Transcellular Transport of a Highly Polar 3+ Net Charge Opioid Tetrapeptide

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ABSTRACT

Oligopeptides are generally thought to have poor permeability across biological membranes. Recent studies, however, suggest significant distribution of [Dmt]DALDA (Dmt-o-Arg-Phe-Lys-NH₂; Dmt is 2',6'-dimethyltyrosine), a 3+ net charge opioid peptide, to the brain and spinal cord after subcutaneous administration. Peptide transporters (PEPT1 and PEPT2) play a major role in the uptake of di- and tripeptides across cell membranes, but their ability to transport tetrapeptides is not clear. The purpose of this study was to determine whether [Dmt]DALDA can translocate across Caco-2 cell monolayers and whether PEPT1 plays a role in the uptake process. Our results show that [3H][Dmt]DALDA can readily translocate across Caco-2 cells, with a permeability coefficient estimated to be 1.24 × 10⁻⁶ cm/s. When incubated with Caco-2 cells, [3H][Dmt]DALDA was detected in cell lysates by 5 min. The internalization of [Dmt]DALDA was confirmed visually with a fluorescent [Dmt]DALDA analog (H-Dmt-o-Arg-Phe-dnsDap-NH₂; dnsDap is β-dansyl-l-α,β-diaminopropionic acid). The uptake of [3H][Dmt]DALDA was concentration-dependent but temperature- and pH-independent. Treatment with diethylpyrocarbonate (DEPC) inhibited [14C]glycine-sarcosine uptake but increased [3H][Dmt]DALDA uptake 34-fold. These findings suggest that PEPT1 is not involved in [Dmt]DALDA internalization. [Dmt]DALDA uptake was also observed in SH-SYSY, human embryonic kidney 293, and CRFK cells, and was independent of whether the cells expressed opioid receptors. The efflux of [3H][Dmt]DALDA from Caco-2 cells was temperature-dependent and was inhibited by DEPC, but was not affected by verapamil, an inhibitor of P-glycoprotein. These data show transcellular translocation of a highly polar 3+ charge tetrapeptide and suggest that [Dmt]DALDA may not only distribute across the blood-brain barrier but also it may even have reasonable oral absorption.

Apart from metabolic instability, oligopeptides are generally considered to be of limited therapeutic potential because of their poor permeability across biological membranes. Although enzymatic degradation can be overcome by a variety of structural modifications, the ability of peptides to distribute to their site(s) of action remains a significant problem. Besides insufficient lipophilicity, this low permeability is also due to the high capacity of the peptide backbone to bind water molecules through hydrogen bonding (Burton et al., 1996). Attempts at modifying polar oligopeptides with lipid groups have only been met with limited success, and such modifications often resulted in a decline of biological activity. It was therefore a surprise when we discovered that a synthetic opioid peptide, [Dmt]DALDA (Dmt-o-Arg-Phe-Lys-NH₂; Dmt is 2',6'-dimethyltyrosine), was 36 times more potent than morphine in antinociceptive tests after subcutaneous administration in mice (Zhao et al., 2002). [Dmt]DALDA has a molecular weight of 640 and carries a 3+ net charge at physiological pH, thus making it a highly polar molecule and rather unlikely to cross the blood-brain barrier. However, repeated subcutaneous administration of [Dmt]DALDA led to supraspinal and spinal tolerance, and binding studies revealed a 30% reduction in [Dmt]DALDA binding sites in both brain and spinal cord (Zhao et al., 2002). These findings suggest significant distribution of [Dmt]DALDA to the brain and spinal cord after subcutaneous administration.

