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Research paper

Enfuvirtide–PEG conjugate: A potent HIV fusion inhibitor with improved pharmacokinetic properties



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ABSTRACT

Enfuvirtide (ENF) is a clinically used peptide drug for the treatment of HIV infections, but its poor pharmacokinetic profile ($T_{1/2} = 1.5$ h in rats) and low aqueous solubility make the therapy expensive and inconvenience. In this study, we present a simple and practical strategy to address these problems by conjugating ENF with polyethylene glycol (PEG). Site-specific attachment of a 2 kDa PEG at the N-terminus of ENF resulted in an ENF–PEG (EP) conjugate with high solubility (≥ 3 mg/mL) and long half-life in rats ($T_{1/2} = 16.1$ h). This conjugate showed similar antiviral activity to ENF against various primary HIV-1 isolates ($EC_{50} = 6\text{--}91$ nM). Mechanistic studies suggested the sources of the antiviral potency. The conjugate bound to a functional domain of the HIV gp41 protein in a helical conformation with high affinity ($K_d = 307$ nM), thereby inhibiting the gp41-mediated fusion of viral and host-cell membranes. As PEG conjugation has advanced many bioactive proteins and peptides into clinical applications, the EP conjugate described here represents a potential new treatment for HIV infections that may address the unmet medical needs associated with the current ENF therapy.

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1. Introduction

Highly active antiretroviral therapy (HAART) that allows for the simultaneous administration of multiple drugs is currently the standard treatment for HIV infection. The drug combinations used in this setting normally consist of several inhibitors that target different enzymes in the viral life-cycle, including reverse transcriptase, protease, and integrase. Although HAART can dramatically decrease the levels of morbidity and mortality associated with HIV infection, the emergence of drug resistance is usually unavoidable because of the high mutation and rapid replication rates

of the virus. The sequential administration of different drugs is therefore required for the treatment of HIV infection, as well as the development of new targeted therapies [1]. Enfuvirtide (ENF) is an important choice for the treatment of patients who have not responded well to HAART. In contrast to traditional small-molecule enzyme inhibitors, ENF is a 36-residue peptide that binds to the viral envelope gp41 protein and inhibits the gp41-mediated membrane fusion between the virus and the host-cell, which is an essential step for the delivery of the viral genome into the cell [2]. ENF has been successfully used in clinic practice to control HIV infection and transmission. However, as is the case with many peptide and protein drugs [3], ENF exhibits poor aqueous solubility and can be rapidly removed from systemic circulation through metabolic pathways and immune response systems. To maintain an efficacious concentration, ENF must be administered twice daily at a high dosage (90 mg) by subcutaneous injections. Furthermore, ENF therapy must be implemented consistently over an extended period of time because of the chronic nature of HIV infection [4],

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which can lead to severe injection-site reactions [5]. ENF treatment is therefore inconvenient to the patient and represents an expensive regimen for the health care provider, and these factors have severely limited its application in the clinic.

A number of different approaches have been developed to address the pharmacokinetic problems associated with ENF and several other peptide analogues, which can inhibit HIV fusion in a similar manner. Peptide engineering strategies, such as the incorporation of salt bridges, can lead to increases in the in vivo stability of a peptide sequence, which can result in extended half-lives [6–10]. The chemical modification of a peptide with a synthetic or natural molecule, such as cholesterol [11] or albumin [12,13], can also lead to a reduction in its immunogenicity and proteolysis [14–20]. Polyethylene glycol (PEG) conjugation is a commonly used method for improving the pharmacokinetics of therapeutic peptides and proteins [21–23], and this method has been applied to a peptide inhibitor of HIV fusion that overlaps ENF by 33 residues [19]. The PEGylated peptide showed a 3.4-fold increase in the half-life against tryptic degradation, although there was a slight decrease in the fusion-inhibitory potency when compared with the unmodified peptide. Despite the positive results reported in this study for the PEGylation of the inhibitor, there have been no reports in the literature to date pertaining to the antiviral activity or the pharmacokinetic properties of a fusion inhibitor conjugated with PEG.

