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A rapid screening system to determine drug affinities for the intestinal dipeptide transporter 1: system characterisation

Vanessa A. Moore [a,1], William J. Irwin [a,*], Peter Timmins [a,c], Saeho Chong [b], Sandra A. Dando [b], Richard A. Morrison [b]

[a] *Pharmaceutical Sciences Research Institute, Aston Pharmacy School, Aston University, Aston Triangle, Birmingham, B4 7ET, UK*

[b] *Bristol-Myers Squibb, Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ, USA*

[c] *Bristol-Myers Squibb, Pharmaceutical Research Institute, Moreton, Merseyside, L46 1QW, UK*

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Abstract

Purpose: To establish an in vitro system for the rapid assessment of the affinities of potential substrates for the di/tri/oligopeptide transport system (DTS). *Methods:* Monolayers of Caco-2 cells were cultured in plastic wells for 7–9 days and the uptake of Gly-[^{3}H]L-Pro, a specific and relatively stable substrate for the DTS was used as an affinity probe. Gly-[^{3}H]L-Pro (50 nM), together with excess L-Pro (10 mM), to suppress uptake of any [^{3}H]L-Pro produced by degradation of the probe, was incubated with the test compound (usually 1 mM) at pH 6 for 3 min. The uptake of radiolabel was determined by liquid scintillation counting. *Results:* High specific-uptake ($>85\%$) of Gly-[^{3}H]L-Pro was obtained with cells grown for 7–9 days. Gly-[^{3}H]L-Pro uptake had a substantial active concentration-dependent component (K_m of 0.39 ± 0.02 mM, V_{max} of 0.98 ± 0.04 nmol min^{-1} (mg protein)$^{-1}$. This process was shown to be specific for the DTS as evidenced by the significant inhibition by compounds reported to be transported by this system and the lack of inhibition by amino acids. The use of low competitor concentrations (1 mM) enabled a range of inhibition values (0–89%) of a series of competitors (amino acids, dipeptides and β-lactam antibiotics) to be estimated, illustrating that structurally similar compounds can be ranked for affinity to the DTS. *Conclusion:* A screening system, using Caco-2 cells and the dipeptide Gly-[^{3}H]L-Pro as a displaceable probe, was developed to assess a variety of compounds for recognition by the di/tri/oligopeptide transport system. This fully describes the first system that allows structurally related compounds to be ranked on the basis of their affinity for the DTS recognition site.

Keywords: Permeability screen; Caco-2 cells; di/tri/oligopeptide transport system; Peptidomimetics; Structure–activity relationships; Uptake mechanisms

* Corresponding author. Tel.: +44-121-3593611; fax: +44-121-3590733.

E-mail address: w.j.irwin@aston.ac.uk (W.J. Irwin).

[1] Present address: AstraZeneca, Loughborough, Leicestershire, LE11 5RH, UK.

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1. Introduction

The intestinal di/tri/oligopeptide transporter system (DTS) plays an important role in the absorption of certain orally administered drug molecules (Hidalgo and Li, 1996) and is involved, at least in part, in the absorption of some of the antihypertensive, angiotensin-converting enzyme (ACE) inhibitors, a group of synthetic peptide-like molecules. There is evidence to suggest that, in the absorption of compounds such as SQ-29852 (ceranapril) (Nicklin et al., 1992a, 1996) captopril, (Hu and Amidon, 1988) enalapril (Friedman and Amidon, 1989; Thwaites et al., 1995), and quinapril (Hu et al., 1995), there is a contributing active transport component mediated by the DTS. In the case of SQ-29852, which is a stable and specific probe of the DTS (Nicklin et al., 1992a; Marino et al., 1996; Nicklin et al., 1996) saturable (carrier-mediated) transport contributing up to 70% of the absorption process in rats, dogs and humans, has been shown to involve this carrier (Foley et al., 1988). Absorption of several cephalosporins (e.g. cefaclor, cephalexin and cephradine) and the penicillins (e.g. amoxicillin and ampicillin) has also been shown to involve the DTS to some extent (Dantzig and Bergin, 1990; Inui et al., 1992; Oh et al., 1992; Gochoco et al., 1994; Dantzig, 1997). Although the absorption mechanisms of these drugs were discovered after their selection as clinical candidates, an understanding of the structure–affinity relationships and the development of a rapid screening system that could be used to enhance the bioavailability of specific classes of orally administered compounds and prodrugs designed to be substrates for the DTS (Kissel and Amidon, 1996).

The prediction of potential cell permeability is an important consideration in selecting candidate molecules from combinatorial libraries and the development of a rapid throughput screening model, to allow fast assessment of a large number of drug candidates for recognition by the DTS, would be advantageous in the identification of potential orally active compounds. The aim of this study was to develop a rapid screening system that could be used to assess quantitatively the affinity of a large number of compounds for their ability to inhibit the binding of a probe compound to the DTS recognition site. The availability of such a model, with controlled experimental conditions to allow compounds to be ranked with regard to interaction with the DTS, would facilitate the early selection of lead compounds and assist in the optimisation processes in drug discovery and development programmes. The recent report of a system using glycyl-sarcosine to study β-lactam affinity (Bretschneider et al., 1999) prompts us to report in detail our own studies with ACE-inhibitors (Moore et al., 1995).

