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Transport of Pharmacologically Active Proline Derivatives by the Human Proton-Coupled Amino Acid Transporter hPAT1

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Running title**:** TRANSPORT OF PROLINE DERIVED DRUGS BY hPAT1

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Abbreviations: hPAT1, human proton-coupled amino acid transporter 1; GABA, γ-aminobutyric acid; CHLP, cis-4-hydroxy-L-proline; CHDP, cis-4-hydroxy-D-proline; THLP, trans-4-hydroxy-L-proline; LACA, L-azetidine-2-carboxylic acid; APSA, 3-amino-1-propanesulfonic acid; GHB, γ-hydroxybutyric acid; MeAIB, α-(methylamino)isobutyric acid.

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ABSTRACT

Several proline derivatives such as L-azetidine-2-carboxylic acid, cis-4-hydroxy-L-proline and 3,4-dehydro-D,L-proline prevent procollagen from folding into a stable triple-helical conformation thereby reducing excessive deposition of collagen in fibrotic processes and the growth of tumors. This study was performed to investigate whether the recently discovered human proton-coupled amino acid transporter 1 (hPAT1) is capable of transporting such pharmacologically relevant proline derivatives and also GABA analogs. Uptake of L-[^{3}H]proline and [^{3}H]glycine in Caco-2 cells was Na^+-independent but strongly H^+-dependent. The L-proline uptake was saturable and mediated by a single transport system (hPAT1) with an affinity constant of 2.0 ± 0.2 mM. The uptake of L-[^{3}H]proline was inhibited by D-proline, trans-4-hydroxy-L-proline, cis-4-hydroxy-L-proline, cis-4-hydroxy-D-proline, 3,4-dehydro-D,L-proline, L-azetidine-2-carboxylic acid, 3-amino-1-propanesulfonic acid, D- and L-pipecolic acid, L-thiaproline and many others. Apical uptake and transepithelial flux of L-[^{3}H]proline across Caco-2 cell monolayers were strongly inhibited by proline derivatives in proportions corresponding to their respective affinity constants at hPAT1. The basolateral to apical flux of L-[^{3}H]proline was only 8 % of that in the opposite direction. Apical uptake of unlabeled L-proline, cis-4-hydroxy-L-proline and L-azetidine-2-carboxylic acid was stimulated by an inside directed H^+ gradient two to three-fold. Total apical to basolateral flux of proline derivatives was moderately correlated with their inhibitory potency for L-[^{3}H]proline uptake and flux inhibition. We conclude that (1) the substrate specificity of hPAT1 is very much broader than so far reported and (2) the system accepts therapeutically relevant proline and GABA derivatives. hPAT1 is a promising candidate for new ways of oral drug delivery.

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A very promising approach for the delivery of drugs across epithelial barriers is the exploitation of physiological transport systems. Because of that, the substrate specificity of carriers, the design of prodrug substrates and the pharmacogenetics relevant to drug transporters gained enormous interest in recent years. Examples are the cephalosporin and prodrug transport via peptide transporters (Daniel and Adibi, 1993; Ganapathy et al., 1998; Bretschneider et al., 1999; Neumann and Brandsch, 2003), the transport of cationic drugs by the organic cation transporters (Koepsell et al., 2003) and the therapeutically relevant activity of efflux systems such as the P-glycoprotein, e.g. for digoxin absorption (Hoffmeyer et al., 2000).

A membrane transport system receiving very much attention at present is hPAT1, the most recent cloned human intestinal amino acid transporter. The system is very likely identical to the transport Thwaites described in the last decade as H^+-driven uptake of glycine, alanine, imino acids, GABA, 3-amino-1-propanesulfonic acid (APSA), D-serine, α-(methylamino)isobutyric acid (MeAIB), ß-amino acids and others at the apical membrane of Caco-2 cells (Thwaites et al., 1993a,b; 1995a,b; Thwaites and Stevens, 1999; Thwaites et al., 2000). In these early studies D.T. Thwaites also established Caco-2 as an in-vitro model for studies regarding intestinal H^+ dependent amino acid transport. A carrier with similar characteristics has been cloned recently from brain (rLYAAT1, rat lysosomal amino acid transporter 1, Sagne et al., 2001). Subsequently, H. Daniels group identified mPAT1 from mouse intestine (Boll et al., 2002). In 2003, the cloning, structure, function and localization of the human PAT1 at the apical membrane of Caco-2 cells has been described comprehensively (Chen et al., 2003). The primary substrates for hPAT1 in the mammalian small intestine are very likely glycine, L-proline and L-alanine. The system was also shown to translocate D-amino acids such as D-serine, D-proline and D-cycloserine with affinity constants similar or even lower than those of the L-isomers. Hence, hPAT1 might be responsible for the intestinal absorption of D-serine

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and D-cycloserine used in the treatment of affective disorders and cancers, respectively (Chen et al., 2003).

hPAT1 is now considered to be the major proline transport system at the intestinal epithelium. In the very recent study by Thwaites` and Ganapathy`s groups the authors discuss extensively the many reports on proline transport at the intestine of several species. They conclude that the hPAT1 is identical to the system known as IMINO carrier.

We investigated in the present study whether the system accepts proline derivatives known as orally available drugs effective in the treatment of serious diseases. Takeuchi and Prockop reported that L-azetidine-2-carboxylic acid (LACA) and cis-4-fluoro-L-proline are incorporated into protocollagen and that this incorporation results in the biosynthesis of abnormal collagen (Takeuchi and Prockop, 1969). Shortly after, Prockop's group also showed that cis-4-hydroxy-L-proline (CHLP) is incorporated into protocollagen and other proteins in place of L-proline and that the resulting unassembled or malfolded procollagen is not extruded into the extracellular matrix at a normal rate (Rosenbloom and Prockop, 1971; Uitto et al., 1975). An intracellular accumulation of polypeptides not folded into a stable triple-helical conformation was observed that is not tolerated by cells (Kao and Prockop, 1977; Tan et al., 1983). Recently it was shown that the retention of procollagen within the endoplasmatic reticulum is mediated by prolyl 4-hydroxylase (Walmsley et al., 1999). In the following years, the therapeutic potential of LACA, CHLP and 3,4-dehydro-D,L-proline was shown convincingly in many studies. The derivatives are effective in the treatment of processes where accumulation of collagen is a major pathological feature such as pulmonary fibrosis, liver cirrhosis, dermal fibrosis, systemic sclerosis, hypertrophic scars and others (Uitto et al., 1984). CHLP inhibits tumor cell growth and even leads to tumor necrosis of some rat mammary tumors at a non-toxic level (Lewko et al., 1981). LACA and CHLP inhibit cardiocyte myofibrillogenesis (Fisher and Periasamy, 1994) and the cytodifferentiation of

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chondrocytes (Berggren et al., 1997). There are patents claiming orally administered CHLP for the treatment of cancers (Hoerrmann, 1986, 2000).

