RESEARCH PAPER

The proton-coupled amino acid transporter, SLC36A1 (hPAT1), transports Gly-Gly, Gly-Sar and other Gly-Gly mimetics

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BACKGROUND AND PURPOSE

The intestinal proton-coupled amino acid transporter, SLC36A1, transports zwitterionic α -amino acids and drugs such as vigabatrin, gaboxadol and δ -aminolevulinic acid. We hypothesize that SLC36A1 might also transport some dipeptides. The aim of the present study was to investigate SLC36A1-mediated transport of Gly-Gly and Gly-Gly mimetics, and to investigate Gly-Sar transport via SLC36A1 and the proton-coupled dipeptide/tripeptide transporter, SLC15A1 in Caco-2 cells.

EXPERIMENTAL APPROACH

Transport of a compound via SLC36A1 was determined by its ability to induce an increase in the inward current of two-electrode voltage clamped SLC36A1 cRNA-injected *Xenopus laevis* oocytes. SLC36A1-mediated L-[³H]Pro uptake in Caco-2 cells was measured in the absence and presence of Gly-Gly or Gly-Sar. In addition, apical [¹⁴C]Gly-Sar uptake was measured in the absence and presence of the SLC36A1 inhibitor 5-hydroxy-L-tryptophan (5-HTP) or the SLC15A1 inhibitor L-4,4'-biphenylalanyl-L-proline (Bip-Pro).

KEY RESULTS

In SLC36A1-expressing oocytes, an inward current was induced by Gly-Sar, Gly-Gly, δ -aminolevulinic acid, β -aminoethylglycine, δ -aminopentanoic acid, GABA, Gly and Pro, whereas Val, Leu, mannitol, 5-HTP and the dipeptides Gly-Ala, Gly-Pro and Gly-Phe did not evoke currents. In Caco-2 cell monolayers, the apical uptake of 30 mM Gly-Sar was inhibited by 20 and 22% in the presence of 5-HTP or Bip-Pro, respectively, and by 48% in the presence of both.

CONCLUSION AND IMPLICATIONS

Our results suggest that whereas Gly-Gly amid bond bioisosteres are widely accepted by the hPAT1 carrier, dipeptides in general are not; and therefore, Gly-Sar might structurally define the size limit of dipeptide transport via SLC36A1.

Abbreviations

5-HTP, 5-hydroxy-L-tryptophan; AEG, β -aminoethylglycine; ALA, δ -aminolevulinic acid; Ala, alanine; β -Ala, β -alanine; APA, δ -aminopentanoic acid; Bip-Pro, L-4,4'-biphenylalanyl-L-proline; GABA, γ -aminobutyric acid; Gly, glycine; Gly-Ala, glycyl-alanine; Gly-Gly, glycyl-glycine; Gly-Phe, glycyl-phenylalanine; Gly-Pro, glycyl-proline; Gly-Sar, glycyl-sarcosine; Leu, leucine; Pro, L-proline; Sar, sarcosine; SEM, standard error of the mean; SLC15A1 (hPEPT1), human proton-coupled di-/tripeptide transporter 1; SLC36A1 (hPAT1), human proton-coupled amino acid transporter 1; TEER, trans-epithelial electrical resistance; Val, valine

