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Pharmacokinetics and Delivery of Tat and Tat-Protein Conjugates to Tissues in Vivo

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The membrane permeation in vivo of therapeutic proteins may be enhanced by conjugation of the protein to cationic import peptides, such as the tat protein of the human immune deficiency virus. The organ uptake, expressed as a percent of injected dose (ID) per gram of tissue, is a function of both membrane permeability and the area under the plasma concentration curve (AUC), which is a function of the plasma pharmacokinetics. The purpose of the present studies was to examine the effect of the tat peptide on the plasma AUC of a model exogenous protein, streptavidin, and to examine the extent to which changes in the plasma AUC influence organ uptake (%ID/g) of the protein. The cationic portion of the tat protein is comprised of a lysine/arginine-rich sequence, designated tat^{48-58}. A biotin analogue of this cationic peptide, tat-biotin, was radioiodinated and injected intravenously into rats with or without conjugation to streptavidin. The unconjugated tat-biotin peptide was nearly instantaneously cleared from plasma by all tissues with a very high systemic clearance of 29 ± 4 mL/min/kg and a high systemic volume of distribution of 4160 m± 450 mL/kg. The plasma clearance of the tat-biotin/streptavidin conjugate, 1.37 ± 0.01 mL/min/kg, was reduced relative to the clearance of unconjugated tat peptide, but was higher than the plasma clearance of the unconjugated streptavidin, 0.058 ± 0.005 mL/min/kg. Conjugation of cationic import peptides such as tat^{48-58} to higher molecular weight proteins results in a marked increase in the rate of removal of the protein from the circulation, which is reflected in the reduced plasma AUC. In summary, tat conjugation of a protein has opposing effects on membrane permeation and the plasma AUC. Therefore, the organ %ID/g is not increased in proportion to the increase in membrane permeation caused by tat conjugation of proteins.

INTRODUCTION

The application of genomics and gene microarray is predicted to lead to the discovery of many new therapeutic proteins (*1*). However, the delivery of large-molecule pharmaceuticals into tissues in vivo is restricted by transport across the capillary microvascular endothelial barrier. Transport of therapeutic proteins to the brain is particularly restricted because of the presence of the blood−brain barrier (BBB).[1] One strategy for mediating the uptake of proteins by cells in culture and by tissues in vivo is cationization of the protein (*2*). The cationic charge forms electrostatic interactions with anionic charges on the cellular surface, and this triggers absorptive-mediated endocytosis and absorptive-mediated transcytosis across endothelial barriers (*3, 4*). Proteins may be cationized directly by conversion of surface carboxyl moieties into extended primary amino groups, and this raises the isoelectric point (p*I*) of the protein from the neutral/acidic range to the cationic range (*5*). Alternatively, a cationic peptide or protein, such as polylysine, may be conjugated to the therapeutic protein (*6*). Polycations that have been used in the past include polylysine, protamine, histone, or import peptides. Import peptides are oligopeptide-containing sequences derived from larger proteins and are arginine (R)- or lysine (K)-rich peptides of strong cationic charge. Import peptides include the R-rich sequence from the third helix of the Antennapedia (Antp) protein (*7, 8*), which is a *Drosophila* transcription factor, the K-rich transportan peptide (*9*), the R/K-rich D-penetran or the R-rich SynB1 (*10*), and the R-rich sequence from the tat protein of the human immune deficiency virus (HIV)-1 (*11, 12*). When arginine and/or lysine residues are clustered in a particular conformation, there is high-affinity binding to anionic proteoglycans such as heparin on cellular surfaces (*13*).

