Transport Characteristics of L-Carnosine and the Anticancer Derivative 4-Toluenesulfonylureido-Carnosine in a Human Epithelial Cell Line

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Purpose. The aim of the present study was to evaluate whether the transepithelial transport of the anticancer compound 4-toluenesulfonylureido-carnosine (Ts-carnosine) and the dipeptide moiety L-carnosine was due to a hPepT1 carrier-mediated flux.

Methods. Transport experiments were conducted using Caco-2 cell monolayers and either reversed-phase HPLC-UV or liquid scintillation counting methods for quantification. pK_a, LogD, and LogP were determined using the Sirius $GlpK_a$ meter.

Results. L-carnosine was transported across the apical membrane with a $K_{m,app}$ of 2.48 ± 1.16 mM and a V_{max} of 2.08 ± 0.34 $nmol \cdot cm^{-2} \cdot min^{-1}$ and across the basolateral membrane with a $K_{m,app}$ of 7.21 ± 3.17 mM and a V_{max} of 0.54 ± 0.10 $nmol \cdot cm^{-2} \cdot min^{-1}$, and transepithelially with a P_{app} of $4.46 \cdot 10^{-2} \pm 6.4 \cdot 10^{-6}$ $cm \cdot min^{-10}$. Ts-carnosine had an affinity (K_i) for hPepT1 of 2.33 ± 0.54 mM; however, the transepithelial transport was low as compared to that of L-carnosine.

Conclusions. L-carnosine was transported across both the apical and basolateral membrane of Caco-2 cell monolayers in a carrier-mediated manner however, the transepithelial transport followed apparent simple non-saturable kinetics. Ts-carnosine had an affinity for hPepT1 but a relatively low transepithelial transport. This indicates that the transepithelial transport of L-carnosine and Ts-carnosine is not hPepT1 carrier-mediated and that L-carnosine is not a suitable dipeptide moiety for hPepT1-mediated absorption of sulfonamide-type anticancer compounds.

KEY WORDS: L-carnosine; PepT1; hPepT1-mediated transport; sulfonamide-type anticancer compounds.

INTRODUCTION

Some recent studies by the group of Supuran and Scozzafava showed that some sulfonamide carbonic anhydrase inhibitors are very potent *in vitro* tumor growth inhibitors in a variety of tumor cell lines (1,2). In another study, a series of toluenesulfonylureido (Ts) derivatives of amines, amino acids, and dipeptides were also shown to possess such an action (3). The dipeptide derivatives of Ts are very interesting from a drug delivery viewpoint, because they might be substrates for the intestinal di/tri-peptide transporter PepT1. PepT1 is a di/tripeptide-proton co-transporter located in the apical membrane of enterocytes in the small intestine. PepT1 mediates the uptake of di- and tripeptides originating from protein hydrolysis in the gut lumen as well as a number of peptidomimetic drug compounds, such as β-lactam antibiotics, bestatin, and valaciclovir (4–6). It has also been shown that some cancer cell lines express PepT1, whereas PepT1 is not expressed in the surrounding normal tissue (8). Recently, Nakanishi *et al.* have developed a mouse model that shows the selective delivery of bestatin and L-carnosine to tumors by specific PepT1 transport activity (11). Thus, the approach of using dipeptide promoieties to target suitable anticancer compounds to cancer cells is appealing especially as it may also improve oral absorption via the peptide transporter.

L-carnosine (β-Ala-His) was one of the dipeptides used in the study of Mastrolorenzo *et al.* (3). Ts was linked via an amide bond to the N-terminus of L-carnosine (Fig. 1). L-carnosine is a commonly used substrate for hPepT1 due to its high stability and hPepT1-mediated uptake and L-carnosine has been characterized in a number of studies (12–15). Several studies have shown that a free dipeptide N-terminus is not absolutely essential for recognition by PepT1 and that various N-terminal modifications are accommodated by the transporter (16). The hypothesis for the present study is thus that Ts-carnosine may be transported across epithelial cells via a hPepT1-mediated process. However, many factors such as ionization state, logD-values and hydrolysis of Ts-carnosine as well as other involved carriers may influence its transepithelial transport.

The aim of the present study was therefore to evaluate whether transepithelial transport of the anticancer compound Ts-carnosine in a human intestinal cell line (Caco-2) was due to a flux via a hPepT1 carrier-mediated pathway. In this study we therefore investigated transport parameters of Ts-carnosine, as compared with those of L-carnosine alone.