Introduction

The proton-coupled amino acid transporter SLC36A1 (hPAT1), is an absorptive intestinal transporter for small zwitterionic α -amino acids such as proline (Pro), glycine (Gly) and alanine (Ala) (Thwaites *et al.*, 1995b; Chen *et al.*, 2003) and osmolytes such as sarcosine and taurine (Thwaites *et al.*, 1995b; Anderson *et al.*, 2009). Moreover, SLC36A1 has been identified as a drug carrier mediating the absorption across the intestinal luminal membrane of a number of γ -aminobutyric acid (GABA) mimetics, such as vigabatrin and gaboxadol (Thwaites *et al.*, 2000; Abbot *et al.*, 2006; Larsen *et al.*, 2009). Other drug substances identified *in vitro* as substrates for SLC36A1 are the antibiotic and N-methyl-D-aspartate receptor agonist D-cycloserine, the orally administered creatine analogue β -guanidinopropionic acid and pharmacologically active proline derivatives such as L-azetidine-2-carboxylic acid, *cis*-4-hydroxy-L-proline and 3,4-dehydro-D,L-proline (Thwaites *et al.*, 1995a; Metzner *et al.*, 2004; 2009). Very interestingly, tryptamine, 5-hydroxy-L-tryptophan (5-HTP) and 5-hydroxytryptamine (serotonin) have been identified as inhibitors of SLC36A1-mediated transport, thus providing inhibitors used as investigational tools. Pharmacokinetic investigations in beagle dogs have recently suggested that the high oral bioavailability of gaboxadol is mediated by its intestinal absorption via SLC36A1 (Larsen *et al.*, 2009). Recently, Anderson *et al.* (2010) and Frølund *et al.* (2010) demonstrated that the dipeptidomimetic drug substance δ -aminolevulinic acid (ALA) is a substrate for SLC36A1. ALA is a δ -amino acid and is referred to as a dipeptidomimetic substance as it was originally identified as a substrate for the proton-coupled di-/tripeptide transporter, SLC15A1 (PEPT1) (Doring *et al.*, 1998). We found that in Caco-2 cell monolayers, ALA is transported via both SLC36A1 and SLC15A1 and estimated that at a concentration likely to be present in the human intestine after oral doses of ALA, SLC36A1 could be the predominant transporter mediating the intestinal absorption into the enterocytes. Preliminary observations also indicated, that the standard marker of SLC15A1-mediated transport, Gly-Sar, might also be a substrate of SLC36A1 (Frølund *et al.*, 2010). Thus, we hypothesized that the proton-coupled amino acid transporter, SLC36A1, could be a transporter of Gly-Gly, Gly-Sar and related Gly-Gly mimetics. The aim of the present study was therefore to investigate a series of Gly-Gly mimetics with respect to their substrate profile for SLC36A1, and to investigate whether dipeptide transport using Gly-Sar via SLC36A1 is abundant in an *in vitro* model of the

intestinal epithelium, that is, Caco-2 cell monolayers. Our findings show that SLC36A1 recognizes and transports Gly-Gly, Gly-Sar and related Gly-Gly mimetics, whereas Gly-Ala, Gly-Pro and Gly-Phe are not translocated. Moreover, we show that SLC36A1 – and not only SLC15A1 – transports Gly-Sar in Caco-2 cell monolayers. These findings extend the current understanding of the structural requirements for SLC36A1-mediated transport and add to the structures currently being identified as substrates for both SLC15A1 and SLC36A1. Furthermore, the present findings may assist in the understanding of drug–drug or drug–food interactions present at the transporter level during intestinal absorption.

Methods

The nomenclature used for drugs and molecular targets follows the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Study design

In order to investigate whether Gly-Gly and Gly-Gly mimetics are substrates for SLC36A1, a series of mimetic compounds (test compounds; see Table 1) were selected and investigated by two-electrode voltage clamp measuring on SLC36A1-expressing *Xenopus laevis* oocytes. Here, we used the term mimetic for the Gly-Gly-related chemical structures, although this distinction may not be clear-cut. In the literature, δ -aminopentanoic acid (APA) has also been referred to as a GABA mimetic. The selection of compounds was based on the recent finding that ALA is a substrate for both the proton-coupled amino acid transporter, SLC36A1 and the di-/tripeptide transporter, SLC15A1 (Anderson *et al.*, 2010; Frølund *et al.*, 2010). ALA is a ketomethylene dipeptidomimetic of Gly-Gly, and has an IC_{50} value of 2 mM and 7–11 mM for SLC15A1 and SLC36A1, respectively (Anderson *et al.*, 2010; Frølund *et al.*, 2010). Gly-Gly is the simplest dipeptide possible, it has a K_i value of 1.0 mM for SLC15A1 (Gebauer *et al.*, 2003) and was included as a test compound. N-methylation of Gly-Gly yields Gly-Sar, which is the most studied substrate for SLC15A1; Gly-Sar has a K_i value of 0.83 mM for SLC15A1 (Gebauer *et al.*, 2003), and was also included as a test compound in the present study. A dipeptidomimetic without a carbonyl group, that is, β -aminoethylglycine (AEG), was also included as a test compound; AEG has a very poor affinity for SLC15A1, with an estimated K_m value above 30 mM (Brandsch *et al.*, 2004). Furthermore, a compound entirely without an amide