The R-rich sequence of the tat peptide encompasses the amino acids from positions 48−58 of the tat protein, which has a sequence of GRKKRRQRRRP (*14*), and the cationic region of the tat peptide was observed to mediate uptake by cells in tissue culture (*15*). The conjugation of synthetic peptides containing the R/K-rich tat sequence to proteins, such as β-galactosidase, results in enhanced tissue uptake in vivo based on histochemical assays (*11, 12*). Histochemistry is a qualitative assay of protein uptake, and does not evaluate the plasma pharmacokinetics of the conjugate. The extent to which tissue uptake of an exogenous protein, which is expressed as percent injected dose (%ID) per gram of tissue, is increased by conjugation of cationic peptides to the protein is a dual function of (a) the increased membrane permeability of the conjugate and (b) the area under the plasma concentration curve (AUC), which is determined by the plasma

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[1] Abbreviations: BBB, blood−brain barrier; p*I*, isoelectric point; Antp, Antennapedia; HIV, human immune deficiency virus; R, arginine; K, lysine; G, glycine; Q, glutamine; P, proline; Y, tyrosine; ID, injected dose; SA, streptavidin; TCA, trichloroacetic acid; *Cl*, systemic clearance; V_{ss}, systemic volume of distribution; AUC, plasma area under the concentration curve; V_d, organ volume of distribution; V_o, plasma organ volume of distribution.

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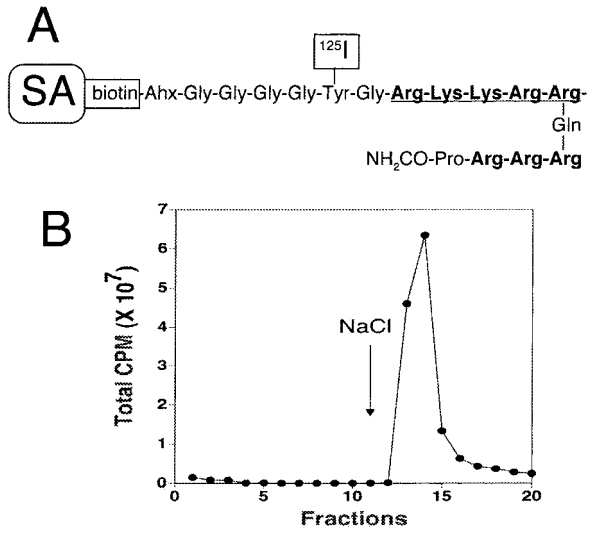

Figure 1. (A) Structure of [^{125}I]tat-biotin/streptavidin conjugate. (B) Elution of [^{125}I]tat$^{48-59}$-biotin with high salt from a cationic exchange column results in a single homogeneous peak. There is no elution of noncationic components from the column prior to the high-salt wash, indicating the absence of radiochemical impurities from the iodinated tat$^{48-58}$ peptide.

pharmacokinetics of the tat-protein conjugate. Conjugation of cationic peptides to protein therapeutics would be expected both to increase membrane permeability and to decrease the plasma AUC, and these could have offsetting effects on the organ %ID/g. The organ %ID/g, the plasma AUC, and membrane permeation were examined in the present study. A biotinylated tat$^{48-58}$ synthetic peptide was prepared and conjugated to streptavidin (SA). The tat peptide contained a tyrosine residue, which allowed for radiolabeling with ^{125}I. The conjugate of the [^{125}I]tat-biotin and SA is designated [^{125}I]tat-biotin/SA, and is shown in Figure 1A. The organ uptake in vivo of [^{125}I]tat-biotin/SA was compared to the organ uptake of native SA, which was labeled with [^{3}H]biotin, and designated [^{3}H]biotin/SA.