We extended our study to therapeutically relevant GABA derivates such as the $GABA_A$ receptor agonist APSA (homotaurine) and γ-hydroxybutyric acid (GHB). APSA and calcium-acetylhomotaurinate restore the normal activity of glutaminergic neurons and are employed for anticraving treatment of alcohol dependence and other pathological conditions (Bartholini, 1985; Olive et al., 2002). GHB is used for treatment of narcolepsy, sleeping disorders, alcohol/opioid withdrawal and as general anastethic adjunct. As an anesthetic it never gained wide-spread acceptance but it is increasingly abused (Hernandez et al., 1998).

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Materials and Methods

Materials. The cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). L-[^{3}H]Proline (specific activity 43 Ci/mmol) and [^{3}H]glycine (specific activity 15 Ci/mmol) were purchased from Amersham International (UK). Cell culture reagents were obtained from Invitrogen (Germany). The amino acids and the derivatives CHLP, cis-4-hydroxy-D-proline (CHDP), THLP, trans-3-hydroxy-L-proline, 3,4-dehydro-D,L-proline, LACA, GABA, piperidine, L-and D-pipecolic acid, L-thiaproline, pyrrolidine, thiazolidine, APSA and GHB were from Sigma (Taufkirchen, Germany).

Cell culture. Caco-2 cells were routinely cultured (passages 76-110) in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution and gentamicin (45 µg/ml) (Knütter et al., 2001). Cells grown to 80% confluence were released by trypsinization and subcultured in 35 mm disposable petri dishes (Becton Dickinson, UK). The medium was replaced every two days and the day before the uptake experiment. With a starting cell density of $0.8 \cdot 10^6$ cells per dish, the cultures reached confluence within 24 h. Uptake was measured 7 days after seeding when cells on plastic dishes reach optimal differentiation. Caco-2 cells were also cultured on permeable polycarbonate Transwell® cell culture inserts (diameter 24.5 mm, pore size 3 µm, Costar GmbH, Bodenheim, Germany). Subcultures were started at a cell density of 43.000 cells/cm^2 and cultured for 23 days as routinely done (Thwaites et al., 1993a; Bretschneider et al., 1999; Thwaites et al., 2000).

Transport studies. Uptake of L-[^{3}H]proline, [^{3}H]glycine and unlabeled derivatives was measured as described earlier for other transporter substrates (Bretschneider et al., 1999; Knütter et al., 2001; Neumann and Brandsch, 2003). The uptake buffer (1 ml) contained either 25 mM MES/Tris (pH 6.0) or 25 mM HEPES/Tris (pH 7.5) with 140 mM NaCl or 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose, the

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radiolabeled reference amino acid and/or concentrations of unlabeled derivatives. After incubation for the desired time (mostly 10 min) the buffer was removed and monolayers were quickly washed with ice-cold uptake buffer four times and prepared for liquid scintillation spectrometry or HPLC, respectively. Protein was determined according to the method of Bradford.

Transepithelial flux of compounds across Caco-2 cells cultured on permeable filters was measured as described (Bretschneider et al., 1999). Uptake was started by adding buffer containing the amino acids and/or derivatives in labeled or unlabeled form to the donor side. At time intervals of 10 to 120 min samples were taken from the receiver compartment and prepared for liquid scintillation counting or HPLC, respectively. After 2 h, the filters were washed, cut out of the plastic insert and also prepared for analyses.

HPLC analysis. Qualitative and quantitative HPLC analyses of unlabeled proline derivatives in apical and basolateral buffer compartments as well as in cell homogenates were performed at an Agilent 1100 Chemstation. Fluorescence detection (exitation 266 nm, emission 305 nm) was done after precolumn derivatization with o-phthalaldehyde and 9-fluorenylmethoxycarbonyl chloride reagents. An ODS Hypersil column (200 x 4.6 mm, 5 µm) with a guard column (20 x 2.1 mm, 5 µm) was used for separation. The eluents were (A) 0.03 M sodium acetate, 0.5 % tetrahydrofuran, (B) 0.10 M sodium acetate, acetonitrile (1:4) applied with a gradient. The injection volume was 1 ml and the flow rate from 1.0 to 1.5 ml/min.

Data analysis. Experiments were routinely done in duplicate or triplicate and each experiment was repeated two to three times. The kinetic constants K_t and V_{max} were calculated by non-linear regression of the Michaelis-Menten plot and confirmed by linear regression of the Eadie-Hofstee plot. IC_{50} values (i.e. concentration of the unlabeled derivatives necessary to inhibit 50% of radiolabeled L-proline or glycine carrier-mediated uptake) were determined

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by non-linear regression procedure using the four parameter logistic equation (Bretschneider et al., 1999; Knütter et al., 2001). Inhibition constants (K_i ± S.E.) were calculated from IC_{50} values. Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver well vs. time (Bretschneider et al., 1999).

