Targeting the Sodium-Dependent Multivitamin Transporter (SMVT) for Improving the Oral Absorption Properties of a Retro-Inverso Tat Nonapeptide

Srinivasan Ramanathan,[1] Shahriar Pooyan,[2] Stanley Stein,[2,3,5] Puttur D. Prasad,[4] Jihong Wang,[2,3] Michael J. Leibowitz,[5,6] Vadivel Ganapathy,[4] and Patrick J. Sinko[1,6–8]

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Purpose. To investigate the potential for delivering large peptides orally by altering their absorptive transport pathways and improving intestinal permeability. The absorptive transport of retro-inverso (R.I.-) K-Tat9 and R.I.-K(biotin)-Tat9, novel peptidic inhibitors of the Tat protein of HIV-1, and their interactions with human SMVT (hSMVT), a high affinity, low capacity transporter, were investigated using Caco-2 and transfected CHO cells.

Methods. Following synthesis on a PAL resin using Fmoc chemistry, the transport of R.I.-K-Tat9 (0.01–25 μM) and R.I.-K(biotin)-Tat9 (0.1–25 μM) was evaluated across Caco-2 cells. The transport and kinetics of biotin, biocytin and desthiobiotin (positive controls for SMVT) were also determined. Uptake of R.I.-K-Tat9 and R.I.-K(biotin)-Tat9 (both 0.1–10 μM) was determined in CHO/hSMVT and CHO/pSPORT (control) cells.

Results. The absorptive transport of R.I.-K-Tat9 was passive, low (P_m~1×10^{-6} cm/sec) and not concentration dependent. R.I.-K(biotin)-Tat9 permeability was 3.2-fold higher than R.I.-K-Tat9 demonstrating active (E_a = 9.1 kcal/mole), concentration dependent and saturable transport (K_m = 3.3 μM). R.I.-K(biotin)-Tat9 uptake in CHO/hSMVT cells (K_m = 1.0 μM) was ~ 500-fold greater than R.I.-K-Tat9 (at 10 μM). R.I.-K(biotin)-Tat9 transport in Caco-2 and CHO/hSMVT cells was significantly inhibited by known substrates of SMVT including biotin, biocytin, and desthiobiotin. Passive uptake of R.I.-K(biotin)-Tat9 was significantly greater than R.I.-K-Tat9 uptake in CHO/pSPORT cells.

Conclusions. These results demonstrate that the structural modification of R.I.-K-Tat9 to R.I.-K(biotin)-Tat9 altered its intestinal transport pathway resulting in a significant improvement in its absorptive permeability by enhancing nonspecific passive and carrier-mediated uptake by means of SMVT. The specific interactions between R.I.-K(biotin)-Tat9 and SMVT suggest that targeting approaches utilizing transporters such as SMVT may substantially improve the oral delivery of large peptides.

KEY WORDS: Tat peptide; oral absorption; biotin; vitamin transporter; Caco-2; CHO.

INTRODUCTION

The successful oral delivery of peptides and peptidomimetics poses numerous challenges. Low permeability, lack of proteolytic stability, and binding to intestinal components are some of the main factors leading to their low oral bioavailability. The proton linked intestinal oligopeptide transporter (PepT1) facilitates the apical transport of smaller peptides (i.e., typically less than 4 amino acid residues) and some peptide-like drugs (1–4). PepT1 is a low affinity, high capacity transporter that is involved with the absorption of relatively large doses (i.e., milligram quantities) of drugs such as the cephalosporins and penicillin antibiotics (4). Larger peptides such as Leu-enkephalin, a pentapeptide, are not substrates for PepT1 and, therefore, are relatively poorly absorbed (5). It is possible to enhance the oral absorption of low permeability, larger peptides by enhancing their stability to proteolytic degradation in the gastrointestinal (GI) tract (6–8). However, net peptide absorption remains relatively low if the effective permeability across the intestinal mucosa is also not enhanced. Using citric acid to reduce intestinal pH and minimize trypsin activity and lauroyl carnitine to enhance permeability, our group recently demonstrated a significant enhancement in the oral bioavailability of a large peptide, salmon calcitonin (9–11). However, like most currently used strategies for enhancing peptide absorption, it is nonspecific or the mechanisms of action are unknown making it difficult to precisely control the resulting *in vivo* effect. Therefore, our laboratory has been investigating alternate strategies for enhancing peptide absorption without altering the GI environment.

