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Pharmacophore Identification of a Specific CXCR4 Inhibitor, T140, Leads to Development of Effective Anti-HIV Agents with Very High Selectivity Indexes

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Abstract—A polyphemusin peptide analogue, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), and its shortened potent analogues, T134 (des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}, L-citrulline^{16}]-T22 without C-terminal amide) and T140 {[L-3-(2-naphthyl)alanine^{3}]-T134}, strongly inhibit the T-cell line-tropic (T-tropic) HIV-1 infection through their specific binding to a chemokine receptor, CXCR4. T22 is an extremely basic peptide possessing five Arg and three Lys residues in the molecule. In our previous study, we found that there is an apparent correlation in the T22-related peptides between the number of total positive charges and anti-HIV activity or cytotoxicity. Here, we have conducted the conventional Ala-scanning study in order to define the anti-HIV activity pharmacophore of T140 (the strongest analogue among our compounds) and identified four indispensable amino acid residues (Arg^{2}, Nal^{3}, Tyr^{5}, and Arg^{14}). Based on this result, a series of L-citrulline (Cit)-substituted analogues of T140 with decreased net positive charges have been synthesized and evaluated in terms of anti-HIV activity and cytotoxicity. As a result, novel effective inhibitors, TC14003 and TC14005, possessing higher selectivity indexes (SIs, 50% cytotoxic concentration/50% effective concentration) than that of T140 have been developed.

Introduction

Recently, combination therapy using multi-types of anti-HIV drugs, such as reverse transcriptase inhibitors and protease inhibitors, has improved the clinical treatment of HIV-1-infected patients.[1] However, this chemotherapy has not yet reached the stage of perfection owing to the possibility of the appearance of drug-resistant virus, side effects, high cost, etc. Thus, further development of different type drugs is required for multiple drug-combination chemotherapy. In 1996, coreceptors for the entry of HIV-1 were identified: a CXC-chemokine receptor, CXCR4, and a CC-chemokine receptor, CCR5, are major coreceptors for the entry of T cell line-tropic (T-tropic) HIV-1[2] and macrophage-tropic (M-tropic) HIV-1,[3] respectively. Since the identification of coreceptors, several compounds targeting chemokine receptors have been reported by us[4] and others.[5] We showed that an anti-HIV peptide, T22 ([Tyr^{5,12}, Lys^{7}]-polyphemusin II), is a CXCR4 inhibitor that blocks T-tropic HIV-1 entry. T22 is a synthetic analogue, which was previously found by us on the basis of self-defense peptides of horseshoe crabs, tachyplesins and polyphemusins.[6] T22 is an 18-residue peptide amide, and takes an antiparallel β-sheet structure that is maintained by two disulfide bridges.[7] In addition, we synthesized several downsized analogues, such as TW70 (des-[Cys^{8,13}, Tyr^{9,12}]-[D-Lys^{10}, Pro^{11}]-T22), which is a 14-residue peptide amide having one disulfide and maintains an antiparallel β-sheet structure possessing a type II′ β-turn with D-Lys^{8}-Pro^{9} at the ($i+1$) and ($i+2$) site.[8] T22 and TW70 are amphiphilic peptides containing both basic residues (five Arg and three Lys residues) and hydrophobic residues, exhibiting total +9 positive charges. The electrostatic and/or hydrophobic interaction of T22 and TW70 with membranes might be