The distribution of substances across a continuous cell layer may proceed via transcellular or paracellular pathways. The transcellular route is highly unlikely for oligopeptides, and previous studies with met-enkephalin analogs have shown that they are primarily transported via the paracellular route, and their permeability across a cell layer

ABBREVIATIONS: [Dmt]DALDA, H-Dmt-o-Arg-Phe-Lys-NH₂, where Dmt is 2',6'-dimethyltyrosine; PEPT, peptide transporter; Gly-Sar, glycine-sarcosine; MEM, minimal essential medium; HEK, human embryonic kidney; HBSS, Hanks’ balanced salt solution; CLSM, confocal laser scanning microscopy; [Dmt],dnsDap][DALDA, H-Dmt-o-Arg-Phe-dnsDap-NH₂, where dnsDap is β-dansyl-l-α,β-diaminopropionic acid; PBS, phosphate-buffered saline; DEPC, diethylpyrocarbonate; P_app, apparent permeability coefficient.
is very low (Lang et al., 1997). For efficient transcellular transport of a highly polar molecule such as [Dmt]DALDA, a sort of a carrier system is likely to be required.

Specific peptide transporters have been reported for the uptake of di- and tripeptides across the plasma membrane of mammalian cells. In the small intestine and kidney, di- and tripeptides are actively transported into the epithelial cells by peptide transporters that are driven by H⁺ gradient (Leibach and Ganapathy, 1996; Daniel and Herget, 1997). Two H⁺-coupled peptide transporters have been cloned, PEPT1 and PEPT2, and they are expressed primarily in the intestinal and renal epithelial cells, respectively (Fei et al., 1994; Liu et al., 1995; Boll et al., 1996; Saito et al., 1996). PEPT2 is also expressed in an appreciable extent in the brain (Wang et al., 1998). Oligopeptides with more than four amino acid residues are generally not recognized by these peptide transporters. Whether PEPT1 or PEPT2 would transport tetrapeptides is less clear. Although the tetrapeptide Tyr-Pro-Phe-Pro was not transported by PEPT2 (Tiruppathi et al., 1991), an H⁺-coupled peptide transporter cloned from sheep oesmal epithelium actively transported tetrapeptides (Pan et al., 1997). It therefore remains possible that [Dmt]DALDA may penetrate cell membranes via proton-coupled peptide transporters such as PEPT1 or PEPT2.

The purpose of this study was to determine whether [Dmt]DALDA can translocate across cell membranes and whether the peptide transporters play a role in the uptake process. Caco-2 cell line, a human colon carcinoma cell line, was chosen for these studies because PEPT1 is expressed on these cells and it has been extensively characterized (Leibach and Ganapathy, 1996). Our results show that [Dmt]DALDA penetrated the Caco-2 cell readily, but its uptake did not involve PEPT1. Instead, the uptake of [Dmt]DALDA was energy-independent and was observed in several other cell types independent of whether the cells expressed opioid receptors. In addition, we found evidence that [Dmt]DALDA can undergo apical-to-basolateral translocation across a Caco-2 cell monolayer, suggesting that this tetrapeptide might not only distribute across the BBB but also might even have reasonable oral absorption.

Materials and Methods

**Drugs and Chemicals.** [Dmt]DALDA and [³H][Dmt]DALDA (47 Ci/mmol) were synthesized according to methods described previously (Schiller et al., 2000; Zhao et al., 2002). [¹⁴C]Gly-Sar (56.7 mCi/mmol) and [³H][N-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (50 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ). All other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** All cell lines were obtained from American Type Culture Collection (Manassas, VA), and cell culture supplies were obtained from Invitrogen (Carlsbad, CA). Caco-2 cells were grown in MEM, whereas SH-SYSY and HEK293 cells were grown in Dulbecco's modified Eagle's medium. Growing media were supplemented with 10% fetal bovine serum, 200 µg/ml penicillin, and 100 µg/ml streptomycin sulfate. CRFK cells were grown in MEM + 10% horse serum, nonessential amino acids, and penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Assay for Peptide Uptake.** Peptide internalization was studied primarily using Caco-2 cells and subsequently confirmed with SH-SYSY, HEK293, and CRFK cells. Monolayers of cells were grown on 12-well plates (5 × 10⁵ cells/well) coated with collagen for 3 days. On day 4, cells were washed twice with prewarmed HBSS, and then incubated with 0.2 ml of HBSS containing either 250 nM [³H][Dmt]DALDA or 50 µM [¹⁴C]Gly-Sar at 37°C for various times up to 1 h. In a separate experiment, cells were incubated with the same concentration of [³H][Dmt]DALDA in the presence of unlabeled [Dmt]DALDA (1 µM–3 nM) for 1 h at 37°C. For uptake studies at 4°C, cells were put on ice for 20 min before incubation with [³H][Dmt]DALDA or [¹⁴C]Gly-Sar. At the end of the incubation period, cells were washed four times with HBSS, and 0.2 ml of 0.1 N NaOH with 1% SDS was added to each well. The cell contents were then transferred to scintillation vials and radioactivity was counted. An aliquot of cell lysate was used for determination of protein content using the method of Bradford (Bio-Rad, Hercules, CA). To distinguish between internalized radioactivity from surface-associated radioactivity, an acid-wash step was included. Before cell lysis, cells were incubated with 0.2 ml of 0.2 M acetic acid/0.05 M NaCl for 5 min on ice.