Choudhury *et al.* (12) synthesized a 10-amino acid tat inhibitor, N-acetyl-L-Arg-L-Lys-L-Lys-L-Arg-L-Arg-L-Gln-L-Arg-L-Arg-L-Arg-L-Cys-NH_2, denoted Tat9-C, consisting of the 9 amino acid RNA binding domain of Tat linked to a C-terminal cysteine, with its amino terminus acetylated and its carboxy terminus amidated. They showed that Tat9-C, upon S-biotinylation on the cysteine residue (Tat9-C(biotin)) was taken up 30-fold more efficiently by Jurkat cells than was Tat9-C (3% vs. 0.1%, respectively). Subsequently, Tat9-K(biotin), which resembles Tat9-C(biotin) except that the cysteine-S-biotin moiety is replaced by lysine-ε-N-biotin, was synthesized (13). Tat9-K(biotin) and Tat9-C(biotin) showed similar ability to compete with Tat protein binding to the TAR domain of viral RNA preventing Tat-dependent gene expression in cultured cells (13). The replacement of L-amino acids by their D-stereoisomers generally resulted in a reduced

[1] College of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, New Jersey 08854.
[2] Center for Advanced Biotechnology and Medicine (CABM), 679 Hoes Lane, Piscataway, New Jersey 08854.
[3] Department of Chemistry, Rutgers, The State University of New Jersey, 610 Taylor Road, Piscataway, New Jersey 08854.
[4] Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, Georgia 30912.
[5] University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854.
[6] Cancer Institute of New Jersey, 203 George Street, New Brunswick, New Jersey 08901
[7] Center for Biomaterials (CBM), 610 Taylor Road, Piscataway, New Jersey 08854.
[8] To whom correspondence should be addressed. (e-mail: sinko@rci.rutgers.edu)

ABBREVIATIONS: R.I.- K-Tat9: N-acetyl-D-Lys-D-Arg-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Lys-D-Lys-D-Arg-NH_2; R.I.-K(biotin)-Tat9: R.I.-K-Tat9 with biotin linked to ε-N of N-terminal D-lys; CHO/hSMVT: Chinese Hamster Ovary cells transfected with the hSMVT gene; CHO/pSPORT: Chinese Hamster Ovary cells transfected with the pSPORT vector control; E_a: Energy of activation; P_m: Passive membrane permeability; P_c: Carrier-mediated membrane permeability; K_m: Michaelis-Menten half-saturation constant.

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ability of Tat9-K(biotin) to bind to the TAR RNA (14). We found that a retro-inverso derivative of Tat9-K(biotin), denoted R.I.-K(biotin)-Tat9, had comparable TAR RNA binding activity to Tat9-K(biotin) (J. Wang, S. Pooyan, M. J. Leibowitz, S. Stein, unpublished results). R.I.-K(biotin)-Tat9, with its lack of L-amino acids and blocked termini, was found to be highly resistant to proteolysis in 10% fetal calf serum (S. Pooyan, M. J. Leibowitz, S. Stein, unpublished results). With its reversed order of mirror-image amino acids, it should have steric similarity to Tat9-K(biotin), differing only in the polarity of the underlying peptide backbone. Therefore, we used R.I.-K(biotin)-Tat9 for the present study.

In the present study, a mechanistic evaluation of the transport of R.I.-K(biotin)-Tat9 was performed to determine its suitability for oral administration. The current results suggest that a novel strategy for enhancing the intestinal absorption of large peptides may be to add a targeting moiety such as biotin to significantly alter its absorption pathway and enhance intestinal permeability.

MATERIALS AND METHODS

Materials

R.I.-K(biotin)-Tat9 and R.I.-K-Tat9, both tritiated at the N-acetyl terminus, were synthesized in Dr. Stein's lab at Center for Advanced Biotechnology and Medicine (CABM, Piscataway, NJ) as described in the section titled 'Peptide Synthesis'. [^{3}H]biotin, [^{14}C]mannitol and [^{14}C]PEG-4000 were obtained from NEN-Life Science Products (Boston, MA). The human sodium-dependent multivitamin transporter, hSMVT, subcloned in the mammalian expression vector, pSPORT, was kindly provided by Dr. Puttur D. Prasad (Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, GA). All medium components and reagents for cell culture and molecular assay were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). All other chemicals were received from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) and used as received.

Peptide Synthesis

R.I.-K(biotin)-Tat9, N-acetyl-D-Lys:(ε-biotin)-D-Arg-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Lys-D-Lys-D-Arg-NH_2, was synthesized manually on a PAL resin by Fmoc chemistry using reagents from PerSeptive Biosystems (Framingham, MA). Biotin was appended to the ε-amine group of the lysine side chain using the reagent, NHS-biotin (N-hydroxy succinamide, Pierce, Rockford, IL), as follows. After assembly of the peptide and while still attached to the solid support, the MTT (3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide) protecting group was removed from the lysine side chain of R.I.-K-Tat9 by drop-wise addition of 1% trifluoroacetic acid (TFA) in dichloromethane at the rate of 1 mL/min for 2 h. The solid support was washed with dimethylformamide, and NHS-biotin in DMF was added at 5-fold molar excess. Conjugation with biotinylation reagent proceeded overnight with vigorous shaking. Peptides were cleaved from the support and deprotected by treatment with a mixture of 90% TFA, 5% thioanisole, 2% anisole and 3% ethanedithiol for 4 h. The peptides were then purified by reverse-phase high-performance liquid chromatography and characterized by mass spectrometry for the molecular ion. Peptide concentration was determined by amino acid analysis. The synthesis of R.I.-K-Tat9 [N-acetyl-D-Lys-D-Arg-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Lys-D-Lys-D-Arg-NH_2] entailed the same procedure as R.I.-K(biotin)-Tat9 except for attachment of biotin.

