Peptide Antivirals Hot Paper

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Cell-Penetrating, Dimeric α-Helical Peptides: Nanomolar Inhibitors of HIV-1 Transcription**

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Abstract: *We constructed dimeric α-helical peptide bundles based on leucine (L) and lysine (K) residues for both efficient cell penetration and inhibition of the Tat–TAR interaction. The LK dimers can penetrate nearly quantitatively into eukaryotic cells and effectively inhibit the elongation of the TAR transcript at low nanomolar concentrations. The effective inhibition of HIV-1 replication strongly suggests that the LK dimer has strong potential as an anti-HIV-1 drug.*

RNA structure is used as a control element in most biological systems.[1] Human immunodeficiency virus-1 (HIV-1) contains a short hairpin RNA, named TAR, located in the long terminal repeat (LTR) region that participates in efficient transcription of the integrated genome.[2] Specifically, the interaction between TAR RNA and the viral Tat protein activates the transcription of viral genes.[2b–d,3] Consequently, this interaction is a plausible target when developing substances that destroy HIV-1.[4] However, no pharmaceutical agents that inhibit Tat binding to hairpin RNA proteins have been commercialized thus far. The likely reason for this lies in the fact that small molecules that can penetrate cells[5] bind ineffectively to TAR and the poor cell-penetrating ability of large molecules that efficiently inhibit the Tat–TAR interaction.[6]

Peptides are attractive biomaterials that can be employed to inhibit the Tat–TAR interaction. Thus, Tat-derived peptide fragments, as well as cyclized and more extended analogues of Tat, have been explored to assess their competitive binding affinities against hairpin RNA.[7] Members of a recently updated list of such peptides were shown to possess single-digit nanomolar affinities against TAR,[8] which suggests that they are potential candidates as therapeutic drugs to target HIV-1. However, although Tat is a well-known cell-penetrating peptide (CPP),[9] the cell permeabilities of Tat analogues are insufficiently low when in-cell concentrations in the nanomolar range are required. As a result of the weak cell-penetrating activities of these peptides, significant differences exist between the dissociation constants (K_d) arising from in vitro binding and cell-based assays.

Recently, we observed that a strong correlation exits between the α helicities and the cell-penetrating activities of amphipathic peptides composed of leucine (L) and lysine (K) residues. Furthermore, we constructed dimeric bundle amphipathic peptides containing two cysteine residues per monomer as replacements for the leucine residues located at the i and $i+7$ positions on the hydrophobic face as well as two disulfide bonds connecting the cysteine residues.[8a] Compared to their monomeric counterparts, the dimeric peptides display higher α helicities as well as stronger affinities (corresponding to sub-nanomolar K_d values) against hairpin RNA targets. In addition, the amphipathic characteristics and high α helicities of the dimeric peptides lead to high cell-penetrating activities and, consequently, the peptides can serve as effective intracellular inhibitors of HIV-1 transcription in mammalian cells (Figure 1). Their high cell penetration and TAR RNA binding abilities not only make these peptides the first agents that inhibit transcription of the integrated HIV-1 genome, but also the first peptides that serve as key leads in the search for drugs for the treatment of HIV-1.

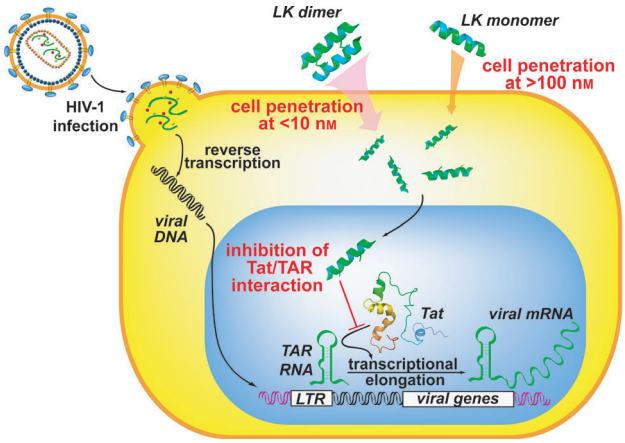

Figure 1. Schematic representation of the inhibition of Tat-TAR-mediated transactivation by using LK peptides.