EXPERIMENTAL PROCEDURES

Materials. Na^{125}I was purchased from Amersham−Pharmacia Biotech (Arlington Heights, IL), and [^{3}H]-biotin was obtained from NEN Life Sciences (Boston, MA). Sep-Pak C_{18} cartridge and CM Bio-Gel were obtained from Waters (Milford, MA) and Bio-Rad (Hercules, CA), respectively. Acetonitrile was purchased from Fisher Scientific (Pasadena, CA), and trifluoroacetic acid was supplied by Pierce (Rockford, IL). Recombinant streptavidin (SA) and all other chemicals were obtained from Sigma−Aldrich, Inc. (St. Louis, MO). Male Sprague Dawley rats were obtained from Harlan Sprague Dawley (San Diego, CA). The tat-biotin peptide with the sequence biotin-Ahx-GGGGYGRKKRRQRRRP-NH_2, where Ahx = 6-aminohexanoic acid, was synthesized and purified to >95% homogeneity on HPLC by Multiple Peptide Systems (San Diego, CA). The molecular mass, 2224 daltons, determined by mass spectrometry, equaled the expected molecular mass. This peptide contains an amino-terminal biotin, an Ahx-$(G)_4$ linker, and the Y^{47}−P^{58} sequence of the tat protein of HIV-1 (*14*). The tyrosine (Y) at position 47 enables radio-iodination (Figure 1A).

Iodination of Tat-Biotin Peptide. The tat-biotin peptide (29 μg, 13 nmol) was iodinated with [^{125}I]iodine (2 mCi, 1 nmol) and chloramine T (10 nmol), and the reaction was quenched by the addition of sodium metabisulfite (62 nmol). The mixture was then applied to an activated C_{18} Sep-Pak cartridge, followed by elution in 40% acetonitrile/0.1% trifluoroacetic acid. The radiochemical purity of this preparation was assayed by measurement of the fractional precipitation of the peptide by cold 10% trichloroacetic acid (TCA) in the presence of 5% carrier albumin, and the TCA precipitation of the peptide was only 65%. A TCA precipitation of <95% is indicative of either radiochemical impurities or an incomplete acid precipitation of the peptide owing to the small size of the peptide. Therefore, the radiochemical purity was assayed further with cation exchange chromatography. The fraction of [^{125}I]tat-biotin eluted from the C_{18} Sep-Pak cartridge was evaporated to 0.5 mL and then applied to a 0.7 × 6 cm Biogel CM column. The column was eluted with 10 mL of 0.01 M Na_2HPO_4, pH 7.4, followed by 10 mL of 0.01 M Na_2HPO_4, pH 7.4, containing 0.5 M NaCl. No radiolabeled impurities eluted in the low-salt fraction, and the [^{125}I]tat-biotin peptide eluted as a single peak with the high-salt fraction, indicating a high degree of radiochemical purity of the iodinated tat-biotin peptide (Figure 1B). The [^{125}I]tat-biotin was added to a molar excess of recombinant SA 30 min prior to injection in rats. Owing to the very high affinity of SA binding of biotin ($K_D = 10^{-15}$ M, dissociation $t_{1/2}$ = 89 days) (*16*), there is nearly instantaneous formation of the [^{125}I]tat-biotin/SA complex.

Pharmacokinetics and in Vivo Biodistribution. Rats (320−350 g) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2 mg/kg) intraperitoneally, and a femoral vein and artery were cannulated with PE50 tubing. A 0.25 mL aliquot of 0.005 M Na_2HPO_4/0.25 M NaCl, pH 7.4, containing either 4 μCi of unconjugated [^{125}I]tat-biotin peptide or 4 μCi of [^{125}I]tat-biotin peptide conjugated to 20 μg of SA was injected into the femoral vein. In addition, SA bound to [^{3}H]biotin was injected in a separate group of rats. Blood was removed from the femoral artery at 0.25, 1, 2, 5, 15, 30, and 60 min after injection of the compound. The volume of blood removed was replaced by an equal volume of 0.9% saline via the femoral artery catheter. The blood samples were centrifuged, serum was counted for total radioactivity, and samples containing ^{125}I were precipitated by 10% TCA. The animals were decapitated at 60 min after the isotope administration, and radioactivity (cpm/g) was measured in brain, liver, kidney, heart, lung, and spleen.