Cell Culture Protocols and Caco-2 Cell Transport Studies

The Caco-2 cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD) at passage 25, cultured as previously described (15), and seeded at a density of 63,000/cm^2 on Snapwell inserts with 0.4 μm pore diameter (Corning-Costar, Cambridge, MA). Cells cultured for 3–4 weeks after seeding (passage 27–30) were used for all experiments. CHO cells were obtained from ATCC at passage 4. After reaching confluency, the cells were transfected with the human sodium-dependent multivitamin transporter, hSMVT, subcloned in the mammalian expression vector, pSPORT. The cells were transfected with hSMVT or pSPORT (vector control) by Lipofectamine using a previously described procedure (16).

The Caco-2 transport studies were performed using an Ussing-type diffusion chamber system (Harvard Apparatus, Natick, MA). The apical (AP) solution was a MES Ringer's buffer (pH 6.5) consisting of 114.0 mM NaCl, 5.0 mM KCl, 1.1 mM $MgSO_4$, 1.25 mM $CaSO_4$, and 15 mM 2-(N-morpholino)ethanesulfonic acid (MES). The basolateral solution was a Ringer's buffer (pH 7.4) consisting of 114.0 mM NaCl, 5.0 mM KCl, 1.65 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, and 1.25 mM $CaSO_4$. Both buffers contained 25 mM glucose and were adjusted to 290 mOsm/kg using a vapor pressure osmometer (Wescor Inc, Logan, UT). Transport studies were performed at 37°C unless otherwise indicated using methods described elsewhere (15).

CHO Cell Uptake Studies

CHO/hSMVT cells were washed twice with 25 mM uptake buffer, containing 25 mM Hepes/Tris pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. The cells were incubated with the permeant at 37°C for 10 min. Uptake was stopped by washing the cells three times with ice-cold buffer. Non-specific uptake was measured in parallel experiments in CHO/pSPORT vector-transfected cells. Finally, the cells were solubilized using 0.1% v/v Triton X-100. Uptake was quantified using scintillation counting. Protein concentration was determined using the Bio-Rad reagent according to the manufacturer's instructions. Bovine serum albumin was used as the standard.

Gene Expression Using Reverse Trancription-Polymerase Chain Reaction (RT-PCR)

The presence of SMVT in Caco-2 cells and transfected CHO cells was determined using RT-PCR. Total RNA from Caco-2, CHO/hSMVT and CHO/pSPORT cells was isolated with TRIzol reagent (Promega, Madison, WI). The first strand of cDNA was synthesized using 3 μg of RNA, 2 pmol of reverse primer, 10 mM dithiothreitol, 0.5 mM dATP, dCTP, dGTP and dTTP, and 200 units of Superscript II reverse transcriptase as described by the manufacturer (Gibco Life Technologies, Inc.). Two specific primers were synthe-

sized based on human SMVT. The sequences for forward and reverse hSMVT primers were 5′-CTG TCC GTG CTG GCC CTG GGC-3′ and 5′-GAC CAG GCC AAT GAG GCA GCC-3′, respectively. PCR was performed using 50 μL of reaction volume containing 10 ng of cDNA, 0.2 mM $MgCl_2$, 0.5 μM primers, and 2.5 units of *Taq* DNA polymerase. The reaction was run for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed through 1.5% agarose gel containing ethidium bromide and images captured using NucleoTech imaging system (NucleoTech, San Mateo, CA).

Functional Assay of SMVT

The functional expression of SMVT in Caco-2 and CHO/hSMVT cells was confirmed by studying the transport of biotin (0.1–100 μM and 0.1–25 μM, respectively). The time-period for optimal uptake post-transfection was determined by performing uptake experiments at 24h, 36h, 48h, and 72h after transfection. At each of these time points, uptake studies were performed for 5 min, 10 min, 20 min, and 30 min and the experimental duration (time course) for optimal uptake was also determined.

Concentration Dependence

The concentration dependence of R.I.-K(biotin)-Tat9 in Caco-2 cell monolayers was determined by evaluating its AP to Basolateral (BL) or absorptive transport at concentrations ranging from 0.1–25 μM. The effective permeability, P_e, (cm/sec) was determined using the equation:

$$P_e = dC/dt * V_r/AC_0$$

where V_r is the volume of the receptor chamber (5 mL), A is the surface area of the filter (1.13 cm^2), C_0 is the initial drug concentration, and dC/dt is the flux (J) determined by the linear slope of receptor drug concentration vs. time plot after correcting for dilution. R.I.-K-Tat9 (0.01–25 μM) was used in the control experiments. The uptake of R.I.-K-Tat9 (0.1–10 μM) and R.I.-K(biotin)-Tat9 (0.1–10 μM) was also studied in CHO/hSMVT and CHO/pSPORT cells. The Michaelis-Menten kinetic parameters of Caco-2 and CHO cell transport were then estimated using non-linear regression.