As part of our ongoing research towards the development of persistent inhibitors against HIV fusion, we previously developed a series of glycosylated ENFs with long in vivo half-lives that completely retained their antiviral activities [20]. In a similar manner to PEGylation, the glycosylation of peptides and proteins leads to an increase in their molecular size and steric bulk, which can have a significant impact on their biophysical and physiological properties. However, current techniques for the construction of glycopeptides and glycoproteins are quite challenging. Recombinant expression systems allow for the rapid and large-scale production of glycopeptides and glycoproteins, but these systems provided only limited control over the glycosylation step [24]. Chemical and chemoenzymatic syntheses can produce structurally well-defined molecules [25,26], but generally require multiple steps and tedious purification procedures that can result in low production yields, thereby limiting their practical application.

Based on our previous experience of the glycosylation of ENF [20], and the fact that PEGylation has advanced many bioactive proteins and peptides into clinical applications [23], we sought to prepare the ENF-PEG (EP) conjugate as a simple alternative. By controlling the modification site of ENF and the molecular weight (MW) of PEG, we successfully synthesized an EP conjugate with a remarkably improved pharmacokinetic profile. This conjugate formed a strong binding interaction with a functional fragment of the gp41 protein and exhibited potent inhibitory activities towards various HIV isolates that are currently in circulation, with in vitro efficacies comparable to that of ENF. It is therefore envisaged that the results presented in this study will provide a basis for the development of further EP conjugates as long-lasting HIV fusion inhibitors for the treatment of HIV infection.

2. Results and discussion

2.1. Synthesis and characterization of EP conjugates

Following our established glycosylation strategy [20], we investigated the site-specific addition of PEG to the terminus of ENF using a thiol-maleimide coupling reaction (Fig. 1). Two ENF derivatives bearing a single cysteine residue at their N- or C-termini (cENF and ENFc) were modified with methoxy-PEG-maleimides