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related to its cytotoxicity.[9,10] Based on the structure–activity relationship (SAR) study of TW70, we synthesized several effective analogues, such as T134 ([L-citrulline[12]]-TW70 without C-terminal amide), which has stronger anti-HIV activity and significantly less cytotoxicity when compared to TW70 or T22.[11] T134 is a 14-residue peptide with one disulfide bridge (Fig. 1), and also takes an antiparallel β-sheet structure. In the course of development of T134, we found that there is an apparent correlation between the number of the total positive charges and anti-HIV activity or cytotoxicity. The reductions of total positive charges resulted in even less cytotoxicity, whereas extreme reductions caused a significant decrease in anti-HIV activity. The number of total +6 or +7 charges seems to be a suitable balance between activity and cytotoxicity. The reductions of the number of total positive charges by substitution with nonbasic polar amino acids, such as Glu or L-citrulline (Cit), were useful for developing effective analogues possessing high activity and low cytotoxicity. Cit is an analogue having an Arg-like isosteric structure without charges. T134 is an analogue with total +7 charges, which contains one Cit substituted for Lys[12] of TW70. According to our previous results from the Glu-substitution study (Glu-substituted analogues have total +6 charges),[11] analogues having two Cit residues (total +6 charges) might be more effective than T134 (total +7 charges). As such, it was our expectation that +6 charged analogues of T134 would provide a good scaffold to develop more effective compounds. Recently, we found a more potent CXCR4 inhibitor, T140 {[L-3-(2-naphthyl)alanine[3]]-T134}, which has L-3-(2-naphthyl) alanine (Nal)[3] instead of Trp[3] of T134 (Fig. 1).[12] Thus, T140 has been used as a new template in the present SAR study. Initially, in order to identify the pharmacophore of T140, the conventional Ala-substitution scanning was conducted for all amino acid residues except two Cys residues in T140. The consequent general information obtained on the importance of each intrinsic residue in T140 allowed us to discover effective anti-HIV peptides with less cytotoxicity through Cit-scanning of basic amino acids.

Synthesis of T140 Analogues

The protected peptidyl resins were constructed by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase methodology on *p*-benzyloxy benzyl alcohol resins (0.1 mmol). Fmoc-protected amino acid derivatives (2.5 equiv) were successively condensed using 1,3-diisopropylcarbodiimide (2.5 equiv) in the presence of *N*-hydroxybenzotriazole (2.5 equiv) according to the reported schedule.[13] The following side-chain protected Fmoc amino acids were used: Cys(Trt), Arg(Pbf) (Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl), Tyr(Bu^t), Lys(Boc) and D-Lys(Boc). The resulting protected peptidyl resins (30 μmol) were treated with 1 M trimethylsilyl bromide–thioanisole/trifluoroacetic acid (5 mL) in the presence of *m*-cresol (250 μL, 100 equiv) and 1,2-ethanedithiol (100 μL, 60 equiv) at 4 °C for 2 h. The crude Cys(SH)-peptides were air-oxidized in the NH_4OAc aqueous solution at pH 7.8. The crude products were purified by preparative HPLC and gel-filtration to afford a white powder following lyophilization. The integrity of peptides was determined by ion spray mass spectrometry analysis, and the purity was confirmed by analytical HPLC (data not shown).

Evaluation of Anti-HIV Activity and Cytotoxicity

The HIV-1(III_B) strain was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1(III_B) cells. Anti-HIV activity was determined based on the protection against HIV-induced cytopathogenicity in MT-4 cells. Various concentrations of test compounds were added to virus infected MT-4 cells.

T134	H-Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
T140	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14001	H-Ala-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14002	H-Arg-Ala-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14003	H-Arg-Arg-Ala-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14004	H-Arg-Arg-Nal-Cys-Ala-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14005	H-Arg-Arg-Nal-Cys-Tyr-Ala-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14006	H-Arg-Arg-Nal-Cys-Tyr-Arg-Ala-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14007	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DAla-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TA14008	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Ala-Tyr-Arg-Cit-Cys-Arg-OH
TA14009	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Ala-Arg-Cit-Cys-Arg-OH
TA14010	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Ala-Cit-Cys-Arg-OH
TA14011	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Ala-Cys-Arg-OH
TA14012	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Ala-OH
TC14001	H-Cit-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TC14002	H-Arg-Cit-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TC14003	H-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TC14004	H-Arg-Arg-Nal-Cys-Tyr-Arg-Cit-DLys-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TC14005	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DCit-Pro-Tyr-Arg-Cit-Cys-Arg-OH
TC14006	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Cit-Cit-Cys-Arg-OH
TC14007	H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Cit-Cys-Cit-OH

Figure 1. Amino acid sequences of T134, T140 and its analogues. A disulfide linkage is shown by a solid line. The substituted amino acids are underlined.

After 5 days' incubation at 37 °C, the number of viable cells was determined using the 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[14] Cytotoxicity of the compounds was determined based on the reduction of the viability of mock-infected cells using the MTT method (test I). Cytotoxicity was also determined by the trypan blue exclusion staining method in human peripheral blood mononuclear cells (PBMC) (test II).[15] 3′-Azido-2′,3′-dideoxythymidine (AZT) was tested for comparison. All data are the mean values for at least three experiments.

