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Synthesis and HIV-1 integrase inhibitory activity of dimeric and tetrameric analogs of indolicidin

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Abstract—We found that indolicidin, a natural antimicrobial peptide, has HIV-1 integrase inhibitory activity. Subsequently, we also discovered analogs of indolicidin with substantially higher inhibitory potency. The dimers and tetramers of the most active sequence (ILPWKWPWWPWPP) were prepared by connection of the monomers' C-terminal ends, using lysine as a linker. The inhibitory potency of the dimeric peptide is higher than the monomeric peptide. The tetrameric peptide, prepared by connection of two dimers at C-ends using again lysine as the linker, is the most potent integrase inhibitor with IC_{50} value of 0.6 μM for both 3′-end processing and strand transfer.

1. Introduction

The HIV-1 integrase plays a crucial role in the HIV-1 virus replication cycle.[1] The ability of integrase–viral DNA complex (pre-integration complex) to migrate into the nucleus allows the virus to infect non-dividing cells.[2] In addition to reverse transcriptase and protease, integrase has also been the focus of attention for HIV antiviral chemotherapy. Although many integrase inhibitors have been reported to date,[3] the progress with the design of effective inhibitors of HIV-integrase is slower than in the case of reverse transcriptase or protease inhibitors, and no clinically useful drugs have yet been approved by FDA. Integrase is an attractive target also because it has no counterpart in mammalian cells; therefore, selective integrase inhibitors should not produce any toxicity. This is very important, because antiviral drugs have to be administered continuously for the rest of patient's life.

In 1998 Robinson's group discovered the anti-HIV activity of indolicidin, and they suggested that this activity is correlated with antimicrobial activity and postulated a membrane-mediated mechanism for antiviral activity of indolicidin.[4] This potent antimicrobial agent, a tryptophan rich 13-mer peptide with sequence—ILPWKWPWWPWRR-NH_2, was isolated from the cytoplasmic granules of bovine neutrophils in 1992.[5]

Based on our experience that tryptophan rich peptides often possess integrase inhibitory activity we anticipated that indolicidin's anti-HIV activity may also involve inhibition of HIV-1 integrase. In this paper we present our studies on the HIV-1 integrase inhibitory activity of indolicidin and new indolicidin analogs.

In our previous paper[6] we presented HIV-1 integrase inhibitory activity data for dimeric analogs of the hexapeptide HCKFWW-NH_2.[7] All dimeric analogs of this hexapeptide were more potent than the hexapeptide itself. To check if dimerization would increase peptide activity also in the case of indolicidin sequence, we synthesized dimeric analogs. But, because there are no cysteine residues in the indolicidin sequence, we chose a different method of dimerization. A lysine residue was used as a linker and the peptide backbones were coupled

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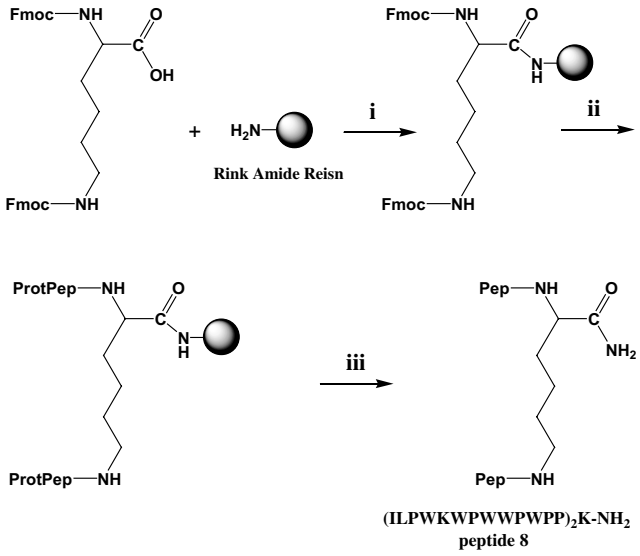
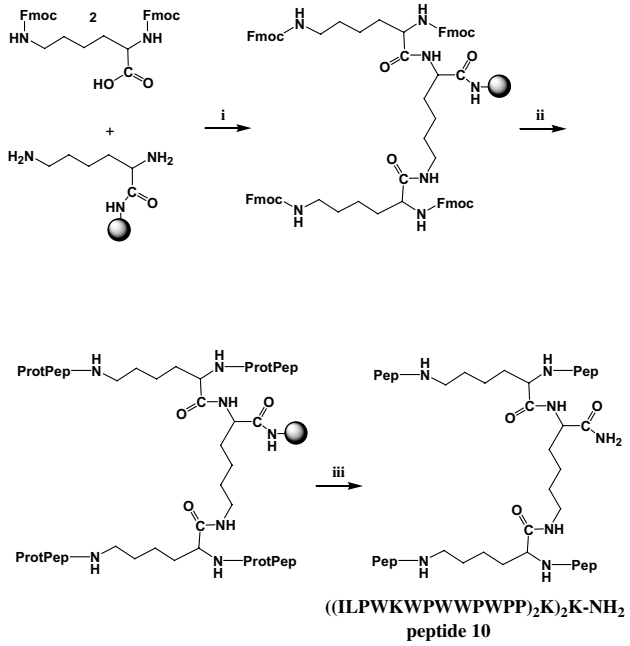