Table 1

Affinity for SLC36A1

Compound	Structure	Affinity (K_m)	(n)
L-Pro		1.8 ± 0.17 mM	(7)
Gly		11 ± 1.0 mM	(11)
GABA		2.4 ± 0.28 mM	(6)
APA		12 ± 1.6 mM	(8)
AEG		37 ± 2.2 mM	(7)
ALA		15 ± 1.6 mM	(8)
Gly-Gly		21 ± 2.2 mM	(6)
Gly-Sar		49 ± 9 mM	(12)

Structures of the compounds included in the present study and their affinity for SLC36A1 obtained by two-electrode voltage clamp measurements in *SLC36A1* cRNA injected *X. laevis* oocytes. The affinities are given as the $K_m \pm$ SEM obtained by analysing the concentration-dependent currents evoked from 6–12 different oocytes ($n = 6$ –12) using Eq. (1).

bond was included, that is, APA; APA has a K_i value of approximately 25 mM for SLC15A1 and a K_m value of approximately 38 mM for SLC36A1 (Boll *et al.*, 2003; Brandsch *et al.*, 2004); although Boll *et al.* (2003) considered that the affinity of APA for SLC36A1 was too low for classifying APA as a SLC36A1-substrate. A total of five Gly-Gly mimetics were included in the study. To validate the oocytes

expression system with respect to function, a panel of positive and negative controls was selected for investigations of SLC36A1 activity. The group of positive controls consisted of the α -amino acids Pro and Gly and the γ -amino acid GABA (Thwaites *et al.*, 1993; 1995b; 2000; Boll *et al.*, 2003). The negative controls included the non-SLC36A1 translocated α -amino acids Leu and Val (Chen *et al.*, 2003), the

osmolyte mannitol, the non-translocated SLC36A1-ligand 5-HTP and a group of dipeptides with side chains other than –H in the C-terminal amino acid, for example, Gly-Ala, Gly-Pro and Gly-Phe. The relative contribution of SLC36A1 and SLC15A1 to the total uptake of Gly-Sar was investigated by inhibition studies on Caco-2 cell monolayers, a model of the small intestinal epithelium cell. The inhibitor used for SLC36A1-mediated Gly-Sar uptake was the non-translocated SLC36A1-ligand 5-HTP (Metzner *et al.*, 2005) and that for SLC15A1-mediated Gly-Sar uptake, the SLC15A1-substrate L-4,4'-biphenylalanyl-L-proline (Bip-Pro) (Knutter *et al.*, 2007).

In vitro synthesis of SLC36A1 cRNA

The open reading frame of the *SLC36A1* cDNA was PCR-amplified using *SLC36A1* specific primers (forward: 5'-cggaattcaccatgtccacgcagagacttc-3', reverse: 5'-ggctctagatcctatatgaaggcacag-3') and the Pfu Ultra II polymerase. The pcDNA3.1-*SLC36A1* (Frolund *et al.*, 2010) was used as template. The PCR product was sub-cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit and verified by sequencing. The *SLC36A1* cDNA was cut from the vector using the PCR-introduced restriction sites (*EcoRI* and *XbaI*) and ligated into the pGEMHE vector. pGEMHE-*SLC36A1* was linearized downstream from the stop codon using *NheI* and cRNA was synthesized by *in vitro* transcription using the mMESSAGE mMACHINE T7 mRNA-capping Kit according to the protocol supplied by the manufacturer.

Two-electrode voltage clamp measurements

The SLC36A1-activity of a compound was determined by its ability to induce an increase in the inward current of two-electrode voltage clamped *SLC36A1* cRNA injected *X. laevis* oocytes as oppose to water-injected oocytes. *X. laevis* handling and oocyte extraction procedures were performed according to national guidelines and approved by the Danish Animal Experiments Inspectorate. Oocyte preparation and *SLC36A1* cRNA injection was performed essentially as described by Jensen *et al.* (2006). Adult female *X. laevis* frogs were anaesthetized by submersion into a 0.04% (w/v) aqueous solution of ethyl-m-amino-benzoate. The removed oocytes were separated by collagenase treatment (0.5 mg·mL⁻¹ collagenase type 1A) and maintained at 18°C in modified Barth's saline (in mM: NaCl, 88.0; KCl, 1.0; HEPES, 15.0; NaHCO₃, 2.4; CaCl₂, 0.41; MgSO₄, 0.82; Ca(NO₃)₂, 0.3) supplemented with 0.1 U·L⁻¹ penicillin and 10 µg·L⁻¹ streptomycin. On the second day, oocytes were injected with 9–23 nL diluted *SLC36A1* cRNA (2.5 µg·µL⁻¹) or