**Assay for Peptide Efflux from CaCo-2 Cells.** Monolayers of Caco-2 cells were grown on 12-well plates (5 × 10⁵ cells/well) for 3 days. On day 4, cells were preloaded with [³H][Dmt]DALDA or [¹⁴C]Gly-Sar for 1 h at 37°C. Cells were then washed four times with 1 ml of ice-cold incubation solution to terminate uptake and then incubated with 0.5 ml of MEM for 1 h at either 37 or 4°C to measure the efflux of peptide from cells to the incubation medium. The amount of radioactivity was determined in cell lysates and in the incubation medium. To examine the role of P-glycoprotein on peptide uptake and efflux from cells, [³H][Dmt]DALDA uptake and efflux were also determined in the presence of 100 µM verapamil (P-glycoprotein inhibitor).

**Assay for Peptide Translocation across Caco-2 Monolayers.** Monolayers of Caco-2 cells were prepared as described previously (Irie et al., 2001). Caco-2 cells (2 × 10⁵) were seeded on microporous membrane filters (24 mm, 0.4 µm) inside Transwell cell culture chambers (Corning Glassworks, Corning, NY). Each Transwell chamber was filled with 1.5 ml of medium in the apical compartment and 2.5 ml in the basolateral compartment. The cell monolayers were given fresh medium every 1 to 2 days and were used on day 28 for transport experiments. Apical-to-basolateral transport of peptides was determined by adding 0.2 µM [³H][Dmt]DALDA or 100 µM [¹⁴C]Gly-Sar to the apical compartment, and 50-µl aliquots were removed from both apical and basolateral compartments at various times after peptide addition for determination of radioactivity counts.

The apparent permeability coefficient was calculated according to the following equation: Papp = Xtr/A·Co, where Xtr is the rate of uptake in the receiver compartment, A is the diffusion area (4.72 cm²), and Co is the initial concentration in the donor compartment.

**Confocal Laser Scanning Microscopy.** The uptake of [³H][Dmt]DALDA into Caco-2 cells was confirmed by confocal laser scanning microscopy (CLSM) using a fluorescent analog of [³H][Dmt]DALDA (Dmt-d-Arg-Phe-dnsDap-NH₂, where dnsDap is β-dansyl-l-α,β-diamino-propionic acid). Cells were grown as described above and were plated on (35-mm) glass bottom dishes (MatTek, Ashland, MA) for 2 days. The medium was then removed, and cells were incubated with 1 ml of HBSS containing 0.1 to 1.0 µM of the fluorescent peptide analog at 37°C for 1 h. Cells were then washed three times with ice-cold HBSS and covered with 100 µl of PBS. Confocal microscopy was performed within 10 min at room temperature using a confocal laser scanning microscope with a C-Apochromat 63×/1.2W corr objective (Nikon, Tokyo, Japan). Excitation was performed at 340 nm by means of a UV laser, and emission was measured at 520 nm. For optical sectioning in z-direction, 5 to 10 frames with 2.0 µm were made.

