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Research Paper

Half-life extension of the HIV-fusion inhibitor peptide TRI-1144 using a novel linker technology

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ABSTRACT

We have previously developed a linker technology for half-life extension of peptides, proteins and small molecule drugs (1). The linkers undergo β-elimination reactions with predictable cleavage rates to release the native drug. Here we utilize this technology for half-life extension of the 38 amino acid HIV-1 fusion inhibitor TRI-1144. Conjugation of TRI-1144 to 40 kDa PEG by an appropriate *β*-eliminative linker and i.v. administration of the conjugate increased the *in vivo* half-life of the released peptide from 4 to 34 h in the rat, and the pharmacokinetic parameters were in excellent accord with a one-compartment model. From these data we simulated the pharmacokinetics of the PEG-TRI-1144 conjugate in humans, predicting a $t_{1/2,\beta}$ of 70 h for the released peptide, and that a serum concentration of 25 nM could be maintained by weekly doses of 8 μmol of the conjugate. Using a non-circulating carrier (2) similar simulations indicated a $t_{1/2,\beta}$ of 150 h for the peptide released from the conjugate and that dosing of only 1.8 μmol/week could maintain serum concentrations of TRI-1144 above 25 nM. Hence, releasable *β*-eliminative linkers provide significant half-life extension to TRI-1144 and would be expected to do likewise for related peptides.

1. Introduction

We have developed an approach for half-life extension in which a drug is covalently attached to a long-lived macromolecule through a linker that is slowly cleaved to release the native drug [1]. The carrier may be a circulating macromolecule such as polyethylene glycol (PEG), for half-life extension of one or two weeks [1], or non-circulating hydrogel depots, for half-life extension of weeks to months [2]. The linkers self-cleave by a nonenzymatic β-elimination reaction in a highly predictable manner.

In this approach, a macromolecular carrier is attached to a linker that is attached to a drug via a carbamate group (1; Scheme 1); the β-carbon has an acidic carbon–hydrogen bond (C–H) and also contains an electron-withdrawing "modulator" (Mod) that controls the pK_a of that C–H. Upon hydroxide ion-catalyzed proton removal to give **2**, a rapid β-elimination occurs to cleave the linker-carbamate bond and release the free drug and a substituted alkene **3**. The rate of drug release is proportional to the acidity of the proton, and that is controlled by the chemical nature of the modulator; thus, the modulator controls the drug release rate. With releasable prodrug conjugates of this type, the half-life of a rapidly cleared drug is transformed into that of the conjugate itself.

The *β*-eliminative half-life extension technology is particularly useful for peptides, where serum residence times are often minutes to hours. Recently, we described circulating PEG and non-circulating hydrogel conjugates of the 39 amino acid peptide exenatide that gave 56- and 156-fold half-life extensions, respectively [1]. As an additional example, we chose to study TRI-1144, a 38-amino acid second-generation fusion inhibitor derived from the HIV gp41 sequence. TRI-1144 shows potent low-nanomolar *in vitro* inhibition of HIV-1 [3] and a remarkable dose-reducing synergistic action when combined with one or two other fusion inhibitors with distinct binding sites [4]. TRI-1144 was at one time in clinical trials [5] but suffered from a short half-life that required daily injections and was superceded by effective orally active anti-HIV agents.

Ac-TTWEAWDRAIAEYAARIEALLRALQEQQE^{30}KNEAALREL-NH_2

TRI-1144

In the present work, we have attached Lys-30 of TRI-1144 to PEG_{40kDa} by *β*-eliminative linkers, and demonstrated significant half-life extension in the rat. Simulations of the pharmacokinetics in humans suggest that therapeutic levels of the drug could be

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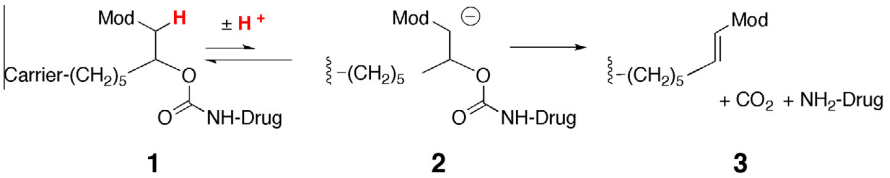


Scheme 1.

achievable with once weekly administration. Although TRI-1144 will likely not be resurrected as a potential drug, it serves as an excellent model for the β-eliminative linker platform technology.

