A Fluorescent hPept1 Transporter Substrate for Uptake Screening

Christopher P. Landowski,[1] Hyo-kyung Han,[1,2] Kyung-Dall Lee,[1] and Gordon L. Amidon[1,3]

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Purpose. To synthesize fluorescent analogues of hPept1 substrates, FITC-Val-OCH_3, Lys-FITC-OH, and Lys-FITC-OCH_3, and to characterize their hPept1 transporter-mediated uptake.

Methods. FITC analogues of amino acids were synthesized using established synthetic procedures, and the extent of their [^{3}H]Gly-Sar uptake inhibition in HeLa/hPept1 cells was determined. The uptake of Lys-FITC-OCH_3 was evaluated in HeLa, HeLa/hPept1, and Caco-2 cells in the presence and absence of Gly-Sar using a fluorescence microscopy-based assay. The uptake and transport of the Lys-FITC analogues were also determined in Caco-2 cells using HPLC assays.

Results. In HeLa/hPept1 cells, [^{3}H]Gly-Sar uptake was significantly inhibited by Lys-FITC-OCH_3 (74%) but not by FITC-Val-OCH_3 (22%). The uptake of Lys-FITC-OCH_3 (100 μM) was approximately 10-fold higher in HeLa/hPept1 cells. Also, Lys-FITC-OCH_3 (100 μM) uptake in HeLa/hPept1 and Caco-2 cells was reduced by 77% and 80%, respectively, in the presence of 1 mM Gly-Sar. Dipeptides and cephalexin significantly reduced Lys-FITC-OCH_3 uptake in Caco-2 cells. The apical permeability of Lys-FITC-OCH_3 (1.5×10^6 cm/s) in Caco-2 cells was significantly lowered in the presence of Gly-Sar. Fluorescence micrographs revealed that this analogue was localized in the cytoplasm and in the nucleus.

Conclusions. The combined results indicate that Lys-FITC-OCH_3 is recognized and transported by hPept1 in HeLa/hPept1 and by peptide transporters in Caco-2 cells. The results also suggest that Lys-FITC-OCH_3 might be a useful fluorescent substrate for rapid assessment of peptide transporter activity in cells of interest.

KEY WORDS: fluorescent substrate; oligopeptide transporter; hPept1; HeLa; Caco-2; permeability.

INTRODUCTION

The human intestinal di/tripeptide transporter, hPept1, plays a nutritional role in transporting dietary peptides; however, in some cases it serves as a carrier for pharmacologically active peptide-like compounds. Because of its broad substrate specificity, the transporter is also known to contribute to the intestinal absorption of several nonpeptide drug compounds including oral β-lactam antibiotics (1), the antineoplastic agent bestatin (2), and an angiotensin-converting enzyme inhibitor (3). This wide substrate specificity allows the design of peptidomimetic prodrugs to increase the intestinal transport of low-permeability drug compounds or to potentially target tumor cells with anticancer drugs. This approach has been successful in increasing the bioavailability of the antiviral drugs acyclovir and ganciclovir. Nucleoside antivirals such as acyclovir and ganciclovir are polar drugs with low membrane permeability and poor oral absorption (4,5). The L-valyl ester prodrugs valacyclovir and valganciclovir increase their parent drug bioavailability three- to fivefold (6) and 10-fold (7), respectively, in humans because of their affinity for hPept1 (8,9). Targeted delivery of drugs to cancer cells would be possible if the peptide transporter exhibits differential expression in cancer cells compared to normal healthy cells. Pancreatic tumors have been shown to express higher levels of Pept1 than normal pancreatic cells (10), thereby providing a potential target for delivery of anticancer drugs. Based on this rationale, the *in vitro* delivery of δ-aminolevulinic acid to rat pancreatic tumor cells via the Pept1 transporter has been reported (11).

The peptide transporters are capable of carrying natural substrates up to at least 522 Daltons (tritryptophan) (12). The promiscuous nature of the hPept1 transporter, together with its ability to carry "large" substrates, makes it an attractive carrier for anticancer compounds. The characterization of the peptide transporter uptake activity in different cell types *in vitro* or different tissues *in vivo* could require the screening of many samples. In particular, if cancer cell libraries need to be screened rapidly, suitable alternatives to tedious HPLC or hazardous radioactive assays would be highly beneficial. Fluorescence-based assays offer high sensitivity and are amenable to rapid assessment with multiwell plate readers. Several studies with fluorescent peptide transporter substrates have been reported. In one such study, fluorescent peptide analogues of Val-Lys and Lys-Sar were synthesized by Abe *et al.* and examined for their transport in Caco-2 cells (13). The authors found that although the fluorescent analogues do not appear to be transported in Caco-2 cells, they did exhibit high affinity for the transporter (13). Groneberg *et al.* reported the synthesis and *ex vivo* uptake studies of D-Ala-Lys-AMCA (7-amino-4-methylcoumarin-3-acetic acid) in murine small intestine (12). The authors demonstrated and visualized mammalian intestinal peptide transport *ex vivo* and identified the cellular sites of Pept1 expression in the intestinal tract and suggest that such visualization assays might be a useful tool to characterize compounds that use Pept1 for uptake into epithelial cells or that inhibit transport activity specifically.

