Molecular Parameters for the Anti-Human Immunodeficiency Virus Activity of T22 ([Tyr\textsuperscript{5,12}, Lys\textsuperscript{7}]-Polyphemusin II)

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T22 ([Tyr\textsuperscript{5,12}, Lys\textsuperscript{7}]-polyphemusin II) was found to exhibit strong anti-human immunodeficiency virus (HIV) activity and exert its effects on a virus-cell fusion process. In the present study, the all-D enantiomer of T22 and its related compounds were synthesized to examine the molecular parameters required for the interaction of T22 with membrane components of cells or viruses in order to exert this anti-HIV activity. The anti-HIV activity of these analogs was investigated in comparison with their membrane permeability with aspect to large unilamellar vesicles (LUV's). The all-D enantiomer of T22 exhibited a 20-fold lower anti-HIV activity compared with T22, whereas they both showed the same membrane permeability. No positive correlation between anti-HIV activity and membrane permeability was observed. These results suggest that the anti-HIV activity of T22 is mediated through the interaction with chiral component(s) of the cell or virus.

Keywords T22; anti-human immunodeficiency virus peptide; chiral component; tachyplesin; polyphemusin

Tachyplesin and polyphemusin, which are highly abundant in the hemocyte debris of the Japanese horseshoe crab (Tachypleus tridentatus) and the American horseshoe crab (Limulus polyphemus), respectively, inhibit the growth of gram-positive and gram-negative bacteria and some fungi.\textsuperscript{2,3} Tachyplesin I and its isopeptides, tachyplesin II and III, are 17-residue peptide amides possessing two disulfide bonds. Both polyphemusin I and polyphemusin II consist of 18 amino acid residues with an additional arginine residue at the amino-terminal end.\textsuperscript{2,3} These peptides have three tandem repeats of a tetrapeptide, hydrophobic amino acid-Cys-hydrophobic amino acid-Arg(Lys), suggesting that these peptides are amphiphilic in nature. Generally, amphiphilic peptide antibiotics interact electrostatically and/or hydrophobically with phospholipid bilayers to exhibit antimicrobial activities.\textsuperscript{4} Indeed, it has been shown that the tachyplesin I-induced enhancement of bacterial membrane permeability may cause its antimicrobial action.\textsuperscript{5} It has also been suggested that depolarization of the cytoplasmic membrane, followed by the outer membrane for gram-negative bacteria becoming permeable, might contribute to the bactericidal activity of tachypleisin I.\textsuperscript{6}

Tachyplesin I exhibits antiviral activity against HIV-1.\textsuperscript{7,8} However, its cytotoxicity is relatively strong and the antiviral activity is only seen at concentrations several times lower than the cytotoxic concentration. While searching for more effective and less cytotoxic tachyplesin or polyphemusin analogs, we found that a novel compound, T22 ([Tyr\textsuperscript{5,12}, Lys\textsuperscript{7}]-polyphemusin II), showed strong anti-HIV activity and low cytotoxicity,\textsuperscript{9} and that T22 exerted its effect on a process, most probably virus-cell fusion, occurring immediately after virus adsorption.\textsuperscript{9}

An all-D configuration amino acid-containing peptide exists in a mirror image conformation to the all-L parent peptide. There are several examples of synthetic all-D peptide antibiotics which have been shown to exhibit antibacterial activity nearly identical with those of the all-L enantiomers.\textsuperscript{10} Additionally, these D enantiomers induce the same electrical conductivity in artificial lipid membranes as their L enantiomers. These results suggest that the formation of the ion-channel pores spanning the membranes, without specific interaction with chiral receptors or enzymes, contribute to the bactericidal activity of these peptides. It has been reported that a retroenantiomer, with the direction of the peptide bonds reversed, is topologically superimposable on its parent compound, and that this isomer, in some cases, can retain biological activity.\textsuperscript{11} In the present study, the all-D enantiomer and retroenantiomers of T22 (Fig. 1) were synthesized to investigate whether a close molecular contact with the chiral components of membranes is required for the expression of the anti-HIV activity of T22. Additionally,

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Fig. 1. Amino Acid Sequences of Tachyplesin I, Polyphemusin II and Their Analogs, with Alignment Based on Their Homology

The disulfide linkages are shown by solid lines. The D enantiomers of these peptides were also prepared.

