Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 473–479

BIOORGANIC & MEDICINAL CHEMISTRY

Downsizing of an HIV–Cell Fusion Inhibitor, T22 ([Tyr^{5,12}, Lys^{7}]-Polyphemusin II), with the Maintenance of Anti-HIV Activity and Solution Structure[1]

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Received 29 September 1997; accepted 10 December 1997

Abstract—T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II) has been shown to have strong anti-human immunodeficiency virus (HIV) activity comparable to that of 3′-azido-2′,3′-dideoxythymidine (AZT). T22, an 18-residue peptide amide, takes an antiparallel β-sheet structure that is maintained by two disulfide bridges. Herein we synthesized several shortened analogs of T22 in order to search for a more suitable lead compound. A 14-residue analog having one disulfide bridge, TW70 (des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}]-T22), was found to have highly potent activity comparable to that of T22, and to take an antiparallel β-sheet structure similar to that of T22. This indicates that the molecular size of T22 can be reduced without loss of activity or significant change in the secondary structure, and that TW70 may represent a novel lead compound. Furthermore, modifying the N-terminal α-amino group of TW70 with a fluoresceinthiocarbamoyl group, and the ε-amino group of D-Lys^{8} at the turn portion with a 5-aminopentanoyl group remarkably increased the selectivity index (50% cytotoxic concentration/50% effective concentration).

Introduction

Tachyplesins (I–III) and polyphemusins (I and II) are 17- and 18-residue peptide amides, respectively, possessing two disulfide bonds, which are highly abundant in the hemocyte debris of Japanese horseshoe crabs (*Tachypleus tridentatus*) and American horseshoe crabs (*Limulus polyphemus*). These peptides have antimicrobial activity against Gram-positive and Gram-negative bacteria and some fungi,[2] and possess antiviral activity against vesicular stomatitis virus, influenza A virus and HIV-1.[3,4] In an effort to derive peptide analogs with enhanced anti-HIV activity, we synthesized more than 100 peptide analogs of tachyplesin and polyphemusin.[5] Among these synthetic peptides, we found a novel compound, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), which showed potent anti-HIV activity and relatively low cytotoxicity in vitro. The effective concentration (EC_{50}) of T22 for 50% protection in an assay of HIV-induced cytopathogenicity was 6.0 nM, a value comparable to that of AZT (1.4 nM). T22 is an 18-residue, Arg-rich peptide amide, which takes an antiparallel β-sheet structure which is maintained by two disulfide bridges.[6]

Key words: Anti-HIV peptide; T22; TW70; NMR; tachyplesin.
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0968-0896/98/$19.00
PII: S0968-0896(97)10055-4

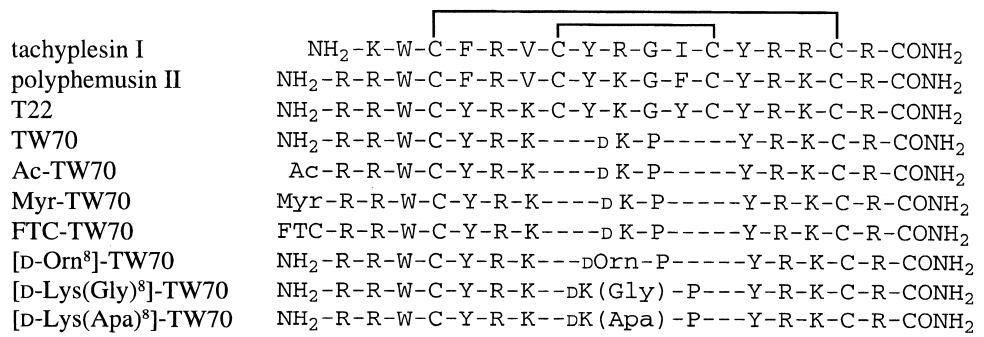

Figure 1. Amino acid sequences of tachyplesin I, polyphemusin II, T22, TW70 and its analogs. Disulfide linkages are shown by solid lines. TW70 and its analogs have one disulfide bridge between Cys^4 and Cys^{13}.