In this study we report the synthesis of fluorescent analogues of the amino acids valine and lysine, FITC-Val-OCH_3, Lys-(N^{ε}-FITC)-OH (Lys-FITC-OH), and Lys-(N^{ε}-FITC)-OCH_3 (Lys-FITC-OCH_3), by conjugation of the α-amino group of Val or the ε-amino group of Lys or its methyl ester to the fluorescent molecule fluorescein isothiocyanate (FITC). The fluorescent analogues were then tested for their ability to inhibit the uptake of [^{3}H]Gly-Sar, a Pept1 substrate, in HeLa cells overexpressing hPept1. We further describe the cellular uptake of Lys-FITC-OCH_3 in normal HeLa cells, in HeLa cells overexpressing hPept1, and in Caco-2 cells in the presence and absence of Gly-Sar using a simplified fluorescence assay. The uptake profiles obtained using fluorescence-based assays were corroborated using conventional HPLC and radioactivity methods. The involvement of hPept1 in mediating active transport of Lys-FITC-OCH_3 and its free acid form was also examined with transport studies in Caco-2

[1] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.

[2] Current address: Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Ann Arbor, MI 48105.

[3] To whom correspondence should be addressed. (email: glamidon@umich.edu)

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monolayers and with conventional fluoroscopic images of Lys-FITC-OCH_3 uptake in HeLa/hPept1 cells.

MATERIALS AND METHODS

Materials

[^{3}H]-Glycyl-sarcosine (sp. act., 400 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cell culture reagents were obtained from Gibco (Grand Island, NY), and cell culture supplies were from Corning (Corning, NY) and Falcon (Lincoln Park, NJ).

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC HTB37, Passage number 30-35) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% MEM nonessential amino acids, 1 mM sodium pyruvate, and 1% L-glutamine. HeLa cells were obtained from ATCC and grown in similar conditions. All cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

RT-PCR Assay for hPept1 mRNA Expression

For RT-PCR, total RNA from the tissue and cell samples was purified using Trizol reagent (Gibco, Grand Island, NY). One microgram of total RNA from each sample was subjected to RT-PCR (PCR Access system, Promega, Madison, WI) using hPept1 specific primers. The primers corresponded to nucleotide positions 342–360 (forward primer) and 1575–1592 (reverse primer) of the cDNA. The first strand cDNA was synthesized using AMV reverse transcriptase (AMV RT) at 48°C for 45 min. This was followed by a 2-min cycle at 94°C to inactivate AMV RT and to denature the primers and cDNA. The PCR was performed for 25 cycles of 94°C for 30 s, primer annealing for 1 min at 54°C, extension at 68°C for 2 min, and a final extension at 68°C for 7 min. The experimental conditions were optimized to ensure linear amplification of the PCR product. The expected hPept1 PCR fragment was 1.2 kb. The reaction mixture was separated on a 4–20% TBE -polyacrylamide gel (Invitrogen, Carlsbad, CA) and visualized with SYBR Green nucleic acid gel stain (Molecular Probes, Eugene, OR).

Adenoviral Infection of HeLa Cells

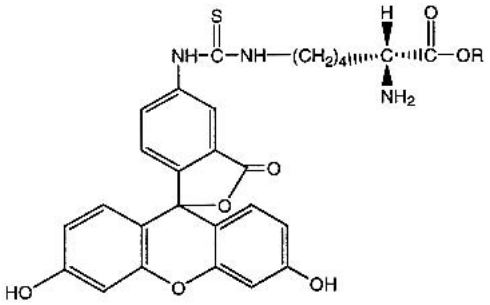
Ad.RSVhPept1 was prepared and was used to infect HeLa cell cultures as described by Hsu *et al.* (14). The HeLa cells were seeded onto six-well plates at a density of 1×10^5 cells/cm^2 and grown for 24 h. The cells were infected with 5000 viral particles per cell, and uptake experiments were conducted at 48 h postinfection.

Lys-(N$^\varepsilon$-FITC)-OH and Lys-(N$^\varepsilon$-FITC)-OCH_3 Synthesis

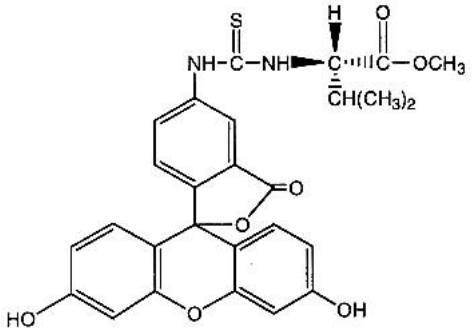
Boc-Lys-OH or Boc-Lys-OCH_3 (0.67 mmol) was dissolved in 20 ml dimethylformamide (DMF) and cooled to 0°C. To this 237 mg (0.61 mmol) fluorescein-5-isothiocyanate (FITC) and 93 μl (0.67 mmol) of triethylamine (Et_3N) were added. After stirring at 0°C for 15 min and then at room temperature for 2 h, the reaction mixture was diluted with 50 ml ethyl acetate and washed successively with 0.6 M citric acid (40 mL), water (30 mL), and brine (20 mL). The organic layer was dried over sodium sulfate and concentrated under vacuum. The resulting residue was purified by column chromatography using a mixture of chloroform:methanol:acetic acid (15:1:0.08, v/v) as solvent. After evaporation of the desired fractions, the obtained yellow intermediate was treated with 10 ml of trifluoroacetic acid /CH_2Cl_2 (1:1, v/v) and stirred at 0°C for 30 min. The excess acid was removed *in vacuo*. The resulting residue was triturated with diethylether (Et_2O) and dried *in vacuo* to obtain the desired product as an orange powder. The overall yield for the two lysine-FITC analogues was ~91%. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weights of the FITC analogues were found to be similar to those predicted from their expected structures. Lys-FITC-OH, ESI-MS m/z = 536.0 $(M+H)^+$; Lys-FITC-OCH_3, ESI-MS m/z = 549.9 $(M+H)^+$. The FITC analogue structures are shown in Fig. 1.