Previous studies in Caco-2 cells that have utilised competitive inhibition of uptake or transport to define absorption processes have principally used high competitor concentration (5–20 mM), where inhibition has been virtually complete. Our intention was to obtain a range of inhibition values without saturating interaction with the DTS, by using a lower concentration of competitor (e.g. 1 mM), thus allowing compounds to be ranked for affinity and the impact of structural variations to be explored. When this work was undertaken (Moore et al., 1995), previous studies had been designed to characterise transporter properties of specific radiolabelled compounds (e.g. glycyl-sarcosine [Gly-Sar]) rather than screen affinity of potential competitors. The adenocarcinoma cell line, Caco-2, which is an established model for intestinal drug absorption (Bailey et al., 1996; Gan and Thakker, 1997) and which has been shown to express the DTS (Nicklin et al., 1992a; Dantzig et al., 1994; Nicklin et al., 1996) was selected as the experimental model. The hydrolysis-resistant dipeptide glycyl-[^{3}H]L-proline (Gly-[^{3}H]L-Pro) (Ganapathy et al., 1980) was selected as the experimental probe.

2. Materials and methods

2.1. Materials

The dipeptide probe, glycyl-3,4-[^{3}H]L-proline (Gly-[^{3}H]L-Pro) (50 Ci mmol^{-1}), was purchased from NEN Research Products (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesul-

phonic acid (HEPES) buffer solution (1 M) were obtained from Gibco BRL, (Grand Island, NY). Foetal bovine serum was purchased from Hyclone Lab. Inc. (Logan, Utah). Ethylenediamine tetra acetic acid (EDTA), L-glutamine solution (200 mM), penicillin, streptomycin and trypsin were obtained from JHR Bioscience (Lenexa, KS). Dimethyl sulphoxide (DMSO), Hank's balanced salts, non-essential amino acids solution (NEAA), 2-[*N*-morpholino]ethanesulphonic acid (MES), phosphate-buffered saline (PBS) tablets, L-proline (L-Pro), sodium azide and Triton X-100 (TX-100) were purchased from Sigma Chemical Co. (St. Louis, MO). The bicinchoninic acid (BCA) protein assay reagent kit was obtained from Pierce (Rockford, IL). The culture flasks and 24-well plates were purchased from Costar (Cambridge, MA). All solvents were analytical grade. SQ-29852 and fosinoprilat were provided by Bristol-Myers Squibb (Princeton, NJ). Sarcosyl-L-proline (Sar-L-Pro) was obtained from Schweizerhall Inc. (South Plainfield, NJ), and L-phenylalanyl-L-proline (L-Phe-L-Pro) was from Aldrich Chemical Company Inc. (Milwaukee, WI). All other chemicals used throughout the study were obtained from Sigma Chemical Co. (St. Louis, MO), and were cell culture grade.

2.2. Cell culture

Caco-2 cells (passage 17) were purchased from the American Type Tissue Culture Collection, Rockville, MD. The cells were grown in plastic tissue culture T-flasks (area = 150 cm^2), and maintained in 20 ml of cell growth media (M1) comprising DMEM supplemented with 4.5 g l^{-1} glucose, 10% v/v foetal bovine serum, 1% v/v NEAA, 1% v/v L-glutamine solution, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.5% v/v HEPES buffer solution. The cultures were grown at 37°C in an atmosphere of 5% CO_2 (95% air) and 90% relative humidity, with M1 being replaced every 48 h. The cultures were passaged every 3–4 days using 0.25% w/v trypsin/EDTA (0.25% w/v in PBS, pH 7.2) and flasks were seeded at approximately 4.7×10^4 cells cm^{-2}. The cells were used between the passages 25 and 40 for uptake studies and seeded onto 24-well tissue culture plates (2 cm^2) at a density of 8×10^4 cells cm^{-2} (1.6×10^5 cells $well^{-1}$). M1 (2 ml) was renewed every 48 h and the monolayers were used after 7 days of growth, unless otherwise stated. The protein content of the cells was determined at various passage numbers, using a BCA kit with bovine serum albumin (2 mg ml^{-1}) as the standard.

3. Characterisation of Gly-[^{3}H]L-Pro uptake and optimisation of the system

The following general method was used for all studies unless otherwise stated; the incubation media (M2) comprised Hank's balanced salts solution (HBSS) with 25 mM MES adjusted to pH 6 using NaOH (1 M), and containing 50 nM (approximately 1 800 000 dpm) of the probe, Gly-[^{3}H]L-Pro, unless otherwise stated. Excess L-Pro (10 mM) was included in the incubation media to maximise specific uptake by blocking the uptake of the small amount of [^{3}H]L-Pro that could be formed from hydrolysis of the probe during the 3 min incubation period. The experiments were conducted at pH 6, as this is the optimum pH for Gly-L-Pro proton-coupled transport into rabbit intestinal and renal brush-border membrane vesicles (BBMV) (Ganapathy and Leibach, 1983). The pH of the M2 solution was checked after addition of any chemicals and corrected to pH 6 with HCl (1 M) or NaOH (1 M) solutions if required. All solutions used were preheated to 37°C unless otherwise stated, with the exception of the PBS stop-solution (containing 0.05% w/v sodium azide) which was kept at 4°C. Cells were washed (500 μl for 5 min) with M2 at 37°C and then incubated with the incubation solution (250 μl) for the required time period (see individual experimental details), at 37°C with agitation by an orbital shaker (50 Hz). At the end of the incubation period, the plates were transferred to a cold-room (4°C) and the apical (Ap) solutions were removed. The monolayers were washed (2×500 μl $\times$ 5 min) with PBS stop-solution (4°C), and then harvested with 1 ml of 1% (v/v) TX-100 in double-distilled water. The Ap solutions, washings and solubilised cells were collected, added to

15 ml Ecolite^{+} cocktail and counted for [^{3}H] by liquid scintillation counting [(LSC), Hewlett Packard, Model 2500].

4. Specificity of Gly-[^{3}H]L-Pro uptake

As initial studies with the screen were planned to involve a range of substances structurally related to ACE-inhibitors, evaluation of Gly-[^{3}H]L-Pro binding to ACE was investigated by co-administration of 1 μM fosinoprilat (a concentration over fivefold in excess of its K_i for ACE), an ACE inhibitor with a high affinity for the enzyme (Krapcho et al., 1988).