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the pairs of enantiomers of tachyplesin I and polyphemusin II were prepared. We wish to report anti-HIV activity of these peptides and membrane permeability against LUVs with respect to these peptides.

MATERIALS AND METHODS

Compounds All the peptides were chemically synthesized by Fmoc-based solid-phase methods as described elsewhere. Fmoc amino acid derivatives bearing protecting groups based on tert-butyl alcohol were employed, together with Arg(Mtr) and Cys(MeOBzl), and in the synthesis of all-α- and retro-all-α-T22, a combination of δ-Cys(MeOBzl) and δ-Cys(Acm) was selected to establish the two disulfide bonds correctly. Deprotection except for the S-Acm group and cleavage of the peptide from a resin were performed by 1 M TMSBr-thioanisole (TFA) treatment in the presence of m-cresol and ethanedithiol. The reduced peptides were subjected to air-oxidation to establish the disulfide bonds and, in the case of all-α- and retro-all-α-T22, the peptides which had one disulfide bridge were treated with iodine to establish the second disulfide bond. The oxidized peptides were purified by reversed-phase high-performance liquid chromatography (HPLC). The compound 3'-azido-2',3'-dideoxycytidine (AZT) was also used in this study to compare its anti-HIV activity with those of the peptides. AZT was obtained from Sigma Chemical Co. (St. Louis, MO.).

Circular Dichroism (CD) CD spectra were recorded on a Jasco J-720 spectropolarimeter. Peptides were dissolved in H2O at a concentration of 10 μM. Measurements were conducted in a 1-cm cell (Fig. 2) at 1-nm intervals, and 5 scans were averaged.

Cells and Culture Human T-cell lines, MT-4 and MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Viruses HIV-1 was obtained from the culture supernatant of MOLT-4/HTLV-IIIb cells, and stored at −80 °C until used.

Anti-HIV-1 Assay The antiviral activity against HIV-1 was determined on the basis of the protection against virus-induced cytopathogenicity in MT-4 cells. Various concentrations of the test compounds were added to each HIV-1 infected MT-4 cell at a multiplicity of infection (MOI) of 0.01, and placed on the wells of a flat-bottomed microtiter tray (2.5 × 10⁶/well). After 5 d incubation at 37 °C in a CO2 incubator, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The cytotoxicity of the compounds was determined on the basis of the viability of mock-infected cells by the MTT method. The anti-HIV-1 activity was also determined as the inhibitory effect on virus-specific antigen expression.

HIV-1-infected MT-4 cells (MOI=0.01) were cultured with various concentrations of test compound, and then viral antigen expression was examined by indirect immunofluorescence (IF), with polyclonal anti-HIV-1 antibody as a probe, and monitored by laser flow cytometry (Epics profile II; Coulter Electronics, Inc., Hialeah, FL).

Calcein Leakage from LUVs The details of the LUV preparation have been described elsewhere. Briefly, a lipid film composed of acidic phospholipid, phosphaditylglycerol (PG), or zwiterionic phospholipid, phosphaditylcholine (PC), was prepared in a round-bottom flask and hydrated with a 70 mM calcein solution (pH 7.0). The suspension was vortexed and extruded through polycarbonate filters (0.6 μm pore size × 5 times, 0.1 μm × 10 times) using an extruder (Lipex Biomembranes Inc.). Untrapped calcein was removed by gel-filtration on Sephadex G-50 in a buffer (10 mM Tris-HCl/150 mM NaCl/1 mM EDTA, pH 7.0). The lipid concentration was determined by phosphorus analysis. The LUV suspension was mixed with peptide solution in a cuvette. The leakage of calcein from the LUVs was monitored by measuring the fluorescence intensity at 520 nm (excited at 490 nm) on a Shimadzu RF-5000 spectrofluorometer. The fluorescence intensity corresponding to 100% leakage was determined by adding 20 μl 10% Triton X-100 solution to 2 ml of the sample solution.