2. Materials and methods

2.1. General

Dibenzocyclooctyne (DBCO) was from Click Chemistry Tools. PEG compounds were purchased from NOF America Corp. The azido-succinimidyl carbonates and four-branch DBCO-Lys(DEAC)-NH-PEG_{40kDa} were prepared as described [1]. HPLC used a Shimadzu Prominence HPLC equipped with an SPD-M20A diode array detector and an RF-10AXL fluorescence detector. C18 HPLC was performed using a Jupiter 5μ C18 300A 150 × 4.6-mm column (Phenomenex) at 40 °C with a 1 mL min^{-1} isocratic flow of 20% acetonitrile (MeCN) – 0.1% TFA followed by a 10 min linear gradient of 20–100% MeCN 0.1% TFA at 1 mL min^{-1}. Size exclusion HPLC was performed with a BioSep SEC 2000 300 × 7.8 mm HPLC column (Phenomenex) with an isocratic flow of 50% MeCN/H_2O 0.01% TFA at 1 mL min^{-1}. UV analyses were performed using a Hewlett–Packard 8453 UV–Vis spectrophotometer.

2.2. Synthesis

2.2.1. $N^{\varepsilon 30}$-[1-(morpholinosulfonyl)-7-azido-2-heptyloxycarbonyl]-TRI-1144

TRI-1144 (8.5 μmol, 38.2 mg) and *O*-[1-(morpholinosulfonyl)-7-azido-2-heptyl]-*O*′-succinimidyl carbonate (17.0 μmol, 7.6 mg) were combined in 3 mL 1:1 DMSO:100 mM $KHPO_4$, pH 7.5. HPLC analysis showed a complete conversion from starting material (RV 7.3 mL) to the acylated product (RV 7.7 mL) after 3 h. The reaction was diluted to 15 mL in column buffer (100 mM Tris HCl, pH 7.5) and loaded on a 1 mL HiTrap FF Q column (GE Healthcare) pre-equilibrated with column buffer. The column was washed with 5 mL each of column buffer containing 0, 100 and 500 mM NaCl. To the final eluent, 500 μL acetonitrile (MeCN)-0.1% TFA was added and the solution applied to a 1 mL C18 Bond Elut column (Varian). The column was washed with 5 mL of 20% MeCN/H_2O/0.1% TFA and the peptide was eluted with 5 mL 80% MeCN/H_2O/0.1% TFA. The peptide was dried under vacuum to give 36.7 mg (7.6 μmol) as determined by absorbance at 280 nm (ε_{280} = 11,000 M^{-1} cm^{-1}); 89% yield, >95% purity by HPLC.

2.2.2. $N^{\varepsilon 30}$-[1-(phenylsulfonyl)-7-azido-2-heptyloxycarbonyl]-TRI-1144

As above, TRI-1144 (1.6 μmol, 7.7 mg) was reacted with O-[1-(phenylsulfonyl)-7-azido-2-heptyl]-O′-succinimidyl carbonate (3.2 μmol, 1.4 mg) in 1 mL 1:1 DMSO:100 mM $KHPO_4$, pH 7.5. HPLC showed complete conversion of the starting material (RV 7.3 mL) to the acylated product (RV 7.8 mL) after 3 h. The purified peptide was dried under vacuum to give 23.8 mg (1.3 μmol); 78%, yield, >95% purity by HPLC.

2.2.3. $N^{\varepsilon 30}$-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyloxycarbonyl]-TRI-1144

TRI-1144 (6.2 μmol, 27.9 mg) was combined with O-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyl]-O′-succinimidyl carbonate (18.7 μmol, 8.8 mg) in 2.4 mL 1:1 MeCN:100 mM bicine pH 8.5. HPLC analysis showed conversion of 85% of starting material (RV 7.3 mL) to the acylated product (RV 7.8 mL) after 18 h. The peptide was purified as described above and dried under vacuum to give 23.8 mg (4.9 μmol); 79% yield, >95% purity by HPLC.