**Radioligand Binding Assay Using Cell Membranes.** Specific binding of [³H][Dmt]DALDA to cell surface receptors was determined using membranes prepared from Caco-2 and SH-SYSY cells. After 4 days of culture, cells were washed twice with PBS buffer and then scraped off. Cells were centrifuged at 500g for 5 min and...
the pellet stored at −80°C. Cells were homogenized in ice-cold 50 mM Tris-HCl buffer (5 μg/ml leupeptin, 2 μg/ml chymostatin, 10 μg/ml bestatin, and 1 mM EGTA, pH 7.4). The homogenate was centrifuged at 36,000 g for 20 min. The pellets were resuspended with 50 mM Tris-HCl buffer. Aliquots of membrane homogenates (~140 μg of protein) were incubated with [3H][Dmt1]DALDA (15–960 pM) for 60 min at 25°C. Nonspecific binding was assessed by inclusion of 1 μM unlabeled [Dmt1]DALDA. Free radioligand was separated from bound radioligand by rapid filtration through GF/B filters (Whatman, Maidstone, UK) with a cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed three times with 10 ml of Tris buffer, and radioactivity was determined by liquid scintillation counting.

Binding affinities (Kd) and receptor number (Bmax) were determined using nonlinear regression (GraphPad Software, San Diego, CA).

**Results**

**Time Course of Uptake of [Dmt1]DALDA and Gly-Sar into Caco-2 Cells.** When incubated with Caco-2 cells at 37°C, [3H][Dmt1]DALDA was observed in cell lysate as early as 5 min, and steady-state levels were achieved by 30 min (Fig. 1A). The total amount of [3H][Dmt1]DALDA recovered in the cell lysate after 1-h incubation represented about 1% of the total drug. In contrast, under the same experimental conditions, [14C]Gly-Sar continued to increase from 5 to 45 min (Fig. 1B). The measured radioactivity is believed to reflect [Dmt1]DALDA levels, because we have previously demonstrated that [Dmt1]DALDA is resistant against peptidase degradation (Szeto et al., 2001). To determine whether the measured radioactivity was associated with cell membranes, cells were subjected to acid-wash to remove surface binding. Figure 1C shows that 80.8% of [3H][Dmt1]DALDA was resistant to acid-wash and therefore presumed to be inside the cell. The uptake of [Dmt1]DALDA was found to be concentration-dependent over a wide range of concentrations (Fig. 1D).

**Temperature Dependence and Effects of pH on Uptake of [Dmt1]DALDA and Gly-Sar.** When the incubation was carried out at 4°C, the uptake of [3H][Dmt1]DALDA was slower compared with 37°C, but reached 76.5% by 45 min (Fig. 1A) and 86.3% by 1 h (Fig. 1A). In contrast, the uptake of [14C]Gly-Sar was completely abolished at 4°C (Fig. 1B). The uptake of Gly-Sar by PEPT1 is known to be pH-dependent, with optimal uptake occurring at pH 6.0 (Terada et al., 1999). This was confirmed in our study (Fig. 2B). In contrast, the uptake of [3H][Dmt1]DALDA was unchanged when pH varied from 4.0 to 7.4 (Fig. 2A). The lack of temperature and pH dependence suggests that the uptake of [Dmt1]DALDA in Caco-2 cells is not mediated via PEPT1.

**Effect of DEPC on [Dmt1]DALDA and Gly-Sar Uptake.** To further demonstrate that PEPT1 is not involved in the transport of [Dmt1]DALDA, we examined the effect of DEPC (diethylpyrocarbonate; 0.2 mM) on [3H][Dmt1]DALDA and [14C]Gly-Sar uptake. DEPC is a histidine residue-modifier reagent that has been shown to inhibit PEPT1 in Caco-2 cells (Terada et al., 1996). The addition of DEPC to the incubation medium significantly inhibited [14C]Gly-Sar uptake (Fig. 2D). Surprisingly, DEPC not only did not inhibit [3H][Dmt1]DALDA uptake but also it actually increased [Dmt1]DALDA uptake by 34-fold (Fig. 2C).

**[Dmt1]DALDA Internalization in Different Cell Types.** To demonstrate that the internalization of
[Dmt]DALDA was not limited to Caco-2 cells, we compared the internalization of [Dmt]DALDA in several different cell lines. An acid-wash step was included to distinguish internalized radioactivity (acid-resistant) from surface-bound radioactivity (acid-sensitive). Figure 3A compares the levels of acid-resistant radioactivity in Caco-2, SH-SY5Y, HEK293, and CRFK cells. The results show that [3H][Dmt]DALDA was taken up extensively in all cell types.