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Results

Characteristics of L-[^{3}H]proline and [^{3}H]glycine uptake in Caco-2 cells. We first investigated the basic characteristics of L-proline and glycine uptake in Caco-2 cells. In the presence of a Na^+ gradient and the presence or absence of an inside directed H^+ gradient, uptake of L-[^{3}H]proline (10 nM) into Caco-2 cells was strictly linear for at least 30 min (data not shown). A 10 min uptake time was chosen for further experiments. At an outside pH of 7.5, L-proline uptake was comparably low and only modestly stimulated by extracellular Na^+ (Figure 1). Excess amount of L-proline (30 mM) inhibited the L-[^{3}H]proline uptake by only 55 % (in the presence of Na^+) or 32% (in the absence of Na^+). The uptake of L-[^{3}H]proline was strongly stimulated by an inwardly directed H^+ gradient (Figure 1) as it has been reported by Thwaites and coworkers already 10 years ago (1993b). At an outside pH 6.0, the uptake rate was increased 6-7fold compared to transport at an outside pH 7.5. This stimulation was observed in the absence and the presence of an inside directed Na^+ gradient. The accumulation of L-[^{3}H]proline in the cells after 10 min of incubation can be estimated by assuming an intracellular volume of 3.6 µl/mg of protein (Blais et al., 1987). L-[^{3}H]Proline is enriched inside Caco-2 cells cultured on dishes against the concentration gradient ≈16-fold. We conclude, that an inside directed H^+ gradient is the driving force for apical L-proline uptake in Caco-2 cells. As expected, the L-proline uptake was found to be saturable: Under pH stimulated conditions, presence of an excess amount of unlabeled L-proline (30 mM) decreased uptake of radiolabeled L-proline in tracer concentration by 87%. This value represents the linear, non-mediated transport, most likely simple diffusion plus tracer binding. To determine the kinetic parameters of specific L-proline uptake, Caco-2 cells were incubated for 10 min with L-[^{3}H]proline (15 nM) and increasing concentrations of L-proline ranging from 0 to 10 mM (Figure 2). Nonmediated uptake was determined by measuring the L-[^{3}H]proline uptake in the presence of 50 mM unlabeled L-proline and subtracted from total

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uptake values. Kinetic analysis performed by nonlinear regression of carrier mediated uptake data revealed an apparent affinity Michaelis-Menten constant (K_t) of 2.0 ± 0.2 mM and a maximal velocity of transport (V_{max}) of 62.1 ± 2.5 nmol/10 min per mg of protein. Kinetically, there was no evidence for the participation of a second saturable transport component.

We also characterized the uptake of glycine under identical conditions. [^{3}H]Glycine uptake was found to be independent on Na^+, stimulated by an inwardly directed H^+ gradient and characterized by K_t = 8.5 ± 0.6 mM and V_{max} = 118.1 ± 7.1 nmol/10 min per mg of protein (data not shown). We thereby confirm the findings by Thwaites and coworkers (1995b) that the H^+-coupled amino acid transporter now known as hPAT1 is the transport system responsible for the apical L-proline and glycine uptake.

Recognition of pharmacologically relevant amino acid derivatives by hPAT1. The uptake of L-[^{3}H]proline (10 nM, pH 6.0) into Caco-2 cells could be inhibited by several other natural amino acids: Unlabeled L-proline, D-proline, THLP, glycine, L-alanine, GABA, sarcosine and taurine (all 30 mM) strongly inhibited L-[^{3}H]proline uptake by more than 65%. Similar inhibitions were observed for N-methyl-L-alanine and MeAIB. In contrast, no significant inhibition was found for trans-3-hydroxy-L-proline and D-tryptophan. In addition, we tested whether certain proline and glycine derivatives, in particular derivatives that are of proven or suggested therapeutically relevance are recognized by hPAT1 (Table 1): 3,4-Dehydro-D,L-proline, CHDP, LACA and APSA strongly inhibited L-[^{3}H]proline uptake. Weak inhibitors were the drugs CHLP and GHB. It is interesting that compounds with related structures such as L- and D-pipecolic acid and L-thiaproline were also recognized. Similarly, the uptake of [^{3}H]glycine was inhibited by (all 30 mM) L-proline to 25 ± 2 %, by CHLP to 71 ± 1 %, by CHDP to 33 ± 2%, by LACA to 25 ± 1%, by glycine to 33 ± 1%, by N-methyl-L-alanine to 29 ± 2 % and by sarcosine to 27 ± 2%.

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Figures 3 A and B show the results of competition assays performed to determine the apparent affinity constants (K_i) of the most relevant compounds vs. L-[^{3}H]proline uptake. It has to be mentioned that a 30 or even 100 mM concentration had no unspecific effect on the cells during the uptake period. This was shown by measuring unchanged uptake of L-[^{3}H]proline in the absence or presence of 100 mM mannitol.

The amino acids and derivatives L-proline, D-proline, CHDP, THLP, LACA, APSA, glycine and N-methyl-L-alanine displayed affinity constants between 1 and 10 mM (Table 2). They can be classified as "high affinity" substrates and/or inhibitors of hPAT1 with constants comparable to those of the known natural substrates. CHLP had a lower affinity to hPAT1. Similar results where obtained for the inhibition of [^{3}H]glycine transport (Table 2). The data collected in Table 2 can be considered as the classical ABC test. According to the criteria of the ABC test, the carrier mediated L-[^{3}H]proline uptake has to be completely inhibited by glycine and the carrier mediated [^{3}H]glycine uptake has to be completely inhibited by L-proline. This was the case in our study. The interaction between the two compounds during uptake was strictly competitive. The K_i value of L-proline vs. L-[^{3}H]proline uptake of 1.6 mM corresponds to its K_t value of 2.0 mM. The same affinity constant was obtained for the inhibition of [^{3}H]glycine uptake by L-proline. Moreover, CHLP and LACA inhibited the uptake of L-[^{3}H]proline and the uptake of [^{3}H]glycine with similar potencies, the K_i values of CHLP being 30 mM (vs. L-[^{3}H]proline) and ≈ 45 mM (vs. [^{3}H]glycine) and the K_i values of LACA being 1.8 mM (vs. L-[^{3}H]proline) and 1.9 mM (vs. [^{3}H]glycine). The same agreement was found for D-proline, CHDP, THLP, APSA and N-methyl-L-alanine (Table 2). Hence, all results strictly meet every requirement of the classical ABC test, thus strongly indicating that L-proline and glycine are transported by the same system, hPAT1, in Caco-2 cells.