Val-FITC-OCH_3 Synthesis

Val-OCH_3 (0.67 mmol) was dissolved in 20 ml DMF and cooled to 0°C. To this 237 mg (0.61 mmol) of FITC and 93 μl (0.67 mmol) of Et_3N were added. After stirring at 0°C for 15 min and then at room temperature for 2 h, the reaction mix-



R = H; Lys-(N$^\varepsilon$-FITC)-OH (M.W.: 535)
R = CH_3; Lys-(N$^\varepsilon$-FITC)-OCH_3 (M.W.: 549)



FITC-Val-OCH_3 (M.W.: 520)

Fig. 1. Structures of Lys-(N$^\varepsilon$-FITC)-OH, Lys-(N$^\varepsilon$-FITC)-OCH_3, and FITC-Val-OCH_3.

ture was diluted with 50 ml ethyl acetate and washed successively with 0.6 M citric acid (40 ml), water (30 ml), and brine (20 ml). The organic layer was dried over sodium sulfate and concentrated under vacuum. The resulting residue was purified by column chromatography using a mixture of chloroform:methanol:acetic acid (15:1:0.08, v/v) as solvent. After evaporation of the desired fractions, the residue was triturated with Et_2O and dried *in vacuo* to obtain the desired product as an orange powder. The overall yield was 90%. Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospray ionization mass spectrometer. The observed molecular weight of the FITC analogue was found to be similar to those predicted from its expected structure. Val-FITC-OCH_3, ESI-MS m/z = 521.1 $(M+H)^+$. The Val-FITC-OCH_3 structure is shown in Fig. 1.

HPLC Assay of FITC Analogues

Samples containing the Lys-FITC analogues were analyzed using a high-performance liquid chromatography (HPLC) system consisting of a pump (Waters, Model 510, Milford, MA), an automatic injector (Waters, WISP model 712), a reversed-phase column (Ultrasphere, 5-μm, C-18, 4.6 × 250 mm, Beckman), and a fluorescent detector (Waters, Model 470) set at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The mobile phase was a (82/18, v/v) mixture of phosphate-buffered saline (pH 7.0) and acetonitrile. The compounds were analyzed over 23 min at a flow rate of 1 ml/min. The pump, data acquisition, and integration were controlled with Millennium® software (Waters).

Fluorescent Uptake Assays

The culture medium in the six-well culture plate was removed, the cells were washed with PBS pH 7.4, and 2 ml of uptake medium containing (25–100 μM) Lys-FITC-OCH_3 was added to the wells. The uptake medium contained 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM MES (pH 6.0). The fluorescent analogue was incubated with the cells for 45 min at 37°C, after which the buffer was removed, and the cells were washed three times with room-temperature PBS. Lys-FITC-OCH_3 uptake was determined by fluorescence measurements using an inverted fluorescence microscope (Zeiss Axiovert 135 TV). For each treatment, multiple images were randomly taken using the 10× objective from independent wells and then quantified using image analysis (Metamorph, Universal Imaging Corp., Downingtown, PA). The cells were then scraped off in water, lysed by probe-sonication for 15 s three times, and protein concentration was determined with a Protein Assay DC kit (BioRad, Hercules, CA). After subtraction of background fluorescence, the fluorescent intensity of each image was integrated and reported as an integrated fluorescence value per milligram of protein (integrated value/mg protein).

Fluorescence Micrographs of Lys-FITC-OCH_3 Uptake in HeLa/hPept1 Cells

HeLa cells were grown on chamber slides (Nalge Nunc International, Rochester, NY) and infected with Ad.RSVhPept1 as previously described (14). Lys-FITC-OCH_3 (50 μM) was incubated with the cells for 45 min at 37°C in uptake buffer, after which the buffer was removed, and the cells were washed three times with cold uptake buffer. The HeLa/hPept1 cells were fixed with 4% paraformaldehyde for 15 min at 4°C and washed 4 times with PBS at room temperature. The slides were mounted in Fluoromount-G medium (Electron Microscopy Sciences, Fort Washington, PA), and micrographs of cell images were obtained using an inverted fluorescence microscope (Zeiss Axiovert 135 TV) with 50× and 100× objectives.

Caco-2 Monolayer Transport and Stability Studies

The Caco-2 permeability of Lys-FITC-OH, Lys-FITC-OCH_3, and FITC-Val-OCH_3 (50 μM) were evaluated as previously described (15). The transport of the FITC analogues in the apical-to-basolateral (AP-to-BL) and in the basolateral-to-apical (BL-to-AP) directions was determined with and without Gly-Sar (10 mM) in collagen-coated transwell pates (0.4 μm pore size, 12 mm diameter, Costar, Cambridge, MA). The concentration of the FITC analogue in the receiver and donor compartments were analyzed using HPLC. The apparent permeability (P_{app}) in either direction was calculated using the following equation:

$$P_{app} = \frac{V_r}{A \times C_0} \times \frac{dC_r}{d_t}$$

where V_r is the receiver volume, A is the surface area of the exposed monolayer, C_0 is the concentration of the FITC analogue in the donor solution, and dC_r/d_t is the rate of change of concentration in the receiver solution.