ProtPep = ILPW(Boc)K(Boc)W(Boc)PW(Boc)W(Boc)PW(Boc)PP

Pep = ILPWKWPWWPWPP

Scheme 1. The synthesis of dimeric peptide **8**. Reagents and conditions: (i) HBTU, HOBt, DIPEA in NMP; (ii) (1) 20% piperidine in NMP, (2) 13 cycles of Fmoc SPPS with HBTU/HOBt/DIPEA coupling, (iii) 1% TIS, 2.5% EDT, 2.5% H_2O in TFA (2 h, rt).



ProtPep = ILPW(Boc)K(Boc)W(Boc)PW(Boc)W(Boc)PW(Boc)PP

Pep = ILPWKWPWWPWPP

Scheme 2. The synthesis of tetrameric peptide **10**. Reagents and conditions: (i) HBTU, HOBt, DIPEA in NMP, (ii) (1) 20% piperidine in NMP; (2) 13 cycles of Fmoc SPPS with HBTU/HOBt/DIPEA coupling, (iii) 1% TIS, 2.5% EDT, 2.5% H_2O in TFA (2 h, rt).

at their C-terminal ends to two NH_2 groups of lysine (Scheme 1).

During our structure–activity studies we found that the C-terminal di-proline containing analog ILPWKWPWWPWPP is a significantly more potent HIV-1 integrase inhibitor than indolicidin. Therefore this sequence was used for synthesis of dimeric and tetrameric peptides.

The peptides were synthesized by an automated SPPS (ABI 433A Peptide Synthesizer), using a Rink amide resin and an Fmoc chemistry (coupling with HBTU/HOBt/DIPEA in NMP). Because the peptides contain a large number of tryptophan residues (up to 16 residues) an Fmoc-Trp(Boc)-OH derivative was used for SPPS, to prevent unwanted side-reactions during peptide cleavage and deprotection. The method of synthesis of dimeric peptide **8** is presented in Scheme 1. In this synthesis Fmoc-Lys(Fmoc)-OH was coupled to the Rink resin, and then, after removing the Fmoc protecting groups, additional amino acids were coupled to the two NH_2 groups of lysine.

For the synthesis of the tetrameric peptide **10** (Scheme 2) we designed a template composed of three residues of lysine (tri-lysine template, $(K)_2K$). The tri-lysine template was synthesized by coupling reaction of Fmoc-Lys(Fmoc)-OH with fully deprotected lysine bound to the Rink resin. Then, after deprotection, additional amino acids were coupled to four NH_2 groups of tri-lysine template.

After synthesis the peptide bound resins were washed six times with NMP, DCM, and MeOH, then dried under reduced pressure overnight. The peptides were cleaved from the resin and fully deprotected by treatment with 1% TIS, 2.5% EDT, 2.5% H_2O in TFA (10 mL/g resin), after 2 h the mixture was filtered into cold diethyl ether. After 30 min at −10 °C the precipitated peptides were separated by centrifugation, washed four times with diethyl ether, dried under reduced pressure (1 h over KOH), dissolved in water/acetonitrile 1:1 mixture, kept at rt 6 h (to complete tryptophan residue deprotection), and lyophilized.

