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Substrate specificity of the amino acid transporter PAT1

L. Metzner[1], **K. Neubert**[2], and **M. Brandsch**[1]

[1] Membrane Transport Group, Biozentrum, Martin-Luther-University Halle-Wittenberg, Halle, Germany
[2] Department of Biochemistry/Biotechnology, Institute of Biochemistry, Martin-Luther-University Halle-Wittenberg, Halle, Germany

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Summary. The proton coupled amino acid transporter PAT1 expressed in intestine, brain, and other organs accepts L- and D-proline, glycine, and L-alanine but also pharmaceutically active amino acid derivatives such as 3-amino-1-propanesulfonic acid, L-azetidine-2-carboxylic acid, and cis-4-hydroxy-D-proline as substrates. We systematically analyzed the structural requirements for PAT1 substrates by testing 87 amino acids, proline homologs, indoles, and derivatives. Affinity data and effects on membrane potential were determined using Caco-2 cells. For aliphatic amino acids, a blocked carboxyl group, the distance between amino and carboxyl group, and the position of the hydroxyl group are affinity limiting factors. Methylation of the amino group enhances substrate affinity. Hetero atoms in the proline template are well tolerated. Aromatic α-amino acids display low affinity. PAT1 interacts strongly with heterocyclic aromatic acids containing an indole scaffold. The structural requirements of PAT1 substrates elucidated in this study will be useful for the development of prodrugs.

Keywords: H^+/Amino acid cotransporter – hPAT1 – Caco-2 cells – Substrate specificity – Proline – Membrane transport

Introduction

At the intestinal epithelium, a variety of different transport systems are responsible for the absorption of amino acids and amino acid derivatives (Ganapathy et al., 1994; Palacin et al., 1998; Steffansen et al., 2004). One of the most recently cloned carriers is the proton coupled low affinity, high capacity transport system PAT1. PAT1 was first cloned from rat brain as a lysosomal proton coupled amino acid transport system LYAAT1, which belongs to the amino acid/auxin permease system and mediates transport of lysosomal proteolysis products (Sagne et al., 2001). Subsequently PAT1 and PAT2 (orthologous to LYAAT) have been cloned from mouse intestine and embryonic tissue (Boll et al., 2002) and assigned to the solute carrier (SLC) family 36 (Boll et al., 2004). The human PAT1 cDNA was cloned from intestinal Caco-2 cells (Chen et al., 2003). Immunolocalization studies showed its expression at the apical membrane of Caco-2 cells, human and rat small intestine (Chen et al., 2003; Anderson et al., 2004). These findings confirmed earlier studies describing proton driven uptake of proline, alanine, and other small neutral amino acids in Caco-2 cells (Nicklin et al., 1992; Thwaites et al., 1993a, b, 1994, 1995c).

Expression of the PAT1 carrier protein was detected in the intestine, brain, liver, colon, lung, spleen, and placenta (Boll et al., 2002, 2003b; Chen et al., 2003). The uphill transport of solutes via PAT proteins is Na^+ independent but stimulated by a transmembrane electrochemical proton gradient. The substrate and the proton are translocated at a coupling stoichiometry of 1:1 (Boll et al., 2002). In Caco-2 cell monolayers the transport of L-proline across the brush-border membrane is mediated by PAT1 with a Michaelis-Menten constant (K_t) of 2.0 mM at an outside pH 6.0 (Metzner et al., 2004).

PAT1 transports small, unbranched apolar amino acids (e.g. proline, alanine, glycine), amino acid derivatives (e.g. aminoisobutyric acid, α-(methylamino)isobutyric acid (MeAIB), γ-aminobutyric acid (GABA)), the osmolytes betaine and sarcosine, certain small D-amino acids, and pharmaceutically relevant compounds (e.g. D-cycloserine, L-azetidine-2-carboxylic acid (LACA), 3-amino-1-propanesulfonic acid, 3,4-dehydro-L-proline) (Thwaites et al., 1993a, b, 1994, 1995b, 2000; Ranaldi et al., 1994; Boll et al., 2002; Chen et al., 2003; Metzner et al., 2004). A bifunctional transport mode of PAT1 (and PAT2) was recently described by its capability for transport of short-chain fatty acids such as acetate, propionate, and butyrate in an electroneutral mode and transport of the homolo-

gous amino acids in an electrogenic mode (Foltz et al., 2004). We have shown recently that the naturally occurring amino acid L-tryptophan and the biogenic amines tryptamine and serotonin are accepted by the carrier protein but not transported. They function as PAT1 inhibitors (Metzner et al., 2005). In this study we systematically analyzed the structural requirements for PAT1 substrates by testing 87 amino acids, proline homologs, indoles, and derivatives.

