Lipid Conjugation Yields Novel HIV-1 fusion Inhibitor that Demonstrates Improved Efficacy and Prolonged Serum Half-life

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ABSTRACT:
Human immunodeficiency virus type 1 (HIV-1) Env subunit gp41, mediates fusion between the viral envelope and target cell membrane. GP41 changes conformation by inserting the fusion peptide into the cell membrane, resulting in the formation of a six-helix bundle (6-HB) between the N- and C-terminal heptad repeats (NHR and CHR) bringing the viral and cell membranes into proximity for fusion. T20 (Enfuvirtide), which is a peptide derived from the CHR, is the only clinically available HIV-1 fusion inhibitor, but it suffers from low potency and short half-life, which urgently calls for next-generation drugs. T-cell lipid rafts are enriched in the receptor (CD4) and co-receptors (CCR5/CXCR4) for HIV. To target these sites of active fusion and increase drug potency, a C-16 lipid moiety was incorporated into the current leading fusion inhibitor YIK. Addition of a lipid motif may also prolong the half-life of the peptide inhibitor through binding to serum albumin. Inhibition of 6-HB formation, cell-cell fusion and infection assays were used to assess the anti-HIV potency of YIK-C16. YIK-C16 was twice as potent as YIK in inhibiting cell-cell fusion and 6-HB formation and 10-fold more effective than YIK at preventing HIV- infection. Importantly it retained biological activity for up to ~15 h while YIK lost activity after 2 h. Cell viability assays revealed no cytotoxic effects of YIK-C16. These results suggest that the lipopeptide YIK-C16 shows promise for further development as a new anti-HIV drug with improved anti-HIV-1 activity and prolonged half-life.

INTRODUCTION

AIDS-related deaths have decreased by more than 51% since the peak in 2004 because of the widespread application of highly active antiretroviral therapy (HAART) (http://www.unaids.org/en/resources/factsheet). The essence of HAART is the combination of various drugs that act on different stages of HIV-1 infection and replication [1]. So far, the U.S. FDA-approved HIV drugs mainly consist of four categories, including nucleoside/nonnucleoside reverse transcriptase inhibitors (NRTIs/NNRTIs), protease inhibitors (PIs), integrase inhibitors (IIs), and entry inhibitors (EIs) (https://www.fda.gov). Among them, NRTIs/NNRTIs, PIs, and IIs take effect by blocking the replication process after HIV enters the target cell, while EIs have the advantage of working at the early stage of HIV entry, thus preventing viral fusion into the host cell [2].

The entry process of HIV is triggered by the binding between the gp120 subunit of the HIV envelope glycoprotein (Env) and the host receptor CD4 and co-receptor, CCR5 or CXCR4, on the target cell. Then, the other Env subunit, gp41 is exposed, and the N-terminal fusion peptide (FP) of gp41 inserts into the target cell membrane. The gp41 N-terminal heptad repeat (NHR) domain then interacts with the homologous C-terminal heptad repeat (CHR) domain to form a hairpin-like 6-helix bundle (6-HB) structure, consisting of a NHR trimer core with three CHR helices packing into the hydrophobic grooves on the surface of the NHR trimer in an antiparallel way [3–5]. 6-HB pulls the viral particle and host cell into proximity and elicits membrane fusion between virus and cells. Fusion inhibitors, a subtype of EIs, are derived from the NHR [6] or CHR [7] domain of gp41 and inhibit the fusion between HIV and target cell by competitively blocking the formation of the homologous 6-HB structure. However, clinical application of the only U.S. FDA-approved HIV fusion inhibitor, enfuvirtide (T20), has been limited by its low potency and susceptibility to drug resistance [8]. Moreover, T20 requires twice-daily injection in clinical use because of its short in vivo half-life [9].