2.2.4. PEG_{40kDa}-coumarin-TRI-1144 conjugates

$MorphSO_2^-$ modulator. $N^{\varepsilon 30}$-[1-(morpholinosulfonyl)-7-azido-2-heptyloxycarbonyl]-TRI-1144 (3.8 μmol, 18.4 mg) was combined with DBCO-Lys(DEAC)-NH-PEG_{40kDa} (2.9 μmol, 117.9 mg) in 4 mL 2:1 DMSO:H_2O at room temperature for 1 h. HPLC showed complete loss of the DBCO absorbance at 308 nm (A_{308}) in the PEG peak and a shift of absorbance at 280 nm (A_{280}) from starting peptide (RV 7.7 mL) to the PEG-conjugate (RV 6.9 mL). The reaction was diluted in 10 mM triethanolamine (TEOA) HCl, pH 7.0, and loaded onto a pre-equilibrated 5 mL HiTrap FF Q column (GE Healthcare). The column was washed with 5 mL of 10 mM TEOA HCl, pH 7.0, containing 0, 50, 100, 150 and 500 mM NaCl, with the product eluting with 150 mM NaCl. The product was exchanged into 10 mM NaOAC, pH 5.0, by repeated concentration and dilution using a 10 kDa MWCO spin concentrator (Millipore). Recovery of the conjugate was measured by the absorbance of the DEAC component at 430 nm (ε_{430} = 44,800 M^{-1} cm^{-1}) to provide 1.2 μmol of conjugate; 41% yield, >95% purity by HPLC.

$ClPhSO_2^-$ modulator. As described above, $N^{\varepsilon 30}$-[1-(4-chlorophenylsulfonyl)-7-azido-2-heptyloxycarbonyl]-TRI-1144 (3.1 μmol, 15.1 mg) was combined with DBCO-Lys(DEAC)-NH-PEG_{40kDa} (2.7 μmol, 109.8 mg) in 3.5 mL 2:1 DMSO:H_2O at room temperature. After 1 h, the reaction was purified and exchanged into 10 mM NaOAc pH 5.0 as described above. Recovery of the conjugate measured as absorbance of DEAC provides 1.3 μmol; 48% yield, >95% purity by HPLC.

2.3. In vitro cleavage kinetics of azido-linker-peptides and PEG-TRI-1144 conjugates

Kinetics of β-elimination were determined under accelerated release conditions using 50 μM TRI-1144 in 100 mM Na borate, pH 9.5, 37 °C. At intervals over at least five half-lives, 40 μL aliquots were removed, quenched with 10 μL of 2 M HOAc and kept at −20 °C until assay. The azido-linker-peptides were analyzed by C18 HPLC, and the PEG-TRI-1144 samples were analyzed by SEC HPLC. Release rates (k_{obsd}) were calculated by fitting the released peptide peak area (A_{280}) vs time to the first-order rate equation. Assuming second-order rate constants are described by $k_2 = k_{obs}/[OH^-]$ [1], k_{obs} values obtained at pH 9.5 were converted to k_{obs} values at pH 7.4.

2.4. Pharmacokinetics of PEG-TRI-1144 conjugates in rats

Samples of the PEG-coumarin-TRI-1144 conjugates were adjusted to 800 μM in 10 mM NaOAc, pH 5.0 to prevent release

of the peptide prior to dosing, and filtered through a 0.2 μ syringe filter (Pall Life Sciences). Samples were administered i.v. to cannulated male Sprague–Dawley rats ($n = 3$ per conjugate) at 800 nmol/kg body weight. Blood samples (300 μL) were drawn at 0, 1, 2, 4, 8, 24, 48, 72, 120, 168 h, added to 30 μL of 1 M citrate/0.1% Pluronic F68, pH 4.5, and centrifuged at 1500*g* for 10 min at 4 °C to provide plasma [6,7].

2.5. Plasma PEG_{40kDa}-TRI-1144 analysis

Proteins were precipitated from 100 μL rat plasma by addition of 300 μL of acetonitrile. The samples were kept on ice for 2 h and centrifuged at 16,000*g* for 10 min at 4 °C. The supernatant was analyzed by HPLC using a Phenomenex Jupiter C18 300A 5-μm column at 40 °C. Mobile phases consisted of 10 mM TEOA HCl, pH 7.0 (solvent A) and MeCN (solvent B). Samples (20 μL) were injected and eluted at 1 mL/min starting at 20% B for the first minute followed by a linear gradient of 20–100% B over 10 min. The PEG-coumarin-TRI-1144 conjugate (RV 6.9 mL) and PEG-coumarin-vinyl sulfone product (RV 7.5 mL) were detected with a fluorescence detector with excitation 417 nm and emission 489 nm. Quantitation was performed by comparison of peak areas to standard solutions of the PEG-coumarin-TRI-1144.

