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# Oligopeptide transporter mediated uptake and transport of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala in filter-grown Caco-2 monolayers

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**Abstract**

The oligopeptide transporter, which contributes to the absorption of di-/tri-peptides and various peptidomimetic compounds across intestinal epithelia, is expressed in mature Caco-2 monolayers. It was shown in our previous report that $\beta$-esterified D-Asp(OBzl)-Ala is efficiently transported across Caco-2 monolayers via the oligopeptide transporter (Taub et al., Int. J. Pharmaceutics 146, 1997b, 205–212). In this paper we demonstrate that two additional peptidase resistant side-chain modified dipeptides, D-Glu(OBzl)-Ala and D-Ser(Bzl)-Ala, are also substrates for the Caco-2 oligopeptide transporter. These three modified dipeptides, chosen due to their sequential difference in number of $CH_2$ groups in the side-chain, demonstrate different affinity ($IC_{50}$) and apparent permeability ($P_{app}$) values, uptake profiles, and pH-mediated release of the benzyl group. Both uptake and transport of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala in Caco-2 monolayers are $>90\%$ inhibitable by the presence of a 20-fold molar excess of Gly-Pro in the apical chamber. The $P_{app}$ of D-Asp(OBzl)-Ala is nearly 2-fold greater than that of D-Glu(OBzl)-Ala and 4-fold greater that that of D-Ser(Bzl)-Ala. The half-life ($t_{1/2}$) for the release of benzyl alcohol (BZ-OH) from each dipeptide is also variable; D-Asp(OBzl)-Ala is labile at pH 6.0 and 7.4 ($t_{1/2} = 26.1$ and 7.8 h, respectively), while D-Glu(OBzl)-Ala is extremely stable at pH 6.0 but unstable at pH 7.4 ($t_{1/2} > 96$ and 2.1 h, respectively). D-Ser(Bzl)-Ala, which has a hydrolysis resistant ether linkage rather than a hydrolysis sensitive ester-linked benzyl group, is highly stable ($t_{1/2} > 96$ h at both pH 6.0 and 7.4). These data indicate that it is possible to construct various side-chain modified, peptidase resistant dipeptides having not only different oligopeptide transporter mediated permeability profiles, but also different release characteristics for the attached side-chain moiety. 

*Keywords:* Caco-2; Dipeptide; Oligopeptide transporter; Transport; Uptake

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## 1. Introduction

Intestinal absorption of digested dietary protein includes the transcellular membrane transport of di- and tri-peptide fragments via a proton-dependent oligopeptide transporter such as PepT1 (Matthews and Adibi, 1976; Ganapathy and Leibach, 1985). Di-/tripeptide absorption via PepT1 proceeds at an extremely high capacity and is a primary source of nitrogen in mammalian systems; additionally, dipeptides follow a far more efficient pathway for intestinal absorption compared with single amino acids (Adibi, 1997). As most dipeptide hydrolysis in the mucosal epithelium occurs intracellularly as opposed to lumenally, the oligopeptide transporter is an integral component in the transfer of dipeptides from a region of low to high hydrolase activity (Adibi, 1997). Various proton-dependent electrogenic transporters exist in other physiologically significant areas, e.g. PepT2, which is expressed in the kidney (Liu et al., 1995), and the recently discovered peptide/histidine transporter, PHT1, which is expressed primarily in the rat brain and retina (Yamashita et al., 1997). PepT1 additionally functions as the transport mechanism responsible for the rapid absorption of various peptidomimetic β-lactam antibiotics across brush border epithelia (Dantzig and Bergin, 1990; Dantzig et al., 1994). Since these oligopeptide transporters are widely distributed physiologically and are inherently broadly specific for a variety of structurally similar molecules, they are considered to have significant potential as targets in the field of drug delivery (Smith et al., 1993; Swaan et al., 1993).

Although Caco-2 cells display two distinct, structurally dissimilar oligopeptide transport proteins, human PepT1 and HPT-1, the primary transporter responsible for peptide or peptidomimetic transport across Caco-2 monolayers is PepT1 (Covitz et al., 1996). Various methods for investigation of the mechanisms involved in oligopeptide transport have been reported, yet the Caco-2 cell line remains one of the most convenient and widely utilized in vitro systems of those currently available (Dantzig and Bergin, 1990; Thwaites et al., 1993; Hidalgo et al., 1995; Delie and Rubas, 1997). There are two distinct membrane-localized peptide carriers in confluent Caco-2 monolayers, one on the apical and another on the basolateral membrane (Saito and Inui, 1993; Thwaites et al., 1993). It is likely that the basolateral arm of the transport pathway represents an exit mechanism following intracellular oligopeptide transport and may indeed be the rate-limiting step (Thwaites et al., 1993; Gochoco et al., 1994). The relationship between a substrate's affinity for the apical oligopeptide transporter and its ability to achieve transepithelial transport has not yet been definitively clarified, although it has been shown that affinity for the apical oligopeptide transporter does not necessarily predicate a substrate's ability to access the transepithelial transport pathway (Tamura et al., 1996).