Inhibition of L-[^{3}H]proline flux by proline derivatives. Transport studies were performed at Caco-2 cells cultured on permeable filters for 23 days. At this stage, the

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transepithelial electrical resistance of the Caco-2 cell monolayers in this study was 617 ± 11 Ω · cm^2. We determined the net transepithelial flux of L-[^{3}H]proline in apical to basolateral direction ($J_{a\text{-}b}$) and the uptake into the cells from the apical side ($J_{a\text{-}c}$) in the absence or presence of unlabeled amino acids and derivatives (Figure 4). The transepithelial L-[^{3}H]proline flux (10 nM) was 3.7 ± 0.3 pmol/h per receiver well. This amount corresponds to 5.3 ± 0.4 %/h per cm^2 and exceeds the [^{14}C]mannitol flux 80-fold (0.07 ± 0.002 %/h per cm^2, Bretschneider et al., 1999). As expected, the total transepithelial flux of L-[^{3}H]proline is mainly carrier-mediated: Addition of 30 mM unlabeled L-proline to the apical compartment inhibited the L-[^{3}H]proline flux remarkably by 85 %. More importantly, tracer flux was also decreased by CHDP, CHLP and LACA (Figure 4). The same rank order of inhibition was observed for intracellular uptake (Figure 4, inset): The L-[^{3}H]proline accumulation within the cells was inhibited by the derivatives by 26 to 83 %. There is complete agreement between the affinity constants of the drugs when inhibiting uptake of L-[^{3}H]proline into Caco-2 cells and their inhibition of L-[^{3}H]proline transepithelial flux. The rank order of flux (and also 2 h filter uptake) inhibition was L-proline > LACA > CHDP > CHLP. As shown in Table 2, the rank order of apparent affinities ($1/K_i$) at hPAT1 is identical. Hence, the derivatives with the highest affinity to hPAT1 are the derivatives with the highest potency to inhibit not only the cellular accumulation but also the transepithelial L-[^{3}H]proline net flux (rank correlation coefficient r_s =1, $p > 0.05$).

We also studied the transepithelial L-[^{3}H]proline flux in basolateral to apical direction. This was done by adding the labeled L-proline (10 nM) with or without unlabeled amino acids and derivatives to the abluminal compartment and taking samples for liquid scintillation counting from the luminal fluid (data not shown). L-proline transport occurs in directed manner: As stated above $J_{a\text{-}b}$ is 3.7 pmol/h per receiver well whereas the flux in the opposite direction ($J_{b\text{-}a}$) is only 7.3 % of that value (0.27 pmol/h per receiver well). This low but

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measurable flux was slightly inhibited by L-proline (by 11%), CHLP (by 10%), LACA (by 19%), L-alanine (by 12%, all $p < 0.05$) but not by L-glutamate (by 0%). L-[^{3}H]Proline uptake from the basolateral compartment ($J_{b\text{-}c}$) is only 17% of apical uptake ($J_{a\text{-}c}$) but inhibited by L-proline (by 60%), CHLP (by 46%), LACA (by 50%), L-alanine (by 35%, all $p < 0.05$) and insignificantly by L-glutamate (by 20%).

Total transepithelial flux and intracellular accumulation of proline derivatives. Inhibition of L-[^{3}H]proline uptake and transepithelial flux by proline-type drugs clearly demonstrate the interaction of these drugs with hPAT1. It does not prove, however, that the derivatives can cross the epithelium via hPAT1 alone. Therefore, uptake and flux studies with unlabeled derivatives combined with HPLC analysis were performed. All derivatives were chemically stable during uptake and sample preparation as shown by HPLC analysis. As expected for PAT1 substrates, uptake of unlabeled L-proline, CHLP and LACA was strongly stimulated by extracellular H^+ (Table 3). Figure 5 shows the following rank order of flux rates across Caco-2 cell monolayers cultured on filters: L-Proline > THLP > LACA = CHDP = D-proline > CHLP. Comparison of this rank order of flux rates with the inhibitory constants of the compounds vs. L-[^{3}H]proline uptake (Table 2) reveals that the transepithelial flux of the amino acids and drugs, respectively, corresponds approximately with their affinity at hPAT1 ($r = 0.811$, $p = 0.05$). The rank order of accumulation in Caco-2 cells is: CHDP > THLP > D-proline = CHLP > LACA > L-proline. Again, assuming an intracellular volume of 3.65 µl/mg of protein and a protein content of 0.23 mg/cm^2 filter (measured in this study), L-proline is enriched inside Caco-2 cells against the concentration gradient ≈2-fold. Because, in Transwell® systems efflux is possible, the major part of the compound is found in the basolateral compartment. Likewise, LACA, CHLP and CHDP are accumulated in the cells at an intra- to extracellular concentration ratio of 8, 11 and 23, respectively. There is no correlation between 2 h uptake accumulation and affinity constants.

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Discussion

The intestinal proline transport has been discussed very controversially in the past 20 years. Conflicting results reported in the literature concern the driving force (Na^+ or H^+ gradients), the localization of the transporters and the contribution of diverse amino acid transporters such as systems A, B, IMINO and others to the overall proline uptake (for review and a very helpful discussion of this subject see Chen et al., 2003). Even among studies using one particular model, the Caco-2 cell, the results are on first sight incompatible (Nicklin et al., 1992; Thwaites et al., 1993b; Berger et al., 2000; Chen et al., 2003). Now, that PAT1 has been cloned and studied functionally the remaining problems will soon be resolved. The currently accepted suggestion is that PAT1 and system IMINO are structurally and functionally identical (Chen et al., 2003). Most results gained on PAT1 confirm the early reports of H^+ gradient driven uptake of amino acids such as proline, glycine and many others and that of amino acid derived drugs such as D-serine, D-cycloserine, GABA and APSA at the apical membrane of Caco-2 cells (Thwaites et al., 1993a,b; Ranaldi et al., 1994; Thwaites et al.,1995a,b; Thwaites and Stevens, 1999; Thwaites et al., 2000). In our study we found that Caco-2 cells take up L-proline at their apical membrane in a strongly H^+-dependent manner via a system with an affinity constant of 2 mM. We found no evidence whatsoever for a Na^+ dependence of proline transport and no evidence for another system involved in L-proline uptake at the apical membrane. Based on our data, we concluded that the system expressed in Caco-2 cells corresponds to PAT1 cloned from mouse intestine last year (Boll et al., 2002). During our final experimental work a detailed and fundamental study was published describing the most relevant characteristics of hPAT1 in Caco-2 cells (Chen et al., 2003).