water (served as negative control). Electrophysiological measurements were performed 4 to 6 days post-injection. The oocytes were voltage clamped at –60 mV using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA, USA) and continuously perfused with Ringer's solution, pH 6.0 (in mM: NaCl, 115; KCl, 2.5; CaCl₂, 1.8; MgCl₂, 0.1; MES, 10). Compounds were dissolved in Ringer's solution, pH was adjusted and the solutions were added to the oocytes by full bath application for 40 s, while the change in membrane current was monitored. Water-injected oocytes served as negative controls and ensured that any change in membrane current was in fact caused by SLC36A1-activity and not due to non-specific responses related to, for example, changes in osmolality.

Caco-2 cell experiments

Caco-2 cells were cultured as previously described (Nielsen *et al.*, 2001; Larsen *et al.*, 2008). Caco-2 cells of passages 23 through 31 were seeded onto Transwell inserts (1.12 cm², 0.4 µm pore size, 10⁵ cells insert⁻¹) and experiments were conducted on day 21–28 after seeding. Before the experiments were started, the barrier properties of the Caco-2 monolayers were assessed by trans-epithelial electrical resistance (TEER, always >100 Ω·cm²).

The concentration-dependent inhibition of SLC36A1-mediated apical uptake of L-[³H]Pro (13 nM; 1 µCi·mL⁻¹) by Gly-Gly or Gly-Sar, and the ability of 0.5 mM Bip-Pro, 10 mM 5-HTP or 0.5 mM Bip-Pro and 10 mM 5-HTP to inhibit the apical uptake of either 1 mM Gly-Sar (containing 18 µM [¹⁴C]-Gly-Sar, 1 µCi·mL⁻¹) or 30 mM Gly-Sar (containing 45 µM [¹⁴C]Gly-Sar, 2.5 µCi·mL⁻¹) were measured in Caco-2 cell monolayers essentially as previously described (Nielsen *et al.*, 2001; Larsen *et al.*, 2008; Frolund *et al.*, 2010). Apical pH was maintained at 6.0 and basolateral pH was maintained at 7.4. In order to avoid an effect on the uptake rate due to changes in osmolality, the osmolality of the solutions within a single experiment were kept constant by adding mannitol to the solutions. The uptake was measured for 5 min and terminated by washing the monolayers three times with ice-cold HBSS. The polycarbonate filters were cut from the Transwell supports, 2 mL Ultima Gold scintillations liquid was added and the radioactivity was quantified by scintillation counting on a Packard TriCard liquid scintillation counter (Meriden, CT, USA). For the uptake studies of Gly-Sar in the absence or presence of Bip-Pro or 5-HTP, the radioactivity counts measured in the Caco-2 cell monolayers were between approximately 1000–6000 DPM per well. In these experiments, 5-HTP and Bip-Pro were considered to be an inhibitor and

a competitive inhibitor of SLC36A1- and SLC15A1-mediated substrate transport (Metzner *et al.*, 2005; Knutter *et al.*, 2007), respectively.

Data analysis

The K_m values were estimated from the concentration-dependent inward currents recorded after addition of substrate. Data were fitted to the Michaelis–Menten equation:

$$I = \frac{I_{\max} \cdot [S]}{K_m + [S]} \quad (1)$$

The inward current I , measured in nA, was used as a measure of the uptake rate, where $I_{\max}$ is the maximal inward current or uptake rate in nA, K_m is the Michaelis–Menten constant in mM and $[S]$ is the concentration of substrate in mM. Due to variability in the expression of SLC36A1 among the oocytes, it is not feasible to compare $I_{\max}$ values obtained for the individual test compounds, and thus, $I_{\max}$ values are not reported in the present study.