We recently reported that, upon side-chain modification of one amino acid in a peptidase resistant D/L-configured dipeptide, it is possible for the modified dipeptide to retain its affinity for PepT1 in Caco-2 monolayers (Taub et al., 1997a). Specifically, benzyl-esterified D-Asp-Ala (D-Asp(OBzl)-Ala) achieves PepT1-mediated uptake and apical-to-basal transport in cultured Caco-2 monolayers (Taub et al., 1997b). In this report, we demonstrate that two additional benzyl-modified dipeptides retain their affinity for PepT1, are taken up and transported intact across Caco-2 cells via PepT1, and display markedly different permeability and stability characteristics.

## 2. Materials and methods

### 2.1. Materials

[$^{14}$C]Glycylsarcosine ([$^{14}$C]Gly-Sar, 60 mCi/mmol) was purchased from Amersham International (Buckinghamshire, UK). 2-(*N*-morpholino) ethanesulfonic acid (Mes), *N*-2-hydroxyethylpiprazine-*N*′-2-ethanesulfonic acid (Hepes), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Boc-D-Glu(OBzl)-OH, Boc-D-Ser(Bzl)-OH, H-Ala-OtBu HCl, H-Glu-Ala-OH, and H-Ser-Ala-OH were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). All solvents used for dipeptide synthesis were obtained from Riedel-de Haën (Seelze,

Germany), all analytical grade solvents used for HPLC analysis were obtained from Gerner and Jensen (Copenhagen, Denmark), and Ultima Gold scintillation fluid was purchased from Packard (Groningen, The Netherlands).

### 2.2. Cell culture

Cell culture was maintained according to a previously published method (Taub et al., 1997a,b). Caco-2 epithelial cells originally obtained from the ATCC (Rockville, MD) were used between passages 21 and 34. Transwell monolayers reached a transepithelial electrical resistance (TEER) of between 600 and 800 Ohms/cm$^2$, and the total amount of protein on each confluent Transwell filter was calculated to be 0.42 mg/cm$^2$ using the Lowry method. The addition of either modified or unmodified dipeptides did not impose any significant influence upon the TEER of the monolayers vs. controls over the course of the binding, uptake, or transport experiments.

### 2.3. Dipeptide synthesis

D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala, D-Asp-Ala, D-Glu-Ala and D-Ser-Ala were synthesized via fully protected intermediates essentially as described previously (Taub et al., 1997a,b) with the modifications given below. Reaction progress and product purity were assessed by analytical $C^{18}$ reversed phase HPLC. Identification of the purified dipeptides was obtained by $^1$H NMR.

#### 2.3.1. D-Asp(OBzl)-Ala and D-Asp-Ala

Boc-D-Asp(OBzl)-Ala-OtBu was synthesized in a mixed solvent of DMF (10 ml) and ethyl acetate (100 ml) by coupling Boc-D-Asp(OBzl)-OH (25 mmol) to Ala-OtBu (25 mmol) with HOBt (25 mmol), TBTU (25 mmol), and DIPEA (75 mmol). After coupling for 2 h, extra ethyl acetate (75 ml) was added and the fully protected Boc-D-Asp(OBzl)-Ala-OtBu isolated as a clear oil (HPLC purity $>98\%$; yield 24 mmol, 96%) by extraction and drying of the ethyl acetate phase (Taub et al., 1997a,b). A portion of the Boc-D-Asp(OBzl)-Ala-OtBu (5 mmol) was dissolved in DCM (20 ml) and selectively deprotected with TFA (20 ml) at room temperature for 2 h. The solvents were removed in vacuo by rotary evaporation leaving an oil which, after extraction with diethyl ether ($3 \times 20$ ml) and drying, gave a white powder of D-Asp(OBzl)-Ala TFA (yield 4.2 mmol, 84%; HPLC purity $>98\%$). The dipeptide D-Asp-Ala TFA was prepared via Boc-D-Asp(OtBu)-Ala-OtBu using a single step deprotection with TFA in DCM as described previously (Taub et al., 1997a).