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Table 1: Binding affinities on TAR RNA and α helicities of LK peptides[a]

Peptide	Sequences of peptide[b]	K_d [nM]	α helicity [%][d]
LK-1	LKKL**L**KLLKKL**L**KLAG	63[c]	24/77
LK-2	LKKL**C**KLLKKL**C**KLAG	9.6[c]	28/77
LK-3	LKKL**C**KLLKKL**C**KLAG LKKL**C**KLLKKL**C**KLAG	0.061[c]	91/99
LK-4	LKKL**C**KLLKKL**C**KLAG LKKL**C**KLLKKL**C**KLAG	0.059	87/92
R9	RRRRRRRRR	n.d.[e]	13/15

[a] Affinities were measured at 20 °C by fluorescence anisotropy using the rhodamine-Rev peptide as a probe. [b] Disulfide linkers are shown in dotted lines in LK-3. *N*,*N*′-Phenylenedimaleimide linkers are shown in solid lines in LK-4. N-terminal and C-terminal modification of all peptides were performed with acetylation (Ac) and amidation (NH_2), respectively. [c] Data are adopted from a previous report.[8a] [d] The α helicities of peptides were measured by circular dichroism (CD) in PBS (pH 7.4; the first value) and in 50% TFE in the same buffer (the second value). [e] n.d.: not determined.

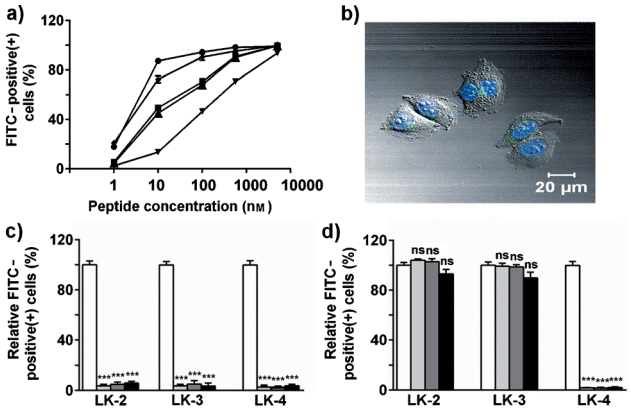

Figure 2. Cell-penetration activity of the LK peptides on HeLa cells after 12 h incubation. a) FACS results for LK-1 (▲), LK-2 (■), LK-3 (●), LK-4 (◆), and R9 (▼) at various peptide concentrations. b) Confocal laser scanning microscopy (CLSM) images of HeLa cells with FITC-labeled LK-3 (10 nM). The nucleus was stained with Hoechst 33442 (blue). c,d) The relative cell-penetration activity of the LK peptides at endocytosis-inhibiting conditions at 10 nM (c) and 500 nM (d). Control (white), wortmannin (gray), amiloride (dark gray), and 4 °C (black). Each error bar represents the standard deviation ($n=3$). The markers (***) and (n.s.) indicate $p<0.001$ and no significant difference compared with the control, respectively.

The current investigation arose from an earlier observation[8] that the peptide LKKL**L**KLLKKL**L**KLAG (LK-1, Table 1) has a strong binding affinity to short hairpin RNAs. In addition, we found that the amphipathic head-to-head dimeric bundle LK-3,[8a] which contains two disulfide bonds in the middle of each chain, has a sub-nanomolar affinity to TAR RNA. LK-1 and LK-3 were selected for our continuing studies aimed at the development of anti-HIV-1 peptides. As the disulfide bonds in LK-3 might be cleaved under cytosolic conditions,[8a,10] the reduced monomeric peptide (LK-2) was generated for use as a control. Furthermore, the nonreducible dimer (LK-4), in which the peptide chains are linked by two *N*,*N*′-phenylenedimaleimide moieties, was also prepared. Inspection of the dissociation constants (K_d) for the binding of the LK peptides to TAR RNA (Table 1) shows that LK-3 and LK-4 display about 100 and 1000 times stronger affinities than the monomeric peptides LK-1 and LK-2. In addition, we determined the α helicities of the LK peptides in phosphate-buffered saline (PBS; pH 7.4) under membrane-mimic conditions (50% trifluoroethanol (TFE) in the PBS). The data show that the dimeric peptides have significantly higher α helicities (about 90%) than their monomeric counterparts.

