol na indicates no significant inhibitory activity detected at 10 μM.

acids (lysine and arginine) at the P_2 position (Table II). In vitro inhibition assay (pH 5.5) results show that analogues (**M** and **N**) carrying these residues were devoid of activity when tested up to 10 μM concentration. Our observation is consistent with a substrate-hydrolysis study, in which a Lys at the P_2 position prevented cleavage of a synthetic substrate [21]. The histidyl analogue **L** retained a weak inhibitory activity probably because the aromatic imidazole group partially compensated the loss of activity caused by a positively charged side chain.

From an examination of our three-dimensional model, it is apparent that an isopropyl side chain of the P_2 residue of an inhibitor fits well in the S_2 pocket without causing steric hindrance. Fig. 1 shows that in **A**, the P_2-valine side chain is surrounded by the enzyme's amino residues Ala-28, Val-32, Ile-47, Ile-84 and Ile-149 which make up the S_2 hydrophobic pocket. Whereas, when the free-energy minimized **A** was converted to **I** (by replacing valine with leucine residue), an obvious steric hindrance of the isobutyl side chain with Ile-84 is then easily seen (Fig. 2). While there are several low energy P_2-Leu side chain conformations in addition to the one shown here, all interfere sterically in some way. Although minimizing the energy reduces some of the unfavorable steric interactions, new strains are created in other regions of the complex. For minimal steric interaction, the distance between the united-atom carbon of Ile-84 and a substrate hydrogen should be greater than the sum of the van der Waals radii which is 3.0 Å. The results of the calculations for the P_2-histidine containing compound **L** are shown in Fig. 3. Our starting conformation for **L** is based on a model for **G** (not shown) in which the phenyl ring in the P_3-position of **A** is replaced by a naphthalene ring. This was further altered to produce a model for **L** by minimizing the two different torsion angles for the histidine alpha-beta bond. Figs. 1 and 3 show that the pocket around the Val in **A** or the one around the His in **L** is essentially hydrophobic. The later pocket is delineated by the hydrophobic side chains of residues Val-32, Ala-28, Ile-84, Ile-149 and Ile-47 (compare with Fig. 1). It appears that the very weak activity of **L** is due to the energetically unfavorable transfer of the hydrophilic histidine from an aqueous environment to the hydrophobic environment of the enzyme. No hydrogen bonds can be made to the histidine side chain without desolvating it, an energetically unfavorable process. In the case of **L**, the histidine residue does not interfere sterically but does not have an opportunity, either, to make effectively hydrogen bonds in the hydrophobic environment. Desolvation of the histidine residue is then an important factor that reduces activity. In the cases where P_2 is a charged residue (as for Lys and Arg in **M** and **N**, respectively), desolvation and steric hindrance would be expected to play a role in deteriorating the potency.

Since compounds **G** and **A** were shown to inhibit the enzymatic function of PR (**G** being most potent and the other moderately active), we also tested their antiviral activity against HIV-1 in CEM and MT-2 cells [6]. Assay results (Table III), indeed, show these compounds were effective antiviral agents although their antiviral potency was lower than those usually reported for AZT (3′-azido-2′,3′-dideoxythymidine) or ddC (2′,3′-dideoxycytidine) [6] which are RT inhibitors. Considering factors like antiviral potency and peptidyl nature, it is unlikely that either **G** or **A** is a desirable clinical agent for the treatment of AIDS. Nevertheless, these two compounds might be economical experimental tools useful in delineating certain antiviral pharmacological principles. Additional modifications at the $P_{2'}$ and $P_{3'}$ positions may further enhance potency [37,38].

Very recently, a combined regimen of more than one RT inhibitor has been shown to elicit synergistic in vitro antiviral effect. Wouldn't it be even more beneficial in antagonizing HIV by means of a dual inhibitory strategy acting on both PR and RT?

Table III

Antiviral effects of synthetic HIV proteinase inhibitors

Compound	CEM		MT-2	
	ID_{50} (μM)	TD_{50} (μM)	ID_{50} (μM)	TD_{50} (μM)
G	1.09	>129	4.12	>129
A	3.37	>138	12.6	>138

Each value is the average result of three to four experiments each of which generated triplicate data.