Materials and methods

Materials

The human colon carcinoma cell line Caco-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). L-[^{3}H]Proline (specific radioactivity 42 Ci/mmol) was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Reagents for cell culture were procured from Invitrogen (Karlsruhe, Germany). Amino acids, biogenic amines, indole structures and derivatives were from Sigma-Aldrich and Fluka (Taufkichen, Germany) and Bachem (Weil am Rhein, Germany). L-Proline methyl ester, L-proline tert.-butyl ester, L-proline benzyl ester, N-benzyloxycarbonyl-L-proline, L-proline amide, N-acetyl-L-tryptophan and L-tryptophan methyl ester were synthesized according to standard procedures (Goodman et al., 2002).

Cell culture

Caco-2 cells (passages 5–69), maintained in 75 cm^2 culture flasks at 37 °C and 5% CO_2, were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution and gentamicin (45 µg/ml) (Metzner et al., 2004). Cells (80–90% confluence) were released by trypsination and subcultured in 35 mm petri dishes (Sarstedt, Nümbrecht, Germany) with a cell density of 0.8×10^6 cells/dish. For the membrane potential (MP) assay Caco-2 cells were subcultured for 7 days in 96-well plates with a starting density of 0.8×10^5 cells/well. Medium was changed the day after seeding, every two days, and the day before the experiment. Uptake was measured 6 days after confluence.

Uptake measurements

L-[^{3}H]Proline uptake was determined as described (Metzner et al., 2004). Uptake buffer was 25 mM Mes/Tris (pH 6.0) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, the radiolabeled L-proline (10 nM) and unlabeled compounds (0–100 mM). After an incubation of 10 min the uptake buffer was removed, monolayers were quickly washed four times with ice-cold uptake buffer and prepared for liquid scintillation spectrometry. Protein was determined according to the method of Bradford.

Membrane potential assay measurements

After washing the cells twice with uptake buffer (pH 6.0) they were incubated with 50 µl red MP dye (Molecular Devices Corp., Munich, Germany) per well at 37 °C (Faria et al., 2004; Metzner et al., 2005). After incubation for 60 min, compounds dissolved in 200 µl uptake buffer at a final concentration of 10 mM were added. Fluorescence was measured immediately at wavelengths of 530 nm (excitation) and 570 nm (emission) at a FLUOstar Galaxy (BMG Labtechnologies Offenburg, Germany).

Data analysis

Experiments were done in duplicate and each experiment was repeated two to three times. Results are expressed as means ± SE. K_i values (i.e. concentration of the unlabeled compound necessary to inhibit 50% of L-[^{3}H]proline carrier-mediated uptake) were determined by non-linear regression of the four parameter logistic equation (Metzner et al., 2004).

Results and discussion

The 87 compounds investigated here were divided into three groups: (A): proline, proline derivatives, and homologs, (B): proteinogenic and non-proteinogenic neutral amino acids, and derivatives, (C): aromatic and heteroaromatic amino acids, and selected indole derivatives. All data were obtained in experiments with the human intestinal cell line Caco-2. Caco-2 is an accepted model for proline uptake studies by PAT1 (Thwaites et al., 1993b; Ranaldi et al., 1994; Chen et al., 2003; Metzner et al., 2004). Very recently, another proline transport system, the Na^+/imino acid transporter SIT1 has been cloned from rat, mouse, and human small intestine (Takanaga et al., 2005; Kowalczuk et al., 2005). We and others found no evidence for a functional contribution of SIT1 to the L-[^{3}H]proline uptake in Caco-2 cells under the conditions used. In Caco-2 cells, L-proline transport is saturable with a Michaelis-Menten constant (K_t) of 2.0 ± 0.2 mM and a maximal transport velocity (V_{max}) of 62.1 ± 2.5 nmol/10 min per mg of protein. Transport is Na^+ independent, energized by an inward directed proton gradient and mediated by a single transport system (Thwaites et al., 1993b; Metzner et al., 2004).

First we determined the effect of the 87 compounds on L-[^{3}H]proline uptake at a fixed concentration of 10 mM to get preliminary information about the grade of interaction with human PAT1 expressed constitutively in Caco-2 cells (Tables 1–3). For strong inhibitors, we then determined affinity constants in substrate saturation studies. This competition assay gives information about the interaction of a compound with the transporter. No conclusions about real translocation can be made. The assay does not allow differentiation between a transported substrate and an interacting inhibitor. Therefore we used a MP assay (Tables 1–3, third column) that is based on the measurement of changes in MP generated by the symport of a proton during substrate transport via PAT1 (Faria et al., 2004; Metzner et al., 2005). It has to be noticed, however, that all transporters present in Caco-2 cells, which function in an electrogenic mode at pH 6.0 in the presence of Na^+, are able to induce changes in MP. Another drawback of this assay is that it would not detect electroneutral transport via PAT1 such as that of short chain

Table 1. Substrate specificity of L-[^{3}H]proline uptake in Caco-2 cells: proline, proline derivatives, and proline homologs