Pharmacokinetic parameters were calculated by fitting serum radioactivity data to either a mono- or a biexponential equation using a derivative-free nonlinear regression analysis (PARBMDP, Biomedical computer P-Series, developed at UCLA Health Science Computing Facilities). For the studies with the tat-peptide, the TCA-precipitable serum radioactivity was used. The data were either unweighted or weighted with weight = 1/(concentration)^2, where concentration = %ID/mL of serum and ID = injected dose, so as to obtain the lowest residual sum of squares. The systemic clearance (*Cl*), the systemic volume of distribution (V_{ss}), and the area under the plasma concentration curve (AUC) were determined as described previously (*17*). The organ clearance was determined as follows:

$$\text{clearance} = (V_d - V_o)^* C_p(60\ \text{min})/\text{AUC}_{0\rightarrow 60\ \text{min}}$$

where V_d is the organ volume of distribution (μL/g), C_p(60 min) is the terminal serum concentration, and V_o are the plasma volumes for the respective organs, which have been reported previously (*17*). The organ uptake, %ID/g, was computed from the product of organ clearance and $\text{AUC}_{0\rightarrow 60\ \text{min}}$. The organ V_d was determined from the

Table 1. Pharmacokinetic Parameters

parameter (units)	[^{125}I]tat-biotin	[^{125}I]tat-biotin/SA[a]	[^{3}H]biotin/SA
A_1 (%ID/mL)	0.39 ± 0.02	3.0 ± 0.2	9.7 ± 0.4
A_2 (%ID/mL)	0.063 ± 0.005		
K_1 (min^{-1})	0.88 ± 0.20	0.014 ± 0.001	0.0056 ± 0.0006
K_2 (min^{-1})	0.0069 ± 0.0012		
$t_{1/2}^1$ (min)	0.87 ± 0.17	49 ± 3	124 ± 12
$t_{1/2}^2$ (min)	107 ± 19		
AUC(60) (%ID·min/mL)	3.6 ± 0.3	123 ± 5	494 ± 50
AUC(∞) (%ID·min/mL)	10 ± 1	215 ± 8	1734 ± 136
V_{ss} (mL/kg)	4160 ± 450	97 ± 5	36 ± 1
Cl (mL/min/kg)	29 ± 4	1.37 ± 0.01	0.058 ± 0.005

[a] SA = streptavidin.

ratio of total dpm/g in the tissue divided by the dpm/μL of TCA-precipitable serum radioactivity. The plasma TCA precipitation data were normalized by dividing by the control TCA precipitation of the uninjected peptide, which was 63% and 85% for the unconjugated tat-biotin peptide and for the tat-biotin/SA conjugate, respectively. This approximation was necessary owing to the incomplete precipitation of the tat$^{48-58}$ peptide by TCA (Figure 1). However, the differences in TCA precipitation of [^{125}I]tat-biotin and the [^{125}I]tat-biotin/SA complex are small compared to the large differences in systemic clearance of these conjugates.

The organ clearance, also called a membrane permeability−surface area (PS) product, is a measure of membrane permeation of the tat-SA conjugate or the unconjugated SA protein. Since organ radioactivity was measured at 60 min after injection, the organ clearance may underestimate the actual PS product if there is significant efflux of radioactivity from the organ after initial uptake.

RESULTS

The serum concentration following intravenous injection of [^{3}H]biotin/SA, of unconjugated [^{125}I]tat-biotin, and of [^{125}I]tat-biotin/SA is shown in Figure 2 (left panel). These data demonstrate marked differences in the rate of removal of the proteins from blood in the rat in vivo. The serum radioactivity data were subjected to pharmacokinetic analysis (Experimental Procedures), and the pharmacokinetic parameters are listed in Table 1. The unconjugated [^{125}I]tat-biotin peptide is rapidly removed from blood following intravenous injection as demonstrated by the extremely high rate of plasma clearance, 29 ± 4 mL/min/kg, and the high systemic volume of distribution, 4160 ± 450 mL/kg (Table 1). The plasma clearance of the [^{125}I]tat-biotin peptide was reduced 21-fold following conjugation to SA (Table 1). The plasma clearance of the tat-biotin/SA conjugate was 24-fold greater than the plasma clearance of biotin/SA (Table 1). In parallel with the rapid rate of removal of the tat peptide from plasma, there was rapid degradation of the unconjugated tat peptide in vivo as demonstrated by the rapid decline in the serum radioactivity that was precipitable by TCA (Figure 2, right panel).