MATERIALS AND METHODS

Materials

The 4-toluenesulfonylureido derivative of L-carnosine (Ts-carnosine) was synthesized by Mastrolorenzo *et al.* as described previously (3). Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell culture media and Hanks balanced salt solution (HBSS) were obtained from Life Technologies (Høje Taastrup, Denmark). All HPLC solvents were of analytical grade, and chemicals used in buffer preparations were of laboratory grade. 2-(N-morpholino) ethanesulfonic acid (MES) and N-[2-Hydroxyethyl] piperazineN′-[2-ethanesulfonate] (HEPES), verapamil, digoxin, L-carnosine, and bovine serum albumin (BSA) were from Sigma (Saint Louis, MO, USA). Potassium hydroxide 0.5M, ISA-water 0.16M KCl, octanol (saturated), methanol (standardized ion strength) were from Merck (Damstadt, Germany). Hydrogen chloride 0.5M was from Bie & Berntsen A/S (Rødovre/Højbjerg, Denmark). [^{14}C] glycylsarcosine ([^{14}C]Gly-Sar) with a specific activity of 49.94 mCi/mmol, [^{14}C]mannitol with a specific activity of 51.50 mCi/mmol, and [^{3}H]testosterone with a specific activity of 7.90 Ci/mmol were from New England Nuclear (Boston,

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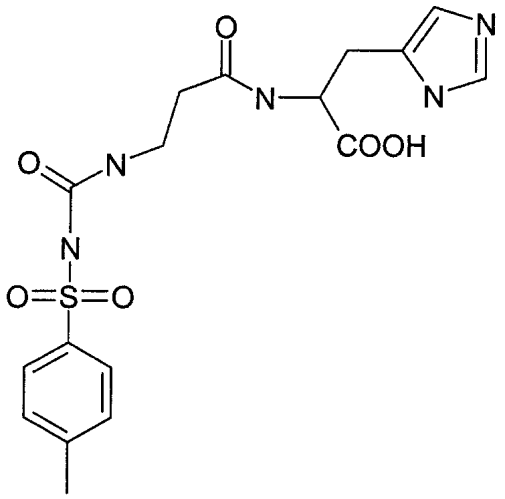

Fig. 1. Structure of 4-Toluenesulfonylureido-β-Ala-His (Ts-carnosine).

Massachusetts). Ultima Gold was from Packard (Groningen, The Netherlands). Human plasma was kindly donated by The State University Hospital (Copenhagen, Denmark).

Apparatus

LogP and pK_a values were determined using Sirius $GlpK_a$-meter from Sirius Analytical Instruments Ltd. (East Sussex, UK) equipped with Sirius pK_aLOGP^{TH}, version 5.2a software from Sirius Analytical Instruments Ltd. (East Sussex, UK). High-performance liquid chromatography (HPLC) was performed with a Waters Spherisorb S5OdS2 reversed-phase column (5 μm, 250 × 4.6 mm) in a Merck/Hitachi system consisting of an L-6000 pump, an L-7450 diode array detector, operated at 225 nm. Transepithelial electrical resistance (TEER) was measured in a tissue resistance measurement chambers (Endohm) with a voltohmmeter (EVOM), both from World Precision Instruments (Sarasota, FL, USA). The shaking plate used for cell culture experiments was from SWIP (SWIP, EB).

Cell Culture

Caco-2 cells were cultured as previously described (21). Briefly, cells were seeded in culture flasks and passaged in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10%fetal bovine serum, penicillin/streptomycin (100 $U \cdot ml^{-1}$ and 100 $\mu g \cdot ml^{-1}$, respectively), 1% L-glutamine, and 1% non-essential amino acids. Cells were seeded onto tissue culture treated Transwells (1.0 cm^2, 0.4 μm pore size) at a density of 10^5 $cells \cdot cm^{-2}$. TEER at room temperature was measured before the experiment. Normal TEER values were from 300–500 $\Omega \cdot cm^2$ depending on passage number. Dipeptide transport activity reached a steady maximal level at day 24–30. Transport experiments were subsequently performed on day 26–28 after seeding.

Affinity Experiments

The buffers used for cell experiments were HBSS supplemented with 0.05% BSA and 10 mM MES (pH 6.0) (MES-Buffer) and 10 mM HEPES (pH 7.4) (HEPES-buffer). Cells were incubated 15 min on the apical and basolateral side with MES- and HEPES-buffer, respectively. The affinity experiment was initiated by adding 0.5 ml MES-buffer containing 0.5 μCi [^{14}C]-Gly-Sar and various amounts of Ts-carnosine (0–10 mM) to the apical side. 1.0 ml HEPES-buffer was added to the basolateral side of each cell monolayer, and the cells were incubated with the buffers for 5 min. During incubation the plates were circularly and continuously shaken. In all experiments, Ts-carnosine was only added on the apical side. The temperature was maintained at 37°C. The uptake of 0.5 μCi [^{14}C]Gly-Sar was terminated by gentle suction of the uptake medium followed by four washes of the monolayers with ice-cold HBSS. Following the washing step the cells were cut from the Transwell support placed into scintillation vials, and 2 ml of scintillation fluid was added. The cell-associated radioactivity was counted using a Packard Tri-Carb 2100TR liquid scintillation analyzer (Meriden, Connecticutt).