The ability of the monomeric and dimeric peptides to penetrate mammalian cells was evaluated next. For this purpose, LK peptides labeled with fluorescein isothocyanate (FITC) were incubated with HeLa cells. R9, a well-known cell-penetrating peptide, was used as a control.[11] The results of fluorescence-activated cell sorting (FACS) experiments, which give the percentage of the FITC-positive cells (Figure 2a), show that the cell-penetration activities at all the concentrations used in the analysis increase in the following order: R9 < LK-1 and LK-2 < LK-4 < LK-3. At high peptide concentrations (>500 nM), both monomeric and dimeric peptides display an almost 100% cell penetration. However, at very low concentrations (10 nM), the dimeric peptides have 70–90% cell-penetration levels, whereas the monomeric analogues display only approximately 40% penetration efficiencies. The control (R9) has only a minimal cell-penetration activity at 10 nM. The combined observations demonstrate that the cell-penetrating abilities of the LK peptides are greatly improved by the formation of dimers. Significant portions of the LK peptides are delivered into cell nuclei (Figure 2b, see also Figures S4 and S5 in the Supporting Information). The dimeric LK peptides also have greatly improved penetration abilities in a mouse leukemic monocyte macrophage cell line (RAW 264.7; see Figure S3 in the Supporting Information).

The cell permeabilities of the LK peptides were examined under various endocytosis-inhibiting conditions. The results show that the mechanism employed is dependent on the peptide concentration. At low concentrations (10 nM), the cell-penetration abilities of all the LK peptides are nearly completely inhibited by lowering the temperature to 4 °C or by treatment with endocytosis inhibitors (wortmannin or amiloride; Figure 2c).[12] Thus, it appears that at low concentrations, LK peptides are internalized by cells through energy-dependent endocytic pathways, even though the activities of each LK peptide differs significantly at these concentrations. At high concentrations (500 nM), where all the LK peptides display >80% cellular uptake, the cell-penetration mechanism varies (Figure 2d). For example, wortmannin and amiloride display almost no inhibition of cell penetration by both monomeric LK-2 and dimeric LK-3, and the internalization efficiencies of these peptides remain unchanged even at 4 °C. Thus, LK-2 and LK-3 penetrate into cells through an energy-independent pathway at high concentrations, not by receptor-mediated endocytosis or macropinocytosis. Interestingly, cell penetration by the 500 nM dimer LK-4, which contains nonreducible maleimide linkers in place of the disulfides, is nearly completely inhibited by lowering the temperature or by treatment with either endocytosis inhibitor. The results suggest that LK-2 or LK-3 undergo energy-independent cell penetration (e.g. hole or carpet formation)[13] at high concentrations, but LK-4 undergoes energy-

dependent cell penetration because of the structural difference.

Since they can be delivered into cells at nanomolar concentrations, the LK peptides were employed in experiments to determine if they inhibit the viral target inside host cells. A reporter system was constructed on HeLa cells by co-transfection with pLTR-luc, a plasmid containing an HIV-1 LTR promoter and a firefly luciferase gene, as well as pTat, a plasmid-containing HIV-1 Tat gene.[14] In this system, expressed Tat proteins should interact with TAR RNA in the LTR promoter as an anti-termination factor to promote transcription of the luciferase gene. After 12 h incubation of the reporter cells with the LK peptides, the relative amounts of mRNA were determined by using RT-PCR (Figure 3). The results show that, compared with those of the two house-keeping genes β-actin and 18S rRNA, the amounts of mRNA in the luciferase gene decreases in a peptide dose dependent manner (Figure 3a,b). Moreover, the amount of the long luciferase gene transcript with total TAR RNA transcribed was evaluated to demonstrate that the LK peptides do not prevent transcription by binding to transfected plasmid DNA. The ratio of TAR-luc (long transcript) to TAR (total transcript) was found to be similar to the ratio of TAR-luc to mRNA in the house-keeping genes. This observation suggests that the Tat–TAR interaction is inhibited by the LK peptides at the transcriptional elongation level (Figure 3c).

