Molecular Size of an Anti-HIV Peptide, T22, Can Be Reduced without Loss of the Activity

Michinori Waki,* Koji Waki, Kenji Miyamoto, Akiyoshi Matsumoto, Hirokazu Tamamura,† Nobutaka Fujii,† Tsutomu Murakami,†† Hideki Nakashima,††† and Naoki Yamamoto††

Tokyo Research Institute, Seikagaku Corporation, Higashiyamato, Tokyo 207

†*Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606*

††*Department of Microbiology, Tokyo Medical and Dental University, School of Medicine, Bunkyo-ku, Tokyo 113*

†††*Department of Microbiology, Yamanashi Medical University, Tamaho-cho, Yamanashi 409-38*

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An 18-peptide, T22, has been shown to have a strong anti-human immunodeficiency virus (HIV) activity comparable to that of 3'-azido-3'-deoxy-thymidine (AZT). Several shortened analogs of T22 were designed, synthesized and evaluated of their anti-HIV activities. A 14-peptide analog with one disulfide bond, TW70 (des-Cys^{8,13}, Tyr^{9,12}-[D-Lys^{10}, Pro^{11}]-T22), showed comparable activity to that of T22, indicating that the molecular size of T22 could be reduced without loss of the activity.

Tachyplesins (Tac) and polyphemusins (Pol) isolated from the hemoctes of the horseshoe crabs, *Tachypleus tridentus* and *Limulus polyphemus*, respectively, have antimicrobial and antifungal activities.[1-3] Furthermore, Tac I (Figure 1) was shown to have an anti-viral activity against HIV-1, vesicular stomatitis virus and influenza A virus.[4,5] Structure-activity relationship (SAR) studies on these horseshoe peptides lead to the findings of a novel peptide, T22 ([Tyr^{5,12}, Lys^{7}]-Pol II) (Figure 1), which exihibited strong anti-HIV activity and relatively low cytotoxicity *in vitro*.[6,7] T22 is a cationic 18-residue peptide amide with two disulfide bridges. T22 was also shown by ^{1}H-NMR analysis to take an antiparallel β-sheet structure with a type II β-turn.[8] In the present study, in order to reduce the molecular size of T22 without loss of the activity and to search for more suitable lead compound for developing anti-HIV agents, several shortened analogs of T22 were designed, synthesized and evaluated of their anti-HIV activities on the basis of the information obtained so far from SAR studies.[9,10]

The SAR studies on the horseshoe antimicrobial peptides and related synthetic peptides including T22 indicate that the existence of antiparallel β-sheet structure with β-turn, two disulfide bonds, two repeats of Tyr-Arg-Lys sequence, two Arg residues at the N-terminus and one Arg residue at the C-terminus is important for exhibiting high anti-HIV activity. The outer disulfide bridge is also shown to be associated with the activity rather than the inner one. Based on these information concerning the structural and conformational requirements for revealing high anti-HIV activity, several shortened analogs of T22 were designed in which essential parts of T22, one disulfide bridge corresponding to the outer one and 14 amino acid residues lacking the inner disulfide bridge are retained to fulfill these requirements. β-Turn part (-Lys-Gly-) of T22 was changed to more rigid -D-Lys-Pro- or -Pro-D-Lys- sequence. It was also intended that the introduction of D-Lys at the turn part might resist attacking of proteases such as trypsin. Modified peptides of a representative shortened analog were also designed to clarify the contribution of amino groups in the analog to the activity. Thus, the N-terminal α-amino group or ε-amino group of D-Lys at the turn part was modified with carboxylic acids or amino acids,

Tac I	K-W-C-F-R-V-C-Y-R-G--I--C-Y-R-R-C-R-NH$_2$
Pol II	R-R-W-C-F-R-V-C-Y-K-G--F-C-Y-R-K-C-R-NH$_2$
T22	R-R-W-C-Y-R-K-C-Y-K-G-Y-C-Y-R-K-C-R-NH$_2$
1	R-R-W-C-Y-R-K------ $_D$K-P-------Y-R-K-C-R-NH$_2$
Ac-**1**	Ac-R-R-W-C-Y-R-K------ $_D$K-P-------Y-R-K-C-R-NH$_2$
Myr-**1**	Myr-R-R-W-C-Y-R-K------ $_D$K-P-------Y-R-K-C-R-NH$_2$
FTC-**1**	FTC-R-R-W-C-Y-R-K------$_D$K-P-------Y-R-K-C-R-NH$_2$
[$_D$Orn^8]**1**	R-R-W-C-Y-R-K----- $_D$Orn-P-----Y-R-K-C-R-NH$_2$
[$_D$K(Gly)^8]**1**	R-R-W-C-Y-R-K----$_D$K(Gly)-P---Y-R-K-C-R-NH$_2$
[$_D$K(Apa)^8]**1**	R-R-W-C-Y-R-K---$_D$K(Apa)-P---Y-R-K-C-R-NH$_2$

Figure 1. Amino acid sequences of Tac I, Pol II, T22, TW70 **1**and modified **1** analogs. The two disulfide bonds are formed between the outer Cys residues and between the inner ones, respectively. **1** and its derivatives have only one disulfide bond. One letter symbols of amino acids are C, Cys; F, Phe; G, Gly; I, Ile; K, Lys; P, Pro; R, Arg; V, Val; W, Trp; Y, Tyr. Other abbreviations: Apa,5-aminopentanoic acid; $_D$K, D-Lys; $_D$Orn, D-ornithine; FTC, fluoresceinthiocarbamoyl; Myr, myristoyl.

respectively: the modification of the α-amino group diminishes the cationic character, but of the ε-amino group retains the cationic one. In addition, the α-amino group was also derivatized with fluoresceins in order to obtain fluorescent peptides with anti-HIV activity. Structures of the horseshoe peptides, T22, a representative designed analog, TW70 (des-Cys^{8,13}, Tyr^{9,12}-[D-Lys^{10}, Pro^{11}]-T22) (**1**), the modified peptides of **1**, and [$_D$Orn^8]**1** are shown in Figure 1. [$_D$Orn^8]**1** was selected to examine the positioning effect of the ω-amino group at the turn part to the activity.