Substrate/inhibitor	L-[^{3}H]proline uptake (%)	K_i (mM)	Fluorescence signal (%)
Control	100 ± 8		
1 L-Proline	32 ± 4[a]	1.6 ± 0.4[b]	100 ± 2[a]
2 D-Proline	24 ± 2	1.2 ± 0.3[b]	100 ± 1
3 cis-4-Hydroxy-L-proline	87 ± 3	30 ± 4[b]	70 ± 1
4 cis-4-Hydroxy-D-proline	39 ± 3	3.3 ± 0.2[b]	99 ± 1
5 trans-4-Hydroxy-L-proline	46 ± 1	9.0 ± 0.9[b]	94 ± 1
6 trans-3-Hydroxy-L-proline	80 ± 1	–	66 ± 2
7 3,4-Dehydro-D,L-proline	42 ± 4	–	–
8 3,4-Dehydro-L-proline	50 ± 7	6.2 ± 0.1	96 ± 1
9 L-Prolinol	85 ± 7	–	–
10 L-Proline methyl ester	63 ± 3	–	–
11 L-Proline tert.-butyl ester	71 ± 2	–	–
12 L-Proline benzyl ester	52 ± 6	–	–
13 N-Benzyloxycarbonyl-L-proline	60 ± 3	–	–
14 L-Proline amide	87 ± 3	–	–
15 Pyrrolidine	82 ± 2	–	19 ± 11
16 Thiazolidine	83 ± 4	–	–
17 L-4-Thiazolidine carboxylic acid (L-Thiaproline)	48 ± 3	7.9 ± 0.7	91 ± 1
18 L-Pyroglutamic acid	75 ± 9	–	45 ± 1
19 D-Pyroglutamic acid	87 ± 7	–	40 ± 1
20 L-2-Oxothiazolidine-4-carboxylic acid	84 ± 4	–	–
21 2-Aminothiazole-4-acetic acid	47 ± 1	4.0 ± 0.1	98 ± 2
22 cis-3-Azabicyclo [3.1.0]-hexane-2-carboxylic acid	35 ± 3	2.7 ± 0.2	–
23 Piperidine	86 ± 5	–	–
24 L-Pipecolic acid (Homoproline)	71 ± 4	11 ± 3	71 ± 2
25 D-Pipecolic acid	58 ± 3	2.5 ± 1.1	81 ± 4
26 L-Azetidine-2-carboxylic acid	24 ± 1	1.8 ± 0.1[b]	102 ± 2

Uptake of L-[^{3}H]proline (10 nM) was measured at pH 6.0 for 10 min in the absence (=100%) or presence of unlabeled compounds (10 mM). For measuring compound mediated fluorescence signals, final concentrations of 10 mM were used. The fluorescence signal elicited by L-proline was set 100%, signals below 30% were considered as not significant. Data are means ± SE (N = 4–8). –: not measured, mostly because of lack of L-[^{3}H]proline uptake inhibition; [a] data from Metzner et al. (2005), [b] data from Metzner et al. (2004)

fatty acids (Foltz et al., 2004). Both assays applied combined, however, are able to filter out substrates and inhibitors. For several compounds, transport was verified or excluded using electrophysiological techniques using *Xenopus laevis* oocytes expressing mouse PAT1 (Metzner et al., 2005).

In Table 1 interactions of proline, proline derivatives, and homologs with PAT1 are summarized. The D-isomer of proline displays an affinity slightly higher than that of L-proline (see also Boll et al., 2002; Chen et al., 2003; Metzner et al., 2004). Hence, the inversion of the configuration seems to be irrelevant. For cis-4-hydroxyproline only the D-isomer shows a high affinity for PAT1 by inhibiting L-[^{3}H]proline uptake by 61%. The position of the hydroxyl group is of importance for PAT1 substrates: Trans-4-hydroxy-L-proline inhibits L-[^{3}H]proline uptake by 54%, trans-3-hydroxy-L-proline only by 20%. A 2,5-dihydropyrrole ring is the scaffold for 3,4-dehydro-L-proline and its racemate which are prototype PAT1 substrates. All these compounds elicit fluorescence signals between 66 and 100% of the L-proline signal and according to these results they are transported electrogenically via PAT1 expressed in Caco-2 cells. Blocking the α-amino group (N-benzyloxycarbonyl-L-proline) or the carboxyl group (L-proline methyl ester, L-proline tert.-butyl ester, L-proline benzyl ester) obviously diminishes the affinity for PAT1. L-Prolinol and L-proline amide as well as the template structure pyrrolidine are not accepted. L-Thiazolidine-4-carboxylic acid is a moderate substrate for PAT1 with an affinity constant of 7.9 mM, showing that the integration of a hetero atom into the pyrrolidine ring is tolerated. In contrast, D- and L-pyroglutamic acid (equivalent to oxoproline) and L-2-oxothiazolidine-4-carboxylic acid are not recognized, inhibition of L-[^{3}H]proline uptake is only about 13, 25, and 16%, respectively. An insertion of a keto group into the pyrrolidine or thiazolidine ring system reduces the affinity. 2-Aminothiazole-4-acetic acid is an unsaturated ring system with a moderate affinity constant of 4 mM. The bicyclic compound cis-3-azabicyclo[3.1.0]-hexane-2-carboxylic acid involves a proline ring and interacts strongly with PAT1 with an affinity constant of 2.7 mM.