The main focus of our investigation was, however, the therapeutic relevance of this carrier. After establishing the experimental techniques for studying L-[^{3}H]proline uptake via hPAT1 cells into Caco-2 cells, we found that D-proline, THLP, CHDP, LACA, GABA, D-

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and L-pipecolic acid (D- and L-homoproline), L-thiaproline, APSA, 3,4-dehydro-D,L-proline, glycine, N-methyl-L-alanine, L-tryptophan, sarcosine, MeAIB, taurine and to a lower extend CHLP, GHB and pyrrolidine are recognized by the system. The new substrates of hPAT1 identified in this study allow conclusions about the essential structural requirements for substrate recognition. Our data support the concept that a primary or secondary amino group of either small aliphatic or heterocyclic amino acids is essential for high affinity. The carrier accepts the 4- , 5- and 6-membered rings of proline derivatives. Hence, in contrast to the proline permease in *Escherichia coli* and salmonella (Rowland and Tristram, 1975; Liao and Maloy, 2001), 6-membered rings are not excluded as long as the compound is not decarboxylated (piperidine). For hPAT1 the carboxy group seems to be essential for a high affinity substrate interaction but can be replaced by a sulfonyl group. The sulfur-containing amino acid thiaproline represents a hPAT1 substrate with comparably high affinity. Removing the carboxy group as in thiazolidine diminishes the affinity. The 10fold higher affinity of CHDP compared to CHLP supports the observation that for some amino acids (serine, cysteine) hPAT1 prefers the D-isomer (Boll et al., 2002; Chen et al., 2003). During final preparation of this manuscript Boll and coworkers published an extension of their studies regarding the hPAT1 substrate specificity using a different class of compounds. They showed that a critical recognition criterion of PAT1 is the backbone charge separation distance and the side chain size, whereas substitutions on the amino group are well tolerated (Boll et al., 2003).

Regarding the therapeutic exploitation of PAT1 for oral CHLP delivery it has to be noted that compared to L-proline the affinity of CHLP is low. Whether or not an affinity constant is in a reasonable range for practical consideration depends among other things on the concentration the compound reaches in the fluid compartment facing the membrane where the carrier is located. The recommended oral dose of CHLP to reach therapeutic blood

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concentrations with significant effects of the drug at the target is 0.05 – 0.2 g/kg body mass per day (Hoerrmann, 1986, 2000). Assuming a dose of 3.5 g given to a human twice a day, a luminal concentration of 30 mM is very conceivable. This concentration corresponds to the affinity constant of CHLP.

We observed a strong direct correlation between the affinity of proline derivatives at hPAT1 and their potency to inhibit both the uptake of L-[^{3}H]proline into Caco-2 cell monolayers in 2 h and the transepithelial net flux of L-[^{3}H]proline. Consequently, L-[^{3}H]proline uptake into the cells and flux through the cells in the presence of inhibitors were also strictly correlated which has to be expected as long as the derivatives interfere only with the L-[^{3}H]proline uptake. Direct measurement of transport of unlabeled drugs and derivatives revealed (i) that transport of L-proline, CHLP and LACA is as expected strongly stimulated by a pH gradient and (ii) that the compounds with the lowest K_i values at hPAT1 show partly the highest flux rate through the monolayers but the lowest accumulation in cells. In other words, there is no correlation between affinity constants at hPAT1 and the amount of derivatives remaining in the cells after 2 h. The low amount of L-proline intracellular is easily explained by its very high total flux through the monolayers (Figure 5). In contrast, the compound with the lowest affinity, CHLP, displays the lowest flux but a concentration in the cells much higher than that of L-proline and LACA. In a two compartment model where hPAT1 represents the only uptake mechanism this should not have been observed. For cells on filters, however, the basolateral efflux is a major factor affecting the intracellular concentration. In cases where a basolateral efflux system with identical substrate specificity as the apical system exists, a correlation between affinity at the apical carrier, intracellular accumulation in filter grown cells and transepithelial flux is observed, e.g. for ß-lactam antibiotics (Bretschneider et al., 1999). The present results let us conclude that the uptake of proline derivatives into Caco-2 cells depends almost completely on their affinity to hPAT1,

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but that the amount remaining in the cell is additionally affected by the substrate specificity of several, very different basolateral carriers. This conclusion is supported by several lines of evidence: First, hPAT1 is expressed in the apical membrane of Caco-2 cells but not in their basolateral membrane (Chen et al., 2003). Second, there are candidates for proline transport systems at the basolateral membrane, mainly system A, subtype ATA2. What has been reported for proline derivatives is that in F98 rat glioma cells cis-4-[^{18}F]fluoro-L-proline used for PET scans is transported by system A in a Na^+-dependent manner (Langen et al., 2002). Fibroblast cell lines most sensitive to CHLP are those in which the activity of the A system is specifically increased (Ciardiello et al., 1988). Concerning the other PAT1 substrates studied so far, certain D-amino acids, alanine, serine and cysteine are transported by the transport proteins 4F2hc/LAT1, 4F2hc/asc-1 and ASCT1. The third line of evidence was obtained in the present investigation: Basolateral flux of L-[^{3}H]proline into Caco-2 cells is very low and weakly inhibited by L-proline, CHLP, LACA or L-alanine but not by L-glutamate which is no system A substrate. Similarly, basolateral L-[^{3}H]proline uptake is low but measurable and to a significant extend only inhibited by L-proline, CHLP, LACA and L-alanine. This result also demonstrates that the transepithelial transport of L-proline and derivatives occurs in a strongly apical to basolateral directed manner.

In some respect, hPAT1 might become of even greater importance for oral drug delivery than hPEPT1: hPAT1 transports its substrates with higher maximal velocity than hPEPT1 and it accepts drugs used in treatment of very different pathological situations. Our results show that the substrate specificity of hPAT1 is much broader than reported so far. Moreover, PAT1 and PAT2 seem to have a tissue distribution at least as wide as PEPT1. Northern blot analyses revealed that PAT1 mRNA in mice and humans is maximal expressed in small intestine with moderate expression in kidney, brain, liver, lung, placenta, testis and colon whereas PAT2 is expressed in lung and heart (Boll et al., 2002; Chen et al., 2003).

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Since the substrate specificity of hPAT1 should be tissue independent one could postulate that uptake of the pharmacologically relevant proline derivatives described in this study occurs though to a lower level at all cell types expressing hPAT1 in their cell membrane; this of course only under the assumption that the derivatives can indeed reach the fluid compartments contacting the respective cell type.