Our previous studies demonstrated that T22 exerts its effect on a process, virus-cell fusion, immediately after virus adsorption,[7] and that it binds specifically to both gp120 (an envelope protein of HIV) and CD4 (a T-cell surface protein).[8] Very recently, we[9] disclosed that T22 exhibits anti-HIV activity through its specific binding to a CXC-chemokine receptor, fusin.[10] At present, it is not clear why T22 interacts with these three proteins which play an important role in virus–cell fusion. A recent structure–activity relationship study of T22 examined contributions of characteristic regions to its activity and cytotoxicity and provided the following information.[5b] The number of Arg residues in the N-terminal and C-terminal regions of T22 is closely correlated with anti-HIV activity. The presence of disulfide rings, especially the major disulfide ring, is indispensable for the maintenance of the secondary structure and for biological activity. Between two repeats of Tyr-Arg-Lys, which represent a characteristic motif in T22, the N-terminal repeat is more closely related to activity.

Based on the above structure–activity consideration, in the present paper we have designed and synthesized several shortened analogs of T22 in order to find more suitable lead compounds. The anti-HIV activity of the new analogs has been correlated with their conformation as analyzed by NMR and CD.

Results

Anti-HIV activity

The amino acid sequences of the synthetic analogs are shown in Figure 1. As shown in Table 1, the anti-HIV activity and selectivity index ($SI = CC_{50}/EC_{50}$) of TW70 (des-[$Cys^{8,13}$, $Tyr^{9,12}$]-[D-Lys^{10}, Pro^{11}]-T22) ($EC_{50} = 7.9$ nM, $SI = 3400$) were comparable to those of T22 ($EC_{50} = 6.0$ nM, $SI = 2900$). Modifications of the N-terminal α-amino group with acetyl and myristoyl groups caused no significant decrease in activity or SI (see Ac-TW70 and Myr-TW70). FTC-TW70, which had a fluoresceinthiocarbamoyl group at the N-terminus of TW70, showed 20-fold higher activity and 10-fold higher SI than TW70. Substitutions for D-Lys^8 at the turn portion by D-Orn and D-Lys(Gly) caused no change in activity or SI (see [D-Orn^8]-TW70 and [D-Lys(Gly)^8]-TW70). [D-Lys(Apa)^8]-TW70 [D-Lys(Apa)^8-substitution for D-Lys^8] showed 3-fold higher SI than TW70.

NMR spectroscopy of TW70

Spin-system identification was achieved by performing DQF-COSY and HOHAHA experiments in H_2O and D_2O. Sequential assignment[11] was subsequently made using two sets of NOESY spectra measured at 28 °C and 10 °C. Both data were useful for the unambiguous assignment of NMR resonances around the water signal, which were bleached due to water suppression. Results are summarized in Table 2. Sequential NOE connectivities and spin coupling constants between

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

Compound	EC_{50} (nM)	CC_{50} (μM)	SI
T22	6.0	17	2900
TW70	7.9	26	3400
Ac-TW70	8.1	26	3300
Myr-TW70	18	35	1800
FTC-TW70	0.30	12	39000
[D-Orn^8]-TW70	7.0	19	2700
[D-Lys(Gly)^8]-TW70	9.7	14	1500
[D-Lys(Apa)^8]-TW70	5.4	45	11000
AZT	1.4	8.5	6100

EC_{50} values are the concentrations for 50% protection of HIV-induced cytopathogenicity in MT-4 cells. CC50 values are based on the reduction of the viability of mock-infected cells. SI is shown as CC_{50}/EC_{50}. All data are mean values for at least three experiments.

NH and α-proton resonances ($^3J_{NH,\ \alpha H}$) are shown in Figure 2. The strong $d_{\alpha,N}$ connectivities associated with a small number of $d_{N,N}$ connectivities suggest the presence of an antiparallel pleated β-sheet structure from residue 4 to 13 with a β-turn formed by residues Lys[7], D-Lys[8], Pro[9] and Tyr[10]. This structure is consistent with the values of spin coupling constants $^3J_{NH,\ \alpha H}$, where all the coupling constants are more than 8 Hz, except for that of Pro[9]. This β-sheet structure was further confirmed by the presence of long-range NOEs between the protons of (Tyr[5] NH, Lys[12] NH), (Lys[7] NH, Tyr[10] NH), (Tyr[5] NH, Cys[13] Hα) and (D-Lys[8] Hα, Tyr[10] NH) (Figure 2). These NOEs are characteristic of protons located in the interior of β-sheets on opposite β-strands. The presence of slow-exchange amide protons ($t_{1/2} > 5$ min) could not be confirmed. Furthermore, we made stereospecific assignments of the prochiral β-methylene protons of Cys[4] and Cys[13]. Intra-residual NOEs between amide and