The peptides were purified by preparative RP HPLC (Vydac C_4 or C_{18} column). The purity of all peptides was between 90% and 95% (RP HPLC on an analytical C_8 column). MALDI-TOF-MS spectra (Kratos Axima-CFR instrument, matrix: α-cyano-4-hydroxycinnamic acid) verified molecular masses of all peptides. The observed monoisotopic masses were in good agreement with theoretical masses (in parentheses): **1** 1905.8 (1906.0 $M + H^+$) Da; **2** 1787.7 (1787.9 $M + H^+$) Da; **3** 1674.5 (1674.9 $M + H^+$) Da; **4** 1690.4 (1689.9 $M + H^+$) Da; **5** 1716.5 (1716.9 $M + H^+$) Da; **6** 1834.7 (1835.0 $M + H^+$) Da; **7** 1709.6 (1709.9 $M + H^+$) Da; **8** 3685.7 (3686.0 $M + H^+$) Da; **9** 3686.1 (3686.0 $M + H^+$) Da; **10** 7486.6 (an average mass) (7487.0 $M + H^+$, an average mass) Da.

2. HIV-1 integrase inhibition assay

The HIV-1 integrase assays were performed as described previously,[8] with the following modifications. The pep-

Table 1. Results of in vitro wt HIV-1 integrase inhibitory assay for peptides **1**–**8**

Peptides	IC_{50} (μM)	
	3′-End processing	Strand transfer
1 ILPWKWPWWPWRR-NH_2*	**60**	**57**
2 ILPWKWPWWPWPP-NH_2	**16**	**13**
3 LPWKWPWWPWPP-NH_2	185	215
4 ILPWKWPWWPWP-NH_2	180	80
5 ILPW**G**WPWWPWPP-NH_2	>333	>333
6 ILPW**G**WPWWPWRR-NH_2	49	19
7 ILAWKWAWWAWPP-NH_2	54	41
8 (ILPWKWPWWPWPP)$_2$K-NH_2	**2.8**	**4.9**
9 (ilpwkwpwwpwpp)$_2$k-NH_2**	**2.3**	**2.3**
10 ((ILPWKWPWWPWPP)$_2$K)$_2$K-NH_2	**0.6**	**0.6**

* Indolicidin.
** All-D-amino acids sequence.

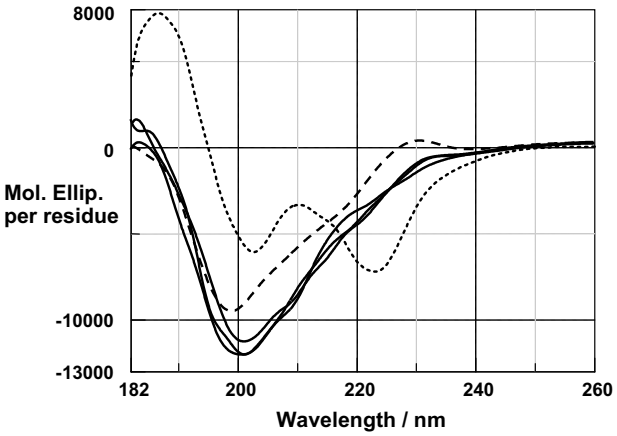


Figure 1. The CD spectra of peptides **2**, **8**, and **10** (solid lines), indolicidin (**1**, dashed line), and peptide **7** (dotted line). Concentrations: 0.1 mM for **1**, **2**, **7**, 0.05 mM for **8**, and 0.025 mM for **10**, in a TFE/water (4:1 vol) mixture, spectra collected at 22 °C.