2.6. Plasma TRI-1144 analysis

Plasma levels of TRI-1144 were determined using a qualified research LC-MS/MS method. For the PEG-TRI-1144 dosed animals, blood samples were collected on citric acid (1 M, pH 4.5) and kept on ice to avoid ex vivo release of TRI-1144 from the PEG-TRI-1144. After protein precipitation (50 μL acidified plasma + 50 μL DMSO + 100 μL H_2O + 250 μL acetonitrile) plasma samples were separated on a reversed phase UHPLC-column (Acquity BEH300 C_{18} 1.7 μm, 2.1 × 50 mm; Waters). Mobile phases consisted of 0.2% formic acid (solvent A) and 0.2% formic acid in acetonitrile (solvent B). Starting conditions were typically from 99% solvent A followed by a linear gradient to 60% solvent A and 40% solvent B over 2.0 min followed by an isocratic hold at 95% B for 0.5 min at a flow rate of 0.4 ml/min. Injection volume was 5 μL. LC-MS/MS analysis was carried out on an API-5500 MS/MS (Applied Biosystems, Toronto, Canada), which was coupled to an UHPLC-system (Nexera; Shimadzu, Japan). The MS/MS, operated in the positive ion mode using the TurboIonSpray™-interface (electrospray ionization) was optimized for different MRM transitions; MRM transitions were as follows: 900.7 > 1152.9; 900.7 > 914.4; 900.7 > 897.1. The limit of quantification was 5.0 ng/ml for plasma. The accuracy (intrabatch accuracy from independent QC samples) was between 85% and 115% of the nominal value over the entire concentration range for plasma samples.

3. Results

3.1. Chemistry

The peptide TRI-1144 was synthesized by SPPS with an acetylated N-terminus and amidated C-terminus. The N^{α}-Ac peptide was acylated at Lys-30 in high yield with *O*-[1-(Mod)-7-azido-2-heptyl]-*O′*-succinimidyl carbonate linkers containing phenylsulfonyl-($PhSO_2$-), 4-chlorophenylsulfonyl-($ClPhSO_2$-) and morpholinosulfonyl-($MorphSO_2$-) pK_a modulators (Scheme 2) [1]. The azido-linker-peptides were attached by strain-promoted alkyne-azide cycloaddition to univalent 4-branched DBCO-Lys(DEAC)-NH-PEG_{40kDa} that contained the fluorescent 7-(N,N-diethylamino)coumarin (DEAC) probe to facilitate HPLC quantitation. PEG conjugates containing the $PhSO_2$- and $MorphSO_2$-modulators are designated as PEG_{40kDa}[$ClPhSO_2$]TRI-1144 and PEG_{40kDa}[$MorphSO_2$]TRI-1144, respectively.

3.2. In vitro cleavage kinetics

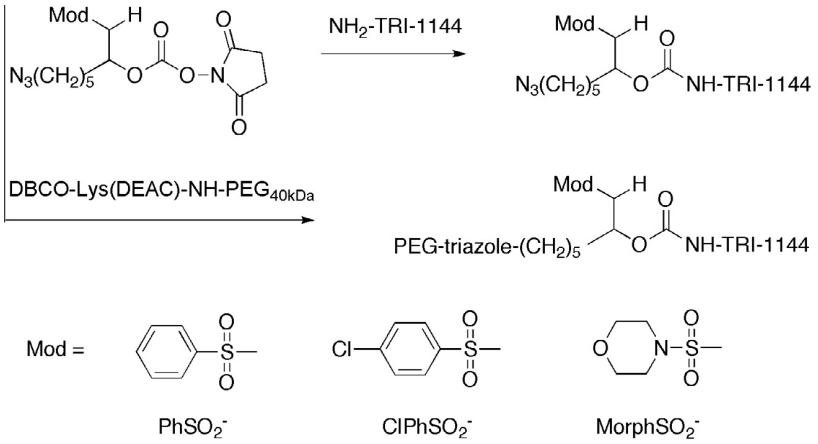
The *in vitro* release rates of the azido-linker TRI-1144 and the PEGylated TRI-1144 conjugates were determined under accelerated cleavage conditions, pH 9.5, 37 °C. Knowing the β-elimination is first-order in hydroxide ion [1], we calculated rates at pH 7.4, 37 °C, by the equation $t_{1/2,\ pH\ 7.4} = t_{1/2,\ pH\ 9.5} \times 10^{(9.5-7.4)}$. The cleavage rate calculated for PEG_{40kDa}[$ClPhSO_2$-]TRI-1144 release at pH 7.4 was $k = 2.04 \pm 0.08 \times 10^{-3}\ h^{-1}$ ($t_{1/2}$ 350 ± 14 h), similar to the azido-linker conjugate, $k = 2.38 \pm 0.23 \times 10^{-3}\ h^{-1}$ ($t_{1/2}$ 290 ± 28 h). For PEG_{40kDa}[$MorphSO_2$]TRI-1144 the calculated cleavage rate was $k = 1.09 \times 0.08 \cdot 10^{-5}\ h^{-1}$ ($t_{1/2}$ 6344 ± 444 h) and 2.48 ± 0.15 $h^{-1} \times 10^{-5}$ ($t_{1/2}$ 2800 ± 168 h) for the corresponding azido-linker-TRI-1144. We obtained $1.12 \pm 0.02 \times 10^{-3}\ h^{-1}$ ($t_{1/2}$ 617 ± 12 h) for azido[$PhSO_2$-]TRI-1144. Based on these results, we concluded that cleavage of the PEG_{40kDa}[$PhSO_2$-]TRI-1144 was too slow to meet our objective of once-weekly administration; we discontinued its further study, and focused on the conjugate with the $ClPhSO_2$-modulator.