L-Carnosine Uptake Experiments

Uptake of [^{3}H]L-carnosine was initiated as described for the affinity experiments. The experiment was then started by adding fresh apical buffer containing the relevant L-carnosine concentration (0–10 mM) and 0.5 μCi[^{3}H]L-carnosine per well, or fresh basolateral buffer containing the relevant L-carnosine concentration (0–20 mM) for basolateral uptake experiments. Apical uptake of L-carnosine into the cells was terminated after 5 min and basolateral uptake after 15 min by gentle suction of the uptake medium, followed by four washes of the monolayers with ice-cold HBSS. For basolateral uptake experiments the uptake of L-carnosine was corrected for the amount of L-carnosine in the extracellular space (using [^{14}C]-mannitol as a marker of the extracellular volume). The polycarbonate filters were cut from the Transwell supports and placed into scintillation vials. Two ml of scintillation fluid was added and the radioactivity was counted in a liquid scintillation analyzer.

Transport Experiments

Apical (A) to basolateral (B) and B to A fluxes of Ts-carnosine was measured in MES- and HEPES buffers. The concentration of Ts-carnosine on the donor (*cis*) side was 15 mM in all experiments. Ten μl samples were taken from the donor solution at t = 0 and 150 min and 100 μl samples at 30 min intervals from the receiver solution and replaced with fresh buffer (t = 60, 90, 120, 150 min). The transport of Ts-carnosine was also measured in the presence of 20 mM Gly-Pro on the donor side; 100 μM of digoxin or verapamil on both donor and receiver side, respectively. Samples were transferred to HPLC vials and analyzed by HPLC-UV as described below. Fluxes were constant after 60 min. The steady state flux values were thus obtained as the means of the flux values at 90, 120, and 150 min. After the experiment the integrity of the Caco-2 cell monolayers was evaluated by [^{14}C] mannitol and [^{3}H]testosterone transport studies. Samples were taken from the donor chamber (10 μl) at 20, 40, and 60 min and from the receiver chamber at 0, 20, 40, and 60 min. The mannitol permeability was calculated as described by Smith *et al.* (22), and the permeability of testosterone was calculated using non-steady-state calculations due to the rapid transport rate of testosterone, which causes a rapid change in

the initial donor concentration. The permeability of both testosterone and mannitol was unaffected by Ts-carnosine, digoxin and verapamil and had P_{app} values of 0.1 ± 0.03 cm/h and 0.005 ± 0.0015 cm/h, respectively. The transport of Ts was evaluated at three concentrations; 0.15, 1.5, and 15 mM where no detectable transport of Ts was observed.

The L-carnosine transport experiment was initiated by adding fresh apical buffer containing varying amounts of L-carnosine (0–15 mM), 0.5 μCi [^{3}H]L-carnosine per well and 0.5 μCi [^{14}H] mannitol per well. Twenty μl samples were taken from the apical solution at t = 0, 60, and 120 min. One hundred μl samples were taken at 15 min intervals from the basolateral solution and replaced with fresh buffer (t = 0, 15, 30, 45, 120 min).

Hydrolysis in Aqueous Transport Buffer Solutions, Porcine Jejunal Homogenate and 80% Human Plasma

The degradation of Ts-carnosine was studied at 37°C in porcine jejunal homogenate, 80% human plasma, and in aqueous MES and HEPES transport buffer solutions as described previously (23). During a 2-day incubation in homogenate and human plasma no detectable decrease in initial concentration was observed; also no degradation was seen for 1 week in transport buffers.

HPLC-UV Analysis of Ts-Carnosine

Ts-carnosine was analyzed using a mobile-phase system consisting of 80% 0.02 M phosphate buffer, 20% methanol, and 0.1% triethylamine (TEA) adjusted to pH 4.5 giving retention times of Ts-carnosine and Ts of approximately 9 min and 19 min, respectively. The flow rate was 1 ml · min. The detection and quantification limits (DL and QL, respectively) for Ts-carnosine and Ts on the L-7450 diode array detector were calculated from the standard curves using: QL: 10 · (Intercept/slope) and DL: 3.3 · (Intercept/slope). The QL was 41, and 111 μM for Ts-carnosine and Ts, respectively, DL was 14, and 37 μM for Ts-carnosine and Ts, respectively.

Analysis of pK_a and LogP Values

The pK_a values for L-carnosine, Ts, and Ts-carnosine were determined by dissolving 2, 2, and 1.5 mg solid compound, respectively, in 10 ml ionic strength adjusted water (ISA-water) adjusted to 0.16 M with respect to KCl. The solution was titrated from pH 1.8 → 12.2. The same procedure was repeated in the pH range 2.5 → 10.5. The LogP values of L-carnosine, Ts, and Ts-carnosine were determined by dissolving 2, 2, and 1.5 mg solid compound, respectively, in 10 ml ISA-water and 10 ml or 15 ml octanol. The solution was titrated in the pH range 2.5 → 10.5. The determinations were done at 25°C in duplicates or triplicates.