The individual organ clearance for the unconjugated tat peptide was high in all tissues (Figure 3). There was a reduction in the organ clearance of the tat peptide following conjugation to SA (Figure 3). However, the organ clearance of the tat-SA conjugate was still greater than the organ clearance of the unconjugated SA, as shown in Figure 4 (top panel). These data demonstrate that conjugation of the tat peptide to SA increases protein uptake in all tissues, particularly kidney. However, when organ uptake was measured as percent injected dose (%ID) per gram of tissue, the increased uptake of SA

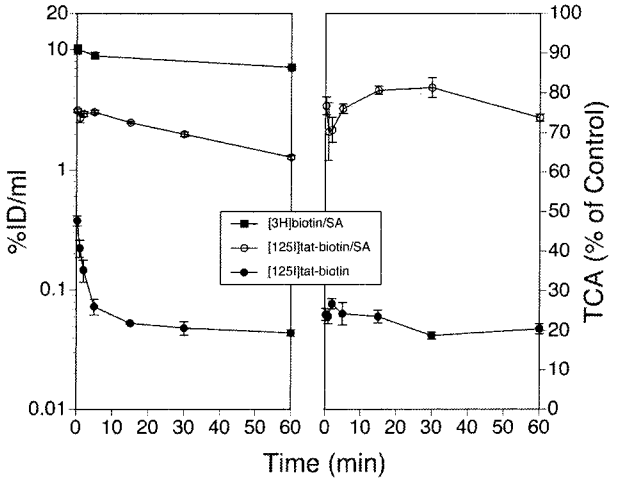

Figure 2. (Left panel) The percent injected dose (%ID) per milliliter of serum is plotted versus time for [^{3}H]biotin/SA, [^{125}I]tat-biotin/SA, and unconjugated [^{125}I]tat-biotin. Data are mean ± SE (n = 3 rats per point). (Right panel) Percent of serum radioactivity that is precipitable by trichloroacetic acid (TCA) is plotted versus time after intravenous injection.

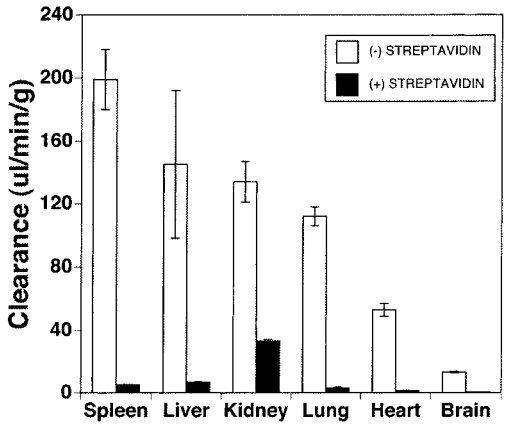

Figure 3. Organ clearance values for [^{125}I]tat-biotin without (−) or with (+) conjugation to streptavidin. These organ clearances are related to membrane permeation of the conjugates. Values were determined at 60 min after a single intravenous injection. Data are mean ± SE (n = 3 rats).

following conjugation of the tat peptide was more modest, as demonstrated in Figure 4 (bottom panel). The differences between the organ clearance and the organ %ID/g values in Figure 4 are due to the difference in the plasma AUC values. The plasma AUC at 60 min for [^{3}H]biotin/SA is decreased 4-fold compared to the plasma AUC of [^{125}I]tat-biotin/SA, and the steady-state plasma AUC of SA is decreased 8-fold following conjugation with the tat peptide (Table 1).