Another aspect of the clinical relevance of a transport protein is the question whether a defect is related to a human disease. In case of hPAT1, it is imperative to investigate possible defects and polymorphisms of this carrier. They might be one cause of iminoglycinuria (Chen et al., 2003). In that case, these patients might also have restricted absorption of therapeutically proline drugs after oral administration.

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This work will be part of the doctoral thesis of L. M.

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Fig. 1. H^+ and Na^+ dependence and saturability of L-[^{3}H]proline uptake in Caco-2 cells. Uptake of L-[^{3}H]proline (10 nM) was measured at pH 7.5 or pH 6.0 for 10 min in the absence or presence of excess amount (30 mM) of unlabeled L-proline. Sodium chloride was isoosmotically replaced by choline chloride. Data are means ± S.E. (*n* = 4 - 5).

Fig. 2. Substrate concentration kinetics of L-proline uptake in Caco-2 cells. Uptake of L-[^{3}H]proline (15 nM, 10 min) was measured over a L-proline concentration range of 0 to 10 mM. Nonmediated uptake was determined by measuring the L-[^{3}H]proline uptake in the presence of 50 mM unlabeled L-proline. Inset: Eadie-Hofstee transformation of the data. v, uptake rate in nmol/10 min per mg of protein; S, L-proline concentration in mM. Data are means ± S.E. (*n* = 4).

Fig. 3. Substrate specificity of L-[^{3}H]proline uptake in Caco-2 cells. Uptake of L-[^{3}H]proline (10 nM, 10 min, pH 6.0) was measured in the absence or presence of increasing concentrations of unlabeled amino acids and derivatives (0 – 100 mM). Uptake of L-[^{3}H]proline measured in the absence of the inhibitors (565.8 ± 74.1 fmol/10 min per mg of protein) was taken as 100%. Data are means ± S.E. (*n* = 4).

Fig. 4. Apical to basolateral transepithelial flux and intracellular uptake of L-[^{3}H]proline at Caco-2 cell monolayers. L-[^{3}H]proline (10 nM) with or without 30 mM of unlabeled inhibitors in buffer (pH 6.0) was added to the apical compartment of the Transwell® systems. L-[^{3}H]proline appearance corrected for buffer replacement is plotted versus time (= transepithelial flux in apical-to-basolateral direction, J_{a-b}). Inset: Uptake of L-[^{3}H]proline into cells on the filter membrane from the apical side (J_{a-c}) in 2 h. A: L-[^{3}H]Proline (control), B:

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Plus cis-4-hydroxy-L-proline, C: Plus cis-4-hydroxy-D-proline, D: Plus L-azetidine-2-carboxylic acid, E: Plus L-proline. Data are shown as means ± S.E. (n = 4).

Fig. 5. Transepithelial flux and intracellular uptake of unlabeled L-proline and derivatives at Caco-2 cell monolayers. Substrates (10 mM) were added to the apical (donor) compartment (1.5 ml) of Transwell® systems in uptake buffer (pH 6.0). After the time intervals indicated, samples (200 µl) were taken from the receiver compartment (pH 7.5, 2.6 ml) and replaced with buffer. Samples were analyzed after precolumn derivatization by HPLC as described in Methods. Inset: Accumulation of L-proline and derivatives in monolayers in 2 h. A: L-Proline, B: trans-4-Hydroxy-L-proline, C: L-Azetidine-2-carboxylic acid, D: cis-4-Hydroxy-D-proline, E: D-Proline, F: cis-4-Hydroxy-L-proline. Data are shown as means ± S.E. (n = 6).

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TABLE 1

Substrate specificity of L-[^{3}H]proline uptake in Caco-2 cells

Uptake of L-[^{3}H]proline (10 nM) was measured at pH 6.0 for 10 min in the presence of unlabeled amino acids and derivatives at a fixed concentration of 30 mM. Data are means ± S.E. (n = 4 - 6).

Inhibitor	L-[^{3}H]Proline Uptake (%)
Control	100 ± 8
L-Proline	16 ± 3
D-Proline	17 ± 2
3,4-Dehydro-D,L-proline	21 ± 2
trans-4-Hydroxy-L-proline	35 ± 2
trans-3-Hydroxy-L-proline	85 ± 9
cis-4-Hydroxy-L-proline	79 ± 3
cis-4-Hydroxy-D-proline	23 ± 1
L-Azetidine-2-carboxylic acid	20 ± 1
3-Amino-1-propanesulfonic acid	30 ± 1
γ-Hydroxybutyric acid	71 ± 1
Glycine	30 ± 2
L-Alanine	31 ± 5
N-Methyl-L-alanine	16 ± 2
L-Phenylalanine	80 ± 5
N-Methyl-L-phenylalanine	81 ± 6
L-Tryptophan	26 ± 2
D-Tryptophan	93 ± 3

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L-Cysteine	87 ± 4
Sarcosine	13 ± 1
γ-Aminobutyric acid	17 ± 1
α-(Methylamino)isobutyric acid	20 ± 2
Taurine	25 ± 2
L-Pipecolic acid	38 ± 2
D-Pipecolic acid	20 ± 1
L-Thiaproline	32 ± 3
Piperidine	80 ± 8
Pyrrolidine	74 ± 2
Thiazolidine	83 ± 4

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TABLE 2

Inhibition constants (K_i) of different derivatives for the inhibition of L-[^{3}H]proline and [^{3}H]glycine uptake in Caco-2 cells

Uptake of L-[^{3}H]proline (10 nM) and [^{3}H]glycine (30 nM) was measured at pH 6.0 for 10 min in the presence of unlabeled amino acids and derivatives. Inhibition curves for calculation of K_i values are shown in Figure 3. Parameters are shown ± S.E. (n = 4).

