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Influence of oligopeptide transporter binding affinity upon uptake and transport of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar in filter-grown Caco-2 monolayers

Mitchell E. Taub [a], Bernard A. Moss [b], Bente Steffansen [a], Sven Frokjaer [a,*]

[a] *Department of Pharmaceutics, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen ∅, Denmark*
[b] *Peptide Technologies, Herredsvejen 2, DK-3400 Hillerød, Denmark*

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Abstract

The oligopeptide transporter, which is responsible for the absorption of various di/tripeptides and several peptidomimetic drugs across the intestinal epithelia, is expressed in mature Caco-2 monolayers. Using certain enzymatically stable dipeptides containing either L- or D-aspartic acid at the amino terminus, we investigated the relationship between a side-chain modified dipeptide's degree of binding affinity for the apically expressed Caco-2 oligopeptide transporter and its ability to undergo uptake and/or apical-to-basal transport. Two β-esterified dipeptides, D-Asp(OBzl)-Ala and Asp(OBzl)-Sar, possess markedly different affinities for the Caco-2 oligopeptide transporter ($IC_{50} = 2.62 \pm 0.35$ and 0.014 ± 0.007 mM, respectively) as determined using a [^{14}C]Gly-Sar cellular uptake displacement assay. D-Asp(OBzl)-Ala undergoes rapid internalization into Caco-2 monolayers (14.33 ± 1.00 nmol/mg protein) during a 15-min uptake study; additionally, D-Asp(OBzl)-Ala is efficiently transported in the apical-to-basal direction across Caco-2 monolayers (14.41 ± 0.91 nmol/h/cm^2). Both uptake and transport of D-Asp(OBzl)-Ala are $> 90\%$ inhibitable by the presence of a 20-fold molar excess of Gly-Pro in the apical chamber. Although Asp(OBzl)-Sar demonstrates a 187-fold lower IC_{50} value than D-Asp(OBzl)-Ala, Asp(OBzl)-Sar does not achieve uptake or transport in parallel experiments. These data indicate that a side-chain modified, enzymatically stable dipeptide, D-Asp(OBzl)-Ala, is actively taken up into and transported across Caco-2 monolayers via the oligopeptide transporter. Additionally, the degree of affinity of a side-chain modified dipeptide for the Caco-2 oligopeptide transporter is not necessarily indicative of its ability to access the oligopeptide transporter-mediated uptake and transport pathway. © 1997 Elsevier Science B.V.

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

* Corresponding author. Tel.: +45 35 376777 ext. 270; fax: +45 35 371277; e-mail: sf@charon.dfh.dk

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1. Introduction

The intestinal oligopeptide transporter(s) is an integral component of the mammalian system for intestinal absorption of both di- and tripeptide fragments derived from the breakdown of dietary proteins; additionally, it is the mechanism by which various peptidomimetic drugs are orally absorbed (Matthews and Adibi, 1976; Ganapathy and Leibach, 1985; Dantzig and Bergin, 1990). In Caco-2 cells, two distinct oligopeptide transporter proteins of human origin are expressed: hPEPT1, which is located throughout the human gastrointestinal tract, pancreas, kidney, and liver; and HPT-1, which is also widely distributed but absent in the kidney and liver (Dantzig et al., 1994; Liang et al., 1995). In various mammalian tissues such as the gastrointestinal tract and kidney, there are most likely multiple mechanisms responsible for peptide transport (Walter et al., 1996); yet, a recent report suggests that the main transporter responsible for peptide or peptidomimetic transport in Caco-2 cells is hPEPT1 as opposed to HPT-1 (Covitz et al., 1996). Although it is not yet clear exactly how many different peptide transporters exist in various types of mammalian intestinal epithelia (Walter et al., 1996), cultured Caco-2 cells remain a convenient system for conducting in vitro uptake and transport studies in efforts to investigate the basic mechanisms of peptide transport (Dantzig and Bergin, 1990; Saito and Inui, 1993; Thwaites et al., 1994; Hidalgo et al., 1995).

It has been reported that 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 is the rate-limiting step for transport of a di-/tripeptide or peptidomimetic (Thwaites et al., 1993; Gochoco et al., 1994). Although 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, 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).

In addition to studying the possibility of transport of peptidomimetic drugs, it has been proposed that substrates for the oligopeptide transporter may be able to function as pro-moieties, thus enhancing the bioavailability of poorly absorbed small drug molecules once stably, yet bioreversibly conjugated to a di- or tripeptide (Smith et al., 1993; Swaan et al., 1993). In our most recent report, it was demonstrated that β-esterified analogs of D-Asp-Ala were able to retain their affinities for the oligopeptide transporter(s) of filter-grown Caco-2 cells (Taub et al., 1997). In this study, we show that although Asp(OBzl)-Sar has an IC_{50} value almost 200-fold less than that of D-Asp(OBzl)-Ala, Asp(OBzl)-Sar does not achieve any detectable uptake into or transport across Caco-2 monolayers, while D-Asp(OBzl)-Ala achieves a considerable degree of oligopeptide transporter-mediated uptake and apical-to-basal transport.