The stability of donor solutions of Lys-FITC-OCH_3 and Lys-FITC-OH bathing either the apical side or the basolateral side was examined at 90 min of the transport experiments. The samples were assayed with HPLC to determine the mode and extent of degradation of the analogues when in contact with Caco-2 monolayers. The receiver solution in AP-to-BL transport experiments was also assayed with HPLC to determine metabolism of the analogues following passage across Caco-2 cells.

Inhibition Studies

For [^{3}H]Gly-Sar uptake inhibition assays, cells were seeded in 12-well culture plates (3.8 cm^2 growth area) at a density of 10^5 cells/cm^2. Two days postinfection, HeLa/hPept1 cells were washed with pH 6.0 uptake buffer before addition of substrates and inhibitors. Solutions containing 1 mM of the test FITC analogue and 20 μM [^{3}H]Gly-Sar (0.4 μCi/ml) was added to each well and incubated for 30 min at 25°C. The cells were then washed with ice-cold pH 6.0 uptake buffer three times to stop cellular uptake. One milliliter of 1 M NaOH/1.5% Triton X-100 was then added to each well and incubated for 30 min at 25°C. The cells were scraped off and transferred into vials containing 4 ml of scintillation cocktail. The samples were analyzed for [^{3}H]Gly-Sar content by scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA), and the protein concentrations determined.

In a second inhibition assay, Lys-FITC-OCH_3 uptake into Caco-2 cells was determined in the presence of the dipeptides Gly-Pro and Gly-Phe and in the presence of Lys or cephalexin. Thus, Lys-FITC-OCH_3 uptake from solutions containing 50 μM Lys-FITC-OCH_3, and known concentra-

tions of the inhibitors were determined and compared to that from a control solution. The inhibitor concentrations used were as follows: 30 mM lysine, 10 mM Gly-Pro, 10 mM Gly-Phe, and 10 mM cephalexin. One milliliter of the test solution was added to cells and agitated on a plate shaker at 25°C. After 30 min incubation, the cells were washed with cold uptake buffer three times, water was added, and cells were scraped and probe-sonicated three times for 15 s. The lysate was treated with TCA (3–5%), vortexed, and centrifuged 5 min at 3000 rpm. The supernatant was filtered through a 0.45-μm membrane filter and analyzed by HPLC. The protein concentration was determined from the pre-TCA-treated sample using the Protein Assay DC kit (BioRad).

RESULTS

RT-PCR Assay for hPept1 mRNA Expression

The hPept1 mRNA expression results from HeLa, HeLa/hPept1, and Caco-2 cells are shown in Fig. 2. A 1.2-kb product was detected in all three samples, confirming the presence of hPept1 in all three cells. A semiquantitative estimate of mRNA expression using RT-PCR suggests that expression was in the order HeLa/hPept1 > Caco-2 cells > HeLa cells. The hPept1 mRNA expression was quite insignificant in normal HeLa cells, whereas its expression in HeLa/hPept1 cells was about 6-fold and 2-fold higher compared to normal HeLa cells and Caco-2 cells, respectively.

[^{3}H]Gly-Sar Uptake Inhibition by FITC Analogues in HeLa/hPept1 Cells

The [^{3}H]Gly-Sar uptake results in the presence and absence of Lys-FITC-OCH_3 or FITC-Val-OCH_3 in HeLa/hPept1 cells are shown in Fig. 3. It is evident that both the FITC analogues were capable of inhibiting Gly-Sar uptake, suggesting their affinity for the hPept1 transporter. However, Gly-Sar uptake inhibition was quite significant with Lys-FITC-OCH_3 (74%, $p < 0.001$), compared to only 22% with FITC-Val-OCH_3. Thus, Lys-FITC-OCH_3 appears to exhibit a much higher affinity for hPept1 than FITC-Val-OCH_3 and was chosen for more detailed uptake and inhibition studies.

Fluorescence Assays of Lys-FITC-OCH_3 Uptake in HeLa and HeLa/hPept1 Cells

The uptake results of 100 μM Lys-FITC-OCH_3 in normal HeLa and HeLa/hPept1 cells determined using a fluorescence assay are shown in Fig. 4. The results in Fig. 4 clearly indicate that Lys-FITC-OCH_3 uptake is approximately 10-fold higher in HeLa cells overexpressing hPept1. The uptake of 100 μM Lys-FITC-OCH_3 in HeLa/hPept1 cells was found to be sig-

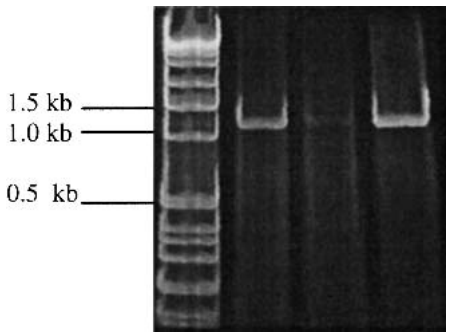


Fig. 2. RT-PCR quantification of hPept1 mRNA in Caco-2 and HeLa cells. Lane 1, MW markers; lane 2, Caco-2 cell RNA; lane 3, HeLa cell RNA; lane 4, HeLa cell RNA from cells infected with adenovirus-containing hPept1.