#### 2.3.2. D-Glu(OBzl)-Ala and D-Glu-Ala

Both dipeptides were obtained from the fully protected Boc-D-Glu(OBzl)-Ala-OtBu intermediate (HPLC purity $>98\%$; yield 23.6 mmol, 94%) which was synthesized as described above for Boc-D-Asp(OBzl)-Ala-OtBu. TFA treatment of a portion of the Boc-D-Glu(OBzl)-Ala-OtBu (5 mmol) in DCM and extraction and drying gave a white powder of D-Glu(OBzl)-Ala TFA (yield 4.8 mmol, 96%; HPLC purity $>98\%$). Another portion of Boc-D-Glu(OBzl)-Ala-OtBu (5 mmol) was selectively deprotected in sequence, firstly by catalytic hydrogenation (2–3 ATM for 14 h in 20 ml of aqueous 75% ethanol with 200 mg of 10% Pd-on-charcoal), then by acidolysis with TFA. After the hydrogenation step, the catalyst was removed by filtration, the ethanol by rotary evaporation, and residual water by azeotroping with absolute ethanol followed by freeze drying. The partially deprotected product (Boc-D-Glu-Ala-OtBu; yield 5 mmol, 100%; HPLC purity $>98\%$) was deprotected further by TFA in DCM, extracted and dried as described above for the other dipeptides, giving a white powder of D-Glu-Ala TFA (yield 4.5 mmol, 90%).

#### 2.3.3. D-Ser(Bzl)-Ala and D-Ser-Ala

Both dipeptides were obtained by the selective deprotection of fully protected Boc-D-Ser(Bzl)-Ala-OtBu (HPLC purity $>98\%$; yield 13.5 mmol, 90%). The latter was synthesized in a 15 mmol scale by procedures as described above, but using D-Ser in place of D-Glu. D-Ser(Bzl)-Ala TFA (yield 4.65 mmol, 93%; HPLC purity $>98\%$) was obtained from 5 mmol of the Boc-D-Ser(Bzl)-Ala-

OtBu by deprotection with TFA in DCM and extraction and drying as above. D-Ser-Ala TFA (yield 4.2 mmol, 84%) was obtained from another 5 mmol of the Boc-D-Ser(Bzl)-Ala-OtBu following catalytic hydrogenation in aqueous 80% acetic acid instead of 75% ethanol and TFA treatment as described above for D-Glu-Ala.

### 2.4. $IC_{50}$ determination experiments

[$^{14}$C]Gly-Sar displacement experiments were performed as follows: Caco-2 monolayers were first rinsed and then incubated with HBSS (apical media = 0.05% BSA, 10 mM Mes, pH 6.0; basal media = 0.05% BSA, 10 mM Hepes, pH 7.4) for 15 min at 37°C under a 5% $CO_2$ atmosphere. Next, [$^{14}$C]Gly-Sar (0.5 $\mu$Ci), and in certain wells, dipeptides of various concentrations were added concomitantly to the apical media of the Caco-2 Transwells. Following a 15 min incubation period, buffer was removed from both the apical and basolateral chambers and the cells were washed four times with ice-cold HBSS, pH 7. Finally, the polycarbonate membrane was cut and placed into a liquid scintillation vial and the [$^{14}$C]Gly-Sar radioactivity was assessed.

### 2.5. Uptake and transport of the benzyl-modified dipeptides in Caco-2 monolayers

Uptake of the benzyl-modified dipeptides via the apical oligopeptide transporter was performed as has been described previously (Taub et al., 1997b). Briefly, uptake was measured at 15 and 120 min, and the apical-to-basal transport of each compound was measured hourly over the course of a 5 h experiment. For all experiments, confluent Caco-2 monolayers were pretreated as described in Section 2.4. Either D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, or D-Ser(Bzl)-Ala (1 mM) was added to the apical chamber of confluent monolayers, and in certain wells, Gly-Pro (20 mM) was added concomitantly as a competitive inhibitor. TEER and the pH gradient were monitored hourly during the 5 h transport studies; both were maintained throughout the course of the experiment. Due to the variability of Caco-2 monolayers over the course of several passages (Delie and Rubas, 1997), the transport of each benzyl-modified dipeptide was conducted in parallel with [$^{14}$C]Gly-Sar (1 mM with the addition of unlabeled Gly-Sar) as an internal control. At the end of each experiment, both apical and basolateral media were removed, cells were washed four times with ice-cold HBSS, 0.25 ml of 0.1% Triton X-100 was added, and then monolayers were incubated at 37°C for 15 min. Cells were then scraped from the Transwell membrane and frozen for future HPLC analysis.