Calculation of K_i, V_{max} and K_m

Affinity for hPepT1 in Caco-2 cells was determined as inhibition of [^{14}C] Gly-Sar uptake in the presence of varying concentrations of competing compound. The degree of inhibition was fitted to a Michaelis-Menten type Equation:

$$1 - (U / U_0) = \frac{(1 - (U / U_0))_{max} \cdot [I]}{IC_{50} + [I]} \quad (1)$$

where U = uptake of [^{14}C]Gly-Sar, U_0 = uptake of [^{14}C] Gly-Sar at zero inhibitor concentration, IC_{50} = the inhibitory constant (mM), [I] = concentration of the competing compound (mM). K_i values were calculated as described by Cheng and Prusoff (24).

Uptake of L-carnosine as a function of apical or basolateral L-carnosine concentration was fitted to a Michaelis-Menten type equation:

$$J = \frac{J_{max} \cdot [S]}{K_m + [S]} \quad (2)$$

where J = flux ($nmol \cdot cm^{-2} \cdot min^{-1}$), J_{max} = maximal flux ($nmol \cdot cm^{-2} \cdot min^{-1}$), K_m = the Michaelis constant (mM), [S] = L-carnosine (mM).

Statistical Analysis

Values are given as means ± SE unless otherwise stated. The statistical significance of the results was determined using two-tailed Students *t* test. Experiments with Caco-2 cell monolayers were performed in duplicates or triplicates in each cell passage, using several passages, where n was the number of cell passages used (n = 2–5). Experiments on stability were performed with n = 3.

RESULTS

In the present study we evaluated the stability, logP and logD and the transcellular transport of the antitumor derivative 4-toluenesulfonylureido-carnosine, and L-carnosine, and compared the transport characteristics of the two compounds in order to investigate whether Ts-carnosine was transported via hPepT1 in Caco-2 cell monolayers.

Determination of pK_a, logP and logD Values of 4-Toluenesulfonylureido-Carnosine

The pK_a, logP, and logD values were determined for L-carnosine, 4-toluenesulfonylurea, and 4-toluenesulfonylureido-carnosine at 25°C (Table I).

L-carnosine has three pK_a values, pK_{a1} due to the terminal amino group, pK_{a2} due to the histidine side chain, and pK_{a3} due to the terminal carboxy group. L-carnosine is predominantly (~85%) on its positive form (net charge +1) at pH 6.0, with a smaller fraction (~15%) being on the zwitterionic form. At pH 7.4 L-carnosine is predominantly (~80%) on the zwitterionic form, with a smaller fraction (~20%) being on the positive form. $logP_1$ is fairly low, while $logP_2$ and $logP_3$ are higher and identical. LogD values of L-carnosine at pH 6.0 and pH 7.4 (the pH values of the experimental solutions) are also similar. Ts has a N-H acidic proton (pK_a ~9.9), and therefore Ts-carnosine has three pK_a values as well. Ts is neutral at pH 6.0 and pH 7.4 and therefore has similar logD values (Table I). Ts-carnosine is predominantly (~70%) on the zwitterionic form at pH 6.0, with a minor fraction (~30%) on the anionic form (net charge –1). At pH 7.4 Ts-carnosine is predominantly (~90%) on the anionic form (net charge –1), with a smaller fraction (~10%) being on the zwitterionic form. The logD value of Ts-carnosine at pH 6.0 and pH 7.4 are fairly similar and Ts-carnosine has a higher lipophilicity than L-carnosine and Ts alone (Table I).

Table I. pk_a, LogP, and Values for L-Carnosine, Ts, and Ts-Carnosine at 25°C

Constant	L-carnosine	4-Ts	Ts-carnosine
pK_{a1}	9.36	9.90	9.56
pK_{a2}	6.78	—	6.62
pK_{a3}	2.76	—	2.56
LogP1	–0.23	0.90	1.85
LogP2	0.23	—	1.59
LogP3	0.23	—	1.29
$LogD_{6.0}$	–0.23	0.90	1.82
$LogD_{7.4}$	–0.23	0.90	1.67

Note: Determinations were performed in duplicate or triplicates. $LogP_{1-3}$ are the logP values for the species of compound titrated from acidic to basic environment. Values are reported as means.

Stability of 4-Toluenesulfonylureido-Carnosine

The aqueous stability of Ts-carnosine in transport buffers (at pH 6.0 and pH 7.4), porcine jejunal homogenate and 80% human plasma was investigated at 37°C. Ts-carnosine was relatively stable since no degradation was observed in the buffers over 1 week. Similarly, no degradation was seen over a 2-day period in porcine jejunal homogenate or 80% human plasma (data not shown).