3.3. Pharmacokinetics

Pharmacokinetic parameters of TRI-1144 and its PEGylated conjugates were determined after IV injection in rats. Plasma samples were prepared by a procedure designed to avoid ex-vivo base-catalyzed *β*-elimination [7]. After precipitation of proteins with MeCN, samples were analyzed by HPLC for the DEAC-labeled PEGylated TRI-1144 conjugates and, if feasible, the PEG-vinyl sulfone (PEG-VS) product, and by LCMS for free TRI-1144. Plasma concentration–time data obtained are shown in Fig. 1, and derived pharmacokinetic parameters are provided in Table 1.

The pharmacokinetic data fit the previously described one-compartment model in Fig. 2 [1]. Here, k_1 is the first-order rate constant for cleavage of the linker *in vivo*, k_2 is the rate of elimination of the peptide and k_3 is the rate of elimination of the conjugate; in this model $k_\beta = k_1 + k_3$ for both the conjugate and the released drug. The single compartment model is in accord with the $V_{d,ss}$ values of the PEG-conjugates that are similar to the expected vascular volume of the rat (~0.05–0.07 L/kg). The $V_{d,ss}$ of TRI-1144 is 2–3-fold higher than the vascular compartment suggesting that the released peptide distributes outside of the vascular compartment.

Fig. 1A shows the plasma concentration–time profiles of PEG_{40kDa}[$ClPhSO_2$]TRI-1144 and the released free TRI-1144. The half-life of the beta-phase of loss from the central compartment ($t_{1/2,\beta}$) of the conjugate and released peptide was 34 h. We estimated k_3 for the PEG_{40kDa}[$ClPhSO_2$]TRI-1144 from the $t_{1/2,\beta}$ of 44 h for the relatively stable PEG_{40kDa}[$MorphSO_2$]TRI-1144, which was in accord with the $t_{1/2,\beta}$ of ~40 h reported for PEG_{40kDa} of similar shapes [7,8]. Although PEG_{40kDa}[$MorphSO_2$]TRI-1144 also slowly released low levels of TRI-1144, there was no fluorescence lost from the conjugate since the DEAC-labeled PEG-vinylsulfone product co-eluted. Using 0.017 h^{-1} for k_3 and the relationship $k_\beta = k_1 + k_3$, we calculated $k_1 = 0.0046\ h^{-1}$ ($t_{1/2}$ 150 h) for PEG_{40kDa}[$ClPhSO_2$]TRI-1144 which is ~4.6-fold faster than the *in vitro* release rate. We have previously reported that, for unknown reasons, *in vivo* release rates of *β*-eliminative linkers are 2- to 3-fold faster than *in vitro* release rates [1]. As expected, the $t_{1/2,\beta}$ of 34 h for released TRI-1144 is the same as the $t_{1/2,\beta}$ for the PEG_{40kDa}[$ClPhSO_2$]TRI-1144, and represents an 8-fold increase over the 4.2 h $t_{1/2,\beta}$ for bolus TRI-1144 in the rat. At 7 days the concentration of TRI-1144 remained above 4 nM. Assuming the PEG-VS product formed upon release of the peptide has the same elimination



Scheme 2.