Temperature Dependence

The temperature dependence studies were performed to determine the presence or absence of an ATP-dependent mechanism in R.I.-K(biotin)-Tat9 transport across Caco-2 cells. Prior to experimentation, diffusion chambers mounted with the Snapwells containing Caco-2 cells were equilibrated at 5°C, 10°C, 15°C, 25°C and 37°C. Transport of R.I.-K(biotin)-Tat9 (1 μM) was then evaluated at the above temperatures and permeability values were calculated.

Inhibition Studies

The involvement of SMVT in the Caco-2 cell monolayer transport of R.I.-K(biotin)-Tat9 was investigated through inhibition studies that were performed by co-incubating R.I.-K(biotin)-Tat9 with known competitive substrates of SMVT in the donor solution (AP) of the diffusion chambers. The tritiated drug was co-incubated with 10 μM and 20 μM concentrations of biotin, biocytin, and desthiobiotin. Further, R.I.-K(biotin)-Tat9 (0.1 μM) uptake was determined in CHO/hSMVT cells in the presence of biotin, pantothenic acid, desthiobiotin, and biocytin (50 μM) and compared to the control (uptake of R.I.-K(biotin)-Tat9 alone).

Data Analysis

The apparent activation energy (E_a) was determined from the slope (E_a/2.303 R, where R (molar gas constant) = 0.001987 kcal/deg mole) of the log P_e vs. 1/T plot (T = absolute temperature (T °C + 273.16 °C)) based on the Arrhenius equation:

$$\log P_e = A - \frac{E_a}{2.303RT}$$

where A is the Arrhenius constant.

The kinetic parameters for the Michaelis-Menten studies were calculated by performing non-linear regression with Scientist software (MicroMath, Salt Lake City, UT) using the following equation:

$$P_e = \frac{P_c}{1 + \frac{C_s}{K_m}} + P_m$$

where P_e is the effective permeability of the substrate transported, C_s is the substrate concentration and P_m and P_c represent the passive and carrier-mediated component of the permeability. All data were initially fitted to a 3-parameter model (P_c, P_m, K_m for Caco-2 studies, J_{max}, P_m, K_m for CHO studies). If P_m was not significantly differently from 0, then data was refit using a 2-parameter model (J_{max}, K_m). The data from inhibition studies were transformed into Lineweaver-Burke plots to determine the nature of the inhibition process (competitive, non-competitive). Subsequently, the inhibition constant (K_i) values were estimated using Dixon plots. Data used for the above plots were weighted using $1/SEM^2$. The kinetic parameters for CHO cell studies were estimated by performing non-linear regression using the equation $J = J_{max} * [C]/(K_m + [C]) + P_m * C$, where J is the rate of permeant uptake, P_m is the passive uptake component and [C] is the substrate concentration. Data were weighted using $1/S.E.M^2$.

Statistical analyses were performed using Jandel SigmaStat, version 2.03. One-way analysis of variance was used to test the difference in the mean values of uptake using $P < 0.05$ as the significance level for all tests.

RESULTS

Functional Assay of SMVT

The absorptive transport of biotin was concentration dependent and saturable in Caco-2 (Fig. 1) and CHO/hSMVT (Fig. 2) cells. The Michaelis-Menten kinetic parameters of biotin transport are shown in Table I. Uptake was significantly lower in the control CHO/pSPORT cells.

SMVT Expression in Caco-2 and CHO Cells

A single amplified DNA band was detected in Caco-2 and CHO/hSMVT cells and was absent in the CHO/pSPORT control cells (Fig. 3). The DNA was ~ 400 base pairs (bp) as

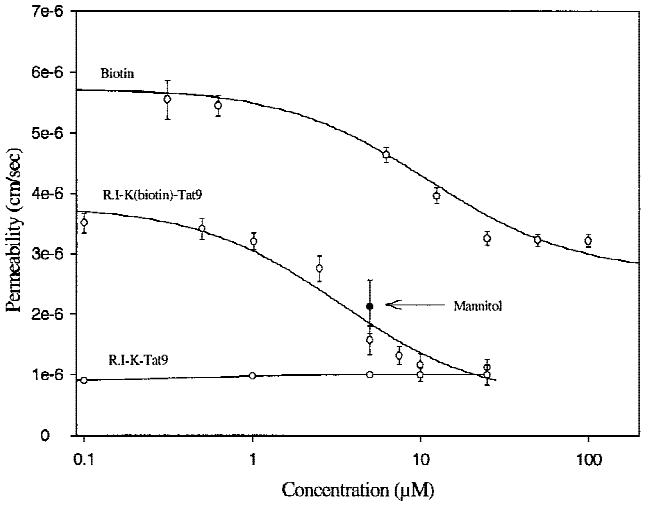

Fig. 1. Absorptive transport of biotin, R.I.-K-Tat9 and R.I.-K(biotin)-Tat9 across Caco-2 cell monolayers. The permeability of mannitol (paracellular transport marker) at 5 μM is also shown. The experimentally observed values [Mean ± S.E.M.] were used to plot the best-fit non-linear regression line (n = 3).

expected from the size of the fragment between the forward (923–943) and reverse (1333–1313) primer positions.