In order to determine that Gly-[^{3}H]L-Pro uptake into Caco-2 cells is via the DTS, specificity was explored by measuring uptake in the presence of 1 mM of the following compounds; glycine (Gly), glycyl-L-prolylglycylglycine (Gly-L-Pro-Gly-Gly), L-lysine (L-Lys), L-phenylalanine (L-Phe), sarcosine (Sar), and L-serine (L-Ser).

To assess whether the present system could be used to rank the affinities of various potential substrates for recognition by the DTS, uptake of Gly-[^{3}H]L-Pro in the presence of two series of potential competitors (dipeptides and cephalosporins) was investigated. Uptake of Gly-[^{3}H]L-Pro was measured in the presence of 1 mM of the following dipeptides; L-aspartyl-L-aspartate (L-Asp-L-Asp), L-glutamyl-L-glutamate (L-Glu-L-Glu), glycyl-DL-methionine (Gly-DL-Met), glycyl-DL-phenylalanine (Gly-DL-Phe), Gly-L-Pro, Gly-Sar, glycyl-L-tyrosine (Gly-L-Tyr), L-lysyl-L-proline (L-Lys-L-Pro), L-Phe-L-Pro and Sar-L-Pro, and 10 mM of the following cephalosporins and penicillins; ampicillin, amoxicillin, cefaclor, cefazolin, cephalexin and cephradine.

The reproducibility of the system was investigated by comparing inhibition (%) values of Gly-[^{3}H]L-Pro uptake in the presence of 1 mM SQ-29852 on 10 separate occasions. The binding mechanism of SQ-29852 to the DTS was explored by measuring Gly-[^{3}H]L-Pro uptake at 0.04, 0.13, 0.4, and 3.6 mM, in the presence of 0, 1 and 3 mM SQ-29852.

5. Data analysis

The data sets are expressed as the mean ± standard deviation (S.D.) from at least 3 monolayers. Results are expressed as uptake of Gly-[^{3}H]L-Pro into the cells (nmol or pmol min^{-1} (mg protein)$^{-1}$), with a BCA protein assay being employed to correct for difference in cell density of individual monolayers, and $K_i \pm$ S.D. (mM) for competitors. In order to quantify the effect of competitors on the active component of Gly-[^{3}H]L-Pro uptake, the inhibitions (%) were corrected for non-specific (passive) uptake (15%), by subtracting the amount of uptake seen in the presence of 5 mM unlabelled Gly-L-Pro.

The transport inhibition constant (K_i mM) is related to the observed inhibition of carrier-mediated uptake using the ratio (r) of the uptake rate in the absence (v_0) of an inhibitor to the value in the presence of (v_i) of an inhibitor ($r = v_0/v_i$) according to Eq. (1) (Nicklin et al., 1995):

$$K_i = \frac{K_m \cdot [I]}{(r-1)\cdot(K_m + [S])} \quad (1)$$

where K_m is the Michaelis constant (mM) and $[S]$ and $[I]$ are the concentrations of substrate and inhibitor (mM), assuming that the compounds inhibit uptake competitively.

At 50% inhibition ($r = 2$, $[I] = IC_{50}$), this provides:

$$IC_{50} = \frac{K_i \cdot (K_m + [S])}{K_m}. \quad (2)$$

In the present study of the inhibition of Gly-[^{3}H]L-Pro uptake, $K_m > [S]$ so that $K_i \approx IC_{50}$. Thus, the IC_{50} of competitors may be estimated from:

$$K_i = IC_{50} = \frac{[I]}{(r-1)}, \quad (3)$$

where K_i values were greater than the test concentration used, results are italicised with the compound ideally requiring further testing at a higher concentration in order to produce a more accurate value; very poor interactions are expressed as > 20 mM.

Uptake of Gly-[^{3}H]L-Pro involved simultaneous active and passive processes. These were modelled

by Eq. (4) and data were fitted using non-linear regression (Fig P, version 2.7; Biosoft, Cambridge).

$$V = \frac{V_{max}[S]}{K_m + [S]} + k_d \cdot [S], \quad (4)$$

where: k_d = diffusional rate constant (nmol min^{-1} (mg protein)$^{-1}$ mM^{-1}), K_m = Michaelis–Menten constant (mM), $[S]$ = substrate concentration (mM), v = flux (nmol min^{-1} (mg protein)$^{-1}$), V_{max} = maximum rate of uptake (nmol min^{-1}(mg protein)$^{-1}$).

Analysis of variance with the Dunnett (comparison with control SQ-29852) or Tukey (comparison of all values with each other) post-tests, or t-tests, were used as appropriate.

6. Results and discussion

One of the most extensively characterised cell systems for absorption studies is the Caco-2 cell model, which is claimed to be a positive predictor of intestinal tract drug absorption (Bailey et al., 1996). The fact that only a small amount of drug is required makes the system applicable to early-stage screening in drug discovery and development programmes. Many absorption studies have used Caco-2 cells in a transport model, however in the present work, an uptake model has been selected for a number of reasons. Firstly, the system has been designed as a potential indicator for recognition by the DTS, with the ability to screen a large number of dipeptide-like analogues. An uptake system has advantages over the transport model in this regard as the system is simple, the time required from seeding to experimentation is reduced (formation of tight junctions is not required) and less cells are required for seeding 24-well plates compared to inserts (transport). Thus the uptake system is more cost- and time-effective. Secondly, recognition by the transporter protein at the apical membrane is the first and primary step in the process of a compound being transported through a cell. The uptake model will provide a good indication of recognition by the transporter, although this may not correlate to actual absorption. However, the present model (Fig. 1) is intended as a screen in order to select possible molecules that are most likely to succeed in having good oral permeability due to absorption via the DTS, as indicated through recognition by the transporter.