2. Materials and methods

2.1. Materials

[^{14}C]Glycylsarcosine ([^{14}C]Gly-Sar, 60 mCi/mmol) and [^{14}C]mannitol (56 mCi/mmol) were 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-Asp(OtBu)-OH DCHA, H-Sar-OtBu, Boc-Asp(OBzl)-OH, H-Asp-Gly-OH, H-β-Asp-Gly-OH, H-Asp-Ala-OH, and H-β-Asp-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 & Jensen (Copenhagen, Denmark), and Ultima Gold scintillation fluid was purchased from Packard (Groningen, The Netherlands).

2.2. Cell culture

Caco-2 epithelial cells were obtained from the ATCC (Rockville, MD), and were used between passages 21 and 40. Cell culture was maintained as has been described previously (Taub et al., 1997). Confluent cell monolayers were obtained 17–21 days post-inoculation, with each well demonstrating a transepithelial electrical resistance (TEER) of between 600–800 $\Omega \cdot cm^2$. Cell monolayers could be maintained at a constant TEER for at least 7 days; and the paracellular leakage between apical and basal compartments, as determined by [^{14}C]mannitol flux, was $< 0.36\%/cm^2/h$. The total amount of protein on each confluent Transwell filter was calculated to be 0.42 mg/cm^2 using the Lowry method.

2.3. Dipeptide synthesis

Synthesis of D-Asp-Ala and D-Asp(OBzl)-Ala were performed as has been described previously (Taub et al., 1997). Synthesis of Asp-Sar and Asp(OBzl)-Sar were performed as follows:

2.3.1. H-Asp-Sar-OH

The dipeptide was synthesized from Boc-Asp(OtBu)-OH DCHA salt via the Boc-Asp(OtBu)-Sar-OtBu intermediate and isolated as its TFA salt. Before starting synthesis, the Boc-Asp(OtBu)-OH DCHA salt (5.5 mmol) was converted to the free acid by a standard procedure (Bodansky and Bodansky, 1994). The ethyl acetate phase was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The resulting oily product (yield: 5 mmol; 91%) was used for coupling to H-Sar-OtBu using a previously described procedure for Boc-D-Asp(OtBu)-Ala-OtBu (Taub et al., 1997). HPLC analysis showed that the reaction was complete within 2 h, and after ethyl acetate extraction and drying the resulting crystalline Boc-Asp(OtBu)-Sar-OtBu product was $> 98\%$ pure (yield: 4.45 mmol; 89%). The Boc-Asp(OtBu)-Sar-OtBu (867 mg; 2 mmol) was dissolved in DCM (10 ml) and deprotected by reaction with TFA (10 ml) for 2 h. The resultant H-Asp-Sar-OH TFA product was isolated in crystalline form (yield: 1.65 mmol; 82.5%; purity $>$ 98%) using a previously described method for H-D-Asp-Ala-OH TFA (Taub et al., 1997).

2.3.2. H-Asp(OBzl)-Sar-OH

The dipeptide, which was isolated in either its TFA or HCl salt form, was synthesized via its Boc-Asp(OBzl)-Sar-OtBu intermediate from Boc-Asp(OBzl)-OH free acid (5 mmol) using the above described procedure. The Boc-Asp(OBzl)-Sar-OtBu was obtained in $> 98\%$ yield and $> 98\%$ purity. TFA treatment of the Boc-Asp(OBzl)-Sar-OtBu (2 mmol) to remove the Boc and OtBu protection gave a product that was not crystalline, was low in yield (1.29 mmol; $< 65\%$), was very unstable and poorly soluble in water. In contrast, deprotection with dry 3 M HCl in ethyl acetate (20 ml for 2 mmol peptide) gave a crystalline product after trituration with ether and drying (yield: 1.89 mmol; $> 95\%$; purity $> 97\%$), which was very soluble in water and considerably more stable.

2.4. IC_{50} determination experiments

[^{14}C]Gly-Sar displacement experiments were performed as has been described previously (Taub et al., 1997). Briefly, 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 in order to equilibrate the cells to the change in pH gradient. Next, [^{14}C]Gly-Sar (0.5 μCi), 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 basal chambers and the cells were washed four times with ice-cold HBSS, pH 7. Following this washing step, the entire polycarbonate membrane was cut from the Transwell support and placed into a scintillation vial, scintillation fluid was added, and the cell-associated radioactivity was counted via liquid scintillation spectrometry.