tides were pre-incubated with 500 nM wild type (wt) HIV-1 integrase for 15 min at room temperature in a buffer containing 50 mM MOPS, pH 7.2, 7.5 mM NaCl, 7.5 mM $MnCl_2$, and 14.3 mM 2-mercaptoethanol. Reactions were started by adding 20 nM of the 5′-end ^{32}P-labeled 21-mer double-stranded DNA template in a final volume of 10 μL, and reactions were carried out for 1 h at 37 °C. Reactions were quenched by adding 10 μL of denaturing loading dye (formamide 99%, SDS 1%, bromophenol blue 0.2 mg/mL, xylene cyanol FF 0.2 mg/mL). Samples were loaded onto 20% (19:1) denaturing polyacrylamide gels. Gels were dried, exposed overnight and analyzed using Molecular Dynamics PhosphorImager (Sunnyvale, CA). The densitometric analysis was performed using ImageQuant v5.2 from Molecular Dynamics software package. Each lane was quantified to determine the amount of 3′-processing and strand transfer products. The results of HIV-1 integrase inhibitory assay are presented in Table 1.

3. Results

The HIV-1 integrase inhibitory assays on peptides **1**–**4** demonstrate that the analog **2**, with two Pro residues at the C-terminal end in the place of two Arg residues, is a substantially more potent inhibitor of both 3′-processing (7 times) and strand transfer (3 times) than indolicidin (peptide **1**), furthermore even small changes in the sequence of peptide **2**, such as removal of one N-terminal residue (peptide **3**) or one C-terminal residue (peptide **4**) significantly reduce inhibitory activity. Peptide **7** is an analog of CP10A (ILAWKWAWWAWRR-NH_2).[9] CP10A is a more potent antimicrobial agent than indolicidin, and has a much higher tendency to form an ordered α-helical structure.

As evidenced by the CD spectra of the peptides, only peptide **7** exhibited a double minimum at 202 and 223 nm along with the maximum at 187 nm, indicating the presence of an α-helical structure. The CD spectrum of peptide **7** in a 4:1 MeOH/water mixture also showed the presence of an α-helical structure, indicating a strong preference of this peptide to adopt the α-helical conformation. However, peptide **7** is significantly less active in HIV-1 integrase inhibitory assays, than peptide **2**. This suggests that the α-helical conformation of peptide inhibitor is not favored for interaction with HIV-1 integrase. The overall shape of the CD spectra for peptides **3** and **4** (for clarity not shown in Fig. 1) is similar to peptides **2**, **8**, and **10** (solid lines). The CD spectrum of indolicidin is slightly different from the spectra of peptides **2**, **8**, and **10**, the main difference being the presence of weak positive Cotton effect at 231 nm. This Cotton effect can be associated with the presence of tryptophan residues.[10] The lower intensity of this maximum (present only as a small shoulder) in the spectra of **2**, **8**, and its absence in the spectrum of **10** may suggest a less defined tryptophan side chain orientation, and more flexible conformations of these peptides.

A comparison of assay results for peptides **1**, **2**, **5**, and **6** shows that the presence a basic amino acid residue (Arg or Lys) is crucial for the inhibitory activity of the peptide. This is supported by the inactivity of peptide **5**, which lacks the lysine residue in the 5-position. The activity data for peptide **6** shows that the presence of arginine residues at the C-terminal end may compensate for the absence of lysine residue, and in fact peptide **6** is even more potent than indolicidin (**1**).

The assay results for peptides **2** and **8** show that the dimeric peptide is much more potent than the monomeric one. The inhibitory potencies of enantiomeric peptides **8** and **9** are similar, suggesting that interactions between HIV-1 integrase and peptide inhibitors are not enantiospecific. The tetrameric peptide **10** is the most potent inhibitor of both 3′-processing (26 times more potent than **2**) and strand transfer (20 times more potent than **2**). The higher potency of dimeric and tetrameric peptides in comparison to the monomeric ones, was also observed in the case of other integrase inhibitory peptide (HCKFWW-NH_2),[6] supporting our hypothesis that multimeric inhibitory peptides may act as multivalent inhibitors, simultaneously occupying two or four

neighboring catalytic sites within the integrase oligomeric complex, with an entropic advantage. From that point of view it is especially interesting, that in human cells HIV-1 integrase exists in the form of homotetramers, and probably at least an octamer of integrase is required to accomplish an effective integration.[11]

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