CD Spectroscopy of T140 Analogues

Peptides were dissolved in H_2O at concentration of 10 μM. CD spectra were recorded on a JASCO J-720 spectropolarimeter (Tokyo, Japan) using 1-cm cells at 1-nm intervals, with five scans averaged for each.

Results and Discussion

The amino acid sequences of T140 analogues are listed in Figure 1. The anti-HIV activity and cytotoxicity of T134, T140 and its analogues are summarized in Table 1. 50% effective concentration (EC_{50}) values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. 50% cytotoxic concentration (CC_{50}) values (test I) are based on the reduction of the viability of mock-infected cells. Selectivity index (SI) is shown as CC_{50} (test I)/EC_{50}. Since the cytotoxicity of T134 and T140 was previously evaluated (both CC_{50}s >40 μM),[12] the examination at high concentrations of T134 and T140 was omitted in this study (test I). Ala-substitution for Arg^2, Nal^3, Tyr^5 and Arg^{14} caused a pronounced decrease in anti-HIV activity, suggesting that these residues are indispensable for activity (see TA14002, TA14003, TA14004 and TA14012). Arg^6, D-Lys^8, Pro^9, Tyr^{10}, Arg^{11} and Cit^{12} could be replaced by Ala (D-Ala for D-Lys^8) without significant reduction of activity, indicating that these residues are not necessarily important (see TA14005, TA14007, TA14008, TA14009, TA14010 and TA14011). All these analogues (TA14001-14012) have low cytotoxicity (CC_{50}s (test I) >40 μM). Ala-substitution for one amino acid residue did not cause any marked increase in cytotoxicity. The results of the Ala-substitution scanning study suggested to us that the basic amino acid residues, Arg^6, D-Lys^8 and Arg^{11}, can be replaced by a nonbasic polar amino acid Cit (D-Cit for D-Lys^8). Next, Cit-substitution scanning was conducted for all Arg and Lys residues in T140. According to our previous results,[11] analogues having two Cit residues (total +6 charges) are thought to be less cytotoxic than analogues having one Cit residue (total +7 charges), such as T140. These analogues (TC14001–TC14007), in fact, did not show significant cytotoxicity even at the highest concentration in the MTT assay (CC_{50}s (test I) >40 μM). Therefore, the cytotoxicity of Cit-substituted analogues (TC14001–TC14007) was precisely examined by the trypan blue dye exclusion method (test II). As a result, all the Cit-substituted analogues (TC14001–TC14007) are less cytotoxic than T140 (TC14001–TC14007: CC_{50}s (test II) = 180–310 μM, T140: CC_{50} (test II) = 96 μM). The Arg^6 or D-Lys^8 residue was replaced by Cit (or D-Cit) maintaining strong anti-HIV activity (see TC14003 (EC_{50} = 2.8 nM) and TC14005 (EC_{50} = 4.0 nM)). In terms of anti-HIV activity, Cit (or D-Cit) is more suitable than Ala (or D-Ala) at the position 6 or 8 (see TC14003 and TA14005,

Table 1. Anti-HIV activity and cytotoxicity of T134, T140 and its analogues[a]

Compound	Charges	EC_{50} (nM)	CC_{50} (μM)		SI	
			(test I)	(test II)	CC_{50} (test I)/EC_{50}	CC_{50}(test II)/EC_{50}
T134	7	8.3	>>1	190	>>120	23,000
T140	7	3.3	>>1	96	>>300	29,000
TA14001	6	56	>40	NT[b]	>750	NT[b]
TA14002	6	1300	>80	NT	>64	NT
TA14003	7	2500	>80	NT	>34	NT
TA14004	7	360	>80	NT	>230	NT
TA14005	6	9.3	>40	NT	>4500	NT
TA14006	6	47	>80	NT	>1800	NT
TA14007	6	16	>80	NT	>5200	NT
TA14008	7	17	>80	NT	>4700	NT
TA14009	7	17	>80	NT	>4500	NT
TA14010	6	18	>80	NT	>4800	NT
TA14011	7	16	55	NT	3300	NT
TA14012	6	400	>80	NT	>210	NT
TC14001	6	15	>80	280	>5300	18,000
TC14002	6	450	> 80	260	> 180	570
TC14003	6	2.8	>80	310	>29,000	160,000
TC14004	6	16	>80	270	>5000	16,000
TC14005	6	4.0	>80	280	>20,000	69,000
TC14006	6	15	>80	310	>5300	20,000
TC14007	6	880	>40	180	>220	200
AZT		48	190	<20	4000	<410