Questions have been raised regarding the suitability of the Caco-2 cell model to predict the permeability of actively absorbed compounds. The transport of certain drugs such as SQ-29852 has been shown to be under-estimated in these cells (Nicklin et al., 1992a, 1996) resulting in lower predicted oral absorption values when using this model compared to those seen in the in vivo situation (Chong et al., 1996). However, despite the reports of underestimation of transport in the Caco-2 cell model, an uptake model remains a valid screening tool by indicating the extent of interaction with the DTS, and thus useful in selecting potential lead molecules for further in vitro and in vivo characterisation.

The importance of characterising cell lines within specific laboratories when screening molecules and predicting absorption has been highlighted in the literature (Yee, 1997). Therefore, the following experiments were carried out in order to validate and characterise the current system:

1. specific uptake of Gly-[^{3}H]L-Pro by the DTS,
2. kinetics of Gly-[^{3}H]L-Pro uptake,
3. the effect of the cosolvent DMSO on total and specific uptake,

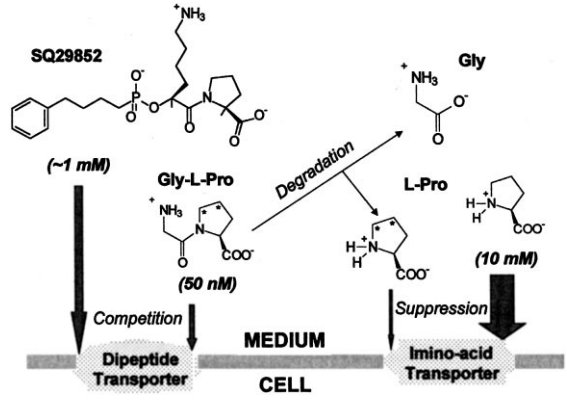


Fig. 1. Schematic representation of the screening system for drug affinities for the intestinal dipeptide transporter. The system is illustrated with the ACE-inhibitor SQ29852.

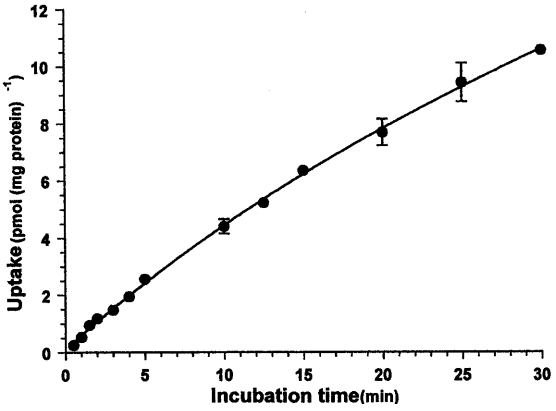


Fig. 2. Uptake of Gly-[^{3}H]L-Pro dependent upon incubation time in 7-day Caco-2 monolayers: The monolayers were washed (1×0.5 ml (Ap) $\times$ 5 min) with M2, and then incubated with M2 containing 40 nM Gly-[^{3}H]L-Pro, 50 nM unlabelled Gly-L-Pro and 10 mM L-Pro over a period of 0.5–30 min. The results (mean $\pm$ S.D. for three monolayers) are expressed as uptake pmol (mg protein)$^{-1}$.

4. concentration dependency of Gly-[^{3}H]L-Pro uptake.

Maximum expression of the DTS in the Caco-2 cells was investigated by monitoring the specific (active) versus non-specific (passive) uptake of Gly-[^{3}H]L-Pro over a 7-day period (age 4–11 days) (data not shown). The passive component was greatest at day 4, contributing $21.30 \pm 2.06\%$ of total uptake at a 5 mM substrate concentration. Throughout the 7-day study period, the non-specific uptake decreased. At 11 days, maximum uptake was observed with the highest specific component (87%) of total uptake, however, after 10 days the monolayers became less adherent to the wells and began to slough off. The specific component was constant between 7–9 days being $> 85\%$ of total uptake, and adhesion to the wells was good; no visible breaks within the monolayer. Therefore, the interval of 7–9 days was selected as the experimental window. The diffusional (passive) component is not saturable and cannot be blocked by excess Gly-L-Pro or competitors and, therefore, the diffusional component for the concentration employed (15%) was subtracted from total uptake when calculating the K_i values.

The kinetic profile of Gly-L-Pro uptake shows a relationship between uptake and time which deviates somewhat from linearity (Fig. 2). After 30 min, only 5.32% of the Gly-[^{3}H]L-Pro in the donor phase had been taken up into the cells, illustrating that the donor phase is not significantly depleted over the time-period studied. A 3-minute incubation period was selected for the competition studies as it falls within the initial linear uptake phase and the relatively short incubation period minimises any hydrolysis of the probe and competitors that may occur. This is important as the radiolabel is found in the proline residue and this amino acid may be taken into the cells by a specific carrier system (Nicklin et al., 1992b) — resulting in intracellular radioactivity not associated with DTS activity. To further minimise this source of error, excess L-Pro (10 mM) was included in the incubation media to block the uptake of the small amount of [^{3}H]L-Pro that could be formed from hydrolysis of the probe during the 3 min incubation period.

DMSO was investigated as a possible cosolvent in order to overcome solubility problems that could be encountered in future experiments with poorly soluble competitors. Concentrations of 1, 2 and 5% DMSO did not significantly reduce the specific uptake of Gly-[^{3}H]L-Pro; however, at 5% DMSO concentration, there was approximately 30% reduction in the total uptake. Therefore, a 2% DMSO concentration was selected due to its having no effect on specific and total uptake of the dipeptide into the cells.