All samples were prepared for HPLC analysis by adding 0.25 ml of acetonitrile, vortexing vigorously to precipitate the protein, and centrifuging for 15 min at 13000 rpm. Membrane samples were first sonicated using a Branson B15 Cell Disruptor for 30 s at 50% power. The mobile phases used were as follows: D-Asp(OBzl)-Ala and D-Ser(Bzl)-Ala: 70% 0.02 M $NaH_2PO_4$, 30% methanol, 0.001 M triethylamine (TEA), pH 6.3; D-Glu(OBzl)-Ala: 80% 0.02 M $NaH_2PO_4$, 15% acetonitrile, 5% methanol, 0.01 M TEA, pH 4.0. The flow rate was 1.0 ml/min, and the column effluent was monitored at 210 nm. Peak elution times for D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala were 10.0, 15.6, and 8.7 min, respectively. BZ-OH elution times were either 12.1 or 13.1 min, depending on the mobile phase employed.

The apparent permeability coefficients were calculated using the following equation: $P_{app} = dQ/dt \cdot A \cdot C_0$, where $(dQ/dt)$ represents the steady-state flux of the dipeptide from the apical to the basolateral chamber following initial lag time; $C_0$ represents the initial concentration of the dipeptide in the apical chamber (1 mM); and $A$ represents the area of the Transwell membrane used for these experiments (4.71 cm$^2$).

### 2.6. Assessment of the stability of D-Glu(OBzl)-Ala and D-Ser(Bzl)-Ala

Analysis of the stability of the modified dipeptides was performed as described previously for D-Asp(OBzl)-Ala (Taub et al., 1997b). Either D-Glu(OBzl)-Ala or D-Ser(Bzl)-Ala (1 mM) was added to pH 6.0 or pH 7.4 BSA-supplemented HBSS (as listed in Section 2.4.), incubated at

37°C, and samples were taken periodically over the course of 96 h. Following sampling, HPLC analysis was done as described in Section 2.5.

## 3. Results

### 3.1. Dipeptide affinities for the apically expressed Caco-2 oligopeptide transporter

The oligopeptide transporter affinities for all dipeptides were determined by measuring their degree of [$^{14}$C]Gly-Sar uptake inhibition ($IC_{50}$) in Caco-2 monolayers when present at various concentrations in the apical media. The $IC_{50}$ values for L/L, D/L, and benzyl-modified Asp-Ala, Glu-Ala, and Ser-Ala were calculated and are shown in Table 1. All modified or unnatural dipeptides demonstrated an increased $IC_{50}$ value relative to their corresponding L/L-dipeptides, and the increase in $IC_{50}$ between each D- or benzyl-modified analog and its corresponding L/L parent dipeptide was also calculated (Table 1).

### 3.2. Benzyl-modified dipeptides exhibit oligopeptide transporter mediated uptake into Caco-2 monolayers

Uptake studies were performed at both 15 and 120 min in order to assess both initial uptake and steady-state intracellular accumulation (Hu et al., 1996). Since the L/L- or D/L-configured dipeptides listed in Table 1 possessed a molar extinction coefficient unacceptable for HPLC analysis via UV detection, the benzyl-modified dipeptides were the only compounds which could be evaluated. As shown in Fig. 1A, D-Asp(OBzl)-Ala demonstrated the most uptake into Caco-2 monolayers in the 15 min study, followed by D-Ser(Bzl)-Ala and D-Glu(OBzl)-Ala. In the 120 min study, D-Ser(Bzl)-Ala demonstrated the most uptake into Caco-2 monolayers, followed by D-Asp(OBzl)-Ala and D-Glu(OBzl)-Ala (Fig. 1B). For each compound in both studies, uptake was $>90\%$ inhibitable by a concomitant 20-fold molar excess of Gly-Pro present in the apical media. For D-Asp(OBzl)-Ala and D-Glu(OBzl)-Ala, the amount of initial uptake (15 min) was approx. 70% of that determined for steady-state intracellular accumulation (120 min); yet, for D-Ser(Bzl)-Ala this value was only 50% (Fig. 1). In addition to the benzyl-modified dipeptides, cells were monitored via HPLC analysis for the presence of the BZ-OH hydrolysis product (Table 4).