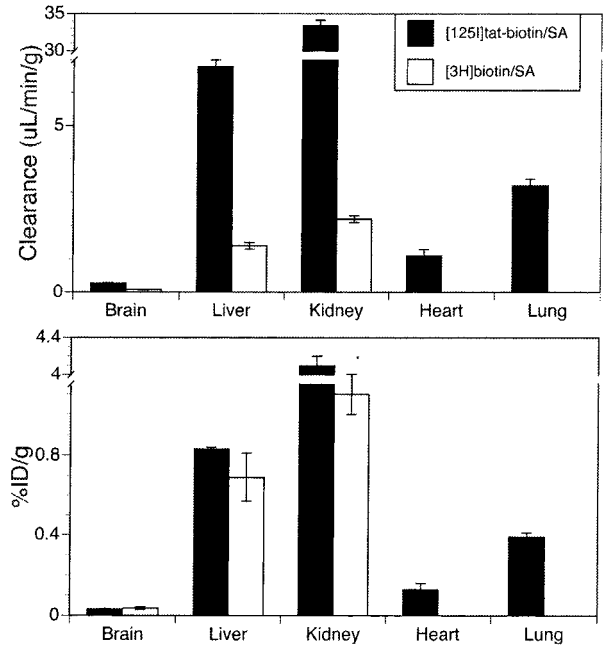

Figure 4. (Top panel) Organ clearance of [^{3}H]biotin/SA (white columns) or [^{125}I]tat-biotin/SA (black columns) at 60 min after intravenous injection. Data are mean ± SE ($n = 3$ rats). (Bottom panel) Organ uptake is expressed as percent injected dose (%ID) per gram of tissue. %ID/g = (clearance) × (60 min plasma AUC). The 60 min AUC values are shown in Table 1. The heart and lung values for [^{3}H]biotin/SA are not visible as these are zero.

DISCUSSION

The results of these studies are consistent with the following conclusions. First, cationic oligopeptides such as tat$^{48-58}$ are removed from blood nearly instantaneously owing to rapid uptake by virtually all tissues. Second, the organ uptake of a model protein, SA, is increased following conjugation with a cationic oligopeptide such as tat$^{48-58}$. Third, conjugation of proteins with cationic peptides such as tat$^{48-58}$ has opposite effects on (i) the membrane permeation, as reflected in the estimate of organ clearance, and (ii) the plasma AUC. These opposing effects on membrane permeation and plasma AUC result in a more modest change in the organ uptake of the tat-protein conjugate, as reflected in the %ID/g (Figure 4). The %ID/g is directly proportional to *both* membrane permeation and the plasma AUC (Experimental Procedures).

Conjugation of cationic import peptides such as polylysine or tat oligopeptides triggers electrostatic interactions with anionic sites on cellular membranes, and this causes absorptive-mediated endocytosis (*2*). The mechanism of cellular uptake of cationic peptides is said not to involve endocytosis, because the uptake is not suppressed by incubations at 4 °C in tissue culture (*18*). However, confocal microscopy investigations of the uptake of tat or Antp import peptides uniformly show sequestration of the peptide in intracellular endosomes (*8, 18, 19*), which suggests the cationic peptides enter cells via absorptive-mediated endocytosis (*2*).