Table 2. ^{1}H chemical shifts of TW-70 at 28 °C and pH 3.0

Residue	Chemical shift (ppm)[a]			
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others
Arg[1]	—	4.07	1.87,1.87	$C^{\gamma}H$ 1.50, 1.50 $C^{\delta}H$ 3.08, 3.08 $N^{\varepsilon}H$ 6.95
Arg[2]	8.68	4.61	1.74,1.74	$C^{\gamma}H$ 1.65, 1.59 $C^{\delta}H$ 3.16, 3.16 $N^{\varepsilon}H$ 7.11
Trp[3]	8.66	4.82	3.31, 3.25	N1H 10.09 C2H 7.25 C4H 7.64 C5H 7.11 C6H 7.20 C7H 7.47
Cys[4]	8.29	5.28	3.00, 2.71	
Tyr[5]	8.73	4.70	3.01, 2.98	C2, 6H 6.98 C3, 5H 6.71
Arg[6]	8.36	4.72	1.81,1.75	$C^{\gamma}H$ 1.58, 1.50 $C^{\delta}H$ 3.14, 3.14 $N^{\varepsilon}H$ 7.16
Lys[7]	8.74	4.54	1.78,1.78	$C^{\gamma}H$ 1.57, 1.57 $C^{\delta}H$ 1.72, 1.72 $C^{\varepsilon}H$ 3.02, 3.02 $N^{\zeta}H$ 7.54
D-Lys[8]	8.43	4.52	1.88,1.88	$C^{\gamma}H$ 1.42,1.42 $C^{\delta}H$ 1.71, 1.71 $C^{\varepsilon}H$ 3.04, 3.04 $N^{\zeta}H$ 7.54
Pro[9]	—	4.46	2.10,1.70	$C^{\gamma}H$ 1.85, 1.25 $C^{\delta}H$ 3.81, 3.62
Tyr[10]	7.72	4.66	3.12, 2.97	C2, 6H 7.19 C3, 5H 6.87
Arg[11]	8.26	4.65	1.75,1.59	$C^{\gamma}H$ 1.43, 1.43 $C^{\delta}H$ 3.13, 3.11 $N^{\varepsilon}H$ 7.72
Lys[12]	8.46	4.49	1.64,1.41	$C^{\gamma}H$ 1.35, 1.35 $C^{\delta}H$ 1.71, 1.71 $C^{\varepsilon}H$ 2.97, 2.94 $N^{\zeta}H$ 7.73
Cys[13]	8.63	5.28	3.00, 2.93	
Arg[14]	8.73	4.49	1.94,1.81	$C^{\gamma}H$ 1.73, 1.73 $C^{\delta}H$ 3.21, 3.21 $N^{\varepsilon}H$ 7.14
$CONH_E$	7.77			
$CONH_Z$	7.26			

[a]Chemical shifts are measured ± 0.01 ppm at 600 MHz frequency.