Transport Parameters of L-Carnosine

To characterize the transport of L-carnosine across Caco-2 cells we measured initial uptake across the apical and basolateral membranes and steady-state transepithelial flux. The apical uptake of ^{3}H-labeled L-carnosine was determined at varying L-carnosine concentrations, as described in the Methods section. Apical uptake as a function of apical L-carnosine concentration obeyed Michaelis-Menten kinetics, implying a carrier-mediated uptake (Fig. 2 upper curve). Data were fitted to the Michaelis-Menten equation [Eq. (2)] and kinetic constants were obtained. $K_{m,app}$ was 2.48 ± 1.16 mM and V_{max} was 2.08 ± 0.34 nmol · cm^{-2} · min^{-1} (n = 4). Basolateral uptake displayed similar kinetics (Fig. 2, middle curve) and fitting of uptake values to Eq. (2) yielded a $K_{m,app}$ value of 7.21 ± 3.17 mM and a V_{max} value of 0.54 ± 0.10 nmol · cm^{-2} · min^{-1} (n = 4). Transepithelial flux of L-carnosine from the apical to the basolateral solution as a function of apical L-carnosine concentration showed a linear relationship, implying simple diffusion (Fig. 2 lower curve), which may be described by Ficks law, flux (J) = permeability (P) · concentration gradient (ΔC). The transepithelial flux, J_{a-b}, (in nmol · cm^{-2} · min^{-1}) is then given by the relationship $J_{a-b} = 0.456 \pm 0.006 \cdot [\text{L-carnosine(mM)}] + 0.12 \pm 0.05$ (R = 0.9996). This corresponds to a P_{app} of $4.46 \cdot 10^{-2} \pm 6.4 \cdot 10^{-6}$ cm · min^{-1}.

Transport Parameters of 4-Toluenesulfonylureido-Carnosine

Uptake of [^{14}C]Gly-Sar in Caco-2 cells was determined in the presence of varying concentrations of Ts-carnosine (Fig. 3). The uptake of [^{14}C]Gly-Sar decreased in the presence of increasing concentrations of Ts-carnosine. The inhibition constant (K_i) of Ts-carnosine was calculated from the data in Fig. 3 using Eq. (1). K_i was 2.33 ± 0.54 mM and the maximal inhibition of [^{14}C]Gly-Sar uptake, calculated from the fit, was 95 ± 9% (Fig. 3). This indicates that Ts-carnosine indeed binds to hPepT1.

Transepithelial flux of Ts-carnosine from the apical to the basolateral (A to B) solution was measured (Fig. 4). The A to B flux of 15 mM Ts-carnosine was relatively low (~0.17 nmol · cm^{-2} · min^{-1}), and was not affected by changing pH from 6.0 to 7.4 or the presence of 20 mM Gly-Pro on the apical side. This could indicate either the absence of an hPepT1-mediated flux component, or the presence of an active efflux mechanism in the apical membrane. Therefore, A to B flux was measured in the presence of 100 μM of verapamil or digoxin. These inhibitors did not affect the A to B transport of Ts-carnosine (Fig. 4)

However, the flux of 15 mM Ts-carnosine from B to A showed a significantly higher flux than in the A to B direction ($p < 0.001$). This flux was decreased slightly in the presence of 100 μM verapamil on both sides of the monolayer, but the flux was still significantly higher than the A to B flux ($p < 0.01$).

DISCUSSION

In the present study, we investigated the transport across Caco-2 cell monolayers of L-carnosine and the L-carnosine derivative Ts-carnosine, which is a compound possessing antitumor activity (3). L-carnosine (β-Ala-His) is a commonly used substrate for hPepT1 due to its high stability and hPepT1 mediated uptake and L-carnosine has been characterized in a number of studies (12–15). In the present study, we confirmed that Caco-2 cells indeed display a carrier-mediated uptake of L-carnosine across the apical membrane. A carrier-mediated uptake across the basolateral membrane was demonstrated as well; however, the K_m and V_{max} were significantly lower than for the apical uptake. Quite surprisingly, we found that the transepithelial transport of L-carnosine in the concentration range of 0–20 mM was nonsaturable, implying that simple diffusion dominates the transepithelial flux. The rate-limiting step in carrier-mediated transcellular L-carnosine transport is likely to be the basolateral exit, via the basolateral peptide transport mechanism. A large body of evidence supports the presence of a basolateral peptide transporter (25–27); however, the transporter(s) have not been cloned. Carrier-mediated transport of L-carnosine across the basolateral membrane of Caco-2 cells has to our knowledge not been investigated previously. We observed a V_{max} of basolateral L-carnosine transport which was lower than that observed for the non-hydrolysable dipeptide Gly-Sar, V_{max} for L-carnosine was 0.54 nmol · cm^{-2} · min^{-1} as compared with 1.2 nmol · cm^{-2} · min^{-1} for Gly-Sar (21). The absence of a carrier-mediated component in the transepithelial flux of L-carnosine is therefore likely to be due to a low basolateral transport capacity, in combination with fairly high passive paracellular permeability.

Ts-carnosine remained stable in aqueous solution for more than 1 week and in jejunal homogenate and plasma for more than 2 days. Evaluating the stability of sulfonamide and sulfonurea derivatives have earlier shown that these compounds are very stable in buffers and biologic media similar to the ones employed in the present study (28). Ts-carnosine showed affinity for hPepT1 and had a K_i-value of 2.33 mM, which is in the range of that observed for other known substrates such as bestatin, valaciclovir and cephradine (29–31).