**Radioligand Binding Assays with [3H][Dmt]DALDA.**

To determine whether [Dmt]DALDA was internalized via receptor-mediated mechanisms, we carried out radioligand ([3H][Dmt]DALDA) binding assays with membranes prepared from Caco-2 cells and SH-SY5Y cells. Figure 3B shows the specific binding of [3H][Dmt]DALDA to SH-SY5Y membranes. The calculated $K_d$ value was 118 pM (range 87–149) and the $B_{max}$ value was estimated to be 96 fmol/mg protein (range 88–104). This is comparable with the values obtained using recombinant human $\mu$-opioid receptor expressed on Chinese hamster ovary cells (G.-M. Zhao and H. H. Szeto, unpublished data). No high-affinity specific binding was observed with Caco-2 membranes (Fig. 3B). It is known that HEK293 cells do not have opioid receptors (Blake et al., 1997).

**Efflux of [Dmt]DALDA and Gly-Sar from Caco-2 Cells.**

The achievement of steady-state [3H][Dmt]DALDA levels in Caco-2 cells after <30 min of incubation suggested that the rate of efflux of the peptide from the cell was equal to the rate of uptake at that time. To examine the efflux of Gly-Sar and [Dmt]DALDA from the cell, Caco-2 cells were preloaded with [14C]Gly-Sar or [3H][Dmt]DALDA and then replaced with fresh medium that did not contain peptide. Figure 4A shows that 39% of [14C]Gly-Sar was found in the medium after 1 h at 37°C. The efflux of [14C]Gly-Sar was significantly reduced at 4°C. The efflux of [3H][Dmt]DALDA from Caco-2 cells was much faster, with 80% of the peptide effluxed into the medium by 1 h (Fig. 4A). In contrast to the internalization of [3H][Dmt]DALDA (Fig. 1A), temperature had a significant effect on the efflux of [3H][Dmt]DALDA from the cell (Fig. 4A). The efflux of [Dmt]DALDA was decreased in cells treated with DEPC (Fig. 4B). The reduction in [3H][Dmt]DALDA efflux by DEPC is consistent with the greatly increased uptake of [3H][Dmt]DALDA in the presence of DEPC (Fig. 2C). On the other hand, the efflux of [3H][Dmt]DALDA was not affected by verapamil, an inhibitor of P-glycoprotein (Fig. 4C). Verapamil also had no effect on cellular uptake of [3H][Dmt]DALDA (Fig. 4D).

**Transcellular Transport of [Dmt]DALDA and Gly-Sar.**

Caco-2 monolayers grown in Transwells were used to study the apical-to-basolateral transport of [3H][Dmt]DALDA and [14C]Gly-Sar. Figure 5 illustrates the transport of [14C]Gly-Sar and [3H][Dmt]DALDA in the basolateral side at various times after loading the peptide in the apical side of the Transwell. The percentage of [3H][Dmt]DALDA translocated from the apical to the basolateral side in 60 min (10.4%) was comparable with the percentage of [14C]Gly-Sar transported (11.9%). The apparent permeability coefficient was estimated to be $1.24 \times 10^{-5}$ cm/s for [Dmt]DALDA and $1.26 \times 10^{-5}$ cm/s for Gly-Sar.

**Visualization of [Dmt]DALDA Internalization Using CLSM.**

To visualize the internalization of [Dmt]DALDA, a fluorescent analog ([Dmt]$^5$dnsDap$^4$DALDA) was synthesized. Figure 6A shows the internalization of the fluorescent peptide into Caco-2 cells after incubation with 0.1 $\mu$M [Dmt]$^5$dnsDap$^4$DALDA for 1 h at 37°C. Figure 6B shows the outline of the cells, and the merged image (Fig. 6C) shows that the fluorescent peptide is clearly localized intracellularly. The fluorescence appeared diffuse throughout the cytoplasm but
incubated with [3H][Dmt1]DALDA (250 nM, 47 Ci/mmol) for 1 h at 37 °C and the was 11.8 pM (range 87 – 149) and the specific binding of [3H][Dmt1]DALDA to cell membranes. Membranes prepared from SH-SY5Y cells and Caco-2 cells were incubated with [3H][Dmt1]DALDA (15–960 pM) for 1 h at 25 °C. Nonspecific binding was assessed by inclusion of 1 µM unlabeled [Dmt1]DALDA. Free radioligand was separated from bound radioligand by rapid filtration. No specific binding was observed with Caco-2 cells. For SH-SY5Y cells, the K_D value was 118 pM (range 87–149) and the B_{max} value was 96 fmol/mg protein.