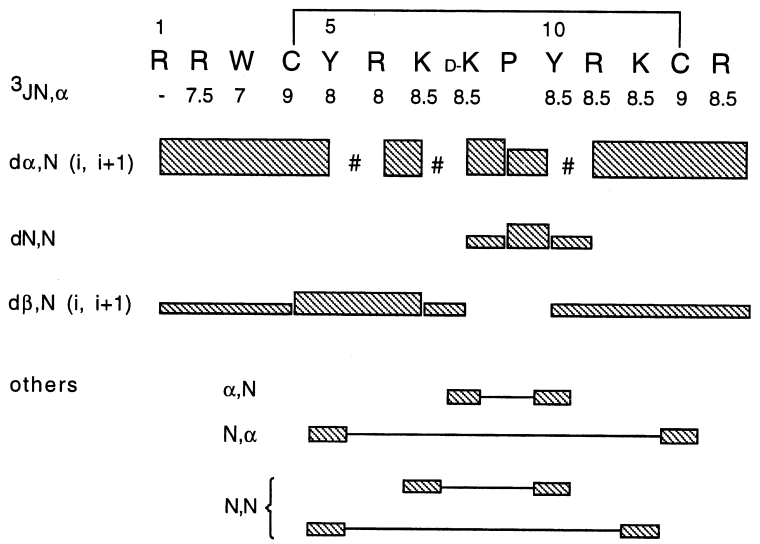

Figure 2. Amino acid sequence of TW70 and main experimental data. Shaded bars indicate that NOEs were observed. Height is related to the relative intensity of cross-peaks. *J*-couplings are indicated in Hz, and $d_{\alpha,N}$ are compiled over two temperatures: 10 and 28 °C. Connectivities to Pro[9] $C_{\delta}H$ resonances are listed in the location for the corresponding backbone amide proton. (#), $d_{\alpha,N}$ could not be determined due to overlapping of signals.

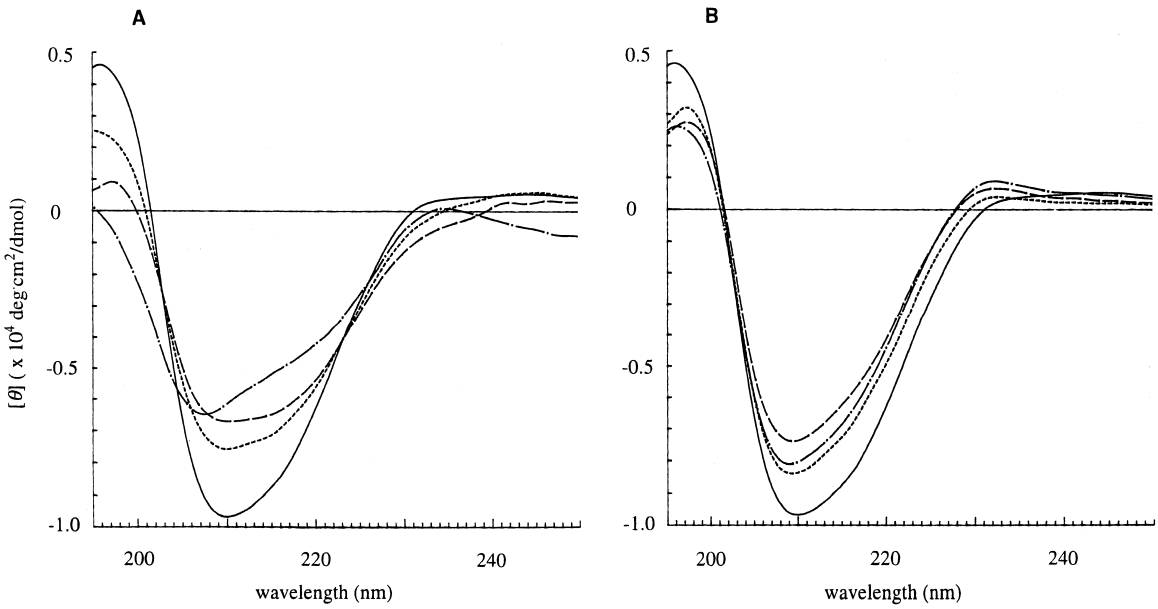

Figure 3. CD spectra of TW70 and its analogs. (A) Solid line: TW70, dotted line: Ac-TW70, dashed line: Myr-TW70, center-dotted line: FTC-TW70. (B) Solid line: TW70, dotted line: [D-Orn^8]-TW70, dashed line: [D-Lys(Gly)^8]-TW70, center-dotted line: [D-Lys(Apa)^8]-TW70.

β-protons and between α- and β-protons led unambiguously to their stereospecific assignments (data not shown). Thus, the conformation of the two Cys residues around the C_α and C_β bonds (χ^1) was found to be a t^2g^3 type, indicating that the conformational structure around the disulfide bridge was fairly rigid.