Concentration Dependence of R.I.-K-Tat9 and R.I.-K(biotin)-Tat9

The absorptive transport of R.I.-K-Tat9 across Caco-2 cell monolayers was low ($P_m = 0.8 \times 10^{-7}$–1×10^{-6} cm/s), not concentration dependent and the probable result of passive diffusion (Fig. 1). Therefore further studies with R.I.-K-Tat9 were not performed. The absorptive transport of R.I.-K(biotin)-Tat9 across Caco-2 cells exhibited concentration dependent and saturable kinetics (Fig. 1). The Michaelis-Menten kinetic parameters for R.I.-K(biotin)-Tat9 transport

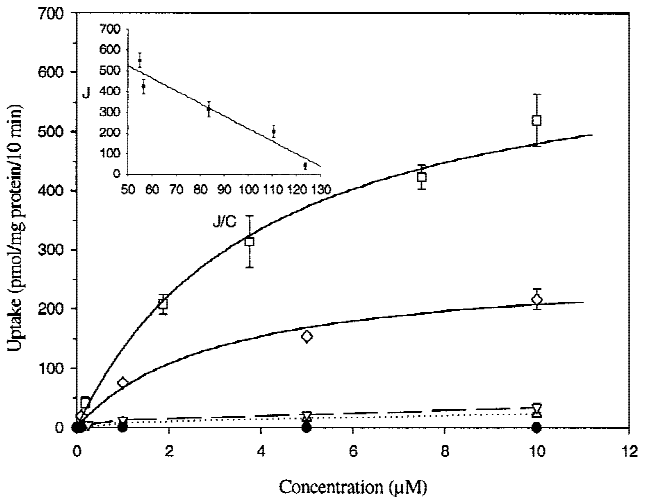

Fig. 2. Uptake of biotin, R.I.-K-Tat9 and R.I.-K(biotin)-Tat9 in CHO/hSMVT and CHO/pSPORT cells. Uptake was measured over a concentration range of 0.1–10 μM at pH 7.5 with 10-minute incubation (n = 3, mean ± S.E.M.); (□) R.I.-K(biotin)-Tat9 in CHO/hSMVT, (◇) biotin in CHO/hSMVT, (▽) R.I.-K(biotin)-Tat9 in CHO/pSPORT, (△) biotin in CHO/pSPORT, and (●) R.I.-K-Tat9 in CHO/hSMVT.

are presented in Table I. The K_m value for absorptive transport was low (3.3 μM), suggesting the potential involvement of a high affinity, low capacity transporter system.

The uptake of R.I.-K(biotin)-Tat9 in CHO/hSMVT cells was concentration dependent and saturable (Fig. 2) with K_m and J_{max} values of 1.0 ± 0.1 μM and 2.3×10^2 ± 0.3 pmol/mg protein/10 min, respectively. Transformation of the uptake data into an Eadie-Hofstee plot (r = 0.96) (Fig. 2 inset) indicated that the kinetics of R.I.-K(biotin)-Tat9 uptake matched a single saturable carrier model. The uptake of R.I.-K-Tat9 in CHO/hSMVT was extremely low (< 1 pmol/mg protein/10 min) and not concentration dependent, indicating the absence of any interaction with hSMVT (Fig. 2). R.I.-K(biotin)-Tat9 uptake in CHO/pSPORT cells (~ 34 pmol/mg protein) was significantly lower than in CHO/hSMVT cells, but higher than R.I.-K-Tat9 uptake in CHO/hSMVT and CHO/pSPORT cells (< 1 pmol/mg protein/ 10 min).

Temperature Dependence

P_e values for R.I.-K(biotin)-Tat9 transport decreased with decreasing temperature. The E_a value estimated from the Arrhenius plot was 9.1 kcal/mole (Fig. 4).

Inhibition Studies

The results suggest competitive inhibition of R.I.-K(biotin)-Tat9 transport by biotin (Fig. 5A) and its structural analogues biocytin (Figure 5B) and desthiobiotin (Figure 5C) with K_i values of 7.9 μM, 3.3 μM, and 1.2 μM, respectively. The interaction of R.I.-K(biotin)-Tat9 with hSMVT was further evident from CHO/hSMVT cell inhibition studies. R.I.-K(biotin)-Tat9 uptake was significantly inhibited by competitive substrates as compared to the control, R.I.-K(biotin)-Tat9 alone (Fig. 6).

DISCUSSION

SMVT, the sodium-dependent multivitamin transporter, which transports the water soluble vitamins biotin, pantothenate and lipoic acid, is a protein of 635 amino acids with 12 transmembrane domains. It is expressed in the placenta, intestine, brain, liver, lung, kidney and heart in rats, rabbits and humans (17). Further, within the intestinal tract, different variants of SMVT (variant I) are also expressed in the duodenum (II), jejunum (II, IV), ileum (II, III), and colon (III) (18). In mammalian cells, the cDNA-induced uptake of physiological substrates biotin, pantothenate and lipoate is electrogenic and sodium-dependent with a Na^+:vitamin stoichiometry of 2:1 (19).