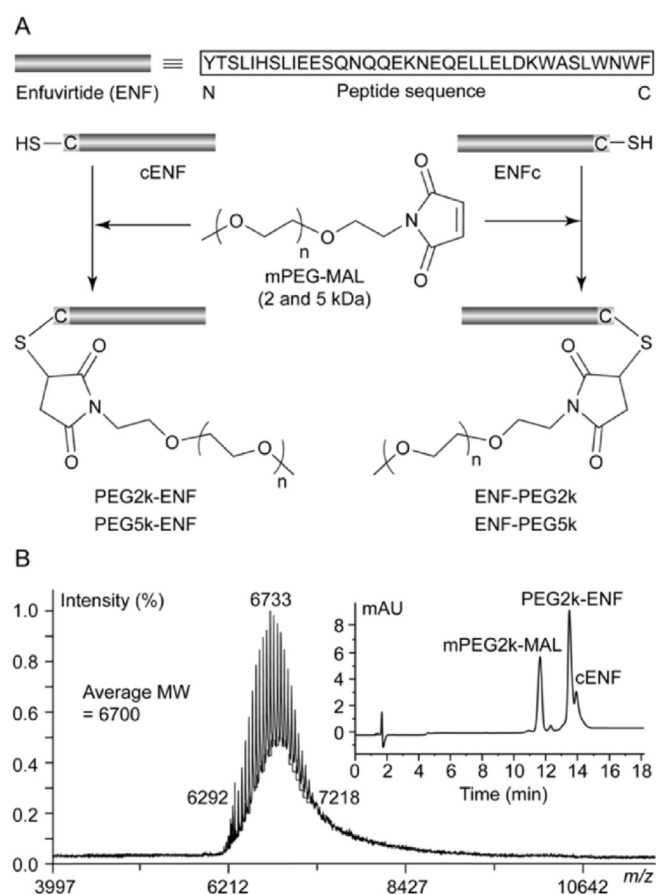


Fig. 1. Synthesis (A) and characterization (B) of EP conjugates (PEG2k-ENF, PEG5k-ENF, ENF-PEG2k, and ENF-PEG5k). All of the peptide sequences were protected by N-terminal acetylation and C-terminal amidation. (A) Reaction conditions: phosphate buffer (pH 7.2), rt, 10 min, 90% for PEG2k-ENF, 85% for ENF-PEG2k, 82% for PEG5k-ENF, and 82% for ENF-PEG5k. (B) MALDI-TOF mass spectrum and HPLC chromatogram (inset) of PEG2k-ENF. All of the other conjugates were characterized in a similar manner. HPLC chromatographic conditions: Phenomenex Synergi Hydro-RP C18 column (4 μ m, 10 \times 250 mm), 40 \rightarrow 70% CH₃CN/H₂O linear gradient over 11 min at 2.5 mL/min, UV detection at 220 nm.

(mPEG-MAL) of 2 and 5 kDa. These reactions proceeded smoothly under near-physiological conditions to give the corresponding EP conjugates bearing the fully intact ENF sequence. The conjugates were purified by preparative HPLC, followed by the lyophilization of the resulting residues to give the desired products as white powders in 82–90% yields (purity >95%). The subsequent characterization of the conjugates by MALDI-TOF MS revealed a single defined attachment of PEG to the peptide backbone. As expected, all of the conjugates were highly soluble in water (≥ 3 mg/mL) as opposed to ENF, which has negligible aqueous solubility.

2.2. Binding of EP conjugates to a functional domain of gp41

To ensure that the modified ENF retained its binding affinity for the HIV gp41 protein, we measured the strength of the binding interaction between the conjugates and a functional fragment of gp41 known as N46, which contains the full binding region for ENF and has been used as a suitable model for the investigations of HIV fusion inhibitors [27–29]. Surface plasmon resonance (SPR) experiments showed that all the conjugates strongly bound to N46 with affinities (K_d) in the range of 307–1410 nM (Fig. 2). Although the affinities of the conjugates were lower than that of ENF (182 nM), we observed a general trend towards increasing affinity as the MW of the PEG portion of the conjugate decreased.

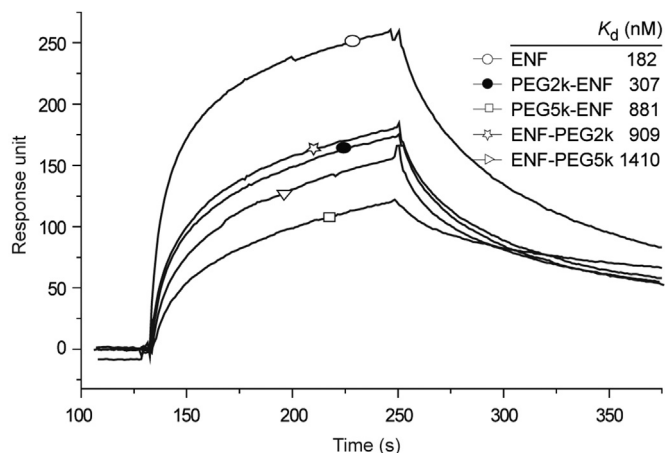


Fig. 2. SPR sensorgrams for the binding of ENF and EP conjugates to N46. Peptide N46 was immobilized on the surface of a Biacore sensor chip. Solutions containing different concentrations of ENF or EP conjugates in running buffer (PBS + 0.05% v/v Tween 20, pH 7.4) were flowed over the surface of the chip. The binding curves of ENF and the conjugates at 1 μ M are shown in this figure. The dose-dependent sensorgrams are shown in the Supplementary data (Fig. S1). The binding kinetic parameters (Table S1) were calculated using Biacore analysis software. The sequence of N46 is as follows: TLTVQARQLLSGIVQQQNNLLRAIEAQQHLIQLTVWGIIKQLQARIL.