Recently, it was discovered that the addition of three residues, Ile654, Asp655, and Leu656 (IDL), which adopt a hook-like (tail-anchor) structure, to the C-terminus of CHR peptides can greatly enhance their binding to NHR and anti-HIV-1 activities [10], providing a new strategy to design and optimize HIV-1 fusion inhibitors. On the basis of the IDL hook structure, a 32-mer peptide inhibitor (HP23-E6-IDL), was designed which exhibited potent inhibitory activity against HIV-1 of diverse subtypes and tropisms, especially those resistant to other HIV-1 fusion inhibitors [11]. However, similar to other peptide-based drugs, HP23-E6-IDL possesses a short in vivo half-life, which significantly attenuates its potential for development into a new anti-HIV drug. Emerging studies have proven that the conjugation of a lipid domain to a peptide substantially enhances its antiviral activity and *in vivo* stability [12–14], possibly resulting from the preferential interaction of the lipid domain with the lipid rafts of membranes where HIV-1 fusion occurs, thereby increasing the topical concentration of the peptide, prompting the exploitation of lipid conjugation to further enhance the inhibitory activity of HP23-E6-IDL.

RESULTS

YIK-C16 was Highly Potent in Inhibiting HIV-1 Infection In Vitro

Palmitic acid (C16) was conjugated to the C-terminus of YIK with a linker (GSG-PEG4-K) between C16 and YIK (Figure 1a). As shown in Figure 1b, the inhibitory activity of YIK against HIV-1 infection was only slightly better than that of HP23-E6-IDL (640 pM versus 813 pM). However, the resultant lipopeptide YIK-C16 possessed dramatically increased anti-HIV-1 activity in vitro. Its half maximum inhibitory concentrations (IC_{50}) for inhibiting HIV-1infection was 61 pM, respectively, which is about 13.3-fold more potent than HP23-E6-IDL.

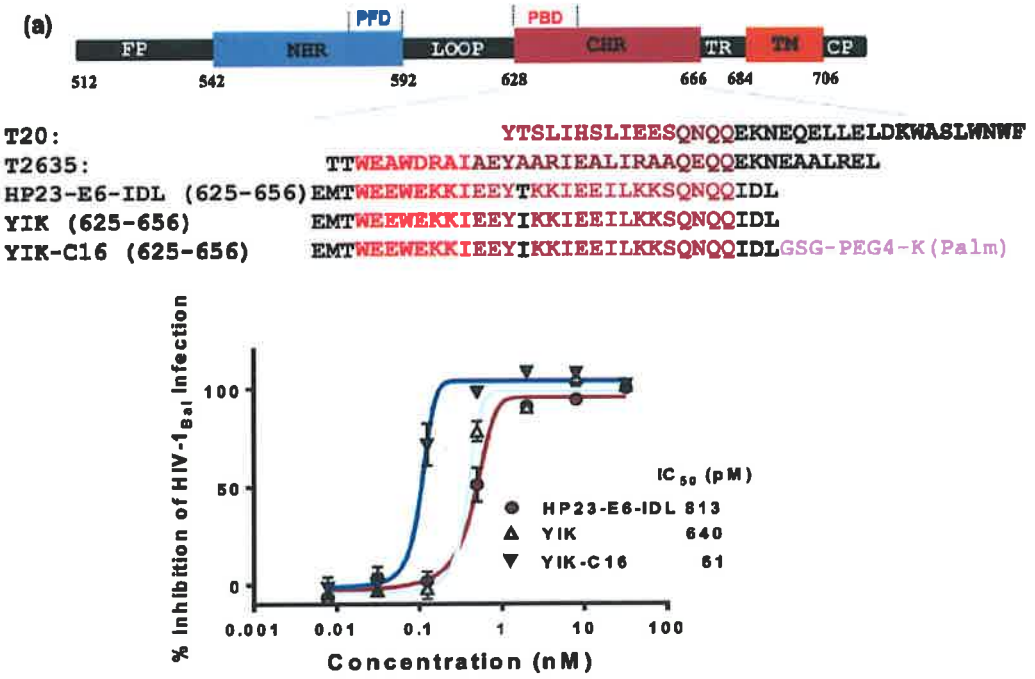

Figure 1. *Sequence and anti-HIV-1 activity of the peptides. (**a**) Schematic diagram of HIV-1 gp41 functional domain and sequences of peptides derived from the gp41 CHR domains. FP, fusion peptide region; NHR and CHR, N- and C-terminal heptad repeats, respectively; PFD and PBD, pocket-forming and binding domains, respectively; TR and TM, tryptophan-rich and transmembrane regions, respectively. (**b**) Inhibitory activities of HP23-E6-IDL, YIK, and YIK-C16 against pseudovirus HIV. Error bars in this figure represent standard deviations.*