Table 2. Substrate specificity of L-[^{3}H]proline uptake in Caco-2 cells: Proteinogenic and non-proteinogenic neutral amino acids, and derivatives

Substrate/inhibitor	L-[^{3}H]proline uptake (%)	K_i (mM)	Fluorescence signal (%)
Control	100 ± 8		
27 Glycine	56 ± 2[a]	5.1 ± 0.9[b]	100 ± 3[a]
28 L-Alanine	50 ± 1	2.8 ± 0.3	74 ± 10
29 D-Alanine	69 ± 3	–	108 ± 1
30 L-Serine	80 ± 4	–	56 ± 2
31 D-Serine	72 ± 4	–	82 ± 1
32 L-Cysteine	84 ± 3	–	54 ± 3
33 D,L- Homocysteine	76 ± 5	–	–
34 L-2-Aminobutyric acid	78 ± 1	>30	59 ± 1
35 α-Aminoisobutyric acid	73 ± 4	23 ± 5	89 ± 3
36 L-2,4-Diaminobutyric acid	71 ± 2	–	51 ± 1
37 L-Valine	87 ± 8	–	54 ± 1
38 D-Valine	96 ± 2	–	50 ± 1
39 L-Norvaline	84 ± 6	–	55 ± 1
40 D-Norvaline	96 ± 4	–	57 ± 1
41 L-Leucine	102 ± 4[a]	–	–
42 L-Isoleucine	90 ± 2	–	–
43 Sarcosine	24 ± 1	1.9 ± 0.1	101 ± 1
44 N-Methyl-L-alanine	35 ± 4	2.2 ± 0.1[b]	107 ± 1
45 α-(Methylamino)-isobutyric acid	31 ± 3	2.2 ± 0.2	91 ± 4
46 Betaine	25 ± 1	1.9 ± 0.3	–
47 L-Cycloserine	52 ± 1	7.4 ± 0.2	98 ± 4
48 D-Cycloserine	28 ± 6	4.4 ± 0.2	112 ± 1
49 β-Alanine	43 ± 2	2.4 ± 0.5	78 ± 10
50 Taurine	36 ± 1	7.8 ± 0.7	102 ± 2
51 γ-Aminobutyric acid	34 ± 3[a]	4.0 ± 0.3[a]	102 ± 2[a]
52 γ-Hydroxybutyric acid	88 ± 3	–	–
53 3-Amino-1-propane-sulfonic acid (Homotaurine)	60 ± 4	7.1 ± 0.6[b]	87 ± 2
54 6-Aminohexanoic acid	81 ± 5	–	57 ± 1
55 D,L-Carnitine	92 ± 4	–	–

Uptake of L-[^{3}H]proline (10 nM) was measured at pH 6.0 for 10 min in the absence (=100%) or presence of unlabeled compounds (10 mM). For measuring compound mediated fluorescence signals, final concentrations of 10 mM were used. The fluorescence signal elicited by L-proline was set 100%, signals below 30% were considered as not significant. Data are means ± SE (N = 4–8). –: not measured, mostly because of lack of L-[^{3}H]proline uptake inhibition; [a] data from Metzner et al. (2005), [b] data from Metzner et al. (2004)

Extension of the pyrrolidine ring system by one methylene unit leads to piperidine-2-carboxylic acid (pipecolic acid, homoproline). D-Pipecolic acid inhibits L-[^{3}H]proline uptake more than the L-isomer (Metzner et al., 2004; Anderson et al., 2005). Compared to the affinity of L-pipecolic acid, the affinity of D-pipecolic acid for PAT1 is nearly five-fold higher (Tables 1–3). Reduction of the ring size to LACA is well accepted by the system. The affinity constant of LACA of 1.8 mM is comparable to the K_i value of L-proline at PAT1.