Table 1
$IC_{50}$ values for the inhibition of [$^{14}$C]Gly-Sar uptake into Caco-2 monolayers by various dipeptides and their modified analogs

| Dipeptide | MW[a] | $IC_{50}$ (mM)[b] | Ratio[c] |
|---|---|---|---|
| Asp-Ala[d] | 204 | $0.33 \pm 0.001$ | — |
| D-Asp-Ala[d] | 204 | $5.75 \pm 0.09$ | 17.4 |
| D-Asp(OBzl)-Ala[d] | 294 | $2.62 \pm 0.35$ | 7.9 |
| Glu-Ala | 218 | $0.44 \pm 0.08$ | — |
| D-Glu-Ala | 218 | $2.58 \pm 0.24$ | 5.9 |
| D-Glu(OBzl)-Ala | 308 | $5.47 \pm 0.13$ | 12.4 |
| Ser-Ala | 176 | $0.21 \pm 0.05$ | — |
| D-Ser-Ala | 176 | $3.78 \pm 0.85$ | 17.9 |
| D-Ser(Bzl)-Ala | 266 | $2.46 \pm 0.33$ | 11.6 |

[a] Not including TFA, if present following synthesis and purification.
[b] Values represent the mean $\pm$ S.D. for $N = 3$ separate filters.
[c] Ratios represent D- or benzyl-modified analog/corresponding L/L-configured dipeptide.
[d] Data taken from Taub et al., 1997a.

### 3.3. Benzyl-modified dipeptides exhibit oligopeptide transporter mediated transport across Caco-2 monolayers

D-Glu(OBzl)-Ala and D-Ser(Bzl)-Ala were transported efficiently in the apical-to-basal direction across Caco-2 monolayers during the course of a 5 h experiment (Fig. 2). D-Asp(OBzl)-Ala transport has been described previously (Taub et al., 1997b). In each transport study, a 20-fold molar excess of Gly-Pro was able to inhibit benzyl-modified dipeptide transport by $>95\%$. The $P_{app}$ values for D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, D-Ser(Bzl)-Ala, and Gly-Sar were calculated and are shown in Table 2. The ratios of benzyl-modified dipeptide $P_{app}$ compared with Gly-Sar $P_{app}$ show that D-Asp(OBzl)-Ala has nearly twice the permeability of D-Ser(Bzl)-Ala, which in turn has about twice the permeability of D-Glu(OBzl)-Ala (Table 2). The hydrolysis product, BZ-OH, is rapidly transported across Caco-2 monolayers via

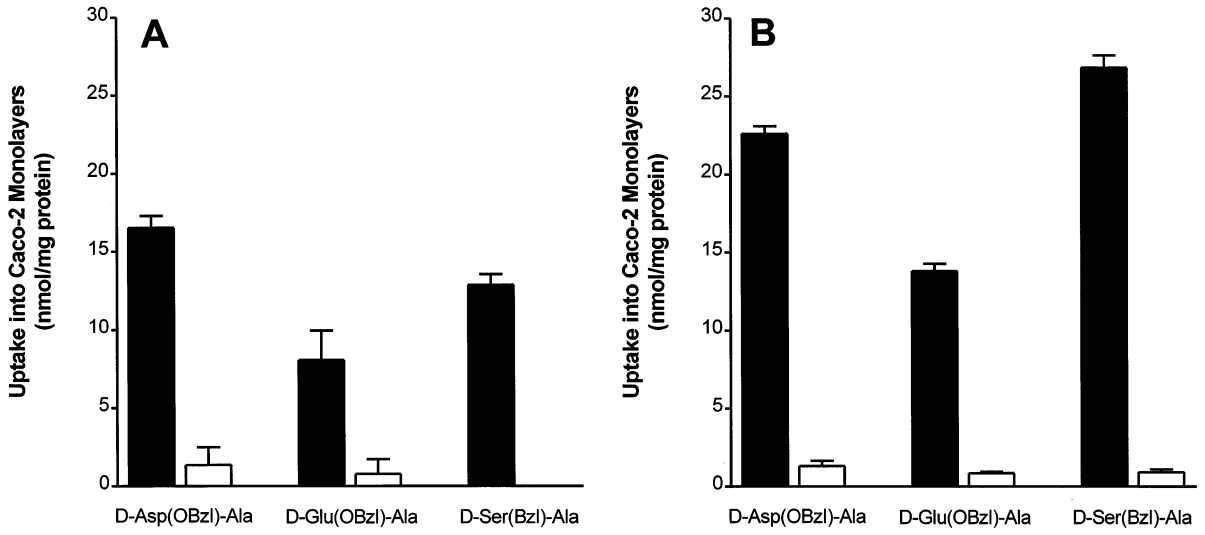


Fig. 1. Apical uptake of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala (1 mM) in the presence or absence of Gly-Pro (20 mM). Each benzyl-modified dipeptide was added to the apical chamber of Caco-2 monolayers either alone (solid bars) or concomitantly with Gly-Pro (open bars). The amount of benzyl-modified dipeptide uptake per mg protein was determined via HPLC following incubation times of either (A) 15 min or (B) 120 min at 37°C. The data represent the means for three separate filters and the S.D. is either indicated by a bar or is smaller than the size of the symbol.