Particularly, the PEG2k-ENF demonstrated an affinity (307 nM) approximating to that of ENF. In contrast, none of the EP conjugates or ENF bound to bovine serum albumin (BSA), confirming that the observed interactions were specific for peptide N46. The SPR experiments measured the binding of ENF or EP conjugates to the chip-immobilized N46, and the assay condition did not exactly match with the membrane environment necessary for the fusion inhibition. However, the potent interaction between EP conjugates and N46 confirmed in this assay suggested that the attached PEG had no significant impact on the intrinsic binding affinity of ENF.

2.3. Secondary structure of EP conjugates and their complex with N46

Peptide conformation can have a remarkable effect on the antiviral activity of fusion inhibitors [27–32]. ENF lacks a defined structure in solution, where it has a low α -helicity. In contrast, N46 adopts a typical α -helical conformation in solution, and its α -helicity is reduced dramatically upon its binding to ENF. The ENF and EP conjugates prepared in this study were analyzed by circular dichroism (CD), and the results revealed that the low α -helical content of ENF was further decreased by the attached PEG in a MW-dependent manner (Fig. 3A). Interestingly, all of the conjugates interacted with N46 to form stable α -helical complexes, which had markedly higher α -helicities than that of N46 alone or in complex with ENF (Fig. 3B). This result is, however, inconsistent with the affinity of the conjugates for N46 which is obviously lower than that of ENF, as the α -helicity of binding complexes is positively correlated to their stability [28,29]. The apparent contradiction between the affinity and the α -helicity likely resulted from the different assay formats. Unlike the chip-based SPR assay in which N46 was immobilized on a solid surface, in CD experiment free N46 and the EP conjugate in solution were able to partake in a dynamic binding process that allowed the attached PEG to interact with the binding complex and the aqueous medium. The mechanism of the improvement in the α -helicity of the complex following PEGylation has not yet been determined. However, the ability of the attached PEG to form an amphiphilic shell capable of stabilizing the coiled-coil peptide core under aqueous conditions [36–38], may provide some evidence in support of our results.