was completely excluded from the nucleus. The uptake of the fluorescent peptide was similar at 37 and 4 °C (data not shown).

Discussion

Caco-2 is an intestinal epithelial cell line that grows in a polarized monolayer, and translocation across this monolayer may occur via transcellular or paracellular pathways. A previous study reported very low permeability for several met-enkephalin analogs across this cell layer (P_{app} = 3.3 × 10^{-8}–9.5 × 10^{-8} cm/s), and the fluorescein-labeled met-enkephalin was observed in the intercellular space only, indicative of paracellular transport (Lang et al., 1997). To our surprise, [Dmt1]DALDA readily translocated across Caco-2 cells via a transcellular pathway. The calculated P_{app} for [Dmt1]DALDA (1.24 × 10^{-5} cm/s) was >100-fold larger compared with the met-enkephalin analogs. The internalization of [Dmt1]DALDA was confirmed visually with CLSM using the fluorescent analog [Dmt1,dnsDap4]DALDA. DnsDap was chosen as the fluorescent amino acid to replace Lys^4 in [Dmt1]DALDA because the dansyl fluorophore is relatively small and because the side chain length of dnsDap is similar to that of Lys. Therefore, the fluorescent peptide analog is similar to [Dmt1]DALDA in terms of its structural characteristics and physicochemical properties. Indeed, [Dmt1,dnsDap4]DALDA has similar affinity and potency as [Dmt1]DALDA for the µ-opioid receptor (P. W. Schiller, unpublished data). [Dmt1,dnsDap4]DALDA was found diffusely throughout the cytoplasm but was completely excluded from the nucleus.

By comparing the uptake of [Dmt1]DALDA with that of Gly-Sar, we further showed that the uptake of [Dmt1]DALDA did not involve PEPT1. Unlike Gly-Sar, the uptake of [Dmt1]DALDA was independent of temperature and pH and was not saturable even at concentrations as high as 3 mM. The uptake of di- and tripeptides via PEPT1 is enhanced at pH 6.0 compared with 7.4 because the transporter is coupled to H^+ (Leibach and Ganapathy, 1996). Furthermore, although the uptake of Gly-Sar was significantly inhibited by DEPC, the addition of DEPC actually greatly increased the internalization of [Dmt1]DALDA. All of these findings indicate that neither PEPT1 nor any other proton-coupled peptide transporter plays a role in the translocation of [Dmt1]DALDA into Caco-2 cells.

The concentration of [Dmt1]DALDA inside Caco-2 cells reached a steady state in less than 30 min, suggesting that the rate of influx was equal to the rate of efflux out of the cell. The efflux of [Dmt1]DALDA was considerably faster than Gly-Sar. Unlike the internalization of [Dmt1]DALDA, the efflux of this peptide was greatly inhibited at 4 °C. In addition, the efflux of [Dmt1]DALDA was inhibited when Caco-2 cells were treated with DEPC. The rapid efflux of [Dmt1]DALDA would keep the concentration of peptide low inside the cell. Indeed, when its efflux was inhibited by the addition of DEPC, the amount of [Dmt1]DALDA inside the cell increased 34-fold. Caco-2 cells express a plethora of active efflux pumps that may play a role in the efflux of [Dmt1]DALDA. One possibility is the basolateral peptide transporter found on the basolateral membrane that is responsible for transport of di- and tripeptides from the epithelial cell to the portal blood (Inui et al., 1992). This basolateral peptide transporter is sensitive to temperature and DEPC (Terada et al., 1999), consistent with what we observed for the efflux of [Dmt1]DALDA.