The physiological aspects involved in the cellular entry of intact HIV-1 Tat protein (86 amino acid residues) and fragments such as residues 1–36 or 36–72 have been extensively documented (20–24). However, the specific transport pathways for cellular uptake were not identified. In the present study, a novel Tat inhibitor, R.I.-K(biotin)-Tat9, with substantial anti-HIV-1 activity (12) was synthesized. The absorptive transport of R.I.-K(biotin)-Tat9 through Caco-2 cell monolayers was found to be concentration dependent and saturable suggesting the involvement of a carrier mediated transport pathway. Inhibition studies implicated the involvement of SMVT. The expression of SMVT in Caco-2 cell monolayers was validated by using a functional assay and

Table I. Michaelis-Menten Parameters for Biotin and R.I.-K(Biotin)-Tat9 Transport Across Caco-2 Cell Monolayers and Uptake in CHO/hSMVT Cells

Cell system	Kinetic parameter	Biotin	R.I.-K(biotin)-Tat9
Caco-2 Cell	P_c ($*10^{-6}$) (cm/sec)[a]	3.4 (0.5)	3.2 (0.3)
Monolayer	P_m ($*10^{-6}$) (cm/sec)	2.7 (0.4)	0.6 (0.3)
Transport	K_m (μM)	11 (6)	3.3 (1.4)
CHO/hSMVT	J_{max} (pmol/mg protein/10 min)	1.7×10^2 (0.5)	2.3×10^2 (0.3)
Cellular	P_m (pmol/mg protein/10 min)	3.5 (3.0)	33 (4)
Uptake	K_m (μM)	1.4 (0.6)	1.0 (0.1)

[a] P_c: Carrier-mediated permeability component; P_m: passive permeability component; J_{max}: maximum uptake rate; K_m: Michaelis constant. Numbers in parentheses represent standard deviation (S.D); n = 3.

known competitive substrates and verified using RT-PCR. The K_m value (11 μM) calculated for biotin transport in the current study is consistent with those obtained from human intestinal brush-border membrane vesicles (5.26 μM), colonic epithelial NCM460 cells (19.7 μM), rat small intestine (8.77 μM) and other reports suggesting the involvement of SMVT (19, 25–28).

Two cell systems were used in these studies to determine the mechanisms of transport of R.I.-K-Tat9. While Caco-2 cell transport entails passage of compounds across the apical and basolateral membranes, uptake into non-polarized CHO cells was used to isolate apical membrane transport processes. The absorptive transport of R.I.-K-Tat9, which lacks biotin, was modest across Caco-2 cell monolayers and not concentration dependent. The addition of biotin to the peptide resulted in a 3.2-fold increase in the absorptive permeability at 1 μM. The carrier-mediated permeability of R.I.-K(biotin)-Tat9 was significantly larger than the passive component, concentration dependent and saturable suggestive of the involvement of a carrier mediated transport pathway. Absorptive transport was temperature dependent with an estimated E_a more than 2 fold greater than that required for passive diffusion, also suggestive of active, carrier-mediated transport (29). The uptake of R.I.-K(biotin)-Tat9 and biotin was significantly higher in CHO/hSMVT cells than in the vector transfected CHO/pSPORT cells with similar K_m values demonstrating the direct involvement of SMVT. The similarity in the K_m values for R.I.-K(biotin)-Tat9 transport from the Caco-2 (3.3 μM) and CHO/hSMVT (1.0 μM) cell studies suggests that the SMVT protein may be located in the apical domain of Caco-2 cells or there are transporters on both cellular domains. Further studies are required to determine the localization of SMVT.

Despite the greater overall permeability of R.I.-K(biotin)-Tat9, its passive permeability was not significantly different from that of R.I.-K-Tat9 in Caco-2 cells. This could be the result of confounding factors such as the presence or absence of other potential transport pathways in the Caco-2 cell model (e.g., active secretory transport). Also, transport occurs through the path(s) of least resistance in intact systems. CHO cells, on the other hand, do not suffer from these drawbacks because they do not represent an intact system and the endogenous expression of transporters is low. R.I.-K(biotin)-Tat9 uptake was significantly higher than R.I.-K-Tat9 in CHO/pSPORT cells, indicating that biotin apparently