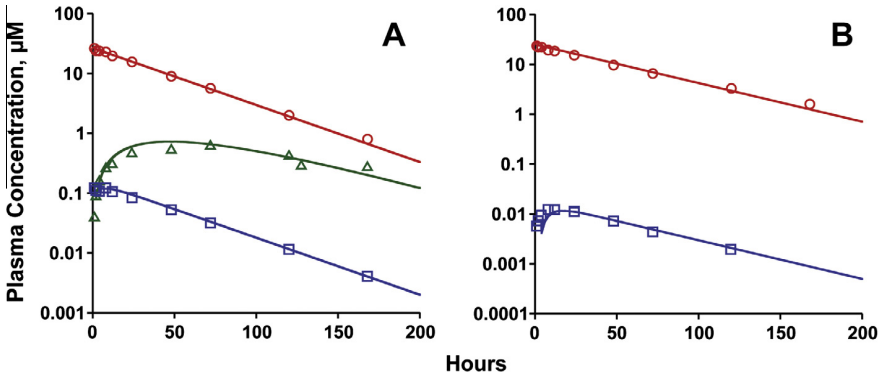


Fig. 1. Pharmacokinetics of PEG-TRI-1144 conjugates. A. $PEG_{40kDa}[ClPhSO_2]$TRI-1144. B. $PEG_{40kDa}[MorphSO_2]$TRI-1144. The plots show plasma concentrations of the conjugates (red circles), released TRI-1144 (blue squares), and in panel A the PEG-VS product (green triangles). Data were overlaid with the calculated concentrations (solid lines) for each component using the one compartment model in Fig. 2. TRI-1144 concentrations from $PEG_{40kDa}[ClPhSO_2]$TRI1144 were corrected at 1- to 4 h for 2% contamination of free peptide in the dosing solution, as verified by HPLC. Each data point represents the average of three independent studies in rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Rat pharmacokinetic parameters for TRI-1144 and releasable PEG-TRI-1144 conjugates.

	$ClPhSO_2$ conjugate	$MorphSO_2$ conjugate	TRI-1144
C_0 PEG-conjugate, µM	25.8	22.5	NA[a]
C_0 TRI-1144, µM	0.14	0.017	NA
V_d, L/kg	0.031	0.035	0.15
$t_{1/2}$, β PEG-conjugate, h	34	44	NA
$t_{1/2}$, β TRI-1144, h	34	41	4.2
$t_{1/2}$ linker cleavage, h	150	–	NA

[a] NA, not applicable.

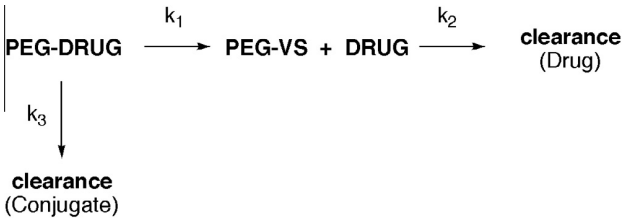


Fig. 2. One-compartment model of the rate of PEG-drug conjugates with releasable linkers.

rate as the PEG-drug conjugate (k_3), the plasma concentration–time curve is described by Eq. (1), and in excellent agreement with the values shown in Fig. 1.

$$[\text{PEG-VS}]_t = [\text{Conj}]_0/V_{\text{conj}} * (e^{-k3t} - e^{-(k1+k3)_t}) \quad (1)$$

Fig. 1B shows the plasma concentration–time profiles of the $PEG_{40kDa}[MorphSO_2]$TRI-1144 and the released TRI-1144. The $t_{1/2,\beta}$ of the released TRI-1144 was 41 h, some 10-fold greater than $t_{1/2,\beta}$ of bolus TRI-1144. Here, unlike the $PEG_{40kDa}[ClPhSO_2]$TRI-1144 conjugate we could not separate the PEGylated TRI-1144 from the PEG-VS product, and hence could not resolve k_β into its k_1 and k_3 components. The $t_{1/2,\beta}$ for the total PEG was 44 h. Because this rate was calculated based on total PEG ($PEG_{40kDa}[MorphSO_2]$TRI-1144 plus PEG-vinyl sulfone) and not the $PEG_{40kDa}[MorphSO_2]$TRI-1144 alone, the $t_{1/2}$ is slower than the $t_{1/2}$ for the released TRI-1144. However, since the difference in the steady state concentration of the conjugate and free drug is $C_{conj}/C_{drug} = (k_2/k_1) * (V_{SS,drug}/V_{SS,conj})$ [1] we could estimate that $k_1 = 5.6 \times 10^{-4}$ h^{-1} ($t_{1/2}$ = 1240 h). This value is 5-fold faster than the *in vitro* cleavage rate and in accord with the *in vivo* rate enhancement observed for Mod = $ClPhSO_2$- and with previous observations [1].