2.5. Uptake and transport of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar in Caco-2 monolayers

Uptake of the compounds via the apical oligopeptide transporter was measured at 15 and 120 min, and the apical-to-basal transport of each compound was measured at various time points 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 or Asp(OBzl)-Sar (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. For uptake experiments, both apical and basolateral media were removed after either 15 or 120 min, cells were washed 4 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. Following incubation, cells could easily be scraped from the Transwell membrane and frozen at −20°C for future HPLC analysis. For transport experiments, 0.25 ml samples were taken periodically from the basolateral chamber and fresh buffer was added as replenishment; these samples were immediately frozen at −20°C for future HPLC analysis. Samples containing cells were sonicated using a Branson B15 Cell Disruptor for 30 s at 50% power. All samples were prepared for HPLC analysis by adding 0.25 ml of acetonitrile, vortexing vigorously, and centrifuging for 15 min at 13 000 rpm. The supernatants were then injected into the following HPLC system: a Merck/Hitachi L-4000 variable wavelength UV detector, a HiCHROM Spherisorb S5ODS2 column (5 μm, 4.6×250 mm^2), a Spherisorb protection column, and a 20-μl loop injection valve. The mobile phase used for Asp(OBzl)-Sar analysis was 60% 0.02 M NaH_2PO_4, 35% methanol, 5% acetonitrile, 0.001 M triethylamine, pH 4.0; and for D-Asp(OBzl)-Ala analysis, a 70% 0.02 M NaH_2PO_4, 10% methanol, 20% acetonitrile, 0.001 M triethylamine, pH 4.0 mobile phase was used. 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, Asp(OBzl)-Sar, and BZ-OH were 5.5, 4.8, and 6.7 min, respectively.

2.6. Assessment of the stability of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar

Analysis of the stability of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar was performed in both pH 6.0 and pH 7.4 BSA-supplemented HBSS. Wells were incubated at 37°C under a 5% CO_2 atmosphere and 100% humidity for 24 h. Either D-Asp(OBzl)-Ala or Asp(OBzl)-Sar (1 mM) was added to the pH 6.0 or pH 7.4 BSA-supplemented HBSS, and media was sampled periodically over the course of 24 h. Following sampling, HPLC analysis was done as described in Section 2.5.

3. Results

3.1. IC_{50} values of various dipeptides for the apically expressed Caco-2 oligopeptide transporter are markedly different

The relative affinities of a series of L/L, D/L, β-configured and side-chain esterified dipeptides for the oligopeptide transporter were determined by calculating their degree of [^{14}C]Gly-Sar uptake inhibition in Caco-2 monolayers when present at various concentrations in the apical media (Table 1). Upon initial evaluation at a concentration of

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	IC_{50} (mM)[a]	Ratio[b]
Asp-Gly	0.91 ± 0.05	—
β-Asp-Gly	4.32 ± 0.59	+4.7
Asp-Ala	0.33 ± 0.001	—
β-Asp-Ala	4.32 ± 0.59	+13.1
D-Asp-Ala[c]	5.75 ± 0.09	+17.4
D-Asp(OBzl)-Ala[c]	2.62 ± 0.35	+7.9
Asp-Sar	0.49 ± 0.18	—
Asp(OBzl)-Sar	0.014 ± 0.007	−35.0

[a] Values represent the means ± S.D. for three separate filters; Asp(OBzl)-Sar represents the means ± S.D. for six separate filters.
[b] Ratios represent β-, D-, or OBzl-esterified analog: corresponding L/L-configured dipeptide.
[c] Data taken from Taub et al., 1997.

20 mM in the apical media, all of the investigated dipeptides demonstrated a $>90\%$ inhibition of [^{14}C]Gly-Sar uptake into the Caco-2 monolayers. Yet, dipeptide inhibitor concentrations between 0.01 and 10 mM demonstrated markedly variable degrees of inhibition of [^{14}C]Gly-Sar uptake. As is shown in Table 1, almost all modified or unnatural dipeptides demonstrated an increased IC_{50} value relative to their corresponding L/L-dipeptides; the only modified/unnatural dipeptide demonstrating a decrease in IC_{50} value when compared to its L/L-configured analog was Asp(OBzl)-Sar.

3.2. D-Asp(OBzl)-Ala, but not Asp(OBzl)-Sar, is taken up into Caco-2 monolayers via the oligopeptide transporter

Uptake studies were performed at both 15 and 120 min in order to assess both initial uptake and intracellular accumulation (Hu et al., 1996). Since most of the dipeptides listed in Table 1 were either too hydrophobic, enzymatically labile, or possessed a molar extinction coefficient unacceptable for HPLC analysis via uv detection, the benzyl-esterified dipeptides were the only compounds which could be evaluated. As is shown in Fig. 1, a significant amount of D-Asp(OBzl)-Ala was taken up into Caco-2 monolayers after both a 15- and 120-min incubation (14.33 ± 1.00 and 18.92 ± 1.08 nmol/mg protein, respectively). D-Asp(OBzl)-Ala uptake into the monolayers was $>90\%$ inhibitable by a concomitant 20-fold molar excess of Gly-Pro present in the apical media (1.61 ± 0.13 and 1.90 ± 0.09 nmol/mg protein for the 15- and 120-min experiments, respectively). Asp(OBzl)-Sar was not taken up to any measurable degree into the monolayers over the course of either the 15- or 120-min uptake study. In addition to the modified dipeptides, cells were monitored via HPLC analysis for the presence of the benzyl alcohol (BZ-OH) hydrolysis product (Table 2).