Secondary Structure of Complex Formed by YIK-C16 and an NHR Peptide

Circular dichroism spectrum (CD) was employed to investigate the secondary structure of the peptides HP23-E6-IDL, YIK, and YIK-C16, either alone, or in complex with NHR peptide N46 overlapping the sequence of the HIV-1 gp41 (residues 536-581). As shown in Figure 2a,

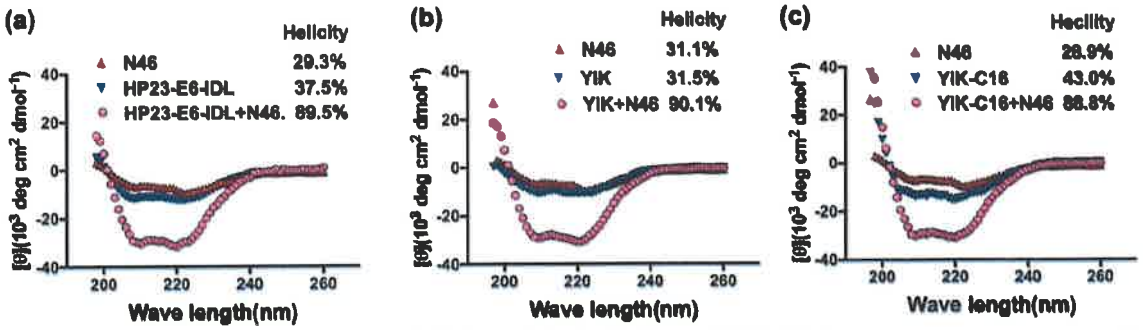

Figure 2. *Secondary structure of HP23-E6-IDL, YIK, and YIK-C16, alone, or in complex with N46. The secondary structures of HP23-E6-IDL (**a**), YIK (**b**), and YIK-C16 (**c**), alone, or in complex with N46, were analyzed with circular dichroism (CD) spectroscopy. The CD spectra of HP23-E6-IDL/N46, YIK/N46, and YIK-C16/N46 complexes displayed typical double minima at 208 and 222 nm for the α-helical feature.*

the helical content of the HP23-E6-IDL/N46 mixture was much higher than that of HP23-E6-IDL, or N46 alone, indicating that HP23-E6-IDL can interact with N46 to form 6-HB. As for

peptide YIK, the replacement of Thr639 by Ile did not change the secondary structure of HP23-E6-IDL, either alone or in complex with N46 (Figure 2b). Similarly, YIK-C16 could also form 6-HB with N46, and the helical content was also similar to that of HP23-E6-IDL and N46 (Figure 2c), suggesting that the addition of a lipid domain to the C-terminus of YIK does not significantly affect its α-helicity and its ability to interact with an NHR peptide to form 6-HB.

Improved Anti-HIV-1 Activity of YIK-C16 may Result from inhibition of cell-cell fusion and 6HB Formation

To investigate why YIK-C16 exhibited much higher potency than YIK and HP23-E6-IDL, we compared their ability to inhibit HIV-1-mediated cell-cell fusion. As shown in Figure 3a, YIK-C16 is about two-fold more potent than YIK and HP23-E6-IDL in inhibiting HIV-1-mediated cell-cell fusion, suggesting that the enhanced anti-HIV-1 activity of YIK-C16 may result from its improved ability to inhibit HIV-1 fusion with and entry into the host cell. Most CHR-derived peptides inhibit HIV-1 infection and cell-cell fusion by binding to the viral gp41 NHR domain and blocking fusion-active 6-HB formation. Thus, we next tested whether YIK-C16 could inhibit 6-HB formation more efficiently. As shown in Figure 3b, YIK-C16 possessed inhibitory activity against 6-HB formation with as much potency as either HP23-E6-IDL or YIK, suggesting that conjugation of C16 to YIK does not affect the peptide's ability to block 6-HB formation.