RESULTS AND DISCUSSION

Chemical and Physical Properties of Synthetic Peptides The amino acid sequences of the peptides studied here are listed in Fig. 1. All peptides were judged to be pure on the basis of HPLC and amino acid analyses. The structures of the monomeric disulfide forms of the peptides were ascertained by fast atom bombardment mass spectrometry (FAB-MS). The disulfide array of the t-form peptides was assigned on the basis of the amino acid analysis of the leucine aminopeptidase digest of tryptic fragments. In the synthesis of all-α- and retro-all-α-T22, the combination of the S-α-MeOBzl group (cleavable with 1 M TMSBr-thioanisole/TFA) and S-Acm group (cleavable by iodine-oxidation with simultaneous disulfide bond formation) was selected for thiol protection to establish two disulfide bonds.

Fig. 2. CD Spectra of Enantiomeric Pairs of T22 and Retro-T22
Solid line, T22; dotted line, all-α-T22; dashed line, retro-T22; center-dotted line, retro-all-α-T22.
bonds regioselectively. The stereochemical antipodal relationships of the all-L and all-D peptides were demonstrated by CD spectropolarimetry (Fig. 2). A nuclear magnetic resonance (NMR) study has revealed that T22 forms a rigid antiparallel β-sheet connected by a type-II β-turn 18 resembling tachyplesin I 19 as depicted in Fig. 3. In aqueous solution, equivalent but mirror image CD spectra were obtained for each enantiomer pair (spectra of enantiomer pairs of tachyplesin I and polyphemusin II not shown), indicating that all of them except for the retroenantiomers of T22 had largely a β-sheet structure. 20 The CD curves for the retroenantiomers of T22, with ellipticity equivalent but opposite sign, indicated that the structure of these peptides was different from those of the others. The β-sheet structure composition of the T22 retroenantiomers (44.4%) was lower than that of T22 and all-D-T22 (73.4%). The preparation of retroenantiomer-isomers which had topologically superimposable relationships with the corresponding parent isomers was unsuccessful.

**Anti-HIV Activity** The anti-HIV activity is summarized in Table I. T22 strongly inhibited HIV-1 induced viral expression in HIV-infected MT-4 cells. Its 50% inhibitory concentration (IC50) was 0.0060 μM, while its 50% cytotoxic concentration (CC50) was 13.4 μM. The IC50 of T22 is nearly equal to that of AZT which is currently used for the treatment of acquired immunodeficiency syndrome (AIDS). However, all-D-T22, the anti-pode of T22, exhibited only weak anti-HIV activity, and retro-all-D-T22 exhibited about one hundredth the anti-HIV activity of T22, since these peptides had neither the same topology as the corresponding parent peptides nor the rigid antiparallel β-sheet structure. As for the other peptides, the D enantiomer was also less potent than the L enantiomer in each pair, except for tachyplesin I. These results suggest that the chirality of T22 is an indispensable factor in determining anti-HIV activity, unlike antibacterial peptides such as cecropin A, magainin 2 amide, and melitin. 19 Thus, the anti-HIV activity of T22 is thought to be mediated through the interaction with chiral component(s) of cells or viruses. The CC50 values of the enantiomeric pairs of both tachyplesin I and polyphemusin II were almost the same, as was that of T22, although the values for these peptides were higher (less cytotoxic) than those for the previous four peptides.