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REFERENCES

[1] Riviere, Y., Blank, V., Kourilsky, P. and Isreal, A. (1991) Nature 350, 625–626.
[2] Baboonian, C., Dalgleish, A., Bountiff, L., Gross, J., Oroszlan, S., Rickett, G., Smith-Burchnell, C., Troke, P. and Merson, J. (1991) Biochem. Biophys. Res. Commun. 179, 17–24.
[3] Shoeman, R.L., Kesselmier, C., Mothes, E., Honer, B. and Traub, P. (1991) FEBS Lett. 278, 199–203.
[4] Oswald, M. and von der Helm, K. (1991) FEBS Lett. 292, 298–300.
[5] Huff, J.R. (1991) J. Med. Chem. 34, 2305–2314.
[6] Hui, K.Y., Manetta, J.V., Gygi, T., Bowdon, B.J., Keith, K.A., Shannon, W.M. and Lai, M.T. (1991) FASEB J. 5, 2606–2610.
[7] Lai, M.H., Tang, J., Wroblewski, V., Dee, A.G., Margolin, N., Vlahos, C., Bowdon, B., Buckheit, R., Colacino, J. and Hui, K.Y. (1993) J. Acquir. Immune Defic. Syndr. 6, 24–31.

[8] Fehrentz, J.A., Chomier, B., Bignon, E., Venaud, S., Chermann, J.C. and Nisato, D. (1992) Biochem. Biophys. Res. Commun. 188, 873–878.
[9] Hui, K.Y., Siragy, H.M. and Haber, E. (1992) Int. J. Pept. Protein Res. 40, 152–160.
[10] Porter, J., Dykert, J. and Rivier, J. (1987) Int. J. Pept. Protein Res. 30, 13–21.
[11] Navia, M.A., Fitzgerald, P.M.D., McKeever, B.M., Leu, C.T., Heimbach, J.C., Herber, W.K., Sigal, I.S., Darke, P.L. and Springer, J.P. (1989) Nature 337, 615–620.
[12] Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J. and Kent, S.B.H. (1989) Science 245, 616–621.
[13] Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J.R., Whittle, P.J., Danley, D.E., Geoghegan, K.F., Hawrylik, S.J., Lee, S.E., Scheld, K.G. and Hobart, P.M. (1989) Nature (London) 342, 299–302.
[15] Miller, M., Schneider, J., Sathyanarayana, B.K., Toth, M.V., Marshall, G.R., Clawson, L., Selk, L., Kent, S.B.H. and Wlodawer, A. (1989) Science 246, 1149–1152.
[16] Billich, A. and Winkler, G. (1991) Arch. Biochem. Biophys. 290, 186–190.
[17] Richards, A.D., Phylip, L.H., Farmerie, W.G., Scarborough, P.E., Alvarez, A., Dunn, B.M., Hirel, P.H., Konvalinka, J., Strop, P., Pavlickova, L., Kostka, V. and Kay, J. (1990) J. Biol. Chem. 265, 7733–7736.
[18] Jupp, R.A., Phylip, L.H., Mills, J.S., Le Grice, S.F. and Kay, J. (1991) FEBS Lett. 283, 180–184.
[19] Konvalinka, J., Strop, P., Velek, J., Cerna, V., Kostka, V., Phylip, L.H., Richards, A.D., Dunn, B.M. and Kay, J. (1990) FEBS Lett. 268, 35–38.
[20] Griffiths, J.T., Phylip, L.H., Konvalinka, J., Strop, P., Gustchina, A., Wlodawer, A., Davenport, R.J., Briggs, R., Dunn, B.M. and Kay, J. (1992) Biochemistry 31, 5193–5200.
[21] Tozser, J., Weber, I.T., Gustchina, A., Blaha, I., Copeland, T.D., Louis, J.M. and Oroszlan, S. (1992) Biochemistry 31, 4793–4800.
[22] Urban, J., Konvalinka, J., Stehlikova, J., Gregorova, E., Majer, P., Soucek, M., Andreansky, M., Fabry, M. and Strop, P. (1992) FEBS Lett. 298, 9–13.
[23] Margolin, N., Heath, W., Osborne, E., Lai, M. and Vlahos, C. (1990) Biochem. Biophys. Res. Commun. 167, 554–560.