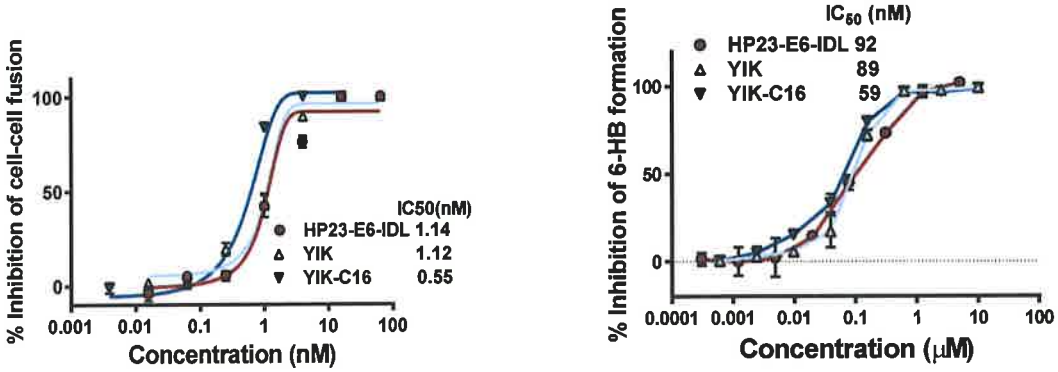

Figure 3. *Inhibitory activity of YIK-C16. (**a**) Inhibitory activities of HP23-E6-IDL, YIK, and YIK-C16 against cell-cell fusion between H9/IIIB cells and MT-2 cells; (**b**) Inhibitory activities of HP23-E6-IDL, YIK, and YIK-C16 against 6-HB formation between N46 and C34 peptides*

YIK-C16 Exhibited Improved Ex Vivo Anti-HIV-1 Activity and Prolonged Serum Half-Life

Previous studies showed that the addition of a lipid domain could enhance in vitro and ex vivo anti-HIV-1 activity and improve the in vivo stability of peptide-based HIV-1 fusion inhibitor [12–14,19]. Here, we investigated whether YIK-C16 could also enhance ex vivo anti-HIV-1 activity, as determined by the highest dilution-fold of the serum causing 50% inhibition of HIV-1 infection (equivalent to the IC_{50}) of the peptide and by extended half-life. Serum samples were collected at different time point post-peptide treatment and tested for their inhibitory activity against HIV-1IIIB infection. As shown in Figure 4a, serum samples treated with HP23-E6-IDL and YIK reached maximum inhibitory activity (about 300-fold of their IC_{50}) at 1 h post-injection and decreased to the background level at about 2 h post-injection. In contrast, sera samples from YIK-C16 showed inhibition peak (about 4500-fold of its IC_{50}) at about 3 h post-injection and maintained high inhibitory activity (>275-fold of its IC_{50}) for 15 h post-injection (Figure 4a). These results confirm that the addition of lipid domain C16 to the YIK peptide can

significantly enhance its in vitro and ex vivo anti-HIV-1 activity. Most recently, Chong et al. have reported that the concentration of an anti-HIV-1 lipopeptide in sera is closely correlated with its ex vivo anti-HIV-1 activity [19]. Therefore, based on the in vitro IC_{50} and ex vivo anti-HIV-1 activity of peptides HP23-E6-IDL, YIK, and YIK-C16 for inhibiting HIV-1 infection

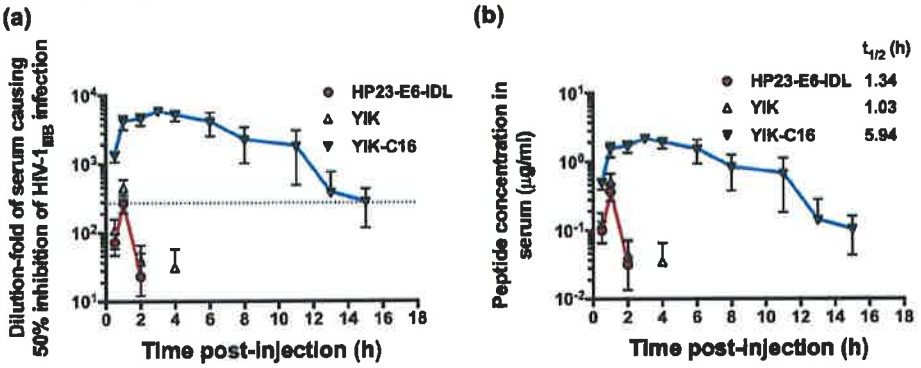

Figure 4. *Ex vivo anti-HIV-1 activity and concentration of the inhibitor peptides in serum samples. (**a**) Anti-HIV activity of the serum samples collected at different time points after administration of HP23-E6-IDL, YIK, and YIK-C16. (**b**) Concentration of the active peptides in the serum samples was estimated using MOD-FIT program.*