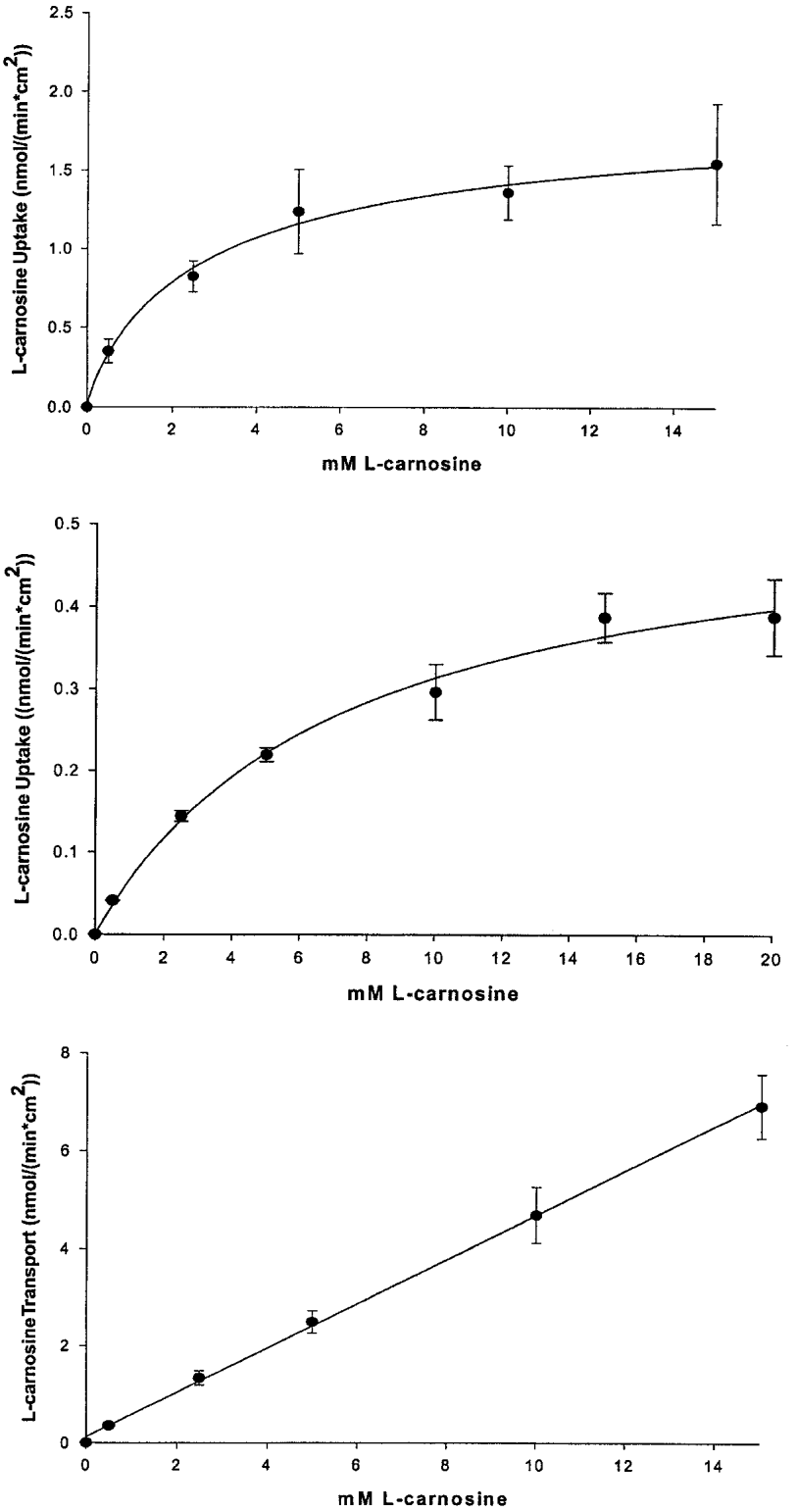

Fig. 2. Upper curve shows the apical uptake of L-carnosine measured as a function of apical L-carnosine concentration. Caco-2 monolayers were incubated with [^{3}H]L-carnosine (0.5 μCi/well) for 5 min. Cells were washed four times in ice-cold HBSS and solubilised. Radioactivity was determined and uptake was calculated. Data were fitted to Eq. (2). Each bar represents mean ± SE of 4 individual passages; experiments were performed in duplicate within each passage. Middle curve shows basolateral uptake of L-carnosine measured as a function of basolateral L-carnosine concentration. Caco-2 monolayers were incubated with [^{3}H]L-carnosine (0.5 μCi/well) for 15 min. Cells were washed four times in ice-cold HBSS and solubilised. Radioactivity was determined and uptake was calculated. Data were fitted to Eq. (2). Each bar represents mean ± SE of four individual passages; experiments were performed in duplicate within each passage. The lower curve shows the transepithelial transport (A-B direction) of L-carnosine measured as a function of apical L-carnosine concentration. Data were fitted to the simple relationship flux (J) = permeability (P_{app}) · concentration gradient (ΔC). Each bar represents mean ± SE of four individual passages; experiments were performed in duplicate within each passage.