CD spectroscopy of TW70 and its analogs

CD spectra of TW70 and its analogs in aqueous solution are shown in Figure 3. TW70 exhibited a strong negative band near 210 nm and a strong positive band near 197 nm. These bands, which are similar to those seen with tachyplesin I and T22, are due to β-sheet structures,[12] and are compatible with the NMR data. The CD spectra of Ac-TW70 and Myr-TW70 were similar to that of TW70, indicating that they formed β-sheet structures (Figure 3A). FTC-TW70 also showed a strong negative band near 210 nm and a strong positive band near 197 nm, which were slightly different from those in the case of TW70. This difference might be due to the fluoresceinthiocarbamoyl group. Three analogs substituted at the turn portion ([D-Orn^8]-TW70, [D-Lys(Gly)^8]-TW70 and [D-Lys(Apa)^8]-TW70) showed CD spectra similar to that of TW70 (Figure 3B).

Discussion

Currently, several effective agents blocking HIV–cell fusion have been investigated.[13] We have sought virus–cell fusion inhibitors derived from tachyplesin and polyphemusin, self-defense peptides of horseshoe crabs, and have found an 18-residue tachyplesin analog, T22, which is an attractive candidate as a new lead compound for anti-AIDS drug development. Subsequently, we have attempted to reduce the molecular size of T22 without loss of the activity or significant change in solution structure, and to develop more efficient compounds.

In the present study, we designed and synthesized shortened analogs, intended to contain the essential pharmacophores of T22, on the basis of the information obtained from a structure–activity relationship study of T22 (as described in the Introduction). Among these peptides, a 14-residue peptide amide, TW70, fulfills the requirements for anti-HIV activity: TW70 contains two Arg residues in the N-terminal region and one Arg residue in the C-terminal region, has only the major disulfide ring and not a minor one, and has two repeats of Tyr-Arg-Lys. The Lys-Gly sequence in the β-turn region of T22 is replaced with the D-Lys-Pro sequence in TW70, anticipating the stabilization of type II′ β-turn conformation. The anti-HIV activity and SI of TW70 were comparable to those of T22 and AZT. The anti-HIV activity of TW70 was also confirmed by the inhibitory effect on the HIV-1 p24 antigen expression examined by indirect immunofluorescence with polyclonal anti-HIV-1 antibody:[14] $EC_{50} = 17$ nM. Furthermore, conformational analysis by NMR and CD showed that the secondary structure of TW70 resembled

that of T22,[5b,6] and that TW70 was composed of an antiparallel β-sheet structure with a β-turn (Fig. 4). While the β-turn of T22 was type II, that of TW70 proved to be type II′ based on the following: The turn of TW70 (Lys^7-D-Lys^8-Pro^9-Tyr^{10}) contains a D-amino acid (D-Lys^8) at the $i+1$ position, and the value of the spin coupling constant ($^3J_{NH,\alpha H}$) of this residue (8.5 Hz) is consistent with a type II′ β-turn structure. In addition, a D-amino acid-Pro sequence accommodates itself to the ($i+1$, $i+2$) positions of the type II′ β-turn structure, represented by Gramicidin S, which consists of antiparallel β-sheet and two type II′ β-turns at both ends of the β-sheet.[15]

As a result, TW70 maintains both the anti-HIV activity and the conformational structure of T22, although it is shortened (18 to 14 amino acids) and has only one disulfide bridge. In our previous study,[5b] [Ala^{8,13}]-T22, which was substituted by Ala for the Cys^{8,13} corresponding to the minor disulfide bridge of T22, showed 6 times less activity than T22, and slightly less β-sheet composition (64%) than T22 (73%), suggesting that the maintenance of activity and conformation requires the minor disulfide bridge. However, since TW70 has a more rigid turn sequence (D-Lys^8-Pro^9) than that of T22 (Lys^{10}-Gly^{11}), the minor disulfide bridge might not be essential for TW70, and two Cys-Tyr sequences in T22 can be deleted. Therefore, TW70 is a more rational and suitable lead compound than T22. The side chains of Tyr^5, Lys^7, Tyr^{10} and Lys^{12} are directed to one side of the plane of the β-sheet, while the side chains of Arg^6 and Arg^{11} and a disulfide bridge are to the other side (Figure 4). T22 is a three residues-substituted analog of polyphemusin II (Phe^5 to Tyr, Val^7 to Lys, Phe^{12} to Tyr), and the side chains of these three amino acids protrude in the same direction from the plane of the β-sheet,[6] as in the case of TW70. Therefore, three amino acids (Tyr^5, Lys^7 and Tyr^{10}) of TW70 and this side of the plane of the β-sheet might be related to the expression of high activity.