Active uptake/transport systems display a saturable process (concentration-dependency). Previous studies using a variety of tissues and models (e.g. human, mouse and rabbit intestinal BBMV, and Caco-2 cells) have revealed that Gly-L-Pro is transported by a Na^+-independent carrier-mediated process (Rajendran et al., 1985a,b; Wootton, 1986; Eddy et al., 1993). Gly-[^{3}H]L-Pro uptake was reduced considerably, but not eliminated (Fig. 3), in the presence of 10 mM unlabelled Gly-L-Pro (10 000-fold molar excess), indicating the presence of a saturable and non-saturable process, which was quantified by fitting the data to the non-linear equation (Eq. (4)). The kinetic parameters of the Gly-L-Pro uptake were: V_{max}, 0.98 ± 0.04 nmol min^{-1} (mg protein)$^{-1}$; K_m, 0.39 ± 0.02 mM; and k_d, 0.25 ± 0.01 nmol min^{-1}

(mg protein)$^{-1}$ mM^{-1} ($r^2 = 0.9999$). Thus, a saturable process which shows a high affinity for Gly-L-Pro is implicated in the uptake of Gly-L-Pro into Caco-2 cells; this is in agreement with a previous study (Eddy et al., 1993) and other data using different experimental models (Rajendran et al., 1985a,b; Wootton, 1986).

The ACE-inhibitor SQ-29852, which has been shown to be a stable and specific probe for the DTS (Cheung et al., 1980; Nicklin et al., 1992a; Marino et al., 1996; Nicklin et al., 1996), produced significant inhibition of Gly-[^{3}H]L-Pro uptake at 1 mM concentration. The mean ± S.D. inhibition (%) and K_i ± S.D. values for SQ-29852 on 10 different experimental days were 60.51 ± 6.73% and 0.68 ± 0.17 mM respectively, illustrating that the system is reproducible with respect to inhibition by a competitor. To distinguish whether the SQ-29852 is displacing the Gly-[^{3}H]L-Pro in a competitive or non-competitive mechanism, a range of Gly-L-Pro concentrations

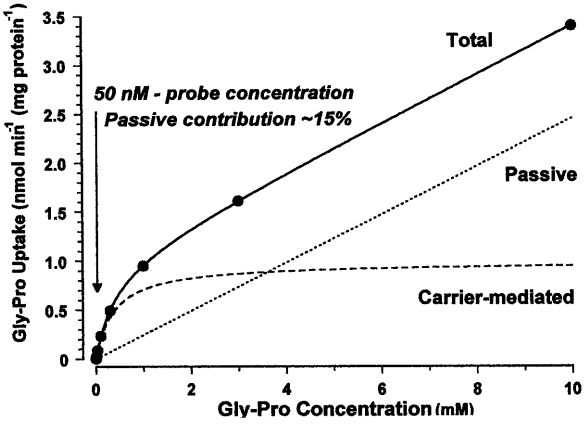


Fig. 3. Concentration-dependency of Gly-[^{3}H]L-Pro uptake: The monolayers were washed (1 × 0.5 ml (Ap) × 5 min) with M2, and then incubated with M2 containing 50 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro in the presence of increasing concentrations of unlabelled Gly-L-Pro (0.001–10 mM). The data points for the total uptake (●) are experimental values and expressed as nmol min^{-1} (mg protein)$^{-1}$, (mean ± S.D. for three monolayers) and provide K_m, 0.389 ± 0.015 mM; V_{max}, 0.983 ± 0.043 nmol min^{-1} (mg protein)$^{-1}$; k_d, 0.245 ± 0.005 nmol min^{-1} (mg protein)$^{-1}$. The carrier-mediated (- - -) and passive (…) components of the total uptake are theoretical plots calculated using the Fig.P computer programme (Biosoft). Error bars are too small to be displayed.

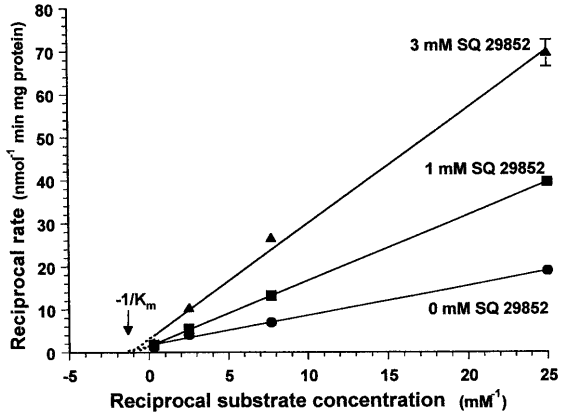


Fig. 4. Lineweaver-Burk plot of SQ-29852 inhibition of Gly-[^{3}H]L-Pro. The monolayers were washed (1 × 0.5 ml (Ap) × 5 min) with M2, and then incubated with M2 containing 50 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro and in the presence of 0 (●), 1(■) or 3 (▲) mM SQ-29852. The results (mean of three monolayers ± S.D.) are corrected for non-specific uptake and are plotted-1/rate (1/v) versus 1/substrate concentration (1/[S])-according to $1/v = K_m/V_{max}\,(1/[S]) + 1/V_{max}$, where [S] = Gly-L-Pro concentration (mM) and the rate units are nmol min^{-1}.

(0.04, 0.13, 0.4, and 3.6 mM) were examined in the presence of 0, (control) 1 and 3 mM SQ-29852. The data were corrected for non-specific uptake (passive) and the results were processed as a Lineweaver-Burk plot. All three lines crossed the y-axis in the same region indicating a probable competitive uptake mechanism (Fig. 4). The equation of the control plot was: $y = 0.681x + 1.42$, giving a K_m of 0.48 mM, which is close to that reported for the concentration-dependency study. The fact that SQ-29852 inhibited Gly-[^{3}H]L-Pro uptake in a competitive manner, supports the use of K_i values, where inhibition is assumed to be competitive.