described above, we estimated the concentration of the active peptides in sera collected at different time points post-injection (Figure 4b). Based on the concentration profile, we calculated the serum half-life of HP23-E6-IDL, YIK, and YIK-C16, using MODFIT software [20]. The serum half-life of YIK-C16 ($t_{1/2}$ = 5.9 h) was about 4.5- and 5.9-fold longer than that of YIK ($t_{1/2}$ = 1.3 h) and HP23-E6-IDL ($t_{1/2}$ = 1.0 h), respectively. These results suggest that conjugation of lipid domain C16 to the YIK peptide can significantly extend the serum half-life of the peptide.

YIK-C16 Exhibited no In Vitro Cytotoxicity

To determine whether lipopeptide YIK-C16 has in vitro cytotoxicity, we incubated lymphocyte cell lines MT-2 and M7 with HP23-E6-IDL, YIK, or YIK-C16 at graded concentrations for three days and tested for their cell viability by CCK8 assay. As shown in Figure 5, none of these peptides exhibited in vitro cytotoxicity to MT-2 or M7 cells at concentrations as high as 8 μM, which is more than 100-fold higher than the IC_{50} of YIK-C16 for inhibiting HIV-1 pseudovirus infection, respectively, suggesting that YIK-C16 has a good safety profile.

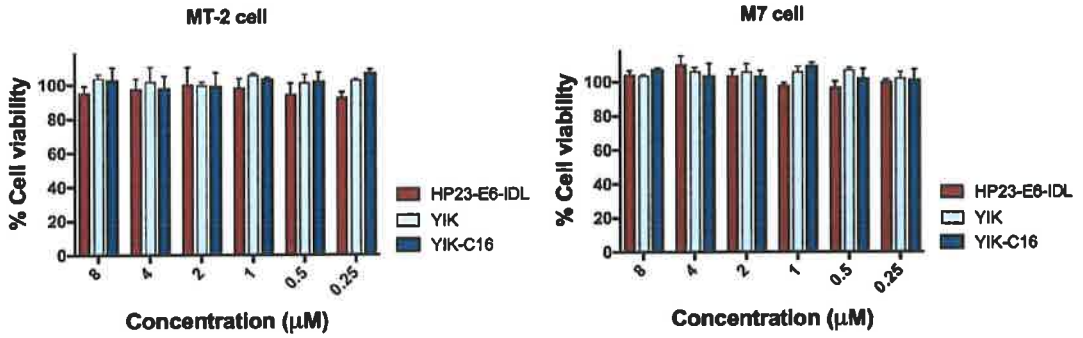

Figure 5. *In vitro cytotoxicity of HP23-E6-IDL, YIK, and YIK-C16. The viability of MT-2 and M7 cells treated with HP23-E6-IDL, YIK, or YIK-C16 at graded concentrations was evaluated by CCK8 assay.*

DISCUSSION

To overcome the limitation of low potency and short in vivo half-life of the peptidic anti-HIV drug enfuvirtide (T20), we introduced a mutation, T639I, and added palmitic acid to HP23-E6-IDL, a peptide-based HIV fusion inhibitor with high potency and good resistance profile [11]. The newly conjugated peptide YIK-C16 is about 10-fold more potent than HP23-E6-IDL against HIV-1 infection, respectively. Mechanistic study suggests that the improved anti-HIV-1 activity of lipopeptide YIK-C16 could result from the enhanced activity of binding to the target cell or viral membranes, not increased inhibitory activity on 6-HB formation. It has been previously reported that lipids, such as cholesterol and sphingolipids, are assembled as lipid rafts on the cell membranes and play an essential role in viral entry and release [21–25]. Meanwhile, alteration in the ratio of these lipids on the viral membrane could significantly impair viral infectivity [26,27]. Even though the exact mechanism of action of T20 is still under debate, it has been reported that T20 inhibits HIV fusion by binding to the viral gp41 NHR domain via the N-terminal portion of T20 and then interacting with the target cell membrane via its C-terminal hydrophobic lipid-binding domain (LBD) [28–30]. Through its lipid domain, a lipopeptide can bind to cell membranes more efficiently than LBD of T20, thus possessing improved antiviral activity [12,13,31]. The highly potent anti-HIV-1 activity of YIK-C16 further certified the feasibility of this strategy and provided more insight into the functions of membrane protein and the roles of lipids in HIV-1 entry.