a passive mechanism (Taub et al., 1997b); thus, apical-to-basal BZ-OH transport was also monitored during these experiments (Table 4).

### *3.4. Stability studies of the benzyl-modified dipeptides at pH 6.0 and 7.4*

The stability of the benzyl-modified dipeptides under experimental conditions was monitored during a 96 h experiment via HPLC analysis. As shown in Table 3, the stability of each parent compound was compared with the generation of the BZ-OH hydrolysis product. No other related hydrolysis products were detected. Since the benzyl modification of D-Ser-Ala resulted in a highly stable ether linkage, D-Ser(Bzl)-Ala remained intact and no benzyl hydrolysis product was detected over the course of the stability experiments.

## 4. Discussion

Since many structurally variable compounds are suitable substrates for the intestinal oligopeptide transporter (Fei et al., 1994; Eddy et al., 1995), targeting peptide drugs, peptidomimetic compounds, or peptide-prodrug systems is considered to be an attractive drug delivery or rational drug design strategy (Smith et al., 1993; Swaan et al., 1993). Structural requirements for substrate interaction with known proton-dependent oligopeptide transporters are similar; however, certain exceptions have been described. For example, recent studies using renal brush border membrane vesicles (BBMV) suggest that an $\alpha$-amino group is essential for oligopeptide transporter interaction (Li and Hidalgo, 1996), while earlier studies using perfused rat intestinal segment suggested that it is not (Bai et al., 1991). Stereospecificity is also a major consideration, e.g. the D-enantiomer of cephalexin is taken up by intact BBMV's while the L-enantiomer, although highly potent as an inhibitor of oligopeptide transporter mediated substrate uptake, is not transported due to its susceptibility to enzymatic degradation (Tamai et al., 1987; Dantzig and Bergin, 1990). Substrate inhibition studies indicate that only one amino acid must be in the L-configuration in order to retain binding affinity for the oligopeptide transporter (Thwaites et al., 1994; Hidalgo et

al., 1995). The inclusion of one unnatural, e.g. D-configured amino acid can render the dipeptide resistant to peptidase activity while maintaining PepT1 interaction (Daniel et al., 1992; Thwaites et al., 1993; Hidalgo et al., 1995).

While it is assumed that there is a size restriction for peptide transport, no systematic studies have investigated the limits of dipeptide modification in relation to oligopeptide transporter affinity conservation (Smith et al., 1993). A model detailing the structural requirements essential for substrate recognition by the intestinal oligopeptide transporter has been proposed (Walter et al., 1996), yet it still remains unclear which substituent groups are absolutely required to ensure affinity. In this study, we have chosen D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala as probes in order to obtain basic information regarding the influence of side-chain length and structure upon oligopeptide transporter mediated uptake and transport in Caco-2 monolayers.

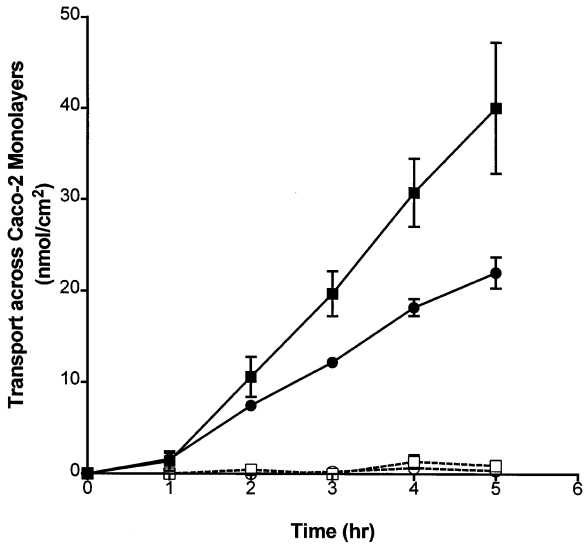


Fig. 2. Apical-to-basal transport of D-Glu(OBzl)-Ala and D-Ser(Bzl)-Ala (1 mM) in the presence or absence of Gly-Pro (20 mM). Benzyl-modified dipeptides were added to the apical chamber of Caco-2 monolayers either alone or concomitantly with Gly-Pro, and the amount appearing in the basal chamber was determined via HPLC at various time points during a 5 h incubation at 37°C. D-Glu(OBzl)-Ala transport in the presence (○) or absence (●) of Gly-Pro and D-Ser(Bzl)-Ala transport in the presence (□) or absence (■) of Gly-Pro are depicted. The data represent the means for three separate filters and the S.D. is either indicated by a bar or is smaller than the size of the symbol.