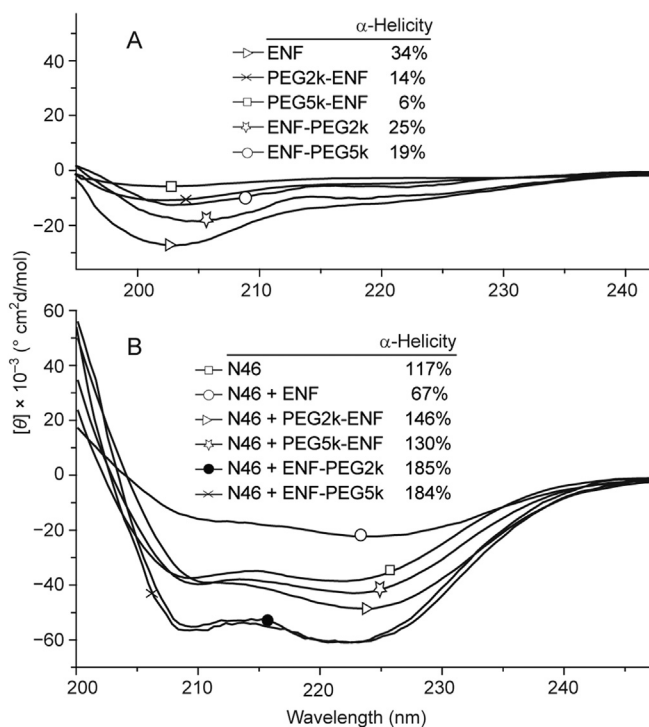


Fig. 3. CD spectra of ENF and EP conjugates in PBS buffers (pH 7.2) at 20 °C. The α -helicities are calculated relative to an ellipticity value of $-33,000^\circ \text{ cm}^2/\text{d/mol}$ (at 222 nm) that is assumed to correspond to a helical content of 100% [33–35] (for details, see Table S2). (A) In the absence of peptide N46, both ENF and the conjugates adopted a random coil structure. (B) In the presence of equimolar amounts of N46, the ENF–N46 complex showed a much lower α -helicity than N46, whereas all of the EP conjugate–N46 complexes were fully α -helical, with their ellipticities below $-42,000^\circ \text{ cm}^2/\text{d/mol}$ (at 222 nm).

2.4. Antiviral activity of EP conjugates against a laboratory-adapted HIV

Having established that the EP conjugates could bind to the gp41 fragment to form high affinity complexes with high α -helicities, we examined their antiviral activities against a laboratory-adapted HIV-1 strain SF33. The replication of the virus in TZM-bl cells [39] was measured in the presence of the conjugates, with ENF as a control (Table 1). ENF and all of the conjugates inhibited the replication of HIV at nanomolar concentrations, with no cytotoxicity observed below the micromolar range ($\text{CC}_{50} > 100 \mu\text{M}$), as determined using an XTT assay [41]. The two conjugates bearing the smaller PEG (2 kDa) showed higher activities than their 5 kDa counterparts. The N-terminally PEGylated peptides showed much greater activity than their C-terminal congeners, with the PEG2k-ENF exhibiting the highest inhibitory activity of all the

Table 1
Antiviral activities of EP conjugates against HIV-1_{SF33}.^a

Inhibitors	EC ₅₀ (nM)	EC ₉₀ (nM)	CC ₅₀ (μM) ^b	SI ^c
ENF	3 \pm 0	23 \pm 2	>100	>33,333
PEG2k-ENF	4 \pm 0	44 \pm 9	>100	>25,000
PEG5k-ENF	8 \pm 1	84 \pm 3	>100	>12,500
ENF-PEG2k	12 \pm 3	250 \pm 7	>100	>3333
ENF-PEG5k	19 \pm 2	362 \pm 2	>100	>5263

^a The EC₅₀ and EC₉₀ values were determined using the Reed and Muench method [40] after the incubation of TZM-bl cells with virus in the presence of ENF or an EP conjugate. All of these experiments were performed in triplicate and the results were reported as the mean value \pm standard deviation (SD).

^b The CC₅₀ values were determined with TZM-bl cells.

^c SI (selectivity index): CC₅₀/EC₅₀.

conjugates. Notably, the EC_{50} value of PEG2k-ENF (4 nM) was comparable to that of ENF (3 nM). The chemical modification at the C-terminus of ENF has an adverse impact on its antiviral activity [11,12,20], because the lipophilic residues (WNWF) in this region are required for the anchoring of the peptide into the membrane interface where the fusion occurs [28,29]. Our data were therefore in agreement with these earlier findings and revealed that the attachment of PEG2k to the N-terminus of ENF did not lead to a significant decrease in its antiviral efficacy.

2.5. Pharmacokinetics of PEG2k-ENF in rats

We next analyzed the pharmacokinetics of PEG2k-ENF in rats following the subcutaneous injection of a single dose of this material (Fig. 4). In contrast to ENF, which was both rapidly adsorbed and rapidly cleared from systemic circulation, the EP conjugate showed a slower but more sustained release profile from the injection site. The calculation of pharmacokinetic parameters using the well-established non-compartmental method [44] revealed that the conjugate had an elimination half-life [45] ($T_{1/2}$) which was over 10-fold longer than that of ENF. The overall pharmacokinetic profile of PEG2k-ENF was also superior to that of ENF, with marked