The representative TW70 **1** was synthesized by the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase synthesis (SPS)[11] on an Applied Biosystems model 430A peptide synthesizer using FastMoc^{TM} system involving 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) activation for coupling and 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink amide resin) for loading the C-terminal amino acid residue. The Fmoc-amino acids with protected side chain were: Lys(Boc), Tyr(Bu^t), Arg(2,2,5,7,8-pentamethylchroman-6-sulfonyl) and Cys(Trt). The protected **1** Rink amide resin obtained was treated with 1 M (1 M = 1 mol dm^{-3}) trimethylsilyl bromide-thioanisole/trifluoroacetic acid (TFA) system following essentially the procedure of Akaji et al.[11] and Tamamura et al.[12] After air-oxidation, the peptide amide was purified by carboxymethylcellulose column chromatography, followed by HPLC to give the homogeneous product. Modified peptide

Table 1. Anti-HIV activity of T22, TW70 **1** and modified **1** analogs

Compound	CC_{50}[a] μg/cm^3	EC_{50}[b] μg/cm^3	SI[c] CC_{50}/EC_{50}
T22	49	0.017	2900
1	65	0.020	3400
Ac-**1**	78	0.024	3300
Myr-**1**	110	0.055	1800
FTC-**1**	39	0.0010	39000
[$_D$Orn^8]**1**	56	0.021	2700
[$_D$K(Gly)^8]**1**	44	0.030	1500
[$_D$K(Apa)^8]**1**	140	0.017	11000
AZT	8.5[d]	0.0014[d]	6100

[a]50% Cytotoxic concentration (CC_{50}) is based on the reduction of the viability of mock-infected cells. [b]50% Effective concentration (EC_{50}) is based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [c]Selectivity index (SI) is shown as CC_{50}/EC_{50}. Mean values are shown in such cases where the assay was carried out more than three times. [d]μM (1M = 1mol dm^{-3}).

amides of **1**, Ac-**1**, Myr-**1**, [$_D$Orn^8]**1**, [D-Lys(Gly)^8]**1** and [D-Lys(Apa)^8]**1** were synthesized by the Fmoc-based SPS and purified in a similar manner to that of **1**. In the cases of [D-Lys(Gly)^8]**1** and [D-Lys(Apa)^8]**1**, Fmoc-D-Lys(Boc-Gly) and Fmoc-D-Lys(Boc-Apa) were used for the introduction of D-Lys having the modified ε-amino group with amino acid, respectively. Fmoc-D-Orn(Boc) was used for [$_D$Orn^8]**1**. FTC-**1** was prepared by the reaction of fluorescein isothiocyanate isomer I (FITC) in phosphate buffered saline (pH 7.5) with **1**, and purified by Sephadex G-25, followed by Sep-Pak C_{18}. The peptide amides thus obtained were characterized and their purity was assessed by amino acid analysis, fast atom bombardment mass spectrometry, analytical HPLC and capillary electrophoresis, respectively.

Antiviral activity of the peptides against HIV-1 was evaluated on the basis of the protection against virus-induced cytopathogenicity in MT-4 cells following the procedure reported previously.[7] The cytotoxicity of the compounds was also determined on the basis of the viability of mock-infected cells.[7] The anti-HIV activity and cytotoxicity of the peptides are summarized in Table 1 together with those of AZT for comparison. Interestingly, the anti-HIV activity of the designed analog, **1**, was comparable to that of T22 with highly potent activity, indicating that the design was rational. Surprisingly, the modifications of the N-terminal α-amino group of **1** with FTC group having aromatic and anionic characters and of the ε-amino group at the turn part with Apa having an ω-amino group at the end of the flexible chain remarkably enhanced the activity. The presence of bulky and hydrophobic FTC group in FTC-**1** may be responsible for its high activity. Although the exact reasons for the potentiation of the activity by these modifications cannot be explained at present time, such modifications may be very useful for designing of the compounds with higher anti-HIV activity.

In conclusion, the molecular size of T22 with strong anti-HIV activity could be reduced without loss of the activity: 18 amino acid residues to 14 amino acid residues and two disulfide bonds to one disulfide bond. Further, the N-terminal α-amino group or ε-amino group at the turn part of the shortened analog, **1**, could also be modified without loss of the activity or even with enhancement of the activity. Furthermore, fluorescence properties of the FTC group in the biologically active modified peptides can be used as a highly sensitive reporter dye for various purposes. For example, fate, metabolism or distribution of such a peptide within cell, tissue, organ or body infected or non-infected with HIV after administration can be observed by fluorescence microscopy. Thus, the information obtained in this study on the shortened peptides and their modified ones may be very helpful for further designing of the compounds possessing high activity, more stability and low cytotoxicity. To obtain conformational information on these shortened peptides, circular dichroism (CD) and ^{1}H-NMR analyses are now in progress.

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