[a]Charges: the number of total positive chages of each peptide. EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. CC_{50} values (test I) are based on the reduction of the viability of mock-infected MT-4 cells. CC_{50} values (test II) are determined by the trypan blue exclusion staining method in PBMC. SI is shown as CC_{50}/EC_{50}.
[b]NT, not tested.

TC14005 and TA14007). Cit (or D-Cit) and Arg (or D-Lys) bring almost the same contribution to anti-HIV activity (see TC14003 and T140, TC14005 and T140). The result suggests that, at the position 6 or 8, a basic residue is not necessarily important; however, at least a hydrophilic polar residue like Cit is preferred to Ala. Taking cytotoxicity into consideration, Cit (or D-Cit) is more suitable than a basic amino acid at the position 6 or 8. When CC_{50} values (test II) are used, the SIs (CC_{50} (test II)/EC_{50}) of TC14003 and TC14005 are 160,000 and 69,000, respectively, both being 2–5 times higher

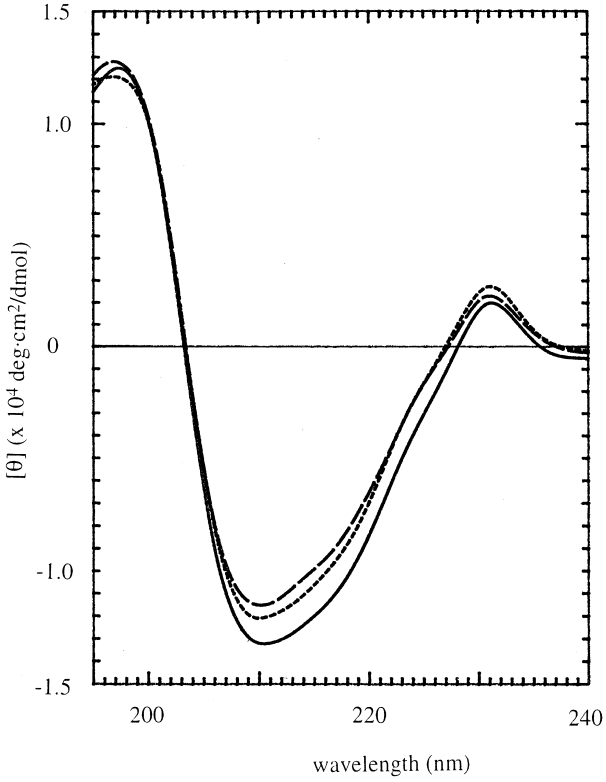

Figure 2. CD spectra of TC14003, TC14005 and T140. Solid line: TC14003, dotted line: TC14005, dashed line: T140.

than the SI of T140 (29,000). TC14006 (Cit for Arg^{11}) has moderate anti-HIV activity (EC_{50} = 15 nM) and a relatively high SI (20,000). TA14010 (Ala for Arg^{11}) also has moderate anti-HIV activity (EC_{50} = 18 nM), which is almost the same potency as that of TC14006 (Cit for Arg^{11}). With regards to anti-HIV activity, basic Arg at the position 11 is required, and even Cit is not satisfactory. TC14001 (Cit for Arg^{1}) and TC14004 (Cit for Lys^{7}) also showed moderate anti-HIV activities (EC_{50}s = 15 and 16 nM, respectively), which are stronger than those of the corresponding Ala-substituted analogues (TA14001 (Ala for Arg^{1}): EC_{50} = 56 nM, and TA14006 (Ala for Lys^{7}): EC_{50} = 47 nM, respectively). In terms of activity, a basic residue (Arg or Lys) is best accommodated at the position 1 or 7, but a nonbasic polar residue Cit is more suitable than Ala. TC14002 (Cit for Arg^{2}) is very weak (EC_{50} = 450 nM), but relatively stronger than TA14002 (Ala for Arg^{2}) (EC_{50} = 1300 nM), suggesting that Arg^{2} is an indispensable residue, which cannot be replaced by Cit (but Cit is still preferred to Ala). TC14007 (Cit for Arg^{14}) and TA14012 (Ala for Arg^{14}) are also very weak (EC_{50}s = 880 and 400 nM, respectively), suggesting that Arg^{14} is an indispensable residue, which cannot be replaced by Cit or Ala.