Modifications of the N-terminal α-amino group and the ε-amino group of the turn portion of TW70 caused no remarkable change in its secondary structure (as described in Results). Thus, increase or decrease in the activity of these modified analogs may not be due to changes in their secondary structures, but due to the functionalities of the modified groups themselves. FTC-TW70 has 20-fold higher activity and 10-fold higher SI than TW70, indicating that its bulky, anionic and hydrophobic fluoresceinthiocarbamoyl group may be responsible for high activity. FTC-TW70 may be used as a labeled marker in various experiments, such as investigations of metabolism and distribution. [D-Lys(Apa)^8]-TW70, with an Apa group at the turn portion, has 3-fold higher SI than TW70. Since [D-Lys(Apa)^8]-TW70 and T22 are of almost the same secondary structure, the distance between the ω-amino group of D-Lys(Apa)^8 and the N- or C-terminal end of [D-Lys(Apa)^8]-TW70 is nearly equal to that between the e-amino group of Lys^{10} and the N- or C-terminal end of T22, providing that the flexible side-chain of D-Lys(Apa)^8 protrudes in a straight line. This distance might be related to the increase in SI of [D-Lys(Apa)^8]-TW70. The exact reasons for the potentiation of activity and SI by these modifications cannot be explained at the present time.

In conclusion, the molecular size of T22 could be reduced without loss of anti-HIV potency and with the maintenance of an antiparallel β-sheet structure, which is thought to be associated with high activity. TW70, which is a shortened analog, is a 14-residue peptide with one disulfide bridge, while T22 is an 18-residue peptide with two disulfide bridges. It may be concluded that the potency possessed by TW70 renders it a novel lead compound of anti-HIV agents and an attractive candidate for the chemotherapy and prophylaxis of HIV infections. In addition, two modified peptides possessing β-sheet structures were found to exhibit higher SI values than that of TW70. The present results will aid in

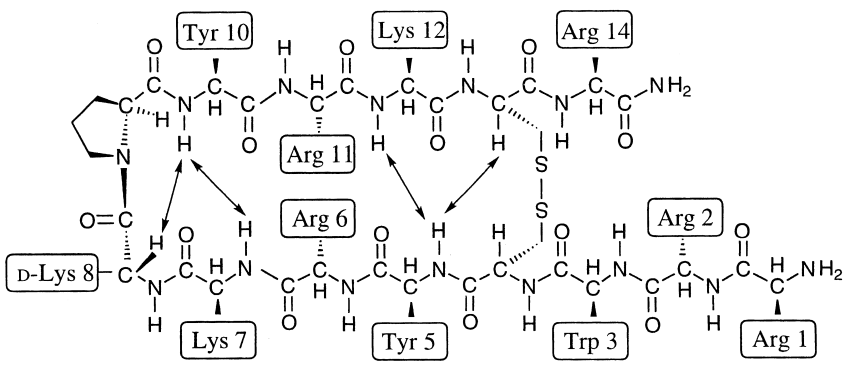

Figure 4. Schematic representation of the structure of TW70 deduced from NMR analysis. The observed inter-strand NOE connectivities between NH and $C_\alpha H$ protons are shown by arrows.

rationally designing efficient compounds having higher potency and lower cytotoxicity.