**Calcine Leakage Assay** The membrane permeability induced by tachyplesin analogs was investigated with respect to the calcine leakage out of the LUVs of PG and PC. T22 and all-D-T22 induced almost the same degree of calcine leakage as did the other enantiomer-pairs of tachyplesin analogs (Fig. 4). In addition, the potency of T22 is ten times lower than that of tachyplesin I and polyphemusin II. All peptides exhibited a lower potency against PC than PG. These results indicate that T22 analogs exert their effects against lipid bilayers without chiral selectivity, that is, these peptides simply interact electrostatically and/or

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**TABLE I. Anti-HIV Activity of Tachyplesin I, Polyphemusin II and Synthetic Analogs**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)a</th>
<th>EC50 (μM)b</th>
<th>CC50 (μM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tachyplesin I</td>
<td>0.70</td>
<td>1.53</td>
<td>6.8</td>
</tr>
<tr>
<td>All-D-tachyplesin I</td>
<td>0.58</td>
<td>1.19</td>
<td>7.1</td>
</tr>
<tr>
<td>Polyphemusin I</td>
<td>0.12</td>
<td>0.27</td>
<td>5.8</td>
</tr>
<tr>
<td>All-D-polyphemusin II</td>
<td>0.63</td>
<td>1.45</td>
<td>7.3</td>
</tr>
<tr>
<td>T22</td>
<td>0.0060</td>
<td>0.046</td>
<td>13.4</td>
</tr>
<tr>
<td>All-d-T22</td>
<td>0.12</td>
<td>0.39</td>
<td>12.8</td>
</tr>
<tr>
<td>Retro-T22</td>
<td>0.24</td>
<td>0.50</td>
<td>13.8</td>
</tr>
<tr>
<td>Retro-all-d-T22</td>
<td>0.86</td>
<td>1.09</td>
<td>6.2</td>
</tr>
<tr>
<td>AZT</td>
<td>0.010</td>
<td>0.015</td>
<td>209.3</td>
</tr>
</tbody>
</table>

a) Indirect IF assay. b) MTT assay.
hydrophobically with the lipid bilayer. The poorer membrane permeability of T22 may be attributable to the lower amphiphilic nature of T22 relative to tachyplesin I or polymethemusin II. Since tachyplesin I and T22 have β-sheet conformations with a distinct dichotomy composed of a hydrophobic surface and a hydrophilic surface, the replacement of Val in the hydrophobic surface (tachyplesin I, polymethemusin II) with Lys(T22) leads to a decrease in the amphiphilic nature of T22. Generally, reducing the amphiphilic nature of antimicrobial peptides interacting with membranes is known to result in loss of activity. These facts suggest that the amphiphilic nature of tachyplesin or T22 is an element in determining their membrane permeability and that there is no positive correlation between anti-HIV activity and membrane permeability. Taken together with the CC₅₀ values of these peptides, the membrane permeability seems to be related to their cytotoxicity.

To develop T22-based drugs for AIDS-therapy, it is important to discover the mechanism of action of T22. In the present study, we found that the anti-HIV activity of T22 was likely mediated through the interaction of this peptide with chiral receptors or enzymes. In addition, the target molecule(s) of T22 with respect to its anti-HIV activity were revealed to be different from those involved in its cytotoxicity: one being chiral and the other achiral. Further study of this mechanism might help in the development of new types of anti-AIDS drugs with novel mechanisms of action.

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REFERENCES AND NOTES

1) The following abbreviations are used: T22=[Tyr₅₋₁₂, Lys₇₋₁₁] polymethemusin II, HIV=human immunodeficiency virus, LUVs=large unilamellar vesicles, Fmoc=9-fluorenylmethoxycarbonyl, Mtr=4-methoxy-2,3,6-trimethylphenylsulfonyl, MeOBzI=4-methoxybenzyl, Acn=acetamidomethyl, TMSBr=trimethylsilyl bromide, TFA=trifluoroacetic acid, CD=cardinal dichroism, AZT=3’-azido-2’,3’-dideoxycytidine, MOI=multiplicity of infection, MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PG=phosphatidylglycerol, PC=phosphatidylcholine.