3.3. D-Asp(OBzl)-Ala, but not Asp(OBzl)-Sar, is transported across Caco-2 monolayers via the oligopeptide transporter

D-Asp(OBzl)-Ala and Asp(OBzl)-Sar were assessed for their ability to achieve apical-to-basal

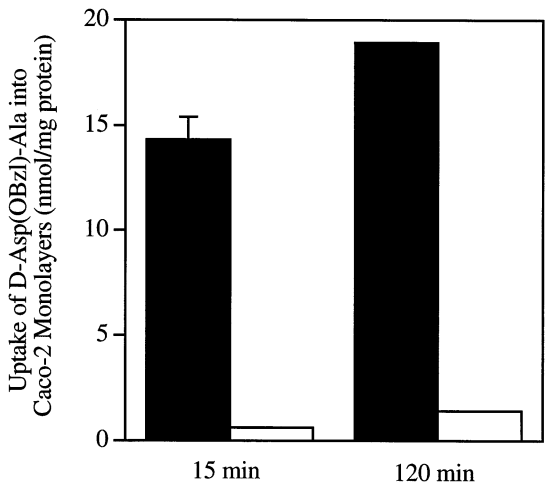

Fig. 1. Apical uptake of D-Asp(OBzl)-Ala (1 mM) in the presence or absence of Gly-Pro (20 mM). D-Asp(OBzl)-Ala was added to the apical chamber of Caco-2 monolayers either alone (solid bars) or concomitantly with Gly-Pro (open bars). The amount of D-Asp(OBzl)-Ala uptake per mg protein was determined following incubation times of either 15 or 120 min at 37°C. Results shown represent the means for three separate filters and the S.D. is indicated by a bar.

transport across Caco-2 monolayers via the oligopeptide transporter. As is shown in Fig. 2A, D-Asp(OBzl)-Ala was efficiently transported across Caco-2 monolayers over the course of a 5-h experiment. A steady-state rate of transport, 14.41 ± 0.91 nmol/h/cm^2, was reached after 2-h incubation. In monolayers treated concomitantly with both D-Asp(OBzl)-Ala and a 20-fold molar

Table 2
Intracellular BZ-OH resulting from the apical administration of either D-Asp(OBzl)-Ala or Asp(OBzl)-Sar (1 mM)

Dipeptide	±Gly-Pro	15 min	120 min
D-Asp(OBzl)-Ala	−	0.62 ± 0.04[a]	1.40 ± 0.06
	+	None detected[b]	None detected
Asp(OBzl)-Sar	−	1.87 ± 0.05	1.26 ± 0.03
	+	0.77 ± 0.01	0.47 ± 0.02

[a] Values represent the mean nmol BZ-OH/mg protein ± S.D. for $N = 2–3$ monolayers.
[b] BZ-OH could be detected via HPLC at ≥ 5 μM.