As Gly-L-Pro is a weak ACE inhibitor (K_i = 450 μM at pH 8.3) (Krapcho et al., 1988) possible binding of the dipeptide to ACE, which is expressed in a heterogeneous mosaic pattern on the apical membrane of Caco-2 cells (Howell et al., 1993) may occur. Uptake in the control (50 nM Gly-[^{3}H]L-Pro) was 0.56 ± 0.08 pmol mg protein^{-1} min^{-1} and in the presence of 1 μM fosinoprilat, an ACE-inhibitor with a high affinity for the enzyme, was 0.61 ± 0.03 pmol mg

protein^{-1} min^{-1}. These results illustrate that, at the concentration used in the current experiments, Gly-[^{3}H]L-Pro is not binding to ACE to a significant extent.

The substrate specificity of the DTS was explored by comparing the uptake of Gly-[^{3}H]L-Pro (50 nM) alone or in the presence of various amino acids and a tetrapeptide (Table 1). Uptake in the presence of 1 mM of the amino acids Gly, L-Phe, L-Lys, Sar and L-Ser was not significantly different from the control value ($P > 0.05$), thus suggesting that uptake does not occur via these specific amino acid transport systems (Christensen, 1990).

The tetrapeptide, Gly-L-Pro-Gly-Gly, produced significant inhibition in Gly-[^{3}H]L-Pro uptake, $41.68 \pm 0.77\%$, giving an K_i value of 1.40 ± 0.04 mM. Literature on the transport of tetrapeptides by the DTS is somewhat controversial. Originally, the DTS has only been reported to transport small peptide units such as di- and tri-peptides (Addison et al., 1975). Three mechanisms may possibly be responsible for the observed inhibition. Tetrapeptides are the preferred substrates for the brush-border proteases (Kania et al., 1972) and it may be that Gly-L-Pro-Gly-Gly is being hydrolysed into subunits (di- and tri-peptides), and it is these that are responsible for inhibition of uptake. However, L-Pro- and Gly-containing peptides are hydrolysis-resistant, suggesting that the tetrapeptide may be a substrate for recognition by the DTS, but not necessarily a substrate for transport. A membrane glycoprotein of 127 kDa, which is directly involved in the uptake of small peptides and orally active cephalosporins has been located in the brush-border membrane of enterocytes of rabbits (Kramer et al., 1990). A protein of similar molecular weight, 120 ± 10 kDa, has also been isolated from the membrane of Caco-2 cells (Dantzig et al., 1994). Tri/tetra/pentaglycine have been shown to inhibit the photoaffinity labelling of the 127 kDa membrane glycoprotein and also cephalexin uptake (Kramer et al., 1995). The inhibitory effect was related to the length of the peptide chain, with higher numbers of amino acid residues resulting in a reduced effect. This evidence suggests that the DTS is capable of transporting tetrapeptides, or that the tetrapeptide may simply be blocking the binding site on the DTS by fitting into the receptor site, and thus preventing the probe from being taken up into the cell.

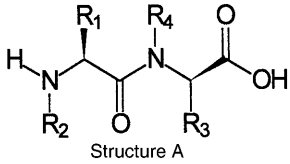
The ability of our method to rank affinity of compounds for interaction with the DTS was investigated by co-administrating a series of dipeptides, at 1 mM, with the probe. All of the dipeptides tested produced significant inhibition of the probe (Table 2) with K_i values ranging from 0.13 ± 0.02 mM to > 1 mM, suggesting that they all share the same dipeptide carrier to some extent. Gly-DL-Met and Gly-DL-Phe caused the greatest inhibition of Gly-[^{3}H]L-Pro uptake, all producing similar degrees of activity, $88.66 \pm 1.39\%$ and $86.11 \pm 2.38\%$ inhibition, respectively,

Table 1
Specificity study, Gly-[^{3}H]L-Pro uptake in the presence of various compounds which have different methods of transport across the intestinal enterocytes[a]

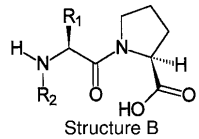
Competitor	Structural type	Mechanism of transport	Inhibition (%) [S.D.]
Gly-[^{3}H]L-Pro (Control)	Dipeptide	H^+/dipeptide system	0.00 [3.14]
Gly	Amino acid	Amino acid transporter	3.43 [7.81]
L-Lys	Amino acid	Amino acid transporter	−2.06 [1.29]
L-Phe	Amino acid	Amino acid transporter	−2.58 [6.24]
Sar	Amino acid	Amino acid transporter	−12.05 [8.47]
L-Ser	Amino acid	Amino acid transporter	0.30 [3.24]
Gly-L-Pro-Gly-Gly	Tetrapeptide	Hydrolysis, absorption Absorption via DTS	41.68 [0.77]

[a] Monolayers were washed (1×0.5 ml (Ap) $\times$ 5 min) with M2. They were incubated with M2 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2% DMSO at pH 6 or in the presence of 1 mM of the competitor. Data are corrected for 15% non-specific uptake and presented as mean% inhibition $\pm$ S.D. for three monolayers. All the amino acids tested had no significant interaction with the DTS. Gly-L-Pro-Gly-Gly has significant inhibition at the confidence limits tested ($P < 0.0001$), producing an K_i value 1.4 mM.