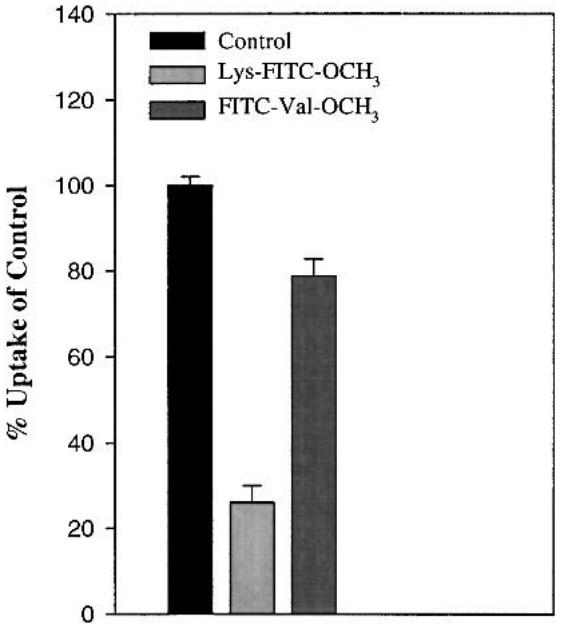


Fig. 3. Lys-FITC-OCH_3 (1 mM) and FITC-Val-OCH_3 (1 mM) inhibition of [^{3}H]Gly-Sar uptake (20 μM, 0.5 μCi/ml) in HeLa/hPept1 cells (mean ± SD, n = 4).

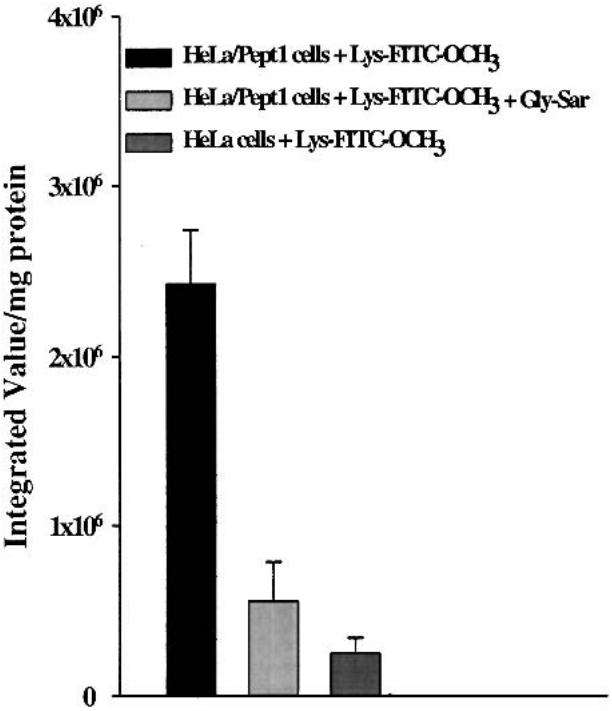


Fig. 4. The uptake of Lys-FITC-OCH_3 (100 μM) in HeLa/hPept1 and normal HeLa cells. Gly-Sar (1 mM) was used as an inhibitor in the virus-infected HeLa/hPept1 cells. HeLa/hPept1 cells treated with 100 μM Lys-FITC-OCH_3 (mean ± SEM, nine wells); HeLa/hPept1 treated with 1 mM Gly-Sar and 100 μM Lys-FITC-OCH_3 (mean ± SEM, six wells); HeLa cells treated with 100 μM Lys-FITC-OCH_3 (mean ± SEM, nine wells).

nificantly inhibited (77%; $p < 0.001$) in the presence of 1 mM Gly-Sar, indicating competitive hPept1 transport (Fig. 4).

The Lys-FITC-OCH_3 uptake in HeLa and HeLa/hPept1 cells at three different concentrations (25, 50, and 100 μM) determined with fluorescence assays is shown in Table I. The Lys-FITC-OCH_3 uptake in HeLa/hPept1 cells increased with Lys-FITC-OCH_3 concentration and appears to show saturable behavior beyond 50 μM. Thus, the enhancement in uptake from 25 μM to 50 μM was about 5.5-fold, whereas it increased only about 1.5-fold with increasing concentration from 50 μM to 100 μM. It was also found that Lys-FITC-OCH_3 uptake in HeLa/hPept1 cells was 10- to 100-fold higher than the uptake in normal HeLa cells at a given concentration. On the other hand, uptake of Lys-FITC-OCH_3 in normal HeLa cells exhibited a relatively large increase at the highest concentration of 100 μM examined compared to that at 25 μM and 50 μM.