It is known that, besides L-proline, many aliphatic neutral amino acids are accepted by PAT1 (Thwaites et al., 1995c). In Table 2 neutral proteinogenic and non-proteinogenic amino acids were tested regarding their substrate specificity for PAT1. Glycine and alanine are common substrates with K_i values of 5.1 mM and 2.8 mM, respectively. L-Serine is no PAT1 substrate. D-Serine elicits a fluorescence signal of 82% indicating that it might be a substrate. Currently, we do not know why the inhibition of L-[^{3}H]proline uptake by D-serine is lower than that reported by others (Thwaites et al., 1995c; Boll et al., 2003a; Chen et al., 2003). L-Cysteine, homocysteine, valine and norvaline (L- and D-configuration), L-leucine, L-isoleucine and L-2-aminobutyric acid, α-aminoisobutyric acid, L-2,4-diaminobutyric acid have no inhibitory potency on L-[^{3}H]proline uptake. The interaction of α-aminoisobutyric acid with PAT1 was studied earlier by Thwaites using also Caco-2 cells (Thwaites et al., 1995c). An insertion of functional groups into the chain (serine, cysteine) is not tolerated. This has also been partially described by Boll and coworkers (Boll et al., 2003a). Methylation of the α-amino group results in an imino group (N-methylglycine/sarcosine, N-methylalanine, α-(methylamino)-isobutyric acid), which enhances substrate affinity in comparison to the corresponding amino acid. These methylated compounds elicit fluorescence signals around 100%. Structures containing a quaternary nitrogen atom and a short methylene chain seem to be high affinity substrates for PAT1 as shown for betaine with a K_i value of 1.9 mM.

Considering the structural limitations for PAT1 substrates it is surprising that the naturally occurring isoxazole derivative cycloserine is a high affinity substrate. The D-isomer is with a K_i of 4.4 mM slightly stronger bound than the L-isomer (K_i 7.4 mM). An active transport mechanism for D-cycloserine, an effective antibiotic, in Caco-2 cells was described several years ago (Ranaldi et al., 1994; Thwaites et al., 1995a). These findings also demonstrate that an insertion of a hetero atom into the pyrrolidine template is well tolerated. The high affinity substrates ß-alanine (K_i 2.4 mM) and GABA (K_i 4.0 mM) show that ω-amino acids with a separation distance of two to three methylene units between the amino and the carboxyl group are accepted by PAT1. Proton dependent transport of β-alanine and GABA in Caco-2 cells has been described earlier by Thwaites and coworkers (Thwaites et al., 1993a, 2000). These compounds elicit MP dependent fluorescence signals of 78 and 102% (Table 1). Sub-

Table 3. Substrate specificity of L-[^{3}H]proline uptake in Caco-2 cells: Aromatic and heteroaromatic amino acids and indole derivatives

Substrate/inhibitor	L-[^{3}H]proline uptake (%)	K_i (mM)	Fluorescence signal (%)
Control	100 ± 8		
56 Pyridine-2-carboxylic acid	68 ± 2	–	72 ± 2
57 Nicotinic acid	61 ± 4	8.0 ± 0.5	65 ± 7
58 Isonicotinic acid	72 ± 2	–	81 ± 2
59 L-Phenylglycine	101 ± 12	–	37 ± 1
60 L-Phenylalanine	82 ± 4	–	37 ± 2
61 N-Methyl-L-phenylalanine	86 ± 3	–	34 ± 1
62 4-Nitro-L-phenylalanine	62 ± 3	–	–
63 4-Fluoro-D,L-phenylalanine	82 ± 2	–	–
64 4-Amino-L-phenylalanine	85 ± 5	–	–
65 L-Tyrosine (2 mM)	94 ± 5	–	–
66 L-Dopa	89 ± 5	–	37 ± 1
67 L-Dopamine	90 ± 4	–	32 ± 1
68 Indole	33 ± 1	–	–
69 Indole-2-carboxylic acid	42 ± 1	4.0 ± 0.3	4 ± 5
70 Indole-3-carboxylic acid	68 ± 6	–	–
71 Indole-5-carboxylic acid	43 ± 7	–	–
72 Indole-3-acetic acid	49 ± 2	–	−3 ± 2
73 Indole-3-propionic acid	37 ± 3[a]	3.9 ± 0.1	−18 ± 6[a]
74 L-Tryptophan	48 ± 4[a]	4.7 ± 0.3[a]	23 ± 3[a]
75 D-Tryptophan	95 ± 7[a]	–	32 ± 2[a]
76 4-Fluoro-D,L-tryptophan	71 ± 4	–	–
77 5-Fluoro-D,L-tryptophan	42 ± 4	–	–
78 6-Fluoro-D,L-tryptophan	38 ± 5	–	–
79 N-Methyl-L-tryptophan	79 ± 7	–	36 ± 1
80 5-Hydroxy-L-tryptophan	30 ± 1[a]	0.9 ± 0.1[a]	13 ± 5[a]
81 N-Acetyl-L-tryptophan	88 ± 6	–	–
82 L-Tryptophan methyl ester	89 ± 1	–	–
83 Tryptamine	30 ± 1[a]	6.1 ± 0.4[a]	12 ± 5[a]
84 Serotonin	45 ± 3[a]	5.7 ± 0.6[a]	23 ± 2[a]
85 Indoline	89 ± 3	–	–
86 L-Indoline-2-carboxylic acid	65 ± 4	–	23 ± 11
87 L-Tetrahydroisochinoline-3-carboxylic acid	81 ± 6	–	–