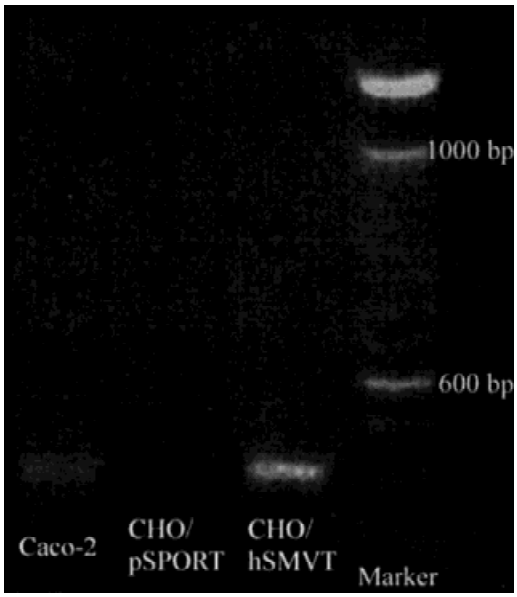

Fig. 3. Images of the PCR products after electrophoresis through a 1.5% agarose gel indicating the presence of the SMVT gene in Caco-2 and CHO/hSMVT cells and its absence in CHO/pSPORT cells. Size of the marker is shown in base pair units.

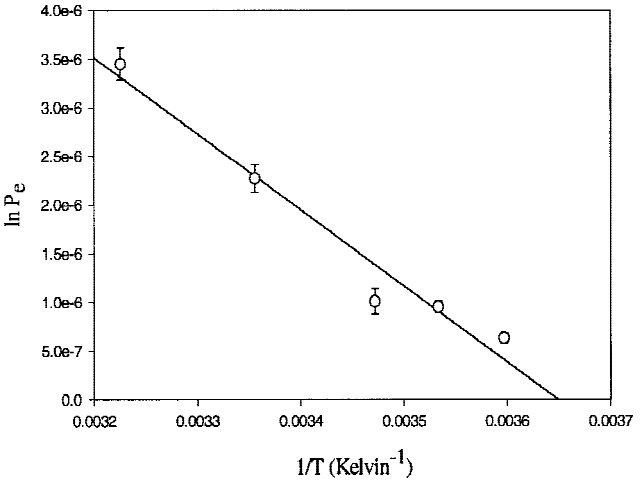

Fig. 4. Temperature dependence of R.I.-K(biotin)-Tat9 transport across Caco-2 cell monolayers in the AP to BL direction. Activation energy, E_a, was estimated to be 9.11 kcal/mole. The study was performed at 5°C, 10°C, 15°C, 25°C, and 37°C. The experimentally observed values [Mean ± S.E.M.] were used to plot the best-fit linear regression line (r = 0.97) (n = 3).

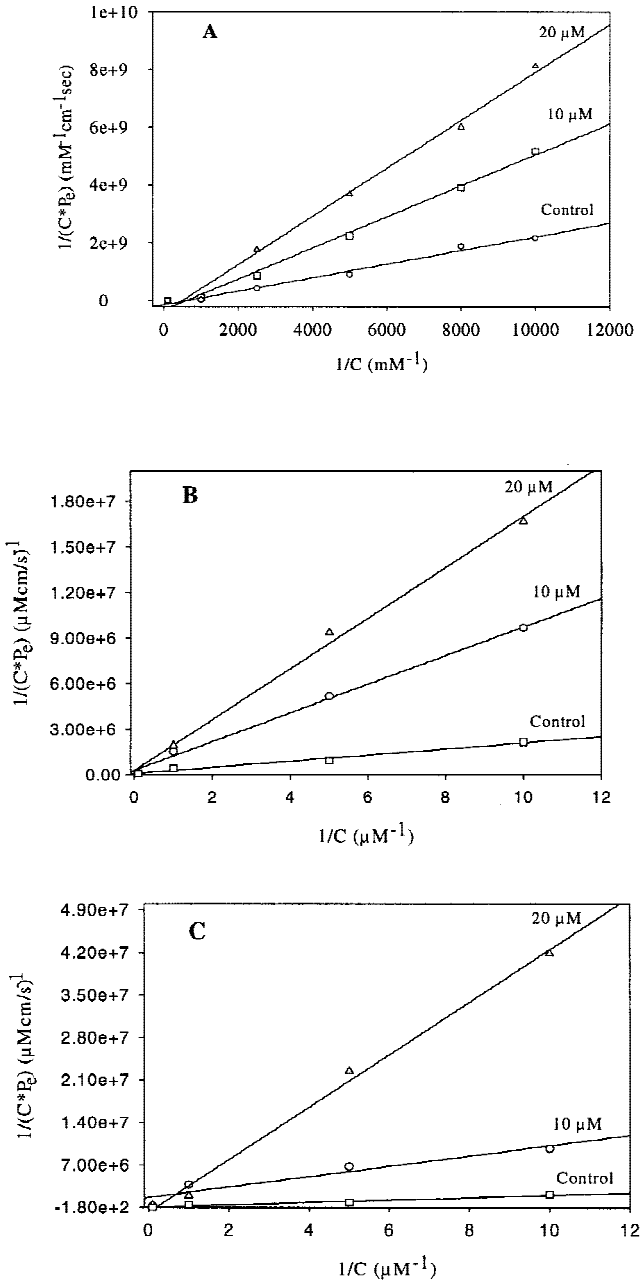

Fig. 5. Lineweaver-Burke plots of R.I.-K(biotin)-Tat9 transport across Caco-2 cell monolayers in the presence of **(A)** biotin, **(B)** biocytin, and **(C)** desthiobiotin, used as inhibitors at 10 and 20 μM. The concentrations of the inhibitors and R.I.-K(biotin)-Tat9 in the apical solution are indicated on the regression lines and *x*-axis, respectively (n = 3).

increased peptide uptake even in the absence of SMVT. This result is consistent with earlier reports demonstrating increased uptake of biotinylated Tat peptides in Jurkat and HL3T1 cells, presumably due to the enhanced hydrophobicity gained by adding biotin to the peptide (12,30).