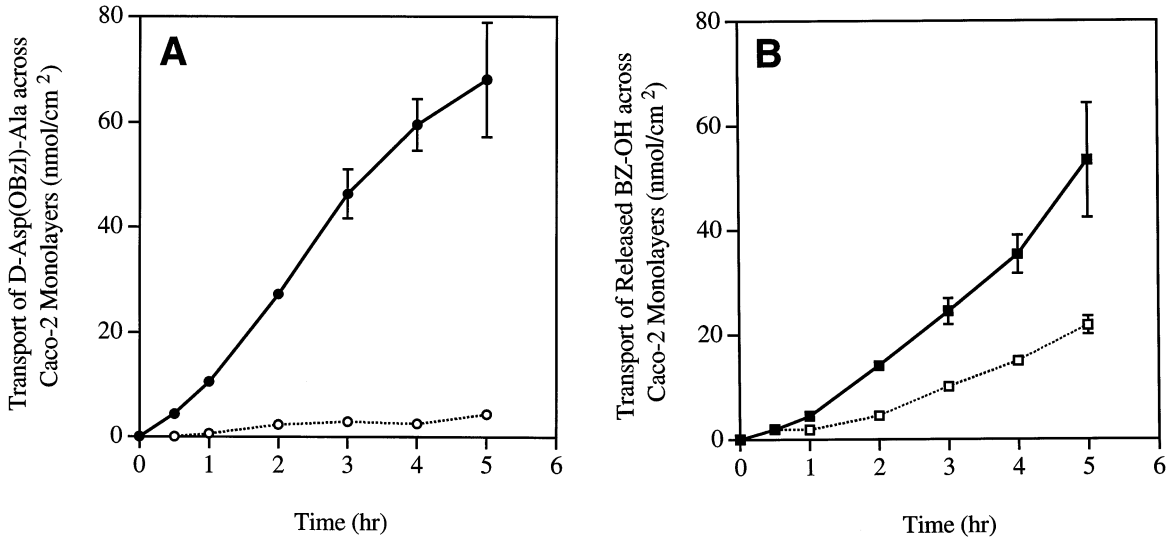

Fig. 2. Apical-to-basal transport of either D-Asp(OBzl)-Ala (1 mM) or BZ-OH released from D-Asp(OBzl)-Ala in the presence or absence of Gly-Pro (20 mM). D-Asp(OBzl)-Ala was added to the apical chamber of Caco-2 monolayers either alone or concomitantly with Gly-Pro, and the amount of either D-Asp(OBzl)-Ala or BZ-OH appearing in the basal chamber was determined at various time points during a 5-h incubation at 37°C. (A) The amount of D-Asp(OBzl)-Ala transported to the basal chamber per cm^2 in the presence (○) or absence (●) of Gly-Pro. (B) The amount of released BZ-OH transported to the basal chamber per cm^2 in the presence (□) or absence (■) of Gly-Pro. Results shown 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.

excess of Gly-Pro, transport of D-Asp(OBzl)-Ala was inhibited by $>90\%$. Benzyl alcohol (BZ-OH) was also transported in the apical-to-basal direction across monolayers treated with D-Asp(OBzl)-Ala (Fig. 2B); yet, the amount of inhibition achieved by the presence of Gly-Pro was only approximately 60%. Asp(OBzl)-Sar was not transported across Caco-2 monolayers to any measurable degree throughout the 5-h experiment. In certain monolayers, the apical-to-basal transport characteristics of apically-administered BZ-OH (1 mM) was monitored. BZ-OH was efficiently transported across the Caco-2 monolayers via a passive mechanism, and it reached a maximal rate of transport of 153.29 ± 26.24 nmol/h/cm^2 after 15 min. After 5 h, BZ-OH reached an equilibrated concentration in both apical and basolateral chambers. In control monolayers treated with both BZ-OH (1 mM) and Gly-Pro (20 mM), there was no significant difference in the amount or rate of BZ-OH transport over the course of a 5-h transport experiment.

3.4. D-Asp(OBzl)-Ala and Asp(OBzl)-Sar stability differ at pH 6.0 and 7.4

The stability of the β-carboxyl ester of both Asp(OBzl)-Sar and D-Asp(OBzl)-Ala under experimental conditions was monitored over 24 h via HPLC analysis of each parent compound compared with generation of the BZ-OH hydrolysis product. The stability of the benzyl ester in both compounds was much greater when incubated in pH 6.0 HBSS (10 mM Mes, 0.05% BSA) versus pH 7.4 HBSS (10 mM Hepes, 0.05% BSA). Under these conditions, the half-lives calculated for Asp(OBzl)-Sar conversion to Asp-Sar and BZ-OH were 20.36 and 4.86 h at pH 6.0 and 7.4, respectively; the half-lives calculated for D-Asp(OBzl)-Ala conversion to D-Asp-Ala and BZ-OH were 26.1 and 7.81 h at pH 6.0 and 7.4, respectively. The stability of D-Asp(OBzl)-Ala and the generation of the BZ-OH hydrolysis product over a 24-h period in either pH 6.0 or 7.4 BSA-supplemented HBSS is shown in Fig. 3.

4. Discussion

Several groups have reported that a diversity of peptides and peptidomimetic drugs possess an affinity for the Caco-2 oligopeptide transporter (Walter et al., 1996). Since such a wide structural variety of compounds are suitable substrates for the oligopeptide transporter, targeting peptide drugs or peptide–prodrug systems could be an attractive drug delivery or rational drug design strategy (Bai and Amidon, 1992; Swaan et al., 1993). With respect to a potential substrate's affinity for the oligopeptide transporter, certain structural constraints have been determined. For example, a potential substrate's N-terminal alpha-amino group, in certain cases, does not appear to be essential for preserving oligopeptide transporter interaction; yet, a terminal carboxyl group is indeed an essential structural requirement for recognition by the oligopeptide transporter (Bai and Amidon, 1992; Hidalgo et al., 1995; Li and Hidalgo, 1996). Due to the fact that hydrolysis of L/L-configured dipeptides in the brush border membrane of intestinal cells renders their analysis quite difficult, the inclusion of unnatural, e.g. D-configured, amino acids may circumvent this problem (Smith et al., 1993; Thwaites et al., 1993; Hidalgo et al., 1995). Substrate inhibition studies indicate that, with respect to dipeptides, only one amino acid must be in the L-configuration in order to retain binding affinity for the oligopeptide transporter (Hidalgo et al., 1995). While it is assumed that there is a size restriction for peptide transport, no systematic investigation has been reported (Smith et al., 1993); yet, a model structure of a molecule containing substituents essential for recognition and transport by the oligopeptide transporter has recently been proposed (Walter et al., 1996).