Table 2
The effect of a series of dipeptides (1 mM) on the uptake of 50 nM Gly-[^{3}H]L-Pro[a]



Structure A



Structure B

Dipeptide	Structure	R_1	R_2	R_3	R_4	Inhibition% [S.D.]	K_i mM [S.D.]
Gly-DL-Met	A	H	H	$(CH_2)_2SCH_3$	H	88.66 [1.39][b]	0.13 [0.02]
Gly-DL-Phe	A	H	H	$CH_2C_6H_5$	H	86.11 [2.38][b]	0.16 [0.03]
Gly-Sar	A	H	H	H	CH_3	72.13 [1.65][b]	0.39 [0.03]
L-Lys-L-Pro	B	$(CH_2)_4NH_2$	H	–	–	64.62 [2.17][b]	0.55 [0.05]
L-Asp-L-Asp	A	CH_2COOH	H	CH_2COOH	H	45.83 [4.02][c]	*1.19 [0.20]*
Gly-L-Tyr	A	H	H	$CH_2C_6H_4OH$	H	41.37 [3.12][b]	*1.43 [0.18]*
L-Phe-L-Pro	B	$CH_2C_6H_5$	H	–	–	36.77 [1.29][b]	*1.72 [0.09]*
L-Glu-L-Glu	A	$(CH_2)_2COOH$	H	$(CH_2)_2COOH$	H	36.05 [2.00][c]	*1.78 [0.15]*
Sar-L-Pro	B	H	CH_3	–	–	22.81 [4.53][d]	*3.51 [0.97]*

[a] Monolayers were washed (1×0.5 ml (Ap) $\times 5$ min) with M2. They were incubated with M2 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2% DMSO at pH 6 or in the presence of 1 mM of dipeptide. Data are corrected for 15% non-specific uptake and presented as mean% inhibition $\pm$ S.D. ($n = 3$) and mean $K_i \pm$ S.D. mM, with superscripts b, c and d demonstrating significant inhibition at $P < 0.0001$, $P < 0.001$ and $P < 0.01$ respectively. K_i values are only indicative as stability studies on the competitors were not undertaken so the absence of degradation of the peptide cannot be confirmed — although the three-minute incubation period should minimise any such changes. It should also be noted that inhibition of Gly-[^{3}H]L-Pro uptake does not necessarily indicate that the competitor is a substrate for the transporter.

which were not significantly different from each other. The observed K_i value for Gly-Sar, 0.39 ± 0.03 mM, was in the same range as previously reported in Caco-2 cells, 0.6 ± 0.3 M (Covitz et al., 1996). Gly-Sar had less affinity for the transporter than Gly-L-Phe when co-administered with Gly-[^{3}H]L-Pro in human intestinal BBMV (Rajendran et al., 1985a). Our results were in agreement with this and the same rank order of inhibition was observed as previously reported (Rajendran et al., 1985a). L-Lys-L-Pro, which possesses a net positive charge, produced a very similar inhibition to that seen with the L-Lys-L-Pro analogue SQ-29852, 64.62 ± 2.17% and 60.51 ± 6.73% inhibition respectively. Sar-L-Pro which lacks a primary N-terminal α-amino group produced inhibition of the Gly-[^{3}H]L-Pro, but to a lesser extent (22.81 ± 4.53% inhibition) than the other compounds tested which all possess this structural feature, agreeing with previous literature that a free α-amino group is not essential for recognition (Bai et al., 1991). The diacidic dipeptides, L-Asp-L-Asp and L-Glu-L-Glu, which have a net negative charge both caused inhibition of Gly-[^{3}H]L-Pro uptake, however to a lesser extent than the zwitterionic dipeptides. This contradicts a previous report (Hidalgo et al., 1995) which states that there is no difference in interaction between linear dipeptides, regardless of charge, with the transporter. However, the study in question used a different radiolabel ([^{3}H]cephalexin) and Caco-2 cells at a higher passage number (60–70). The competitors were used at a concentration of 10–20 mM which may saturate inhibition, masking the differential activity of dipeptides, producing results which appear to be the same for the linear dipeptides tested. L-Asp-L-Asp, Gly-L-Tyr, L-Phe-L-Pro, L-Glu-L-Glu and Sar-L-Pro all produced K_i values which were greater than the test concentration of 1 mM.

The present system allows apparent affinity differences to be seen by using a lower competitor

concentration, and highlights the importance in selecting the appropriate screening concentration of competitors. The importance of using the correct competitor concentration is demonstrated by comparing the present study to previous data, which have suggested that there are no differences in the affinities of linear dipeptides for the transporter (Hidalgo et al., 1995). However, in the current system, the use of lower concentrations (1 mM) of dipeptides has allowed differences in affinities of diacidic and zwitterionic dipeptides for the transporter to be demonstrated. Results for the series of dipeptides illustrate that structurally similar compounds can be ranked with regard to affinity for the recognition site of the DTS. However, experimental design and conditions must be such as to allow competitors to be administered at concentrations that do not produce saturation of the carrier. This provides a system where structure-activity relationships of the DTS can be evaluated with the possibility of identifying the optimum structure for interaction.

The cephalosporins and penicillins, which share several physicochemical features with dipeptides such as the α-amino and carboxyl groups, and the zwitterionic state at physiological pH, have been reported to have an active uptake component mediated by the DTS (Dantzig and Bergin, 1990; Inui et al., 1992; Oh et al., 1992; Gochoco et al., 1994; Dantzig, 1997). However, unlike the dipeptides, they are resistant to hydrolysis by proteases. There is evidence to suggest that, for these groups of orally administered antibiotic drugs, the DTS contributes to their absorption (Dantzig, 1997). However, they have been reported to have a lower affinity for the carrier (e.g. cephalexin $K_m = 7.25 \pm 2.8$ mM) compared to its natural dipeptide substrates (Dantzig and Bergin, 1990).