Table 2
Permeability coefficients of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, D-Ser(Bzl)-Ala, and Gly-Sar across Caco-2 monolayers

| | $P_{app} \times 10^6$ (cm/s)[a] | Gly-Sar control[b] | Ratio[c] |
|---|---|---|---|
| D-Asp(OBzl)-Ala | $4.06 \pm 0.47$ | $4.36 \pm 0.22$ | 0.93 |
| D-Glu(OBzl)-Ala | $1.31 \pm 0.09$ | $5.29 \pm 0.23$ | 0.25 |
| D-Ser(Bzl)-Ala | $2.36 \pm 0.34$ | $4.36 \pm 0.22$ | 0.54 |

[a] Means for $N = 3$ experiments $\pm$ S.D. All compounds 1 mM in the apical compartment.
[b] Gly-Sar $P_{app}$ calculated for each experiment as an internal control.
[c] Dipeptide $P_{app}$/Gly-Sar control $P_{app}$.

The $IC_{50}$ values for L/L-configured Asp-Ala, Glu-Ala, Ser-Ala, and their D/L and benzyl-modified counterparts were determined (Table 1). It is clear that, although affinity constants do not necessarily predicate PepT1-mediated cellular uptake and transepithelial transport (Tamura et al., 1996; Taub et al., 1997b), unnatural or side-chain modified dipeptides generally demonstrate a reduction in oligopeptide transporter affinity compared with their corresponding natural or unmodified analog. It is not clear why the relative $IC_{50}$ values for the D/L- or benzyl-modified dipeptides vary so greatly, but it has been suggested from stoichiometric and pH dependent uptake

Table 3
Hydrolysis of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, and D-Ser(Bzl)-Ala to the corresponding parent dipeptide and BZ-OH

| Compound | pH | $t_{1/2}$ (h)[a] |
|---|---|---|
| D-Asp(OBzl)-Ala[b] | 6.0 | 26.1 |
| | 7.4 | 7.8 |
| D-Glu(OBzl)-Ala | 6.0 | >96 |
| | 7.4 | 2.1 |
| D-Ser(Bzl)-Ala | 6.0 | >96 |
| | 7.4 | >96 |

[a] Values represent the means for $N = 3$ experiments.
[b] Data taken from Taub et al. (1997b).

Table 4
Intracellular or transported BZ-OH resulting from the apical addition of D-Asp(OBzl)-Ala, D-Glu(OBzl)-Ala, or D-Ser(Bzl)-Ala[a]

| Dipeptide (1 mM) | ±Gly-Pro (20 mM) | 2 h Uptake (nmol/mg protein) | 5 h Uptake (nmol/mg protein) | 5 h Transport (nmol/cm$^2$) |
|---|---|---|---|---|
| D-Asp(OBzl)-Ala | − | 4.27 ± 0.30 | 12.76 ± 0.98 | 35.18 ± 0.90 |
| | + | 0.14 ± 0.01 | 1.42 ± 0.11 | 14.41 ± 1.48 |
| D-Glu(OBzl)-Ala | − | 0.06 ± 0.01 | 2.10 ± 1.40 | 12.58 ± 0.25 |
| | + | <0.02b | <0.02 | 4.84 ± 0.90 |
| D-Ser(Bzl)-Ala | − | <0.02 | <0.02 | <0.05 |
| | + | <0.02 | <0.02 | <0.05 |

[a] Values represent the means ± S.D. for $N = 3$ monolayers. The 5 h uptake values represent the remaining intracellular BZ-OH following a 5 h transport study.
[b] BZ-OH was detectable via HPLC at ≥0.02 nmol/mg protein or ≥0.50 nmol/cm$^2$.

studies in PepT1-transfected *Xenopus laevis* oocytes that PepT1 prefers neutral and acidic peptides over basic peptides and their proton-substrate coupling ratios differ with respect to overall charge (Steel et al., 1997). When acidic D-Asp-Ala is modified to neutral D-Asp(OBzl)-Ala, its $IC_{50}$ compared with the native L/L-dipeptide decreases 2-fold; yet, when acidic D-Glu-Ala is modified to D-Glu(OBzl)-Ala, its relative $IC_{50}$ increases 2-fold (Table 1). Thus, perhaps this relationship between dipeptide charge and PepT1 affinity does not apply to unnatural or side-chain modified dipeptides.