Experimental

Materials

TW70 and its analogs were prepared according to the previous procedure.[1,16] Briefly, the representative TW70 was synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase synthesis with FastMoc™ strategy involving 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) activation using an Applied Biosystems model 430A peptide synthesizer. 4-(2′, 4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink amide resin)[17] was used in combination with the following Fmoc-protected amino acids: Lys(Boc), D-Lys(Boc), Tyr(Bu^t), Arg(Pmc) and Cys(Trt). Crude Cys(SH)^{4,13}-TW70 was cleaved from the resin along with deprotection of the protecting groups by the trimethylsilyl bromide system,[18] and was then air-oxidized and purified by preparative HPLC to give the pure peptide (TW70). Modified peptides of TW70 (Ac-TW70, Myr-TW70, [D-Orn^8]-TW70, [D-Lys(Gly)^8]-TW70 and [D-Lys(Apa)^8]-TW70) were synthesized in a similar manner to that of TW70. During the synthesis of Ac-TW70 and Myr-TW70, N-terminal α-amino groups of the protected TW70-resins were acetylated and myristoylated, respectively. In the synthesis of [D-Lys(Gly)^8]-TW70 and [D-Lys(Apa)^8]-TW70, Fmoc-D-Lys(Boc-Gly) and Fmoc-D-Lys(Boc-Apa) were used for the introduction of D-Lys^8 having the ε-amino group acylated with Gly and Apa, respectively. FTC-TW70 was prepared by the reaction of TW70 with fluorescein isothiocyanate isomer I (FITC) in phosphate buffered saline (pH 7.5), then purified by Sephadex G-25, followed by Sep-Pak C_{18}. The peptides thus obtained were characterized and their purity was assessed by amino acid analysis, mass spectrometry, HPLC and capillary electrophoresis.

Anti-HIV assay

A strain of HIV-1, HIV-1(III_B), was used for the anti-HIV assay. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1(III_B) cells. Antiviral activity against HIV-1 was determined based on the protection against virus-induced cytopathogenicity in MT-4 cells. Various concentrations of the test compounds were added to HIV-1 infected MT-4 cells at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (2.5×10^4/well). After 5 days' incubation at 37°C in a CO_2 incubator, the number of viable cells was determined using the 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[7] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method.

NMR spectroscopy of TW70

TW70 was dissolved at 10 mM concentration in either 99.9% D_2O or a mixture of H_2O and D_2O (9:1). The pH was adjusted to 3.0 in the NMR tube with μL increments of 0.1 M DCl in D_2O. Spectra of TW70 were recorded on a Bruker AM 600 spectrometer at 600 MHz ^1H frequency at 28 °C and 10 °C. Chemical shifts were quoted relative to the internal standard 4, 4-dimethyl-4-silapentane-1-sulfonate. DQF-COSY,[19] HOHAHA[20] and NOESY[21] were all recorded in the phase-sensitive mode according to States' method.[22] For the H_2O solution sample, the huge water resonance was suppressed by selective saturation using irradiation. Two-dimensional data of TW70 were displayed as $2\,K\times512\,W$ matrix points. Mixing times for NOESY experiments were set at 250 ms and 400 ms.

CD spectroscopy of TW70 and its analogs

Peptides were dissolved in H_2O at concentrations of 10 μM. CD spectra were recorded on a Jasco J-720 spectropolarimeter using 1 cm cells at 1 nm intervals, with five scans averaged for each.[12]

Acknowledgements

The authors are grateful to Dr. Terrence R. Burke, Jr., NCI, NIH, for valuable discussions during the preparation of this manuscript.

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3. Abbreviations used: HIV, human immunodeficiency virus; T22, [Tyr^{5,12}, Lys^7]-polyphemusin II; EC_{50}, 50% effective concentration; AZT, 3′-azido-2′,3′-dideoxythymidine; NMR, nuclear magnetic resonance; CD, circular dichroism; TW70, des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}]-T22; Ac, acetyl; Myr, myristoyl; Orn, ornithine; Apa, 5-aminopentanoic acid; FTC, fluoresceinthiocarbamoyl; DQF-COSY, double quantum filtered correlated spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; SI, selectivity index; CC_{50}, 50% cytotoxic concentration; NOE, nuclear Overhauser effect; AIDS, acquired

immunodeficiency syndrome; Fmoc, 9-fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bu^t, *tert*-butyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt, triphenylmethyl; MTT, 3′-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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