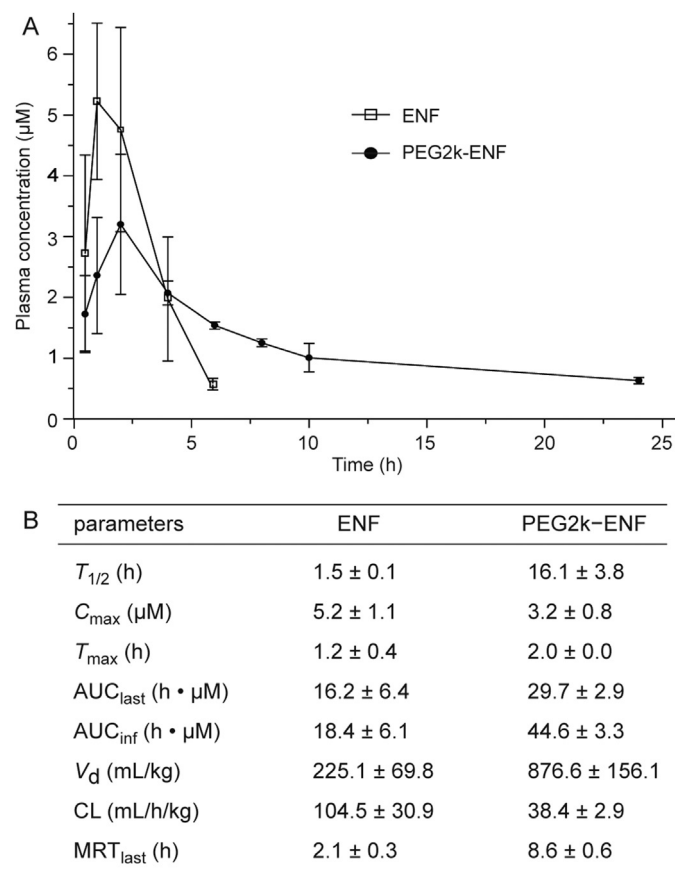


Fig. 4. Pharmacokinetic analysis in Sprague–Dawley rats. ENF and PEG2k-ENF were subcutaneously administered at 1.7 µmol/kg (in physiological saline). Four animals per group were used. (A) Pharmacokinetic curves. The concentrations of ENF and the conjugate in plasma were determined using a known HPLC analysis [42,43]. Error bars indicate the SD. (B) Pharmacokinetic parameters. Values were calculated using the non-compartmental analysis [44] (NCA, WinNonlin, version 6.2, Pharsight Cary, NC) and expressed as the mean values ± SD. $T_{1/2}$ (elimination half-life or terminal half-life) [45] refers to the time required for the concentration of remaining drug in plasma to be reduced by 50% after the pseudo-equilibrium of drug distribution in the body has been reached.

increases in the area under the plasma concentration time curve (AUC), apparent volume of distribution (V_d), and mean residence time (MRT), as well as a marked decrease in systematic clearance rate (CL). This improvement in pharmacokinetic profile, as generally seen for many other PEGylated protein or peptide therapeutics [21–23], makes the EP conjugate attractive as a long-lasting fusion inhibitor to address the unmet medical needs associated with the treatment of HIV infection.

2.6. Antiviral spectrum of PEG2k-ENF

To further evaluate the therapeutic potential of PEG2k-ENF, we examined its antiviral spectrum using fourteen HIV-1 viruses of different subtypes, including five B' (also known as Thai B), five BC, and four AE isolates, all of which represent the predominant strains currently circulating in China [46–49]. The PEG2k-ENF inhibited the replication of these viruses in TZM-bl cells as efficacious as ENF, with activities (EC_{50}) in the low-nanomolar range (Table 2) and selectivity indices (SI) greater than 1000. Taken together with its remarkably improved pharmacokinetic properties, the broad and potent antiviral activities of PEG2k-ENF show that the EP conjugates warrant further preclinical and clinical evaluations.