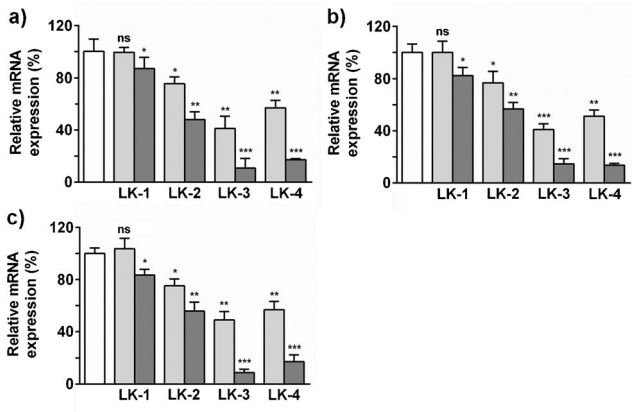

Figure 3. Inhibition of the Tat-mediated transcriptional elongation by LK peptides in HeLa cells. Relative mRNA expression of a) TAR-luc/β-actin, b) TAR-luc/18S rRNA, and c) TAR-luc/TAR at 10 nM and 100 nM of each LK peptide. Control (white), 10 nM (gray), 100 nM (dark gray). Each error bar represents the standard deviation ($n=3$). The markers (*), (**), (***), and (n.s.) indicate $0.01 \le p < 0.1$, $0.001 \le p < 0.01$, $p < 0.001$, and no significant difference compared with the control, respectively.

The inhibitory activity against Tat-mediated transcription varies among the peptides, with monomeric LK-1 and LK-2 exhibiting less than 50% inhibition even at 100 nM. In contrast, dimeric peptides LK-3 and LK-4, which possess higher cell-penetration abilities, display about 50% inhibition at 10 nM, and more than 80% inhibition at 100 nM. As the HeLa cell penetration activities of LK-1 and LK-2 are similar (Figure 2a), the stronger inhibitory effect of LK-2 is likely a consequence of its stronger binding affinity to TAR RNA (Table 1). As disulfide bonds are readily degraded under reductive cytosolic conditions, it is likely that, after internalization, LK-3 is reduced in the cytosol to form monomeric LK-2, which still binds to the TAR RNA with nanomolar affinity. Therefore, the enhanced cell-penetration activity of LK-3 over LK-2 is the main source of its outstanding ability to inhibit the Tat–TAR interaction at the transcriptional level. Although its cell-penetration ability is slightly less than that of LK-3, LK-4 displays a similar inhibitory activity as LK-3 because of the effect of the nonreducible linker that enables the peptide to retain its dimeric form in the cytosol and high TAR RNA binding affinity.

The IC_{50} values of the LK peptides, determined by using the luciferase assay (Figure 4), were found to decrease in the order LK-1 (110 nM) > LK-2 (50 nM) > LK-4 (35 nM) > LK-3 (10 nM). The IC_{50} value of dimeric LK-3, which is more than tenfold lower than that of monomeric LK-1, is a consequence of its enhanced cell-penetration ability as well as the twofold increase in the concentration of the monomeric peptide that takes place when LK-3 undergoes cytosolic cleavage. The IC_{50} value of LK-3 is almost the same as the dissociation constant of LK-2 ($K_d = 9.6$ nM), which indicates that cell-membrane penetration no longer serves as the barrier for in-cell activity, a unique feature not shared by other peptide drugs that display large discrepancies between the K_d and IC_{50} values. Similar results (IC_{50} of LK-3 = 18 nM) are also seen in studies using macrophage cells (RAW 264.7; see Figure S6 in the Supporting Information). Moreover, the decrease in the inhibition of the LK peptides corresponds to the increase in Tat expression (see Figure S7 in the Supporting Information). The result also suggests that the competitive binding between Tat and LK peptides on TAR RNA is the main factor for the inhibition.