Uptake of L-[^{3}H]proline (10 nM) was measured at pH 6.0 for 10 min in the absence (=100%) or presence of unlabeled compounds (10 mM). For measuring compound mediated fluorescence signals, final concentrations of 10 mM were used. The fluorescence signal elicited by L-proline was set 100%, signals below 30% were considered as not significant. Data are means ± SE (N = 4–8). –: not measured, mostly because of lack of L-[^{3}H]proline uptake inhibition; [a] data from Metzner et al. (2005), [b] data from Metzner et al. (2004)

stitution of the carboxyl group by a sulfonic acid group results in moderate affinity substrates such as taurine and homotaurine with K_i values of 7.8 mM and 7.1 mM, respectively. In contrast, substitution of the amino group by a hydroxyl group leads to a decrease in affinity as shown by γ-hydroxybutyric acid which inhibits L-[^{3}H]proline uptake by 32%. 6-Aminohexanoic acid is not a PAT1 substrate probably because of the separation of the two functional groups by five methylene groups confirming the findings by Boll and coworkers (2003a). D,L-Carnitine containing a quaternary nitrogen shows no affinity for PAT1. The functionalization of the molecule as in serine or cysteine seems to be responsible for the lack of inhibitory potency.

Investigation of the substrate specificity of PAT1 was extended by aromatic and heteroaromatic amino acids, and their respective derivatives (Table 3). Pyridine-2-carboxylic acids which are weak substrates served as starting point. The position of the carboxyl group relative to the tertiary nitrogen atom (ortho, meta, para) seems to play a minor role. Aromatic α-amino carboxylic acids such as L-phenylglycine, L-phenylalanine, L-tyrosine, and the derivatives shown in Table 3 are weak inhibitors of L-[^{3}H]proline uptake, except 4-nitro-L-phenyalanine. N-Methyl-L-phenylalanine elicits no significant fluorescence signal and can also be classified as non-substrate. Compared to N-methyl-L-alanine, the phenyl ring seems to be the decisive substrate affinity limiting structure. Heterocyclic

aromatic amino acids containing an indole scaffold (tryptophan and derivatives) give very different results. Indole and indole carboxylic acids except indole-3-carboxylic acid strongly inhibit L-[^{3}H]proline uptake by more than 50%. Addition of a carboxyl group to the indole ring system does not seem to be crucial for an interaction with PAT1. The same applies for auxine derivatives such as indole-2-acetic acid and indole-3-propionic acid. Elongation of the distance between the carboxyl group and the indole system is well tolerated by PAT1 (Table 3).

L-Tryptophan as well is a moderate PAT1 inhibitor with a K_i of 4.7 mM (Metzner et al., 2005). L-Tryptophan derivatives which are substituted at the indole ring system (5-, 6-fluoro-D,L-tryptophan) show a similar interaction. Blocking the α-amino group and the carboxyl group, respectively, causes a decline in binding affinity (N-methyl-L-tryptophan, N-acetyl-L-tryptophan, L-tryptophan methyl ester). For glycine and alanine Boll and coworkers showed that the methyl-substitution of the carboxyl group (O-methyl-glycine, O-methyl-alanine) strongly diminishes affinity and transport rate at PAT1 (Boll et al., 2003a). The decarboxylated L-tryptophan, tryptamine and its 5-hydroxy derivative, the neurotransmitter serotonin, interact with PAT1 with moderate affinities of 6.1 mM and 5.7 mM, respectively. 5-Hydroxy-L-tryptophan, indole-3-propionic acid, and L-tryptophan interact with PAT1 in a K_i value range of prototype substrates from 0.9 mM to 4.7 mM. The fluorescence signals elicited by these compounds are negligible. These findings and measurements at PAT1-expressing *Xenopus laevis* oocytes let us conclude that these compounds function as effective non-transported PAT1 inhibitors (Metzner et al., 2005). All other indole derivatives investigated in this study are so far considered to be potential inhibitors because of lack of fluorescence signals in the MP assay. Detailed studies using PAT1 expressing oocytes have yet to be performed. It could also be shown that a partial hydrogenolysis of the indole template at 2,3-position (indoline) is not accepted by PAT1 whereas an insertion of a carboxyl group in α-position to the imino group leads to a slightly enhanced binding affinity (L-indoline-2-carboxylic acid). Extension of the indole ring system by one methylene unit is not tolerated. L-Tetrahydroisochinoline-3-carboxylic acid shows no substrate properties.

Figure 1 illustrates the relation between L-[^{3}H]proline uptake inhibition vs. effects on MP for many of the compounds listed in Tables 1–3. The plot reveals two clusters of compounds: As expected the inhibitory potency of competing substrates correlates well with their effect on MP ($P < 0.0001$). In strong contrast, there is no correlation between the inhibition of L-[^{3}H]proline uptake and the fluorescence signals for non-transported inhibitors ($P = 0.350$).