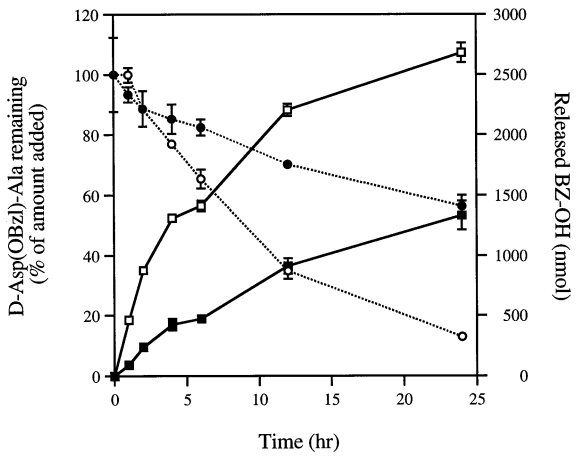

Fig. 3. Time-course of the release of BZ-OH from D-Asp(OBzl)-Ala in experimental buffer. D-Asp(OBzl)-Ala (1 mM) was added to either pH 6.0 or 7.4 HBSS and incubated for 24 h at 37°C. The amount of D-Asp(OBzl)-Ala remaining at pH 6.0 (●) and 7.4 (○) was determined at various time points during the incubation; additionally, the amount of released BZ-OH at pH 6.0 (■) and 7.4 (□) was also monitored. Results shown represent the means for three separate experiments under each condition and the S.D. is either indicated by a bar or is smaller than the size of the symbol.

Using ligand displacement studies, we investigated the ability of various enzymatically stable configurations of certain dipeptides to retain their affinity for the Caco-2 oligopeptide transporter, and additionally, what their degree of affinity retention was. In our previous report, it was demonstrated that D-Asp-Ala and its β-carboxyl cyclohexyl and benzyl esters are substrates for the Caco-2 oligopeptide transporter (Taub et al., 1997). In this study, Asp-Ala and its analogs (β-Asp-Ala, D-Asp-Ala, and D-Asp(OBzl)-Ala) were investigated; Asp-Gly, β-Asp-Gly, Asp-Sar, and Asp(OBzl)-Sar were evaluated as well. As is shown in Table 1, the L/L-configured dipeptides demonstrate the lowest IC_{50} value for the Caco-2 oligopeptide transporter. With the exception of Asp(OBzl)-Sar, the IC_{50} value for all β-/D-configured or side-chain modified dipeptides was greater than that determined for the corresponding L/L-configured parent dipeptide. These results suggest that, with the exception of Asp(OBzl)-Sar, unnatural or side-chain modified dipeptides generally demonstrate a reduction in oligopeptide transporter affinity vs. their corresponding natural or unmodified analog. Although inhibition constants measured for these molecules are not necessarily indicative of their ability to undergo cellular uptake and enter the transepithelial transport pathway (Eddy et al., 1995; Tamura et al., 1996), the extremely low IC_{50} value calculated for Asp(OBzl)-Sar suggested that it might undergo oligopeptide transporter-mediated uptake. Hence,

although not completely unexpected, it was a bit surprising that Asp(OBzl)-Sar was unable to demonstrate any detectable uptake or transport. Alternatively, D-Asp(OBzl)-Ala did achieve uptake into (Fig. 1) and transport across (Fig. 2A) Caco-2 cells via the oligopeptide transporter. As shown in Fig. 1, the uptake of D-Asp(OBzl)-Ala into Caco-2 monolayers reached a steady-state level quite rapidly; at 15 min the intracellular amount was $>75\%$ of that amount taken up at 2 h. This was considered to be indicative of an active process; yet, as can also be seen in Fig. 1, uptake at both 15 min and 2 h was $>90\%$ inhibitable by a 20-fold molar excess of Gly-Pro. From Fig. 2A, the apical-to-basal transport of D-Asp(OBzl)-Ala was approximately 90% inhibitable by Gly-Pro competition. It has been reported that although the D/L-configured dipeptide D-Val-Val is able to inhibit [^{3}H]cephalexin uptake in Caco-2 monolayers, it is only transported across Caco-2 membranes via a passive paracellular route (Tamura et al., 1996). These data, taken together, demonstrate that uptake of D-Asp(OBzl)-Ala into Transwell-grown Caco-2 monolayers proceeds actively via the oligopeptide transporter.