	K_i values	
Inhibitor	L-[^{3}H]Proline Uptake	[^{3}H]Glycine Uptake
	mM	
L-Proline	1.6 ± 0.4	2.2 ± 0.2
D-Proline	1.2 ± 0.3	1.9 ± 0.1
cis-4-Hydroxy-L-proline	30 ± 4	≈ 45 ± 13
cis-4-Hydroxy-D-proline	3.5 ± 0.1	3.2 ± 0.2
trans-4-Hydroxy-L-proline	9.0 ± 0.9	5.3 ± 0.6
L-Azetidine-2-carboxylic acid	1.8 ± 0.1	1.9 ± 0.4
3-Amino-1-propanesulfonic acid	7.1 ± 0.6	10.5 ± 0.5
Glycine	5.1 ± 0.9	9.2 ± 1.0
N-Methyl-L-alanine	2.2 ± 0.1	2.0 ± 0.1

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TABLE 3

Effect of extracellular H^+ on the uptake of unlabeled L-proline and derivatives into Caco-2 cells

Substrates (10 mM) were added to cell monolayers cultured on dishes. After 30 min monolayers were washed and prepared for HPLC analysis as well as protein determination. Data are means ± S.E. (n = 3).

Compounds	pH 7.5	pH 6.0	
	μmol/mg of protein	μmol/mg of protein	%
L-Proline	124 ± 2	256 ± 9	206
cis-4-Hydroxy-L-proline	32 ± 2	76 ± 2	236
L-Azetidine-2-carboxylic acid	128 ± 5	274 ± 10	214

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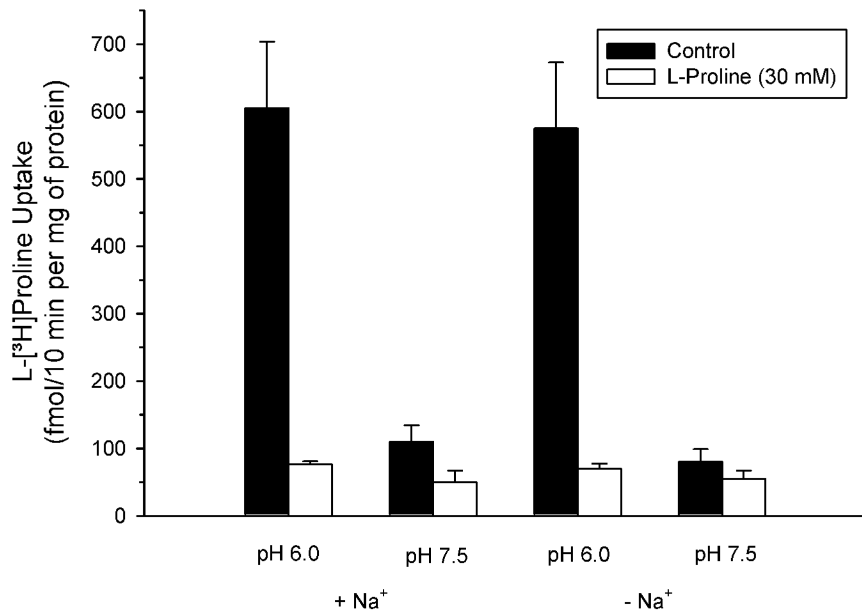
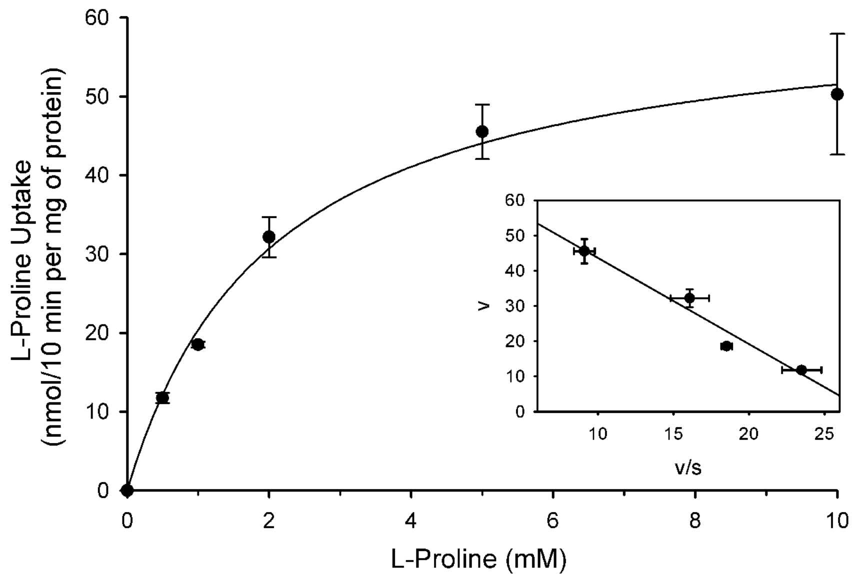
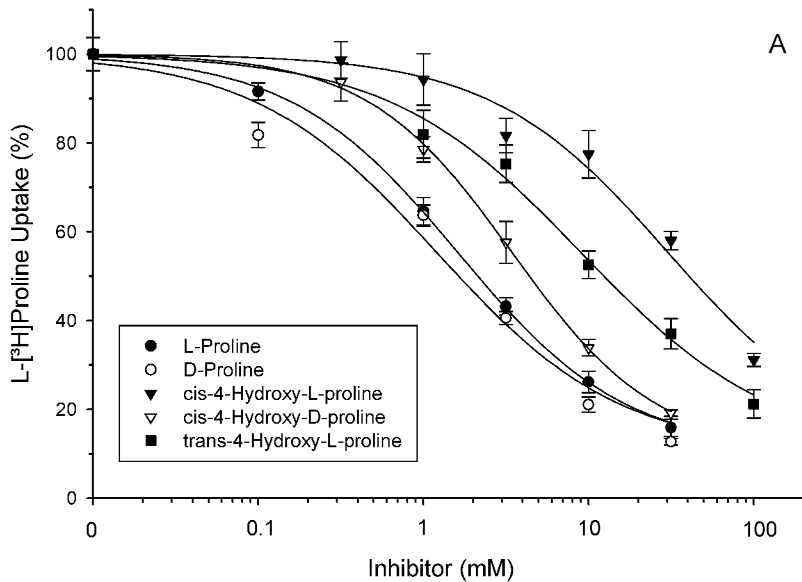