Fluorescence Assays of Lys-FITC-OCH_3 Uptake in Caco-2 Cells

The uptake of 100 μM Lys-FITC-OCH_3 in Caco-2 cells determined with fluorescence assays in the presence and absence of 1 mM Gly-Sar is shown in Fig. 5. The extent of Lys-FITC-OCH_3 uptake inhibition by 1 mM Gly-Sar (80%), was similar to the Lys-FITC-OCH_3 uptake inhibition seen in HeLa/hPept1 cells (Fig. 4). More interestingly, the fluorescent uptake intensities of 100 μM Lys-FITC-OCH_3 in HeLa, Caco-2, and HeLa/hPept1 cells determined under similar experimental conditions (integrated value/mg protein values of 250,000, 500,000 and 2,450,000) appear to be related to hPept1 mRNA expression levels (Fig. 2). Although the hPept1 mRNA expression levels are not quite quantitative, the enhanced fluorescent intensities with increased hPept1 expression in the three cells further supports cellular uptake of the FITC analogue via hPept1-mediated transport.

HPLC Assays of Lys-FITC-OCH_3 Uptake Inhibition in Caco-2 Cells

The Caco-2 uptake results of 50 μM Lys-FITC-OCH_3 in the presence of several inhibitors that were quantified using HPLC assays are shown in Fig. 6. Thus, the small dipeptides Gly-Pro and Gly-Phe, as well as cephalexin, known hPept1 substrates, significantly reduced Lys-FITC-OCH_3 uptake ($p < 0.01$). The extent of inhibition was 44% by Gly-Pro, 68% by Gly-Phe, and 67% by cephalexin. Uptake inhibition by lysine was found to be insignificant (5%). These results obtained through assay of cellular Lys-FITC-OCH_3 content using HPLC corroborate that cellular uptake of the FITC analogue occurs via a peptide transporter-mediated pathway.

Table I. Lys-FITC-OCH_3 Uptake in Normal HeLa and HeLa/hPept1 Cells Determined Using Fluorescence Assays[a]

Lys-FITC-OCH_3 concentration (μM)	Integrated fluorescence intensity/mg protein ($\times 10^7$)	
	HeLa	HeLa/hPept1
25	0.03 ± 0.01	3.03 ± 1.83[b]
50	0.16 ± 0.13	16.58 ± 3.43[b]
100	2.51 ± 0.88	24.24 ± 3.14[b]

[a] All values expressed as mean ± SEM of experiments from 9–10 wells.

[b] p-value < 0.001 compared to corresponding uptake in normal HeLa cells.

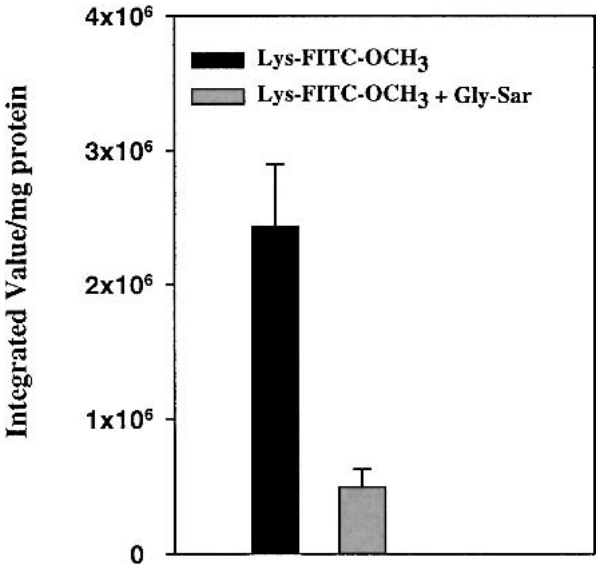


Fig. 5. Caco-2 cell uptake of 100 μM Lys-FITC-OCH_3 with and without 1 mM Gly-Sar inhibitor (mean ± SEM, nine wells each); $p < 0.001$.

Fluorescence Micrographs of Lys-FITC-OCH_3 Uptake in HeLa/hPept1 Cells

Typical fluorescence micrographs of HeLa/hPept1 cells incubated with Lys-FITC-OCH_3 using cell microscopy under high magnification (50×) with an inverted fluorescence microscope are shown in Fig. 7. The micrographs indicate that the chromophore is localized in the cytoplasm as well as in the nucleus. In contrast, no fluorescence was evident with normal HeLa cells, indicating negligible uptake into cells or cell surface binding.

Caco-2 Permeability and Lys-FITC Analogue Stability

The apparent permeabilities of the three FITC analogues in the apical-to-basolateral (AP-to-BL) direction are shown in Table II. The AP-to-BL permeability of Lys-FITC-OCH_3

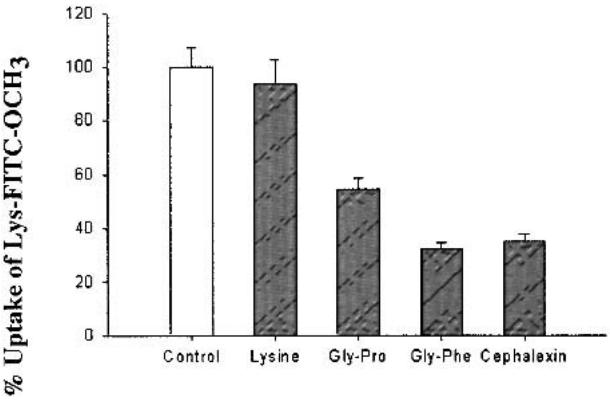


Fig. 6. Inhibition of Lys-FITC-OCH_3 uptake in Caco-2 cells. Control 0.05 mM, Lysine 30 mM, Gly-Pro 10 mM, Gly-Phe 10 mM, cephalexin 10 mM (mean ± SEM, four wells).