The IC_{50} value of Gly-Gly or Gly-Sar was the concentration at which the apical uptake of Pro was reduced to 50% of the control value. The IC_{50} value was determined by fitting the data to Eq. (2) giving a sigmoidal dose–inhibition curve:

$$U = U_{\min} + \frac{U_{\max} - U_{\min}}{1 + 10^{(\log[I] - \log IC_{50})}} \quad (2)$$

U is the specific cellular uptake of Pro (0–100%) at concentration $[I]$ of Gly-Gly or Gly-Sar. $U_{\min}$ is the minimal uptake of Pro (at the highest value of $[I]$), and $U_{\max}$ is the control uptake ($[I] = 0$), both given as % values. All data fitting were obtained using GraphPad Prism (GraphPad Prism Software, version 4.03, San Diego, CA, USA).

In order to estimate the relative contribution of SLC36A1 and SLC15A1 to the total uptake of Gly-Sar in Caco-2 cells, the following assumptions were made. The apical uptake of Gly-Sar is only mediated by SLC36A1 and SLC15A1, and 5-HTP and Bip-Pro can, under our experimental conditions, be considered as an inhibitor and a competitive inhibitor of only SLC36A1 and SLC15A1 (Metzner *et al.*, 2005; Knutter *et al.*, 2007), respectively. The total uptake rate of Gly-Sar V_{total} , measured in the absence or presence of various inhibitors is thus given by the following expression, derived from a Michaelis–Menten type equation:

$$V_{total} = \left(\frac{V_{\max} \cdot [S]}{[S] + K_m \left(1 + \frac{[I]}{K_i} \right)} \right)_{SLC36A1} + \left(\frac{V_{\max} \cdot [S]}{[S] + K_m \left(1 + \frac{[I]}{K_i} \right)} \right)_{SLC15A1} \quad (3)$$

$[S]$ is the apical concentration of Gly-Sar in mM, $[I]$ is the apical concentration of an inhibitor of SLC36A1 or SLC15A1 with the inhibition constant

K_i , K_m is the Michaelis–Menten constant in mM, and $V_{\max}$ is the maximal transport capacity of the substrate via the particular transporter.

The maximal transport capacity of Gly-Sar via SLC15A1 has been estimated to be $1.9 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ and the K_m value to 1 mM, similar to the $V_{\max}$ and K_m values previously obtained for Gly-Sar uptake in Caco-2 cells grown under similar conditions (Nielsen *et al.*, 2003). Since there are no K_m or $V_{\max}$ values available for the transport of Gly-Sar via SLC36A1 in Caco-2 cells, we used the previously obtained maximal transport capacity of Pro across the apical membrane in Caco-2 cells, $V_{\max} = 5.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ (Larsen *et al.*, 2008) and the K_m obtained in the present study for the electrogenic transport of Gly-Sar into oocytes injected with SLC36A1 cRNA (49 mM). The K_i of 5-HTP and Bip-Pro was 2.3 mM and 24 μM , respectively (Knutter *et al.*, 2007; Larsen *et al.*, 2008). Using these assumptions, the total uptake of 1 mM Gly-Sar in the absence of inhibitors should be approximately $1.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$, whereas the uptake should be reduced to 91% ($1.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$), 16% ($0.18 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) and 9% ($0.10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) in the presence of 10 mM 5-HTP, 0.5 mM Bip-Pro or 10 mM 5-HTP and 0.5 mM Bip-Pro, respectively. The total uptake of 30 mM Gly-Sar should be $3.7 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ in the absence of inhibitors. In the presence of 10 mM 5-HTP, 0.5 mM Bip-Pro or 10 mM 5-HTP and 0.5 mM Bip-Pro, the uptake should be reduced to 65% ($2.4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$), 81% ($3.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) and 43% ($1.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$), respectively.

Statistical analysis

Statistical analysis was performed in GraphPad Prism (GraphPad Prism Software, version 4.03). Differences between means were analysed using ANOVA followed by Dunnett's multiple comparison test. ** Denotes a levels of significance of $P < 0.01$.