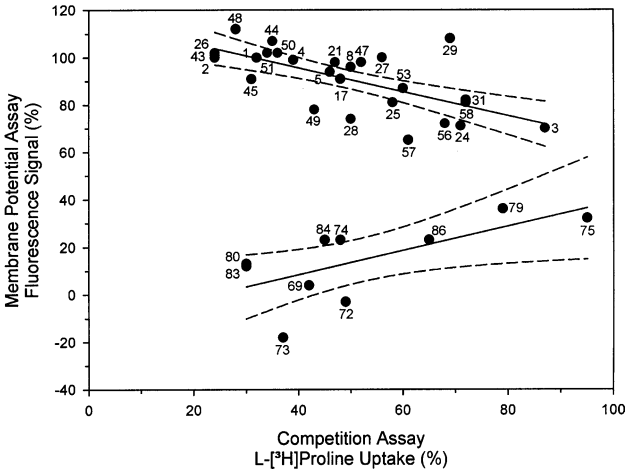

Fig. 1. Correlation between L-[^{3}H]proline uptake inhibition and effect on membrane potential of PAT1 substrates and inhibitors. X-axis: L-[^{3}H]Proline uptake (10 nM, 10 min, pH 6.0) measured in the absence or presence of unlabeled compounds (10 mM). Y-axis: Compound mediated fluorescence signals in relation to that elicited by L-proline (=100%), measured at 10 mM, pH 6.0. Numbers of compounds and data correspond to Tables 1–3. Dashed line: 95% confidence interval of linear regression; N = 4–8

Detailed studies regarding the substrate specificity of the second member of the SLC36 family, the proton coupled amino acid transporter 2 (PAT2), were performed by H. Daniels group and by Kennedy and coworkers (Boll et al., 2002; Foltz et al., 2004; Kennedy et al., 2004). This system is, compared to PAT1, a high affinity carrier which mediates the transport of L-proline with a Michaelis-Menten constant of 172 ± 41 μM (Kennedy et al., 2004). Transport measurements using *Xenopus laevis* oocytes expressing PAT2 showed that PAT2 displays similar structural substrate requirements as PAT1. There are some exceptions, e.g., PAT2 does not accept GABA.

In summary, the transport data of the 87 compounds collected in this study let us conclude that the structural requirements for PAT1 substrates are the following:

1. The carrier prefers small, unbranched neutral amino acids.
2. The carrier does not differentiate between the L- and D-isomers of several amino acids; in some cases PAT1 prefers the D-isomer.
3. Proline derivatives with 4-, 5- and 6-membered ring templates are accepted.
4. Incorporation of sulfur into heterocyclic structures does not diminish affinity for PAT1.
5. A free imino group or a quaternary amino group is essential for high affinity and is preferred over a free

primary amino group. ω-Amino acids comprising up to three methylene units between the functional groups are well tolerated.

6. A free negatively charged carboxyl group preferably in α-position to the imino- or amino-group is necessary. Substitution by a sulfonic group is possible.
7. L-Tryptophan and other heterocyclic compounds containing an indole scaffold are high affinity non-transported inhibitors.

The structural requirements of PAT1 substrates elucidated in this study will be useful for the development of small prodrugs. Their transport by PAT1 across the intestinal epithelium could allow oral administration.

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References

Anderson CM, Grenade DS, Boll M, Foltz M, Wake KA, Kennedy DJ, Munck LK, Miyauchi S, Taylor PM, Campbell FC, Munck BG, Daniel H, Ganapathy V, Thwaites DT (2004) H^+/Amino acid transporter 1 (PAT1) is the imino acid carrier: An intestinal nutrient/drug transporter in human and rat. Gastroenterology 127: 1410–1422

Boll M, Daniel H, Gasnier B (2004) The SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. Pflugers Arch 447: 776–779

Boll M, Foltz M, Anderson CM, Oechsler C, Kottra G, Thwaites DT, Daniel H (2003a) Substrate recognition by the mammalian proton-dependent amino acid transporter PAT1. Mol Membr Biol 20: 261–269

Boll M, Foltz M, Rubio-Aliaga I, Daniel H (2003b) A cluster of proton/amino acid transporter genes in the human and mouse genomes. Genomics 82: 47–56

Boll M, Foltz M, Rubio-Aliaga I, Kottra G, Daniel H (2002) Functional characterization of two mammalian electrogenic proton-dependent amino acid cotransporters. J Biol Chem 277: 22966–22973

Chen Z, Fei YJ, Anderson CM, Wake KA, Miyauchi S, Huang W, Thwaites DT, Ganapathy V (2003) Structure, function and immunolocalization of a proton-coupled amino acid transporter (hPAT1) in the human intestinal cell line Caco-2. J Physiol 546: 349–361

Faria TN, Timoszyk JK, Stouch TR, Vig BS, Landowski CP, Amidon GL, Weaver CD, Wall DA, Smith RL (2004) A novel high-throughput PepT1 transporter assay differentiates between substrates and antagonists. Mol Pharmacol 1: 67–76