The amount of internalized peptide may also be kept low by efflux transporters present on the apical surface of the Caco-2 cell. P-glycoprotein is expressed on the apical surface of Caco-2 cells and has been shown to play a role in the efflux of morphine and paclitaxel (Crowe, 2002). In our study, incubation with verapamil, a P-glycoprotein inhibitor, had no effect on the uptake of [Dmt1]DALDA, suggesting that P-glycoprotein was not involved in the efflux of [Dmt1]DALDA. In contrast, verapamil increased the uptake of paclitaxel >100-fold in Caco-2 cells (Crowe, 2002).

The cellular uptake of such a highly polar tetrapeptide without energy requirement was surprising and its mechanism remains unclear. This uptake of [Dmt1]DALDA seemed to be universal, because it was also observed with a neuronal (SH-SY5Y), a renal epithelial (CRFK), and a human embryonic kidney cell line (HEK293). Generally, the most crucial step in transcellular transport is the partitioning of the drug
from the extracellular aqueous environment into the lipophilic cellular membrane. Hydrogen bonding to water molecules is the major problem with partitioning of peptides into the lipid membrane. The high number of positive charges at physiological pH would further make passive diffusion through the membrane highly unlikely for [Dmt1]DALDA. In addition to active transporter systems, peptide internalization often involves receptor-mediated endocytosis. [Dmt1]DALDA binds with high affinity to μ-opioid receptors (Schiller et al., 2000; Zhao et al., 2002). However, receptor-mediated endocytosis can be ruled out for [Dmt1]DALDA in Caco-2 cells because of the absence of [3H][Dmt1]DALDA binding and the lack of temperature dependence. In SH-SY5Y cells, receptor-mediated endocytosis may play a small role at 37°C because these cells express μ-opioid receptors (Kazmi and Mishra, 1987). Adsorptive-mediated endocytosis is another mechanism for peptide transport into cells, especially for peptides that carry positive net charge. However, adsorptive endocytosis can also be ruled out because [Dmt1]DALDA was internalized at 4°C.

More recently, a number of hydrophilic peptides have been found to penetrate the cell membrane in an energy-independent manner, including several peptides derived from the human immunodeficiency virus Tat protein and the homeodomain of Antennapedia (for review, see Lindgren et al., 2000). These hydrophilic peptides contain >12 amino acid residues and yet internalized readily into cells, even at 4°C. Internalization of Tat (48–60) was reported at concentrations of 0.1 μM (Vives et al., 1997). CLSM showed clear internalization of [Dmt1, dnsDap4]DALDA after incubation at this concentration. Interestingly, a common feature among these cell-penetrating peptides is the high number of basic amino acids (Arg and Lys). For example, Tat (48–60) contains six arginine and two lysine residues out of a total of 13 amino acid residues. Several arginine-rich peptides, including RNA-binding peptides derived from virus proteins, also penetrate cell membranes readily (Futaki et al., 2001). Little homology in amino acid sequences was found among these peptides, except that they all have 5 to 11 arginine residues. Using arginine polymers, it was found that six to eight arginine residues exhibited maximal internalization (Futaki et al., 2001). Structural studies even suggest the absence of a common secondary structure among these cell-penetrating hydrophilic peptides (Futaki et al., 2001). It has been suggested that hydrogen bond formation of arginine with lipid phosphates or interaction with extracellular matrices such as heparan sulfate may be involved in the translocation process (Suzuki et al., 2002). Although the mechanisms involved in cell penetration by these highly charged peptides remain obscure, much excitement has been generated by the ability of these cell-penetrating peptides to serve as carrier systems for delivery of protein cargoes into living cells (Schwarze and Dowdy, 2000).