The inhibitory activity of YIK-C16 reached picomolar level. Even more promising, its serum half-life in mice is 4.5- and 5.9-fold longer than that of HP23-E6-IDL and YIK, respectively. Notably, C16 can bind to human serum albumin (HSA) [32,33], a human protein with wide distribution, with an in vivo half-life of 15 to 19 days [34]. Albuvirtide (ABT), a 3-maleimidopropionic acid (MPA)-modified HIV fusion inhibitor, is the only long-acting anti-HIV drug approved by the China Food and Drug Administration in 2018 for clinical use once a week because MPA can irreversibly bind to HSA, resulting in an extended half-life of the peptide ($t_{1/2}$ = 25.8 h) [35]. Since YIK-C16 may also bind to HSA via its C16 domain, its half-life may be further extended in humans. Meanwhile, YIK-C16 is about 10-fold more potent in inhibiting HIV-1 infection than ABT [36], making it a promising candidate for further development as a new long-acting anti-HIV drug for clinical use. Recently, stearic acid (C18)-modified anti-HIV peptide showed even higher potency and longer half-life time [19] than the C16-modified HIV fusion inhibitor. Therefore, it is possible that we could also modify YIK with C18 to further extend its in vivo half-life time. Many studies have revealed that only single-site mutation can elicit significant alteration of HIV-1 Env structure [37,38]. Conjugation of a tail anchor (IDL) to helical peptide (HP23-E6) may slightly alter the conformation of the original peptide, thus resulting in the mismatch of T639 to the original target residue on NHR. Addition of hook-like structure to both N- and C-termini has already rendered peptide HP23-E6-IDL as potent as C34-T639I [15].

In sum, it has been demonstrated that addition of a lipid domain, palmitic acid (C16), to YIK peptide, a potent HIV fusion inhibitor, results in significant enhancement of in vitro and ex vivo anti-HIV-1 activity and remarkably prolonged in vivo half-life, making it a good candidate for development into a new HIV fusion inhibitor-based, long-acting anti-HIV drug.

MATERIALS AND METHODS

Peptides and Cell Lines

Peptides (Figure 1a) were synthesized with purity >95%. Peptides were dissolved in PBS, and their concentrations were determined by using Nanodrop TM 2000 spectrophotometers (Thermo Fisher Scientific Inc., Waltham, MA) and calculated based on a theoretical molar-extinction

coefficient according to the peptide sequences as described previously [39]. MT-2 cells and H9/IIIB cells were obtained from ATCC or commercial entities.

Inhibition Against HIV-1 Infection by Peptides

Inhibitory activities of peptides on infection by one cycle pseudo virus HIV-1 was determined as previously described [42]. For each well of a 96-well plate, 50 μL of a peptide were mixed with 50 μL of 100 × TCID50 (50% tissue culture infective doses) of HIV-1 pseudo virus and incubated at 37 °C for 30 min. Afterwards, 2×10^4 MT-2 were added. After overnight culture, the supernatant was replaced with fresh RPMI-1640 medium containing 10% fetal bovine serum (FBS). After further culture at 37 °C for three days, 50 μL of the culture medium were collected and mixed with equal volume of 5% (*v*/*v*) Triton X-100. The p24 antigen, which represents HIV-1 quantity, was detected by ELISA. Briefly, the collected mixtures were added to a plate coated with anti-HIV Immune Globulin (HIVIG) from the NIH AIDS Reagent Program. Anti-p24 mAb 183, rabbit anti-mouse IgG-HRP (Dako, Glostrup, Denmark) and substrate 3,3,5,5-TMB (Sigma-Aldrich, New York, NY) were added and washed away sequentially. The absorbance at 450 nm (A450) was determined by a Multi-Detection Microplate Reader (Ultra 384, Tecan, Tokyo, Japan). IC_{50}s were calculated using Calcusyn software (Biosoft, Ferguson, MO), and the lines of best fit were drawn using GraphPad Prism 8 software (La Jolla, CA).