Cationic import peptides such as tat$^{48-58}$ are rapidly cleared from blood following intravenous injection, and more than 90% of the injected dose is removed from blood within the first 60 s after intravenous administration (Figure 2, left panel). The V_{ss} of the [^{125}I]tat-biotin peptide, 4160 ± 450 mL/kg (Table 1), is 50-fold higher than the blood volume, which is indicative of rapid sequestration of the peptide in peripheral tissues following the initial extravascular distribution of the [^{125}I]tat-biotin peptide. In the present studies, a radio-iodinated form of the tat peptide was used. It is possible that the organ transport of the tat peptide is altered by the addition of the ^{125}I-labeled nuclide. However, the ^{125}I moiety comprises only 5% of the mass of the iodinated tat peptide. In addition, prior work has shown that the pharmacokinetics of peptide clearance from blood are identical whether the peptide is labeled with ^{125}I versus another radionuclide, such as ^{111}In (*20*). The identical clearance of a peptide labeled with either ^{125}I or ^{111}In indicates that surface deiodination of the peptide in vivo is not significant. In the case of the tat peptide, surface deiodination of the peptide in vivo would have to be nearly instantaneous to explain the rapid removal of the radioactive peptide from blood (Figure 2), and this has not been described for iodinated peptides in vivo.

The tat$^{48-58}$ is an oligopeptide with a molecular mass of 2200 daltons, and this oligopeptide rapidly traverses the microvascular endothelial barrier in tissues in vivo. The rapid extravascular distribution of the unconjugated tat peptide is exemplified by the very high systemic volume of distribution, 4160 ± 450 mL/kg (Table 1). There is a marked reduction in the systemic volume of distribution of the tat peptide following conjugation to SA, but the V_{ss} of the tat-biotin/SA conjugate is still nearly 3-fold greater than the V_{ss} of the unconjugated SA (Table 1). The increased V_{ss} of the tat-biotin/SA conjugate relative to biotin/SA is paralleled by the organ clearance data shown in Figure 4 (top panel). These data indicate that conjugation of the tat$^{48-58}$ import peptide enhances the organ clearance of streptavidin, a 50 000 dalton protein.

The dose of the streptavidin delivered to the tissue, as reflected in the %ID/g parameter, is not increased in proportion to changes in the organ clearance parameter (Figure 4). This is because the %ID/g is an equal function of the organ clearance and the 60 min plasma AUC. Owing to the rapid uptake of the tat-biotin/SA conjugate in all tissues, there is a substantial decrease in the 60 min plasma AUC of the SA protein following tat conjugation (Table 1). Therefore, conjugation of SA with the tat peptide increases the organ permeability (clearance) for the exogenous protein, but the conjugation of the tat peptide causes a parallel decrease in the plasma AUC, owing to enhanced uptake in nearly all tissues.

The rapid rate of systemic clearance of conjugates of proteins and cationic peptides such as tat may require the systemic administration of relatively large amounts of the conjugate in order to exert pharmacologic effects in vivo. For example, to detect β-galactosidase enzyme activity in tissues in vivo in mice, it was necessary to inject 7−25 mg/kg doses of the conjugate of β-galactosidase and either tat$^{37-72}$ or tat$^{48-58}$ (*11, 12*). The injection of mg/kg doses of cationic peptide, which are required because of the low plasma AUC of the cationic peptide conjugate, may have toxic effects in vivo. Comparable doses of cationic peptides increase membrane permeability in vivo (*21, 22*).

In summary, cationization of a protein therapeutic has effects on the plasma pharmacokinetics and tissue uptake that are similar to the effects of lipidization of small-molecule drugs. Assuming no significant plasma protein binding, increasing the lipid solubility of a small molecule, or increasing the cationic charge of a large molecule, has two parallel effects: (i) the membrane permeation (organ clearance) parameter is increased, and (ii) the plasma AUC is decreased. The increase in the membrane permeation and the decrease in the plasma

AUC have opposing effects on the tissue %ID/g. Therefore, the organ %ID/g is not increased in proportion to changes in membrane permeation following drug lipidization (*23*). Similarly, attachment of a cationic import peptide, such as tat^{48-58}, results in an increase in membrane permeation, but also causes a parallel decrease in the plasma AUC, and these have offsetting effects on the organ %ID/g. Pharmacological effects in vivo are determined not by the organ clearance parameter, but by the %ID/g.

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