[24] Billich, S., Knoop, M.-T., Hansen, J., Strop, P., Sedlacek, J., Mertz, R. and Moelling, K. (1988) J. Biol. Chem. 263, 17905–17908.
[25] Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Krohn, A., Lambert, R.W., Merrett, J.H., Mills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L., Thomas, G.J. and Machin, P.J. (1990) Science 248, 358–361.
[26] Rich, D.H., Green, J., Toth, M.V., Marshall, G.R. and Kent, S.B. (1990) J. Med. Chem. 33, 1285–1288.
[27] Dreyer, G.B., Metcalf, B.W., Tomaszek Jr., T.A., Carr, T.J., Chandler III, A.C., Hyland, L., Fakhoury, S.A., Magaard, V.W., Moore, M.L., Strickler, J.E., Debouck, C. and Meek, T.D. (1989) Proc. Natl. Acad. Sci. USA 86, 9752–9756.
[28] Dreyer, G.B., Lambert, D.M., Meek, T.D., Carr, T.J., Tomaszek Jr., T.A., Fernandez, A.V., Bartus, H., Cacciavillani, E., Hassell, A.M., Minnich, M. et al. (1992) Biochemistry 31, 6646–6659.
[29] Young, S.D., Payne, L.S., Thompson, W.J., Gaffin, N., Lyle, T.A., Britcher, S.F., Graham, S.L., Schultz, T.H., Deana, A.A., Darke, P.L. et al. (1992) J. Med. Chem. 35, 1702–1709.
[30] Thompson, W.J., Fitzgerald, P.M., Holloway, M.K., Emini, E.A., Darke, P.L., McKeever, B.M., Schleif, W.A., Quintero, J.C., Zugay, J.A., Tucker, T.J. et al. (1992) J. Med. Chem. 35, 1685–1701.
[31] Erickson, J., Neidhart, D.J., VanDrie, J., Kempf, D.J., Wang, X.C., Norbeck, D.W., Plattner, J.J., Rittenhouse, J.W., Turon, M., Wideburg, N., Kohlbrenner, W.E., Simmer, R., Helfrich, R., Paul, D.A. and Knigge, M. (1990) Science 249, 527–533.
[32] Swain, A.L., Miller, M.M., Green, J., Rich, D.H., Schneider, J., Kent, S.B. and Wlodawer, A. (1990) Proc. Natl. Acad. Sci. USA 87, 8805–8809.
[33] Fitzgerald, P.M., McKeever, B.M., VanMiddlesworth, J.F., Springer, J.P., Heimbach, J.C., Leu, C.T., Herber, W.K., Dixon, R.A. and Darke, P.L. (1990) J. Biol. Chem. 265, 14209–14219.
[34] Richards, A.D., Roberts, R., Dunn, B.M., Graves, M.C. and Kay, J. (1989) FEBS Lett. 247, 113–117.
[35] Prasad, J.V.N.V., Sun, C.Q., Houseman, K., Mueller, R.A. and Rich, D.H. (1992) in: Proceedings of the Twelfth American Peptide Symposium. Peptides Chemistry and Biology (Smith, J.A. and Rivier, J.E., Eds.) pp. 721–722, ESCOM Science Publisher, Leiden, The Netherlands.
[36] Thaisrivongs, S., Tomasselli, A.G., Moon, J.B., Hui, J., McQuade, T.J., Turner, S.R., Strohbach, J.W., Howe, W.J., Tarpley, W.G. and Heinrikson, R.L. (1991) J. Med. Chem. 34, 2344–2356.
[37] Lyle, T.A., Wiscount, C.M., Guare, J.P., Thompson, W.J., Anderson, P.S., Darke, P.L., Zugay, Z.A., Emini, E.A., Schleif, W.A., Qunitero, J.C., Dixon, R.A.F., Sigal, I.S. and Huff, J.R. (1991) J. Med. Chem. 34, 1228–1230.
[38] deSolms, S.J., Giuliani, E.A., Guare, J.P., Vacca, J.P., Sanders, W.M., Graham, S.L., Wiggins, J.M., Darke, P.L., Sigal, I.S., Zugay, J.A., Emini, E.A., Schleif, W.A., Quintero, J.C., Anderson, P.S. and Huff, J.R. (1991) J. Med. Chem. 34, 2852–2857.