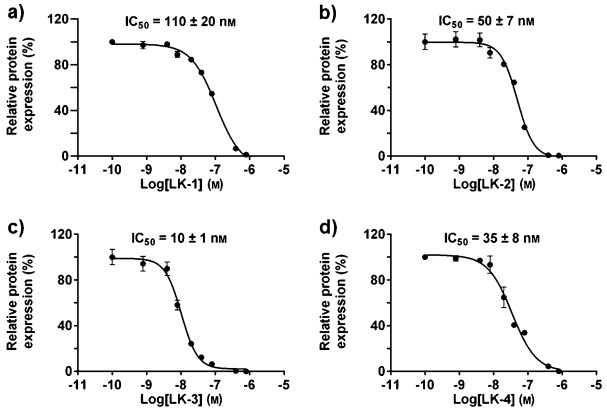

Figure 4. Inhibition of luciferase expression by a) LK-1, b) LK-2, c) LK-3, and d) LK-4 peptides in HeLa cells. Each error bar represents the standard deviation ($n=3$).

The membrane destabilization and cytotoxicities of the peptides were determined by using lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively (see Figures S8 and S9 in the Supporting Information). The results demonstrate that none of the LK peptides cause membrane destabilization

up to 2 μM concentrations, and only LK-3 brings about a small membrane destabilization at 8 μM. Also, the MTT assay results show that LK-3 is not cytotoxic at concentrations below 10 μM, similar to the previous report on oligoarginine peptides.[15] These observations suggest that the high cell-penetrating activities of the LK peptides is not caused by disruption of the plasma membrane, which would induce cell lysis and eventually cell death.

In the final phase of this effort, the inhibitory effects of the peptides on HIV-1 replication in acutely infected T-lymphoblastoid cells (MOLT-4/CCR5)[16] were evaluated (Figure 5). The IC_{50} values of LK-3 and LK-4 for HIV-1 replication were

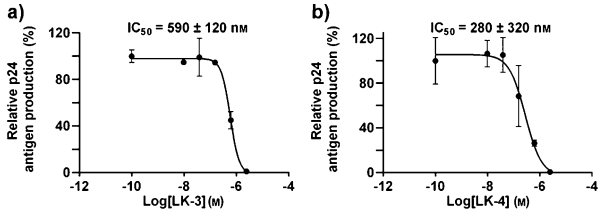

Figure 5. Inhibition of HIV-1 replication by a) LK-3 and b) LK-4 peptides in T-lymphoblastoid cells (MOLT-4/CCR5). Each error bar represents the standard deviation ($n = 3$).

590 and 280 nM, respectively, while that of LK-1 was > 2 μM (data not shown). The larger IC_{50} values, compared with those arising from experiments with HeLa or RAW 264.7 cells, may be partially attributed to the reduced penetration activity of the peptides in T-lymphoblastoid cells. However, LK-3 did not show significant cytotoxicity to the host cells at concentrations up to 2.6 μM, which is considerably higher than its IC_{50} value for HIV-1 replication. LK-4 was found to be more active than LK-3, but it was also more cytotoxic to the host cells (data not shown). These findings suggest that the LK dimers, especially LK-3, have potential as a therapeutic against HIV-1.

In summary, we have demonstrated that α-helical LK dimeric bundles have large cell-penetrating abilities and that they target intracellular hairpin RNA. Approximately 90 % of the LK dimers exist in a helical form in an aqueous buffer, and hence have significantly enhanced cell-penetration activities compared to those of the corresponding monomer. This feature results in near quantitative uptake of the dimeric peptides in HeLa and macrophage cells at low nanomolar concentrations. We showed that the LK dimers display dose-dependent inhibition of the Tat–TAR interaction, with IC_{50} values that are about 10 nM. In addition, the dimeric peptides are not cytotoxic at concentrations < 2 μM. The experiments using HIV-1-infected cells show that LK-3 and LK-4 inhibit viral replication at nanomolar concentrations. This finding strongly suggests that the LK dimers have the potential to serve as effective anti-HIV-1 drugs. Target selectivity, stability, and long-term toxicity of the LK dimers will be further investigated for the future development of new anti-HIV-1 drugs based on the LK dimers.

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