3. Conclusions

We have demonstrated that the site-specific PEGylation of ENF improved its pharmacokinetic profile without discernible decrease in its antiviral activity. The coupling of cENF to mPEG-MAL through a concise thiol-maleimide reaction allowed for the large-scale synthesis of the EP conjugate, which could be important for clinical applications that require a sufficient supply of material. The EP conjugate exhibited much better aqueous solubility and in vivo half-life than ENF, which could lead to less-frequent dosing regimens and a reduction in adverse injection-site reactions. In addition, the EP conjugate displayed potent inhibitory activity towards the most common primary HIV strains circulating in China, further highlighting its potential as an effective and long-acting inhibitor of HIV fusion. It is noteworthy that a number of other enveloped viruses, including Ebola virus, Middle East respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome

Table 2
Antiviral spectrum of PEG2k-ENF against different HIV-1 subtypes.^a

Viruses	EC_{50} (nM)		SI ^b	
	ENF	PEG2k-ENF	ENF	PEG2k-ENF
Subtype B'				
20100259	38 ± 4	44 ± 2	>2632	>2273
20100968	54 ± 5	64 ± 6	>1852	>1563
20100096	51 ± 4	68 ± 2	>1961	>1471
20100311	14 ± 2	20 ± 2	>7143	>5000
20100104	44 ± 2	47 ± 1	>2273	>2128
Subtype BC				
XJDC6371	53 ± 8	58 ± 6	>1887	>1724
CJB256	40 ± 1	46 ± 4	>2500	>2174
XJDC6291	50 ± 9	42 ± 1	>2000	>2381
CJB257	85 ± 7	91 ± 8	>1177	>1099
XJDC6331	50 ± 7	59 ± 5	>2000	>1695
Subtype AE				
CYM033	65 ± 3	71 ± 4	>1539	>1409
BJMSM376	24 ± 1	26 ± 3	>4167	>3846
BJMSM543	56 ± 4	60 ± 1	>1786	>1667
CYM015	5 ± 1	6 ± 1	>20,000	>16,667

^a EC_{50} was determined using the Reed and Muench method [40] after the incubation of TZM-bl cells with virus in the presence of ENF or PEG2k-ENF. Values are from triplicate assays and shown as the mean ± SD.

^b SI = CC_{50}/EC_{50} . The CC_{50} values of both peptides were greater than 100 µM, as determined with TZM-bl cells.

coronavirus (SARS-CoV), have similar mechanisms of virus–cell fusion to that observed in HIV infection. With this in mind, it is envisaged that the peptide inhibitors currently under investigation against these viruses [50–53] could also be subjected to the PEGylation approach described in this study.

4. Experimental procedures

4.1. Materials and instrumentation

All peptides used in this study were synthesized using a solid-phase Fmoc method at Beijing Scilight Biotechnology LLC (Beijing, China), purified by HPLC (purity >95%), and characterized by MALDI–TOF MS. The sources of HIV-1_{SF33} and TZM-bl, as well as the methods used for their preservation and passage have been described previously [20,41]. The clinical isolates of HIV-1 subtypes B', BC, and AE were obtained from treatment-naïve patients and stored at our laboratory in Chinese Center for Disease Control and Prevention [46,47]. These viruses represent the predominant strains circulating in China from 2005 to 2008. Sprague-Dawley rats (male, 7 weeks old, and 150–180 g) were purchased from Beijing HFK Bioscience (Beijing, China) and fed for 2 days before being used for pharmacokinetic analysis. mPEG-MAL (2 and 5 kDa) was purchased from Beijing Kaizheng Biotech Development (Beijing, China). All of the other chemicals used in this study were purchased as the analytical grade from various commercial sources. HPLC analyses were performed on an Agilent 1200 system (Agilent, USA) equipped with a UV detector. MALDI-TOF mass spectra were recorded on a Bruker BIFLEX III spectrometer (Bruker Daltonics, Germany). All experiments with the viruses were performed in an approved biosafety level (BSL-3) containment laboratory.