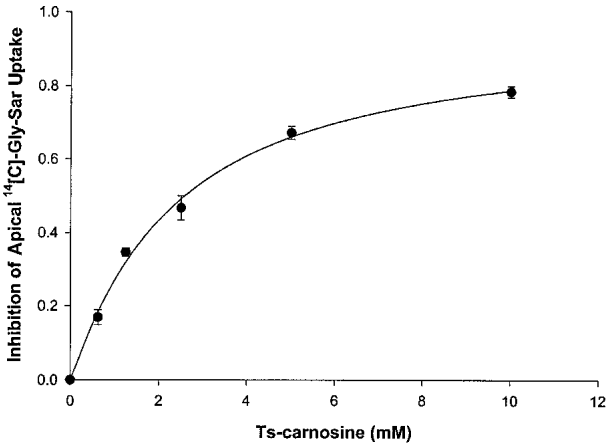

Fig. 3. Affinity for hPepT1 in Caco-2 cells of Ts-carnosine. The values were obtained as inhibition of [^{14}C]Gly-Sar uptake into Caco-2 cells in the presence of various concentrations of Ts-carnosine. The line represents a fit to Eq. (1). Experiments were performed in duplicates in 3 different Caco-2 cell passages.

The transepithelial A-B flux of Ts-carnosine at an apical concentration of 15 mM was 0.17 nmol * cm^{-2} * min^{-1}. This is a rather low flux as compared with that of L-carnosine (~6.5 nmol * cm^{-2} * min^{-1} at 15 mM) or Gly-Sar (V_{max} = 2.61 nmol * cm^{-2} * min^{-1}) (21). From the data it is evident that coupling of Ts to L–Carnosine yields a compound with a low permeability as compared to L-carnosine alone. Experiments were performed to evaluate the possible involvement of hPepT1 in the A to B flux of Ts-carnosine. hPepT1 is a di/tripeptide-proton cotransporter and therefore dependent on the pH gradient across the apical membrane; however, removing this gradient by changing the pH of the apical media from 6.0 to 7.4 did not significantly decrease the flux of Ts-carnosine. Neither did addition of 20 mM of the dipeptide Gly-Pro, a known substrate for hPepT1, affect the A-B flux. This indicates that even though Ts-carnosine has an affinity for hPepT1, the A-B transport across Caco-2 cells is not carrier-mediated. $LogD_{6.0}$ and $LogD_{7.4}$ values for Ts-carnosine and L-carnosine, respectively, are similar and subsequently a changed flux would not be expected if the transport process were non-saturable. The lack of a carrier-mediated transport component could be due a number of factors; a) Ts-carnosine might bind to hPepT1 and act as an inhibitor of Gly-Sar uptake without being translocated and released in the cytosol. There are indeed a few examples of inhibitors of PepT1; however, their affinities are in the μM range (32). b) Ts-carnosine might be translocated across the apical membrane, but then effluxed immediately via an apical efflux mechanism, such as P-glycoprotein multidrug resistance transporter (P-gp) or Multidrug resistance protein (MRP)-1. However, the A-B transport was not increased by adding 100μM of the efflux inhibitors digoxin or verapamil. The flux of Ts-carnosine in the B to A direction was approximately four times greater than the A to B flux, and when verapamil was added the B to A flux showed a tendency to decrease. This indicates that Ts-carnosine may be a substrate for one or more efflux mechanisms, because earlier functional studies in Caco-2 cells, rat and rabbit ileum, and distal colon have shown that B-A transport of vinblastine and etoposide in the presence of 100 μM verapamil decreases without increasing the A-B flux (34). In placental BeWo cells, it has also been shown that cyclosporine A decreases the B-A transport of both vincristine and digoxin without increasing the A-B transport (35). Cyclosporine A is a well-characterized peptide substrate for efflux mechanisms, but also hydrophobic cytotoxic tripeptides, such as N-acetyl-leucyl-leucyl-norleucinal (ALLN) and N-acetyl-leucyl-leucyl-methioninal (ALLM), have been shown to interact with the P-gp (36). MRP-1 has also been established as a transporter for small hydrophobic peptides. such as ALLN (37). Ts-carnosine might have a minor structural resemblance to a tripeptide with a peptide bond and a peptide-like bond. Ts-carnosine has a $logD_{7.4}$ value of approximately two, indicating that the molecule is relatively hydrophobic. Ts-carnosine might be taken up by the cells via hPepT1 and accumulate in the cells but have a low basolateral exit rate, due to a low affinity for the basolateral peptide transport mechanism. It was not possible for us to measure cell content of Ts-carnosine, due to the detection limit of the method (see Methods section). However, our studies on L-carnosine indicate that this could be an explanation. The dipeptide moiety of Ts-carnosine, L-carnosine is taken up into the cells via hPepT1, as discussed previously, but transcellular transport via the carrier mediated pathway seems to be limited by the low basolateral exit and the overall transepithelial transport is dominated by a passive, presumably paracellular flux. At present it is not possible to distinguish between the above-mentioned possibilities. When viewed retrospectively, L-carnosine is probably not ideal as a peptide-moiety when an increased and PepT1-dependent oral absorption is wanted. Although it has a high transepithelial flux, probably due to its passive permeation properties through tight junctions, modifications of the dipeptide are likely to decrease this flux due to an increase in size, as clearly seen with Ts-carnosine, and its transcellular carrier-mediated component is small due to a low basolateral exit. Future studies will therefore be centered on the possibilities of optimizing the affinity for hPepT1 for sulfonamide carbonic anhydrase inhibitors linked to other dipeptide moieties (yielding anticancer compounds) with the aim of yielding derivatives that can be absorbed orally via the peptide transport pathway. A concern will be to avoid efflux mechanisms to assure an effective oral absorption. Once this is established the focus will be on the possibilities of potential accumulation of dipeptide-based anticancer compounds in cancer cells expressing PepT1.