Circular Dichroism (CD) Spectroscopy

The secondary structure of the single peptides HP23-IDL, YIK, YIK-C16 or N46, or the complexes HP23-IDL/N46, YIK/N46, or YIK-C16/N46, were assessed by CD spectroscopy as described previously [44]. Briefly, N46 and/or CHR-peptide in PBS (final concentration: 10 μM) was incubated at 37 °C for 30 min and then measured on a Jasco spectropolarimeter (Model J-815; Jasco, Inc., Easton, MD), using a 1-nm bandwidth with a 1-nm step resolution from 195 to 260 nm at room temperature. The baseline curve was determined on PBS alone.

HIV-1-Mediated Cell-Cell Fusion Assay

A dye transfer assay was performed to detect HIV-1 Env-mediated cell-cell fusion as described previously [42]. Briefly, for each well of a 96-well plate, 2×10^3 H9/IIIB cells labeled with the fluorescent reagent Calcein AM (Molecular Probes, Inc., Eugene, Oregon) were incubated with a CHR-peptide at 37 °C for 30 min. Afterwards, 10^4 MT-2 cells were added and incubated at 37 °C for 2 h. The fused and unfused Calcein-labeled H9/IIIB cells were counted under an inverted fluorescence microscope. The IC_{50} values were calculated by using the Calcusyn computer program (Biosoft, Ferguson, MO).

Inhibition of 6-HB Formation by Peptides In Vitro

The inhibitory activity of a peptide on gp41 6-HB formation was detected as previously described [45]. Briefly, 50 μL of a peptide were incubated with 50 μl of N46 (4 μM) at 37 °C for 30 min. Then, 100 μL of C34 (2 μM) were added and incubated at 37 °C for another 30 min. Then,50 μL of the mixture were added to a 96-well plate coated with 4 μg/mL polyclonal antibody against gp41 6-HB, NY364. Afterwards, a mAb specific for 6-HB, NC-1, rabbit anti-mouse IgG-HRP and substrate 3,3,5,5-TMB (Sigma-Aldrich, New York, NY) were added and washed away sequentially. The absorbance at 450 nm (A450) was determined by a Multi-Detection Microplate Reader. IC_{50}s were calculated using Calcusyn software, and the lines of best fit were drawn using GraphPad Prism software.

Ex Vivo Anti-HIV-1 Activity and Serum Half-Life of Peptides

Evaluation of ex vivo anti-HIV-1 activity of a peptide was performed as described recently [46]. Serum samples were collected before (0 h) and after injection (0.5, 1, 2, 4, and 6 h for peptides HP23-E6-IDL and YIK; 1, 3, 7, 11, 13, 15, 17, and 19 h for lipopeptide YIK-C16). Anti-HIV-1 activities of the mouse serum samples were determined in the same way as described above. The highest dilution-fold of the serum causing 50% inhibition of HIV-1_{IIIB} infection (IC_{50}) was calculated. The concentration of an active peptide in serum of a mouse treated with this peptide was estimated based on the IC_{50} value of the peptide in the mouse serum for inhibiting pseudovirus infection activity as described above. Then, the serum half-life and other pharmacokinetic parameters of the peptide were calculated using MODFIT software [18] based on the estimated concentration of the active peptide in serum samples collected from the mouse at the different time points post-injection of the peptide.

Cytotoxicity of YIK-C16

The cytotoxic effects of HP23-E6-IDL, YIK, and YIK-C16 to MT-2 and M7 cells were determined as previously described [47]. A peptide at graded concentration was incubated with 2×10^5/mL cells at 37 °C for 3 days before adding 10 μL of CCK8 reagent (Sigma-Aldrich). After another round of incubation at 37 °C for 2 h, A450 was measured with the Multi-Detection Microplate Reader. Cell viability was calculated by dividing A450 of untreated cells by A450 of cells treated with a peptide.

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