4.2. Synthesis of EP conjugates

An aqueous solution of disodium hydrogen phosphate (5 mM) was added in a dropwise manner to a stirred mixture of cysteine-incorporated ENF (cENF or ENFc, 10 mg, 2 μ mol) and mPEG-MAL (2 or 5 kDa, 2 equiv) in a sodium phosphate buffer (5 mL, 5 mM, pH 7.5) to adjust the pH of the mixture to 7.2. The resulting mixture was then stirred for 10 min at rt before being directly purified by preparative HPLC to afford PEG2k-ENF (12 mg, 90%), ENF-PEG2k (11 mg, 85%), PEG5k-ENF (15 mg, 82%), or ENF-PEG5k (15 mg, 82%) as a white powder. The HPLC chromatographic conditions are provided in Fig. 1.

4.3. SPR assay

The binding affinities of EP conjugates to peptide N46 were analyzed by a Biacore biosensor system (Biacore 3000, Biacore Co., Ltd, Sweden), using a previously described procedure [20]. Briefly, N46 was immobilized on a carboxymethyl dextran-coated sensor chip (CM-5, research grade) using a standard primary amine-coupling protocol. Solutions of EP conjugates in PBS containing Tween 20 (0.05% v/v) at different concentrations (0.125–4 μ M) were then flowed over the surface of the sensor at a rate of 20 μ L/min, and the responses were recorded. ENF was used as a positive control instead of EP conjugate. BSA was used as a negative control instead of peptide N46. The binding kinetic parameters were calculated using the Biacore evaluation software (version 4.1), and the results are shown in Table S1.

4.4. CD analysis

The secondary structures of ENF and EP conjugates were analyzed both in the absence and the presence of peptide N46 using

a Chirascan spectropolarimeter (Applied Photophysics Ltd, UK). Measurements were conducted using solutions of the peptides in PBS (5 mM, pH 7.2) as described previously [20]. A concentration of 35 μ M was used for both ENF/EP conjugates and peptide N46.

4.5. HIV inhibition assay

The antiviral activities of ENF and EP conjugates against both the laboratory-adapted HIV-1_{SF33} and the clinical HIV-1 isolates (subtypes B', BC, and AE) were determined in TZM-bl cell cultures, as described previously [20,46]. Briefly, TZM-bl cells grown on a tissue culture plate (10⁴/well) were inoculated with 200 TCID₅₀ of the virus in the presence and absence of ENF or an EP conjugate (100 μ L, in DMEM, at different concentrations from 1.2 to 1000 nM). After incubation at 37 °C for 48 h, the cells (100 μ L) were harvested and lysed using a lysing reagent (100 μ L, Promega). The activity of the luciferase expressed by TZM-bl cells was then measured, and the EC₅₀ and EC₉₀ values were calculated using the Reed and Muench method [40] based on the percentage inhibition of the luciferase activity.

4.6. Cytotoxicity test

The cytotoxicity of EP conjugates was measured in TZM-bl cells following a standard XTT assay protocol [20,41,54].

4.7. Pharmacokinetic analysis

Sprague-Dawley rats (n = 4) were given a single subcutaneous injection of ENF or PEG2k-ENF at a dosage of 1.7 μ mol/kg (in physiological saline). The collection and processing of blood sample, the determination of peptide concentration, and the evaluation of pharmacokinetics were performed as described previously [20]. A known HPLC method [42,43] was used to identify the plasma concentrations of ENF and PEG2k-ENF. The HPLC conditions were as follows: Waters RP-HPLC column (XSELECT CSH C18, 5 μ m, 4.6 \times 150 mm, Waters), 30 \rightarrow 52% CH₃CN/H₂O (containing 0.1 v/v% TFA) gradient at 0.6–1.2 mL/min, UV detection at 220 nm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2016.05.027>.

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