The substrate specificity of SMVT and its specific interaction with R.I.-K(biotin)-Tat9 were demonstrated by studying the inhibition of R.I.-K(biotin)-Tat9 transport across Caco-2 cells and uptake in CHO/hSMVT cells in the presence of biotin, biocytin and desthiobiotin. While all three compounds competitively inhibited the Caco-2 cell transport of

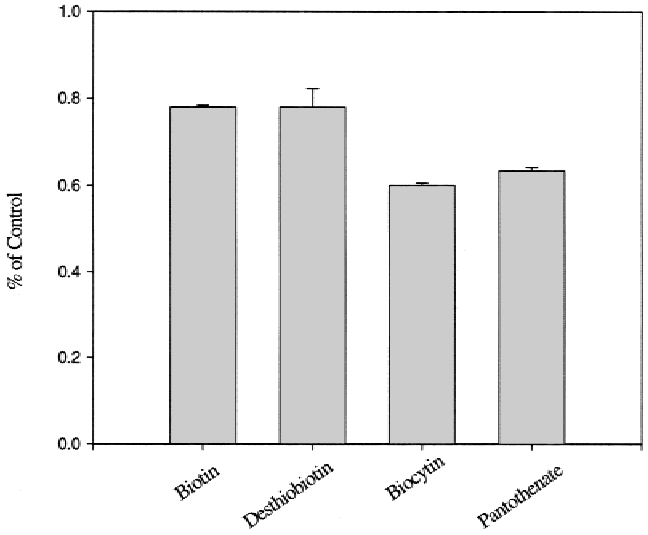

Fig. 6. Uptake of 0.1 μM R.I.-K(biotin)-Tat9 in CHO/hSMVT cells measured for 10 minutes in the presence of 50 μM of other known SMVT substrates. R.I.-K(biotin)-Tat9 uptake is expressed as a percent of control (uptake of 0.1 μM R.I.-K(biotin)-Tat9 ~ 42 pmol/mg protein) (n = 4, mean ± S.E.M.).

R.I.-K(biotin)-Tat9, they also significantly lowered R.I.-K(biotin)-Tat9 uptake in CHO/hSMVT cells, thereby confirming a specific interaction with SMVT. Previous reports have indicated the requirement of a free carboxyl group on substrates for efficient interactions with SMVT (25,27,31). They showed that biotin uptake was significantly reduced by unlabeled biotin, pantothenate, thioctic acid and desthiobiotin compared to biotin analogues that lacked or had a blocked carboxyl group (e.g., biocytin, biotin methyl ester, and thioctic acid amide). Other reports suggest that the keto group at the second position of the imidazole ring is essential for substrate-SMVT interactions, based on the inability of iminobiotin and diaminobiotin to inhibit biotin uptake (17,19,26). It is believed that SMVT interacts primarily, but not exclusively, with the carboxylic side-chain, which is observed in the known substrates biotin, lipoic acid (containing a valeric carboxyl group) and pantothenic acid (containing a propionic carboxyl group) (19). In the current studies, we observed that R.I.-K(biotin)-Tat9, a compound in which the valeric carboxyl group is blocked, was indeed a substrate for SMVT. Additionally, we observed that biocytin (biotin conjugated to lysine through an amide bond) substantially inhibited R.I.-K(biotin)-Tat9 transport across Caco-2 cells. Although R.I.-K(biotin)-Tat9 does not meet the hypothesized requirements of SMVT substrates as outlined in earlier reports (25,27,31), our results point toward the involvement of SMVT in its absorptive transport. The reason behind this apparent incongruity could be the inconclusive characterization of SMVT substrates in the previous reports. For example, the requirement of a free carboxyl group on the valeric acid moiety has been emphasized. Yet, pantothenic acid and short chain fatty acids, compounds with non-valeric carboxyl groups, have been reported to significantly inhibit biotin transport (19,32).

In conclusion, we have demonstrated that the biotinylation of a Tat nonapeptide altered its transport pathways. Nonspecific passive transport increased presumably due to the enhanced hydrophobicity of the peptide resulting from the

addition of biotin. It was also shown that the predominant transport pathway of the biotinylated peptide was facilitated by means of SMVT. Furthermore, these results illustrate the utility of designing peptide prodrugs that target specific intestinal transporters such as SMVT to potentially enhance their bioavailability and therapeutic utility.

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