3.4. Simulation of pharmacokinetics in humans

We have previously presented evidence that *in vivo* rates of β-eliminative linker cleavage, k_1, in PEG conjugates are species

independent [1]; here, we have determined k_1 for PEG_{40kDa}[$ClPhSO_2$]TRI-1144 to be 0.005 h^{-1} in the rat. The clearance rate of the conjugate, k_3, in humans may be estimated as ~0.006 h^{-1} using a nominal $t_{1/2,\beta}$ of ~5 days for a typical branched 40 kDa PEG conjugate. Thus, the PEG_{40kDa}[$ClPhSO_2$]TRI-1144 and TRI-1144 elimination rate, $k_1 + k_3$, in humans is estimated to be 0.01 h^{-1} ($t_{1/2,\beta}$ 69 h), equivalent to a 4.6-fold extension over bolus TRI-1144. Using a $t_{1/2}$ for TRI-1144 in humans of 15 h [5], and a scaled V_d of 0.12 L/kg [9] we could estimate that an initial dose of 9.7 μmol (560 mg conjugate, 44 mg TRI-1144) of PEG_{40kDa}[$ClPhSO_2$]TRI-1144 would give a C_{max} of free TRI-1144 of 76 nM and maintain a 25 nM concentration for 1 week. This level could be maintained by weekly doses of 8.0 μmol (465 mg conjugate, 36 mg TRI-1144). Further simulated modifications of k_1 with different linkers did not significantly improve the pharmacokinetic parameters.

With slowly cleaving linkers, the $t_{1/2,\beta}$ of a circulating conjugate and released drug is often limited by the rate of renal clearance of the conjugate, k_3. For example, using the simulated values for k_1 and k_3 for PEG_{40kDa}[$ClPhSO_2$]TRI-1144 in humans, a single administration would result in about 56% (i.e. $k_3/(k_1 + k_3)$) of the conjugate lost through renal elimination. To mitigate this problem, we recently reported the development of non-circulating hydrogels as sub-cutaneous carriers for drugs tethered by β-eliminative linkers [2]. Here, since there is no elimination of the carrier over the duration of drug release (i.e. in Fig. 2 $k_3 = 0$), the rate of terminal elimination of the released free drug, k_β, is equal to k_1. Using this system, we estimate that administering 4.1 μmol (18.2 mg) TRI-1144 tethered to a hydrogel by a linker with Mod = $ClPhSO_2$ would result in a C_{max} of 53 nM and a $t_{1/2,\beta}$ of 150 h. This represents a 10-fold half-life extension over bolus TRI-1144 and would keep plasma drug concentrations ≥25 nM for 1 week. The level could be continuously maintained by steady state dosing of 2.8 μmol (13 mg) TRI-1144 tethered to the hydrogel.

4. Discussion

Over the past two decades, a large effort has been aimed at developing potent anti-human immunodeficiency virus type 1 (HIV-1) peptides that inhibit the fusion of viral and human membranes necessary for infection [for recent comprehensive reviews see [10,11]]. Enfuvirtide (Fuzeon; TR20), a 36 amino acid peptide, was the first to be approved by the FDA and represents a first-generation HIV fusion inhibitor. It was quite effective in inhibiting HIV-1 infection but resistance rapidly emerged; also, the drug is expensive and treatment requires an inconvenient twice-daily s.c. administration. Currently, enfuvirtide is relegated for use as salvage therapy. Further work led to the discovery of the 39-amino acid peptide T-1249 that showed higher potency than enfuvirtide and was active against many enfuvirtide-resistant strains. T-1249 entered clinical development but was withdrawn because of emergent resistant HIV-1 strains, high cost and the inconvenience of a twice-daily subcutaneous injection. TRI-1144, a 38-mer, was designed to increase favorable structural features, overcoming resistance and to improve pharmacokinetic properties [10]; it had a comparatively favorable half-life of 12 to 15 h in humans, permitting once-daily administration [5].

A common problem in all of the peptidic HIV-1 fusion inhibitors is their short half-lives and inconvenient once- or twice-daily s.c. administration schedules. In the present work, we describe attempts to extend the half-lives of such inhibitors to allow a more convenient and compliant once-weekly administration of peptidic fusion inhibitors, using TRI-1144 as an illustrative example.