In uptake and transport experiments a 20-fold molar excess of Gly-Pro in the donor compartment inhibited uptake and transport by 90–95%, indicating that all three side-chain modified dipeptides are taken up into Caco-2 cells actively via PepT1 (Figs. 1 and 2). It is likely that the unique uptake profile demonstrated by D-Ser(Bzl)-Ala is related to its ether-linked benzyl group as opposed to the ester linkage in D-Asp(OBzl)-Ala and D-Glu(OBzl)-Ala. This suggests that PepT1 mediated uptake of side-chain modified dipeptides can be significantly influenced by the type of linkage employed between the dipeptide and the attached molecule; such a relationship could be a crucial consideration in future drug–dipeptide conjugate studies.

The $P_{app}$ values calculated for each benzyl-modified dipeptide were normalized with respect to the $P_{app}$ of Gly-Sar, which was used as an internal control in parallel experiments (Table 2). Since Caco-2 monolayers vary considerably between passage number (Delie and Rubas, 1997), including receptor or transporter density upon differentiation, employment of a dependable transport marker as an internal control allows one to properly compare inter-experimental data between cells of different passage number. As D-Asp(OBzl)-Ala and D-Glu(OBzl)-Ala possess identical charge yet differ by only one $CH_2$ group in the side-chain, it is possible that a sufficient difference is present in either size or conformation to render D-Glu(OBzl)-Ala 4-fold less permeable in Caco-2 monolayers (Table 2). D-Ser(Bzl)-Ala, possessing the smallest side-chain of the three compounds, has a $P_{app}$ twice that of D-Glu(OBzl)-Ala and half that of D-Asp(OBzl)-Ala (Table 2). These data suggest that both side-chain size and structure are influential with regard to a modified dipeptide's interaction with PepT1 in Caco-2 monolayers.

The release of BZ-OH from either D-Asp(OBzl)-Ala or D-Glu(OBzl)-Ala was different in both uptake and transport studies (Table 4). Since BZ-OH is released from D-Glu(OBzl)-Ala at pH 6.0 with a $t_{1/2}$ which is >4-fold that of D-Asp(OBzl)-Ala, it is reasonable that there is a 6-fold greater degree of BZ-OH uptake for D-Asp(OBzl)-Ala compared with D-Glu(OBzl)-Ala following both the 2 h uptake and 5 h transport studies. Additionally, the 3-fold greater degree of apical-to-basal BZ-OH transport following D-

Asp(OBzl)-Ala compared with D-Glu(OBzl)-Ala administration directly reflects the 3-fold difference in $P_{app}$ between these modified dipeptides (Table 2). As BZ-OH is rapidly transported via a passive mechanism across Caco-2 monolayers, the amount of BZ-OH released at pH 6.0 is an important consideration when determining the stoichiometry of BZ-OH compared with benzyl-modified dipeptide uptake and transport (Taub et al., 1997b).

There are several drug targeting possibilities which can be envisioned using dipeptide-drug carrier systems. For example, it has been shown recently that in the human fibrosarcoma cell line HT1080 there exists a proton-dependent oligopeptide transporter that is morphologically similar but not identical to PepT1 or PepT2 (Nakanishi et al., 1997). This transporter has been shown to exist in fibroblast-derived tumor cells but not in normal fibroblasts; thus, this tumor-specific transporter could provide a novel strategic target for the delivery of either peptidomimetic anticancer drugs or conventional anticancer compounds reversibly linked to dipeptide carriers (Nakanishi et al., 1997). The use of stable dipeptides as drug carriers is attractive for two basic reasons: (a) the oligopeptide transporter, possessing a broad affinity for a variety of structurally dissimilar dipeptides, may be more apt to provide an uptake and transport vehicle for modified substrates than other known types of transporters or receptors; and (b) as these pro-moieties would simply be amino acids, there is a low potential for toxicity following release of the drug. Future studies will be directed at further examination of the size and structural restrictions limiting interaction of side-chain modified dipeptides with PepT1, substituting an active drug for BZ-OH, and optimization of the linkage between drug and dipeptide carrier.

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