No significant inhibition of Gly-[^{3}H]L-Pro uptake was seen with cephalexin at a 1 mM concentration (data not shown), suggesting that it has a lower affinity for the DTS, as suggested in the literature. Therefore, this series of compounds were tested at a higher concentration (10 mM) to enhance potential inhibition. All of the compounds demonstrated some degree of inhibition of the probe, producing a large range of K_i values from 4.77 ± 1.33 mM to >20 mM (Table 3), although activity was less than that seen with the dipeptide series. This may be due to the fact that a portion of the antibiotic structure resembles that of the unnatural dipeptide, D-alanine-D-alanine (D-Ala-D-Ala), and the DTS has been shown to have a preference for the dipeptides containing the L-enantiomers of the amino acids (Hidalgo et al., 1995; Moore et al., 1997).

The cephalosporins produced a greater extent of inhibition (68.08 ± 5.83–33.47 ± 6.89%) than the penicillins. Our results are in agreement with a previous study where cefaclor, cephalexin and cephradine have been reported to have K_m values in the same range in the Caco-2 model, 7.6, 7.5 and 8.3 mM respectively. Cefaclor, a close analogue of cephalexin, was the most active compound with a K_i of 4.77 ± 1.33 mM. Cephradine showed slightly better affinity for the transporter than cephalexin (K_i of 7.72 ± 0.69 mM compared to 9.55 ± 0.51 mM). Surprisingly, the penicillins, ampicillin and amoxicillin produced inhibition (%) values that were not significantly different from one another ($P > 0.05$). In a study using a rat intestine model, Oh et al. (1992) demonstrated amoxicillin to have a K_m of 0.058 ± 0.026 mM, and ampicillin to have $K_m = 15.8 \pm 2.92$ mM. The large difference in affinities (K_m values) was not shown in the present study. However, a recent study has shown that mucosal to serosal permeation rates of ampicillin and amoxicillin across rat jejunum are similar, 0.477 ± 0.066 nmol min^{-1} and 0.548 ± 0.031 nmol min^{-1} respectively (Saitoh et al., 1997).

Cefazolin has been reported to be passively absorbed in Caco-2 cells (Thwaites et al., 1994) yet our system illustrates that it appears to have an affinity for the DTS recognition site similar to that of the actively transported penicillins. This suggests that cefazolin may be blocking uptake of the probe by binding to the recognition site, or possibly the presence of another uptake system for the penicillins. Ampicillin, amoxicillin and cefazolin all produced K_i values which were greater than 20 mM (test concentration), therefore further testing, at a higher concentration of competitor, is required in order to produce accurate K_i values.

7. Conclusion

Gly-L-Pro, a relatively stable probe, is a substrate for the DTS in Caco-2 cells, displaying a high affinity for the transporter, as indicated by the concentration-dependency study, which gives a K_m of 0.39 ± 0.02 mM. The inhibition profile produced with a series of dipeptides and β-lactam antibiotics indicated that a common uptake mechanism is being shared. The ACE-inhibitor, SQ-29852, produced inhibition of the Gly-[^{3}H]L-Pro in a competitive manner, and the K_m value from the control of this study (0.48 mM) is in agreement with that from a concentration-dependency study. The system is reproducible with regard to inhibition by a probe compound of the DTS (SQ-29852) and has been developed as a rapid and unique screening system for evaluating the structural features implicated in recognition by the DTS, by measuring the ability of large numbers of dipeptide-like analogues to inhibit uptake of the probe (Gly-[^{3}H]L-Pro). This system to allows structurally related compounds to be ranked with regard for interaction with the DTS recognition site by using a lower competitor concentration to prevent saturation of the system and, thus, will prove useful in the selection and screening of possible oral drug candidates.

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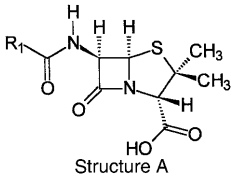
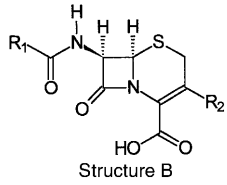
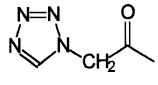
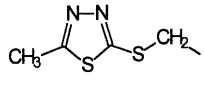
Table 3
The effect of a series of β-Lactam antibiotics (10 mM) on the uptake of 50 nM Gly-[^{3}H]L-Pro[a]



Structure A



Structure B

Compound	Structure	R_1	R_2	Inhibition% [S.D.]	K_i mM [S.D.]
Cefaclor	A	$C_6H_5CH(NH_2)$	Cl	68.08[5.83][b]	4.77 [1.33]
Cephradine	A	$C_6H_7CH(NH_2)$	CH_3	56.49[2.24][b]	7.72 [0.69]
Cephalexin	A	$C_6H_5CH(NH_2)$	CH_3	51.19[1.32][b]	9.55 [0.51]
Cefazolin	A			33.47[6.89][c]	>20
Ampicillin	B	$C_6H_5CH(NH_2)$	–	21.62[6.20][d]	>20
Amoxicillin	B	4-HO-$C_6H_4CH(NH_2)$	–	18.97[4.06][c]	>20

[a] Monolayers were washed (1×0.5 ml (Ap) $\times$ 5 min) with M2. They were incubated with M2 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2% DMSO at pH 6 or in the presence of 10 mM cephalosporin. Data are corrected for 15% non-specific uptake and presented as mean% inhibition $\pm$ S.D. for three monolayers and mean $K_i \pm$ S.D. mM, with superscripts b, c and d demonstrating significant inhibition at $P<0.0001$, $P<0.01$ and $P<0.05$ respectively. K_i values are only indicative as stability studies on the substrates were not undertaken so the absence of degradation of the lactams cannot be confirmed — although the three-minute incubation period should minimise any such changes. It should also be noted that inhibition of Gly-[^{3}H]L-Pro uptake does not necessarily indicate that the competitor is a substrate for the transporter.

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