The PEG-linker-TRI-1144 conjugates were prepared in excellent yield by reported procedures [1]. Upon β-elimination TRI-1144 was released from the polymer in quantitative yield. However, the rates of release were about 3- to 4-fold higher than anticipated from model studies of lysine conjugates [1]. As we accumulate data on these β-eliminative linkers, we are observing some dependence of cleavage rates on the structure of leaving groups. Although we do not know the reason for the slow cleavage of PEG-TRI-1144 conjugates, we speculate it is associated with the peptide's high helical structure and tendency to self-associate [3].

Pharmacokinetic data of the PEG_{40kDa}[$ClPhSO_2$]TRI-1144 conjugate in the rat gave an excellent fit to the one-compartment model in Fig. 2. From these, we could calculate a $t_{1/2,\beta}$ of 34 h for released free TRI-1144, which represents an 8-fold half-life extension over bolus TRI-1144. One feature of β-eliminative linkers is that they allow use of pharmacokinetic data obtained in one species to simulate parameters in another. Here, the assumptions are that, as shown in the rat, mouse and monkey, the rate of *in vivo* linker cleavage is species independent [1,7], whereas the rate of renal elimination of the conjugate is species dependent.

Using the pharmacokinetic data of PEG_{40kDa}[$ClPhSO_2$]TRI-1144 in the rat, we could adjust for the longer $t_{1/2,\beta}$ values of PEG (i.e. k_3) in the human and estimate a $t_{1/2,\beta}$ of 70 h for released TRI-1144 in humans. However, an unknown parameter needed to estimate dosage is the target serum concentration necessary to maintain viral inhibition. For the present study we targeted a 25 nM concentration that is ~5-fold higher than most *in vitro* EC_{50} values [3,4]; a higher or lower target concentration can be met by modifying drug dose. We estimate that weekly administration of 8.0 μmol (470 mg conjugate, 36 mg TRI-1144) of PEG_{40kDa}[$ClPhSO_2$]TRI-1144 would maintain a 25 nM target serum concentration of free TRI-1144 in humans. This is a large amount of PEG_{40kDa} to administer subcutaneously but it could be reduced to a practical 120 mg by using a 40 kDa tetravalent PEG as carrier.

An attractive alternative to circulating PEGylated TRI-1144 is a non-circulating hydrogel-TRI-1144 conjugate. Advantages of the hydrogel drug delivery format are that there is no loss of the carrier over the period of drug release so the drug is more efficiently utilized, and the $t_{1/2,\beta}$ of the released drug should be the same as the half-life of linker cleavage. The net result is that $t_{1/2,\beta}$ of a released drug is increased and the system is more efficient at delivering the drug. Simulations of a hydrogel[$ClPhSO_2$-]TRI-1144 conjugate in humans indicate that the $t_{1/2,\beta}$ of free TRI-1144 would be increased to 150 h, and that weekly administration of only 13 mg of TRI-1144 in the hydrogel would maintain ≥25 nM serum concentration.

Thus, the optimal systems for delivering TRI-1144 via β-eliminative linkers to achieve a constant ≥25 nM serum concentration in humans would be (a) as a conjugate using the $ClPhSO_2$-modulator with tetravalent PEG_{40kDa} or (b) with a non-circulating tetra-PEG hydrogel using the same linker.

One of the benefits of β-eliminative linkers is in the delivery of peptide combinations for the purpose of coordinating the pharmacokinetics of the components. Because the $t_{1/2,\beta}$ of a drug released from a carrier is dictated by the linker used, two or more peptides can be attached to a carrier by the same linker and the released free peptides should have the same effective half-life; the relative amounts of drug delivered are simply controlled by the amounts loaded on the carrier. Pan et al. [4] have shown that combinations of peptidic HIV fusion inhibitors that have distinct primary binding sites show highly potent synergistic activity against membrane fusion and infectivity of HIV-1 strains. For example, a combination of enfuvirtide with TRI-1144 had combination index values indicating high synergy and allowed a 5- to 20-fold dose reduction in either drug to obtain the same inhibition of infection of HIV-1 strains. Such dose reductions would mitigate the aforementioned potential problems in overloading the carrier systems used here. A major important issue in executing combination chemotherapy is how to deliver two drugs with different half-lives (~3.5 h for enfuvirtide [12] vs 15 h for TRI-1144 [5]) in an effective and practical schedule. It would appear that a hydrogel conjugate containing

appropriate amounts of both drugs tethered by a linker with a suitable cleavage rate might serve this purpose well.

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