There are some differences between [Dmt1]DALDA and these cell-penetrating peptides. [Dmt1]DALDA is a much smaller peptide, consisting of only four amino acid residues, with one arginine and one lysine. Although the cell-penetrating peptides distribute throughout the cytoplasm and nucleus, [Dmt1]DALDA was clearly excluded from the nucleus. The inability of [Dmt1]DALDA to penetrate into the nucleus may be explained by its short amino acid sequence and the lack of nuclear localization signals. In addition, the larger arginine-rich peptides are retained in the cell after internalization (Futaki et al., 2001) whereas [Dmt1]DALDA could readily translocate across the cell. As discussed earlier, this efflux out of the cell seems to be dependent on a transporter, and the amount of [Dmt1]DALDA found in the cell can be increased dramatically by inhibiting the efflux transporter.
The ability of [Dmt\textsuperscript{1}]DALDA to serve as a carrier peptide for protein cargoes has not been evaluated. The rapid efflux of [Dmt\textsuperscript{1}]DALDA out of the cell may be beneficial if upon enzymatic cleavage after cellular uptake of the [Dmt\textsuperscript{1}]DALDA-protein conjugate, [Dmt\textsuperscript{1}]DALDA is rapidly effluxed out of the cell while the protein cargo remains inside.

The ability to translocate across the entire cell is important for peptide distribution across biological membranes such as the intestinal epithelial barrier or the blood-brain barrier. The Caco-2 cell line has long been recognized as a model for predicting oral bioavailability of a drug in vivo. The minimum \( P_{app} \) required to anticipate 100% absorption in humans has been estimated between \( 1 \times 10^{-6} \) and \( 6 \times 10^{-5} \) cm/s (Rubas et al., 1993; Gres et al., 1998). Thus, with a \( P_{app} \) calculated to be \( 1.24 \times 10^{-5} \) cm/s, we may anticipate [Dmt\textsuperscript{1}]DALDA to have good oral absorption. In contrast, other met-enkephalin peptide analogs have shown significantly lower \( P_{app} \) (\( \approx 10^{-8} \) cm/s) (Lang et al., 1997).

The findings of this study help explain the unexpected potency of subcutaneous [Dmt\textsuperscript{1}]DALDA. We recently found that [Dmt\textsuperscript{1},Orn\textsuperscript{4}]DALDA, where lysine was substituted with ornithine, was just as potent after subcutaneous administration (G.-M. Zhao, D. L. Wu, Y. Soong, and H. H. Szeto, unpublished data). This would suggest that it is the charged amino acid, rather than lysine specifically, that lends the ability for these tetrapeptides to penetrate cell membranes. In contrast, a much more lipophilic tetrapeptide (Dmt-D-Ala-Phe-Phe-NH\textsubscript{2}) that has similar affinity for the \( \mu \)-opioid receptor was found to be much less effective after subcutaneous administration (G.-M. Zhao, D. L. Wu, Y. Soong, and H. H. Szeto, unpublished data). In summary, small peptides may have the potential of being highly potent systemic and oral drugs.

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![Fig. 5. Transport of [\textsuperscript{3}H][Dmt\textsuperscript{1}]DALDA and [\textsuperscript{14}C]Gly-Sar across a Caco-2 monolayer. Caco-2 cells (2 \( \times \) 10\textsuperscript{5}) were seeded on microporous membrane inside Transwell cell culture chambers. Apical-to-basolateral transport of peptides was determined by adding [\textsuperscript{3}H][Dmt\textsuperscript{1}]DALDA or [\textsuperscript{14}C]Gly-Sar to the apical compartment, and 20-\mu l aliquots were removed from both apical and basolateral compartments at various times after peptide addition for determination of radioactivity.](image1)

![Fig. 6. Internalization of [Dmt\textsuperscript{1}, dnsDap\textsuperscript{4}]DALDA into Caco-2 cells as visualized by CLSM. Caco-2 cells were incubated with 1 \( \mu \)M [Dmt\textsuperscript{1},dnsDap\textsuperscript{4}]DALDA for 1 h at 37°C. Cells were then washed and covered with PBS. Microscopy was carried out within 10 min at room temperature. Excitation was performed at 340 nm and emission was measured at 520 nm. A, image of dansylated peptide (green). B, differential interface contrast of the same field showing the outline of the cells. C, overlay of A and B. The fluorescence appeared diffuse throughout the cytoplasm (C) but was completely excluded from the nucleus (N). All images are 0.14 \( \times \) 0.14 \( \mu \)m.](image2)
References


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