In conclusion, stability, lipophilicity, and transport of the anticancer compound 4-toluenesulfonylureido-carnosine were investigated and compared with that of L-carnosine. 4-toluenesulfonylureido-carnosine was stable in biologic solutions, more lipophilic than the dipeptide moiety L-carnosine and had an affinity to the apical di/tri-peptide transporter, hPepT1, when investigated in a human intestinal cell line, Caco-2. However, transepithelial transport of 4-toluenesulfonylureido-carnosine was extremely low when compared with L-carnosine (which surprisingly turned out to be transported mainly in a passive manner) or other non-hydrolyzable peptides. When taken together, data indicate that L-carnosine is not a suitable dipeptide moiety for hPepT1-mediated absorption of sulfonamide-type anticancer compounds.

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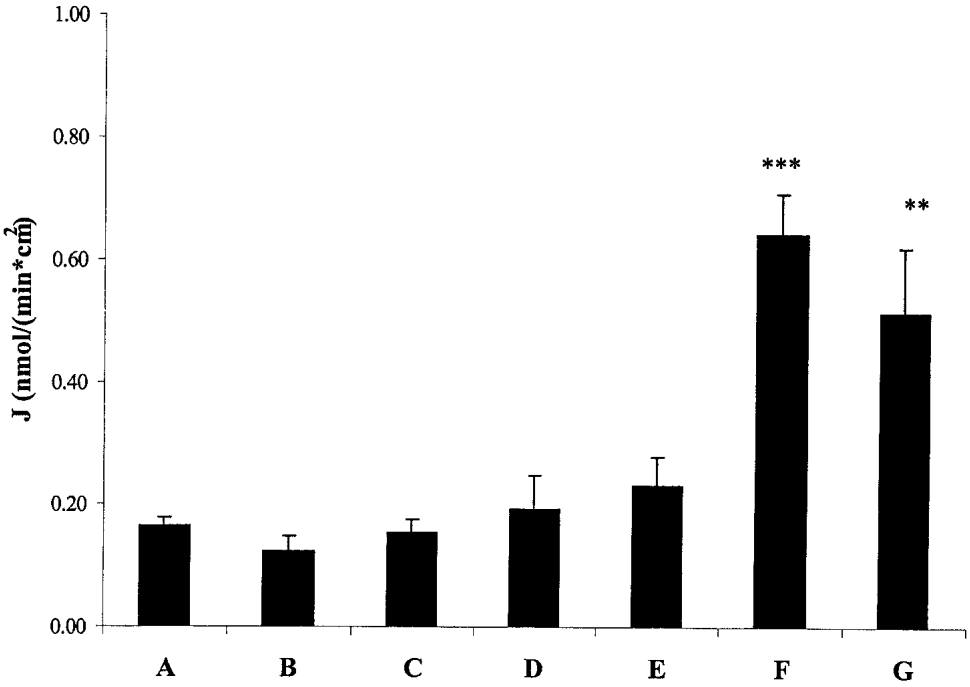

Fig. 4. The transepithelial flux of Ts-carnosine across Caco-2 cell monolayers. In all experiments the concentration of Ts-carnosine was 15 mM on the donor (*cis*) side. Samples were taken from the receiver (trans) side and analyzed by HPLC-UV, as described in the methods section. A: Apical (pH 6.0) to basolateral (pH 7.4) transport, B: Apical (pH 6.0 and 20 mM Gly-Pro) to basolateral (pH 7.4) transport, C: Apical (pH 7.4) to basolateral (pH 7.4) transport, D: Apical (pH 7.4) to basolateral (pH 7.4) transport in the presence of 100 μM verapamil on both sides. E: Apical (pH 7.4) to basolateral (pH 7.4) transport in the presence of 100 μM Digoxin on both sides. F: Basolateral (pH 7.4) to apical (pH 7.4) transport. G: Basolateral (pH 7.4) to apical (pH 7.4) transport in the presence of 100 μM verapamil on both sides. Experiments were performed in duplicates or triplicates in 2–4 different Caco-2 cell passages. **$p < 0.01$ and ***$p < 0.001$ compared with A.

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