Foltz M, Boll M, Raschka L, Kottra G, Daniel H (2004) A novel bifunctionality: PAT1 and PAT2 mediate electrogenic proton/amino acid and electroneutral proton/fatty acid symport. FASEB J 18: 1758–1760

Foltz M, Oechsler C, Boll M, Kottra G, Daniel H (2004) Substrate specificity and transport mode of the proton-dependent amino acid transporter mPAT2. Eur J Biochem 271: 3340–3347

Ganapathy V, Brandsch M, Leibach FH (1994) Intestinal transport of amino acids and peptides. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 3rd ed. Raven Press, New York, pp 1773–1794

Goodman M, Felix A, Moroder L, Toniolo C (eds) (2002) Houben-Weyl: methods of organic chemistry. Thieme, Stuttgart, vol. E22A

Kennedy DJ, Gatfield KM, Winpenny JP, Ganapathy V, Thwaites DT (2005) Substrate specificity and functional characterisation of the H^+/amino acid transporter rat PAT2 (SLC36A2). Br J Pharmacol 114: 28–41

Kowalczuk S, Bröer A, Munzinger M, Tietze N, Klingel K, Bröer S (2005) Molecular cloning of the mouse IMINO system: an Na^+- and Cl^--dependent proline transporter. Biochem J 386: 417–422

Metzner L, Kalbitz J, Brandsch M (2004) Transport of pharmacologically active proline derivatives by the human proton-coupled amino acid transporter hPAT1. J Pharmacol Exp Ther 309: 28–35

Metzner L, Kottra G, Neubert K, Daniel H, Brandsch M (2005) Serotonin, L-tryptophan, and tryptamine are effective inhibitors of the amino acid transport system PAT1. FASEB J 19: 1468–1473

Nicklin PL, Irwin WJ, Hassan IF, Mackay M (1992) Proline uptake by monolayers of human intestinal absorptive (Caco-2) cells in vitro. Biochim Biophys Acta 1104: 283–292

Palacin M, Estevez R, Bertran J, Zorzano A (1998) Molecular biology of mammalian plasma membrane amino acid transporters. Physiol Rev 78: 969–1054

Ranaldi G, Islam K, Sambuy Y (1994) D-Cycloserine uses an active transport mechanism in the human intestinal cell line Caco 2. Antimicrob Agents Chemother 38: 1239–1245

Sagne C, Agulhon C, Ravassard P, Darmon M, Hamon M, El Mestikawy S, Gasnier B, Giros B (2001) Identification and characterization of a lysosomal transporter for small neutral amino acids. Proc Natl Acad Sci USA 98: 7206–7211

Steffansen B, Nielsen CU, Brodin B, Eriksson AH, Andersen R, Frokjaer S (2004) Intestinal solute carriers: an overview of trends and strategies for improving oral drug absorption. Eur J Pharm Sci 21: 3–16

Takanaga H, Mackenzie B, Suzuki Y, Hediger MA (2005) Identification of a mammalian proline transporter (SIT1, SLC6A20) with characteristics of classical system IMINO. J Biol Chem 1: 67–76

Thwaites DT, Armstrong G, Hirst BH, Simmons NL (1995a) D-Cycloserine transport in human intestinal epithelial (Caco-2) cells: mediation by a H^+-coupled amino acid transporter. Br J Pharmacol 115: 761–766

Thwaites DT, Basterfield L, McCleave PMJ, Carter SM, Simmons NL (2000) Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers. Br J Pharmacol 129: 457–464

Thwaites DT, McEwan GT, Brown CD, Hirst BH, Simmons NL (1994) L-Alanine absorption in human intestinal Caco-2 cells driven by the proton electrochemical gradient. J Membr Biol 140: 143–151

Thwaites DT, McEwan GT, Hirst BH, Simmons NL (1995b) H^+-coupled alpha-methylaminoisobutyric acid transport in human intestinal Caco-2 cells. Biochim Biophys Acta 1234: 111–118

Thwaites DT, McEwan GT, Simmons NL (1995c) The role of the proton electrochemical gradient in the transepithelial absorption of amino acids by human intestinal Caco-2 cell monolayers. J Membr Biol 145: 245–256

Thwaites DT, McEwan GTA, Brown CDA, Hirst BH, Simmons NL (1993a) Na^+-independent, H^+-coupled transepithelial ß-alanine absorption by human intestinal Caco-2 cell monolayers. J Biol Chem 268: 18438–18441

Thwaites DT, McEwan GTA, Cook MJ, Hirst BH, Simmons NL (1993b) H^+-coupled (Na^+-independent) proline transport in human intestinal (Caco-2) epithelial cell monolayers. FEBS Lett 333: 78–82

Authors' address: Matthias Brandsch, Membrane Transport Group, Biozentrum of the Martin-Luther-University Halle-Wittenberg, Weinbergweg 22, D-06120 Halle, Germany,
Fax: +49-345-552-7258, E-mail: matthias.brandsch@biozentrum.uni-halle.de