Stability studies performed on D-Asp(OBzl)-Ala and Asp(OBzl)-Sar indicate that the release of BZ-OH from both compounds was much faster at pH 7.4 than at pH 6.0 (Fig. 3). Considering that the linkage between BZ-OH and D-Asp-Ala is an ester, these were the expected results. As BZ-OH was rapidly transported via a passive mechanism across Caco-2 monolayers, the amount of BZ-OH measured in the basolateral compartment following the apical-to-basal D-Asp(OBzl)-Ala transport study could represent BZ-OH released from D-Asp(OBzl)-Ala either: (a) in the apical media or at the apical membrane surface, followed by passive paracellular or transcellular transport across the cells; (b) intracellularly, followed by export into the basolateral media; or (c) in the basolateral media or at the basolateral membrane surface following transcellular transport of the intact D-Asp(OBzl)-Ala. From the stability studies depicted in Fig. 3, the half-life of BZ-OH release from D-Asp(OBzl)-Ala at pH 6.0 is 26.1 h, while at pH 7.4 it is approximately 7.8 h; thus, the lability of BZ-OH in both apical and basolateral media could effectively contribute to BZ-OH apical-to-basal transport. Since a 20-fold molar excess of Gly-Pro was able to inhibit D-Asp(OBzl)-Ala transport by approximately 90% (Fig. 2A) while only inhibiting BZ-OH transport by approximately 60% (Fig. 2B), and a significant amount of intact D-Asp(OBzl)-Ala was transported in the apical-to-basal direction, the increase in BZ-OH versus D-Asp(OBzl)-Ala apical-to-basal transport in the presence of Gly-Pro could be partially accounted for by release of BZ-OH from D-Asp(OBzl)-Ala within the basolateral media following transcellular transport of the intact molecule. The fact that BZ-OH was detected intracellularly following both a 15- or 120-min uptake study (Table 2) suggests that intracellular hydrolysis of D-Asp(OBzl)-Ala, followed by export of BZ-OH into the basolateral media, was a contributing pathway.

Interestingly, although intact Asp(OBzl)-Sar was not taken up into Caco-2 cells, intracellular BZ-OH was detected following both 15- and 120-min uptake studies (Table 2). As opposed to D-Asp(OBzl)-Ala, BZ-OH uptake is slightly inhibited but not eliminated by Gly-Pro competition. Since there was significantly less BZ-OH detected following the 120- versus the 15-min uptake study, with or without Gly-Pro competition, it is possible that BZ-OH released from Asp(OBzl)-Sar enters the cells or is transported across the cells, thus leaving Asp-Sar bound to the oligopeptide transporter at the apical membrane. The bound Asp-Sar could serve as an additional competitive factor, inhibiting further Asp(OBzl)-Sar binding, release of BZ-OH, and uptake of BZ-OH into the cells. This suggests that interaction with the apical membrane via oligopeptide transporter-mediated affinity participates in BZ-OH release from Asp(OBzl)-Sar. Studies conducted in kidney BBMV indicate that there are two oligopeptide transporters present, one characterized as a high-affinity/low-capacity transporter and the other as a low-affinity/high-capacity transporter (Daniel et al., 1992). Similar results have been shown for Caco-2 cells, and it has been suggested that one component could represent either a distinct second carrier or, perhaps more likely, a second

binding site on a carrier that is accessible under varying external conditions, e.g. proton gradient modulation (Hu et al., 1996). Thus, it is possible that the 187-fold IC_{50} value discrepancy between D-Asp(OBzl)-Ala and Asp(OBzl)-Sar, in addition to their different uptake and transport properties, could be related to their interaction with either another binding site or a different transporter altogether.

Although BZ-OH is not an active drug compound, we feel that these results present valuable information which may be used in future rational drug design studies, i.e. a side-chain modified, enzymatically stable dipeptide is able to undergo oligopeptide transporter-mediated transepithelial transport, followed by release of the molecule reversibly attached to the side-chain of one amino acid in the dipeptide. This prodrug approach 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 (Walter et al., 1996). A related drug delivery system using a prodrug of L-Dopa, i.e. pGlu-L-Dopa-Pro, has shown significant uptake when tested in rat perfusion studies (Bai, 1995). These data also indicate that D-Asp(OBzl)-Ala is recognized by both the apically and basolaterally located oligopeptide transporter. These results suggest that it may be possible, using carefully designed drug–dipeptide conjugates, to increase the transepithelial transport of a poorly bioavailable small drug molecule.

References

Bai, J.P.F., 1995. pGlu-L-Dopa-Pro: A tripeptide prodrug targeting the intestinal peptide transporter for absorption and tissue enzymes for conversion. Pharm. Res. 12, 1101–1104.

Bai, J.P.F., Amidon, G.L., 1992. Structural specificity of mucosal-cell transport and metabolism of peptide drugs: Implication for oral peptide drug delivery. Pharm. Res. 9, 969–978.