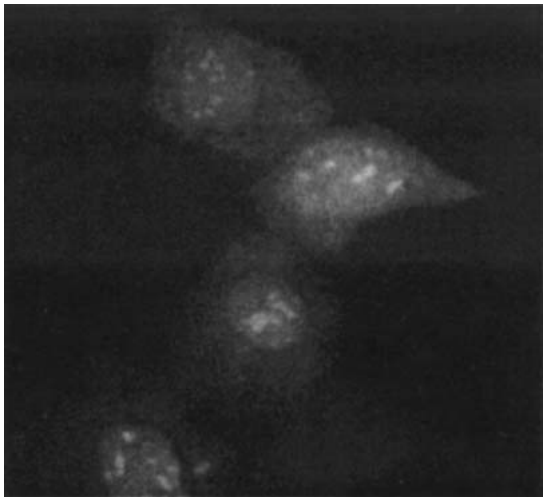


Fig. 7. Lys-FITC-OCH_3 uptake into HeLa/hPept1 cells.

was similar to that observed for Lys-FITC-OH, indicating that both analogues are equally permeable. In comparison, the AP-to-BL permeability of Val-FITC-OCH_3 was about 10-fold lower than that of the Lys-FITC analogues. Further, the presence of 10 mM Gly-Sar significantly inhibited the permeability of both Lys-FITC analogues ($p < 0.001$). The basolateral-to-apical (BL-to-AP) permeabilities of the two Lys-FITC analogues were similar to each other and roughly 7-fold lower than their AP-to-BL permeability (Table II). Interestingly, the AP-to-BL permeability of Lys-FITC-OCH_3 in the presence of 10-mM Gly-Sar was similar to its BL-to-AP permeability. The effect of 10 mM Gly-Sar on AP-to-BL permeability as well as BL-to-AP permeability of Val-FITC-OCH_3 was not determined.

The stability results on the donor Lys-FITC analogue solutions bathing either the apical or basolateral side are shown in Table III. It is seen that the free acid analogue is stable over 90 min with almost no degradation or breakdown of the thiourea linkage when in contact with either side of the Caco-2 monolayers. The methyl ester analogue, however, degraded to the free acid form on the apical side (~53% in 90 min). The degradation of Lys-FITC-OCH_3 was lower when placed on the basolateral side. Further, only the free acid form, Lys-FITC-OH, was detected in the receiver solution in AP-to-BL experiments.

Table II. Transport of FITC Analogues in Caco-2 Cell Monolayers[a]

Compound	Permeability[b] ($\times 10^6$ cm/s)	
	AP to BL	BL to AP
Lys-FITC-OCH_3	1.47 ± 0.10	0.20 ± 0.03
Lys-FITC-OCH_3 + GlySar	0.26 ± 0.03[c]	nd
Val-FITC-OCH_3	0.12 ± 0.02	nd
Lys-FITC-OH	1.51 ± 0.17	0.21 ± 0.03
Lys-FITC-OH + GlySar	0.71 ± 0.08[c]	nd

[a] All values expressed as mean ± SD of triplicate determinations at 37°C.
[b] FITC-Lys analogue concentration, 50 μM; Gly-Sar concentration, 10 mM.
[c] $p \leq 0.002$ compared to control.

Table III. Stability of FITC Analogues in Caco-2 Cell Monolayers[a]

Compound	Stability[b]	
	AP donor	BL donor
Lys-FITC-OCH_3	47.6 ± 5.3	70.4 ± 1.5
Val-FITC-OCH_3	nd	nd
Lys-FITC-OH	~100.0	~100.0

[a] All values expressed as mean ± SD of triplicate determinations at 37°C.
[b] Percentage compound remaining in donor bathing solution at 90 min.

DISCUSSION

There are a wide range of substrates that exhibit affinity for the hPept1 oligopeptide transporter. This substrate flexibility allows the design of prodrugs that are targeted to hPept1 using substrates with an equally wide range of substrate moieties. Thus, it is anticipated that following prodrug design and synthesis, the characterization of hPept1 transporter uptake activity in different cell types *in vitro* or different tissues *in vivo* would require the screening of numerous samples. In these situations, suitable alternatives to tedious HPLC or hazardous radioactive assays would be highly beneficial. Fluorescence-based assays not only obviate the use of hazardous materials but also are amenable to rapid screening with multiwell plate readers. The present study demonstrates this applicability with fluorescently labeled analogues using cells with widely differing levels of hPept1 expression. Thus, HeLa cells, HeLa cells overexpressing hPept1, and normal Caco-2 cells were analyzed for hPept1 mRNA expression levels. The semiquantitative RT-PCR results in Fig. 2 indicate that uptake of hPept1 substrates should be consistent with the order of hPept1 mRNA expression, HeLa < Caco-2 < HeLa/hPept1.