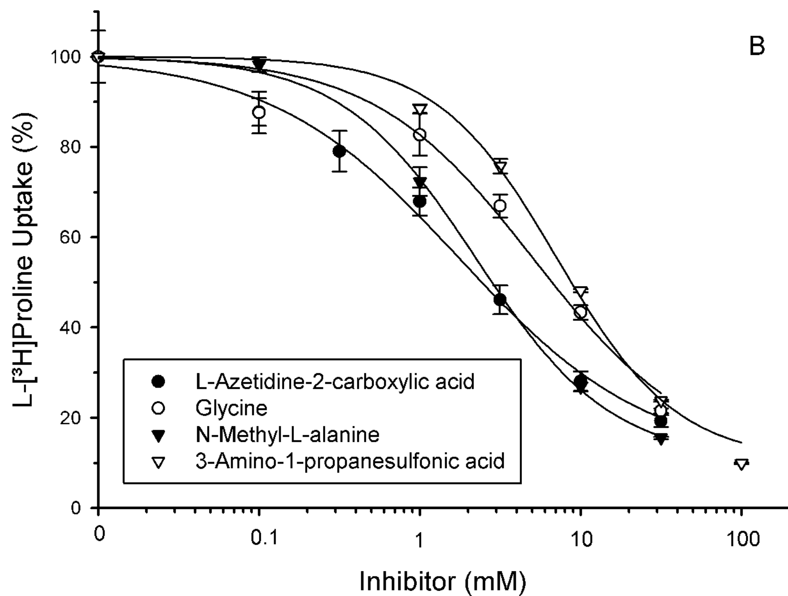
L-[³H]Proline Uptake
(fmol/10 min per mg of protein)
700
600
500
400
300
200
100
0
Control
L-Proline (30 mM)
pH 6.0
pH 7.5
pH 6.0
pH 7.5
+ Na+
- Na+

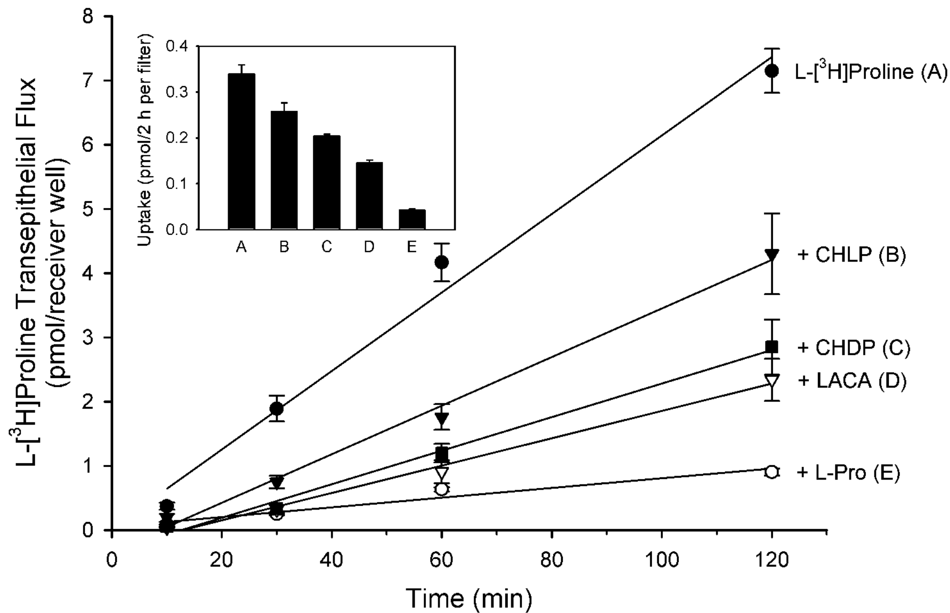


L-Proline Uptake
(nmol/10 min per mg of protein)
60
50
40
30
20
10
0
0
2
4
6
8
10
L-Proline (mM)
v
v/s
60
50
40
30
20
10
0
10
15
20
25

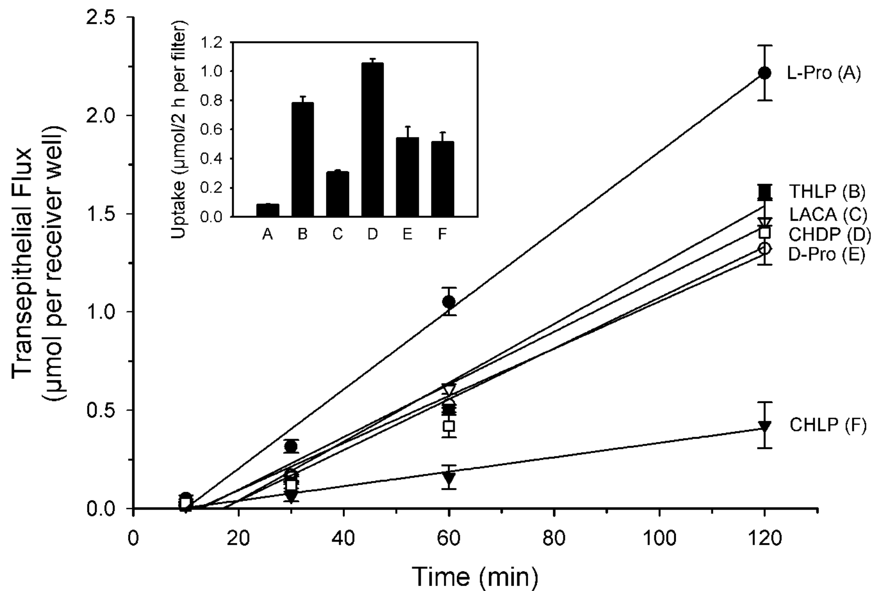

A
L-[³H]Proline Uptake (%)
100
80
60
40
20
0
0
0.1
1
10
100
Inhibitor (mM)
L-Proline
D-Proline
cis-4-Hydroxy-L-proline
cis-4-Hydroxy-D-proline
trans-4-Hydroxy-L-proline

3





L-[3H]Proline Transepithelial Flux (pmol/receiver well)
Time (min)
0
20
40
60
80
100
120
0
1
2
3
4
5
6
7
8
L-[3H]Proline (A)
+ CHLP (B)
+ CHDP (C)
+ LACA (D)
+ L-Pro (E)
Uptake (pmol/2 h per filter)
0.0
0.1
0.2
0.3
0.4
A
B
C
D
E



Transepithelial Flux
(µmol per receiver well)
Time (min)
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
100
120
L-Pro (A)
THLP (B)
LACA (C)
CHDP (D)
D-Pro (E)
CHLP (F)
Uptake (µmol/2 h per filter)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
A
B
C
D
E
F