The conformation of the analogues was analyzed by CD. All the Ala- or Cit-substituted analogues (TA14001–TA14012 and TC14001–TC14007) except TA14008 were found to form β-sheet structures as T140.[12] The CD spectra of TC14003 and TC14005 in aqueous solution are shown as being representative with that of T140 (Fig. 2). These analogues 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 TW70, are due to β-sheet structures.[8] The CD spectrum of TA14008 (Ala for Pro^{9}) showed no characteristic pattern probably due to a disordered secondary structure (data not shown). The stable type II′ β-turn structure of TA14008 seems to be impaired by Pro to Ala substitution. However, TA14008 has

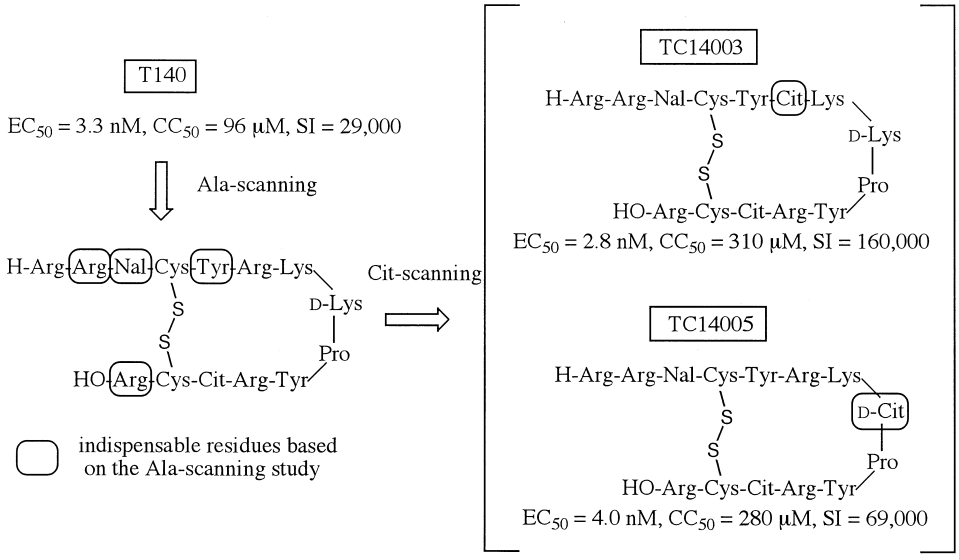

Figure 3. Effective anti-HIV peptides based on the pharmacophore identification of T140.

moderate anti-HIV activity (Table 1). It seems that a type II′ β-turn structure is not necessarily required for anti-HIV activity, if an alternative turn structure is adopted.

In conclusion, from the Ala-scanning study, the indispensable amino acid residues of T140 were proven to be Arg^2, Nal^3, Tyr^5 and Arg^{14}, which are closely located to each other, since T140 takes an antiparallel β-sheet structure. It is expected that another region of T140 can be modified without substantial loss of activity. Thus, novel effective compounds, TC14003 ([Cit^6]-T140) and TC14005 ([D-Cit^8]-T140), have been developed without significant change in the secondary structure by Cit-substitution at the position 6 or 8 of T140 (Fig. 3). These compounds, which have total +6 positive charges, possess almost the same anti-HIV activity and less cytotoxicity when compared to T140 (total +7 positive charges). Further information has been obtained on the correlation between the number of the total positive charges and associated CC_{50} values: analogues having two Cit residues (total +6 charges, such as TC14001-TC14007) are less cytotoxic than those having one Cit residue (total +7 charges, such as T140). A reduction of total positive charges by Cit-substitution was very useful for developing effective anti-HIV peptides. These findings will assist in the rational design of new type agents against HIV coreceptors.

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