In initial studies, fluorescent analogues of the amino acids valine and lysine were synthesized and tested in HeLa/hPept1 cells. The fluorescent moiety fluorescein isothiocyanate (FITC) was linked to the methyl ester of valine via the N-terminal amino group of valine or to the N^{ε} amino group of lysine or its methyl ester. The rationale for this synthesis was based on the previously successful amino acid prodrug strategy used with valacyclovir. Modifications were made with the knowledge that the basic structural requirement for peptide transport is the terminal carboxyl group of the amino acid and that the N-terminal of the amino acid could be altered without affecting affinity for the hPept1 transporter (16). The [^{3}H]Gly-Sar inhibition results shown in Fig. 3 clearly suggest that both methyl ester FITC analogues have affinity for hPept1. However, the lower affinity of FITC-Val-OCH_3 (22% inhibition), a neutral molecule, may be caused by its enhanced lipophilicity. The 10-fold lower AP-to-BL permeability of FITC-Val-OCH_3 in Caco-2 cells compared to that of Lys-FITC-OCH_3 is consistent with its lower peptide transporter affinity. The charged lysine analogue Lys-FITC-OCH_3, however, exhibited a much higher hPept1 affinity judging from its ability to inhibit 74% of [^{3}H]Gly-Sar uptake in HeLa/hPept1 cells. The similar AP-to-BL permeabilities of Lys-FITC-OH and Lys-FITC-OCH_3 may suggest that the structural requirements of a peptide bond and a free carboxyl group may not be necessary for hPept1 recognition and trans-

port. It is important to note, however, that the permeability of Lys-FITC-OCH_3 was affected to a greater extent in the presence of Gly-Sar, indicating some preference for the methyl ester form by the peptide transporter (Table II).

The high stability of Lys-FITC-OH in Caco-2 monolayer bathing solutions clearly suggests that the thiourea linkage between FITC and lysine remains intact. Thus, the loss of Lys-FITC-OCH_3 from bathing solutions resulted from conversion of the methyl ester to its free acid form. This observation coupled with the finding that AP-to-BL permeability of the two forms in Caco-2 monolayers is similar indicates that such degradation may not be a major factor in uptake determinations. The inability to detect the methyl ester analogue in receiver solutions in AP-to-BL experiments suggests its rapid hydrolysis by brush border membrane enzymes and cytosolic enzymes during transit.

The fluorescence uptake intensities of 100 μM Lys-FITC-OCH_3 in HeLa, HeLa/hPept1 cells (Fig. 4), and in Caco-2 cells (Fig. 5) determined under similar experimental conditions is consistent with the order of hPept1 mRNA expression levels in the three systems (Fig. 2). Further, the significant reduction of Lys-FITC-OCH_3 fluorescence intensities in the presence of Gly-Sar in both HeLa/hPept1 (Fig. 4) and Caco-2 (Fig. 5) cells provides strong evidence for hPept1-mediated uptake. The saturable uptake behavior of Lys-FITC-OCH_3 with HeLa/hPept1 cells at higher Lys-FITC-OCH_3 concentrations also suggests hPept1 carrier-mediated transport of the analogue. It is possible that Lys-FITC-OCH_3 might also be a substrate for hPept2 transporter or other peptide transporters. However, the predominance of hPept1 expression in hPept1 overexpressed HeLa cells and the absence of hPept2 in Caco-2 cells indicate that in the two cell systems examined, the contribution of hPept2 to uptake and transport of Lys-FITC-OCH_3 is quite negligible. Thus, the results suggest that the fluorescent analogue Lys-FITC-OCH_3 exhibits high affinity for hPept1 and that Lys-FITC-OCH_3 uptake in test candidate cells or tissues via the hPept1 transporter can be conveniently monitored with simple fluorescence measurements.

The Lys-FITC-OCH_3 uptake into Caco-2 cells determined using HPLC assays shown in Fig. 6 and uptake into HeLa/hPept1 cells using cell microscopy shown in Fig. 7 provide complementary evidence for uptake of the fluorescent analogue. Further, the inhibitory effects of known hPept1 substrates such as Gly-Sar, Gly-Pro, Gly-Phe, and cephalexin on Lys-FITC-OCH_3 uptake and transport across Caco-2 cells determined by HPLC analysis as well as the negligible effect of Lys on uptake (Fig. 6 and Table II), clearly suggest that Lys-FITC-OCH_3 is transported via the hPept1 transporter. The HPLC results also suggest that monitoring of Lys-FITC-OCH_3 uptake in cells with fluorescence measurements would be a reliable alternative.

CONCLUSIONS

In this study, we have demonstrated that the fluorescent analogue Lys-FITC-OCH_3 is a substrate for the hPept1 transporter. Thus, fluorescent intensities of Lys-FITC-OCH_3 uptake in HeLa, Caco-2, and HeLa/hPept1 cells corresponded to the extent of hPept1 mRNA expression in the cells. The effect of known hPept1 substrates on Lys-FITC-OCH_3 uptake in HeLa/hPept1 cells determined using fluorescence measurements was also consistent with hPept1-mediated uptake of the fluorescent analogue. The Lys-FITC-OCH_3 uptake in Caco-2 cells and its inhibition by known hPept1 substrates determined with HPLC analyses provided evidence for the reliability of the fluorescence method. The inhibition of Lys-FITC-OCH_3 transport in the presence of Gly-Sar in Caco-2 cells and the direct evidence of chromophore localization in the cytoplasm and nucleus corroborate the fluorescence uptake studies. Finally, although the fluorescence uptake methodology described in this study is not set up as a high-throughput strategy, it can be easily adapted for such an approach. It is anticipated that with such a high-throughput method, large numbers of cell samples can be screened rapidly and accurately for hPept1 uptake activity.

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