Bodansky, M., Bodansky, A., 1994. The Practice of Peptide Synthesis, 2nd ed. Springer-Verlag, Berlin.

Covitz, K.-M.Y., Amidon, G.L., Sadee, W., 1996. Human dipeptide transporter, hPEPT1, stably transfected into Chinese Hamster ovary cells. Pharm. Res. 13, 1631–1634.

Daniel, H., Morse, E.L., Adibi, S.A., 1992. Determinants of substrate affinity for the oligopeptide/H^+ symporter in the renal brush border membrane. J. Biol. Chem. 267, 9565–9573.

Dantzig, A.H., Bergin, L., 1990. Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. Biochim. Biophys. Acta 1027, 211–217.

Dantzig, A.H., Duckworth, D.C., Tabas, L.B., 1994. Transport mechanisms responsible for the absorption of loracarbef, cefixime, and cefurixime axetil into human intestinal Caco-2 cells. Biochim. Biophys. Acta 1191, 1–13.

Eddy, E.P., Wood, C., Miller, J., Wilson, G., Hidalgo, I.J., 1995. A comparison of the affinities of dipeptides and antibiotics for the di-/tripeptide transporter in Caco-2 cells. Int. J. Pharm. 115, 79–86.

Ganapathy, V., Leibach, F.H., 1985. Is intestinal peptide transport energized by a proton gradient?. Am. J. Physiol. 249, G153–G160.

Gochoco, C.H., Ryan, F.M., Miller, J., Smith, P.L., Hidalgo, I.J., 1994. Uptake and transepithelial transport of the orally absorbed cephalosporin cephalexin in the human intestinal cell line, Caco-2. Int. J. Pharm. 104, 187–202.

Hidalgo, I.J., Bhatnagar, P., Lee, C.-P., Miller, J., Cucullino, G., Smith, P.L., 1995. Structural requirements for interaction with the oligopeptide transporter in Caco-2 cells. Pharm. Res. 12, 317–319.

Hu, M., Chen, J., Zheng, L., Dantzig, A.H., Stratford, R.E., 1996. Uptake characteristics of loracarbef and cephalexin in the Caco-2 cell culture model: Effects of the proton gradient and possible presence of a distinctive second component. J. Pharm. Sci. 85, 767–772.

Li, J., Hidalgo, I.J., 1996. Molecular modeling study of structural requirements for the oligopeptide transporter. J. Drug Target. 4, 9–17.

Liang, R., Fei, Y.-J., Prasad, P.D., Ramamoorthy, S., Jan, J., Yang-Feng, T.L., Hediger, M.A., Ganapathy, V., Leibach, F.H., 1995. Human intestinal H^+/peptide cotransporter. J. Biol. Chem. 270, 6456–6463.

Matthews, D.M., Adibi, S.A., 1976. Intestinal absorption of peptides. Physiol. Rev. 55, 537–608.

Saito, H., Inui, K.-I., 1993. Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line Caco-2. Am. J. Physiol. 265, G289–G294.

Smith, P.L., Eddy, E.P., Lee, C.-P., Wilson, G., 1993. Exploitation of the intestinal oligopeptide transporter to enhance drug absorption. Drug Deliv. 1, 103–111.

Swaan, P.W., Stehouwer, M.C., Blok, R.I.C., Tukker, J.J., 1993. Prodrug approach using the intestinal peptide carrier. Pharm. Res. 10, 295.

Tamura, K., Bhatnagar, P.K., Takata, J.S., Lee, C.-P., Smith, P.L., Borchardt, R.T., 1996. Metabolism, uptake, and

transepithelial transport of the diastereomers of Val-Val in the human intestinal cell line, Caco-2. Pharm. Res. 13, 1213–1218.

Taub, M.E., Larsen, B.D., Steffansen, B., Frokjaer, S., 1997. β-Carboxylic acid esterified D-Asp(OBzl)-Ala retains a high affinity for the oligopeptide transporter in Caco-2 monolayers. Int. J. Pharm. 146, 205–212.

Thwaites, D.T., Brown, C.D.A., Hirst, B.H., Simmons, N.L., 1993. Transepithelial glycylsarcosine transport in intestinal Caco-2 cells mediated by expression of H^+-coupled carriers at both apical and basal membranes. J. Biol. Chem. 268, 7640–7642.

Thwaites, D.T., Hirst, B.H., Simmons, N.L., 1994. Substrate specificity of the di/tripeptide transporter in human intestinal epithelia (Caco-2): Identification of substrates that undergo H^+ coupled absorption. Br. J. Pharmacol. 113, 1050–1056.

Walter, E., Kissel, T., Amidon, G.L., 1996. The intestinal peptide carrier: A potential transport system for small peptide-derived drugs. Adv. Drug Deliv. Rev. 20, 35–58.

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