Chemicals and reagents

The chemicals used were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Gly-Gly, Gly-Sar, Gly-Ala and Gly-Pro were all from Sigma and according to the certificate of analysis they are 99.5, 100, 99.5 and >99% pure, respectively. For the Gly-Sar batch used, we have previously reported that this batch contains 0.1% free Gly with no detectable amounts of Sar (Frolund *et al.*, 2010). N- β -aminoethyl-Gly-OH and H-Gly-Phe-OH was from BaChem AG (Budebdorf, Germany). L-4,4'-biphenylalanyl-L-proline was a generous gift from Dr Matthias Brandsch (Biozentrum of the Martin-Luther-University, Halle-Wittenberg, Germany) (Knutter *et al.*, 2007). Penicillin and streptomycin

were obtained from Invitrogen (Taastrup, Denmark). Hanks' Balanced salt solution (HBSS) with calcium and magnesium was from Gibco, Invitrogen (Paisley, UK). The *SLC36A1* specific primers were bought at Eurofins MWG Operon (Ebersberg, Germany). Restriction enzymes were purchased at New England Biolabs (Ipswich, MA, USA). The pCRII-TOPO vector and the TOPO TA Cloning Kit was from Invitrogen (Carlsbad, CA, USA). Pfu Ultra II Fusion HS DNA Polymerase was from Stratagene (Heidelberg, Germany). pGEMHE, a vector containing 5' and 3' untranslated regions from the *X. laevis* globin gene which stabilizes transcripts in oocytes (Liman *et al.*, 1992), was a gift from H. Lundbeck A/S (Valby, Denmark). The mMESSAGE mMACHINE T7 mRNA-capping kit was obtained from Ambion (Austin, TX, USA). The *X. laevis* frogs were from the African Reptile Park (Tokai, South Africa). Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture plastic ware was obtained from Corning Inc., Life Sciences (Wilkes-Barre, PA, USA). [Glycine-1- 14 C]-glycylsarcosine (Gly-Sar; 56 mCi mmol $^{-1}$, 98% pure, contains $2.2 \pm 0.01\%$ of the total radioactivity as [14 C]-Gly) was from GE-Healthcare (Freiburg, Germany). We have previously shown that under the experimental conditions described above, it is not possible to detect SLC36A-mediated uptake of the 2.2% free [14 C]-Gly present in the [14 C]-Gly-Sar (Frølund *et al.*, 2010). L-[2,3,4,5- 3 H]-proline (Pro; 75 Ci·mmol $^{-1}$) and Ultima Gold scintillations liquid were purchased from PerkinElmer (Boston, MA, USA).

Results

Gly-Gly, Gly-Sar and Gly-Gly mimetics induce SLC36A1-mediated inward currents in X. laevis oocytes

In order to determine whether Gly-Gly and its mimetics are substrates for SLC36A1, a series of experiments were designed to investigate the translocation via SLC36A1 using the two-electrode voltage clamp technique on SLC36A1-expressing *X. laevis* oocytes. As shown in Figure 1, 20 mM of the standard SLC36A1-substrates Pro, Gly and GABA induced similar inward currents in oocytes injected with *SLC36A1* cRNA, whereas no changes in currents were induced in water-injected oocytes (data not shown). The SLC36A1 specificity of the signal was verified by the inability of Leu or Val to evoke changes in current. Furthermore, the inward current was not a result of a changed osmolarity as mannitol failed to induce significant changes in the current. The non-translocated ligand of SLC36A1 5-HTP, as

Figure 1

Functional expression of SLC36A1 in *Xenopus laevis* oocytes. Normalized currents obtained by application of 20 mM compound unless otherwise stated (5-HTP, 10 mM; Gly-Gly and Gly-Sar, 20, 50 and 100 mM; Gly-Ala, Gly-Pro and Gly-Phe; 20 and 100 mM). Each column represents mean $\pm$ SEM for $n = 6$ –15 different oocytes.

expected, did not induce a change in the membrane current upon application at a concentration of 10 mM (Figure 1). However, when 20 mM Pro was applied in the presence of 10 mM 5-HTP, the observed inward current was approximately 25% of the current induced in the absence of 5-HTP (data not shown). APA, AEG, ALA, Gly-Gly and Gly-Sar were then evaluated for their possible interaction with SLC36A1. At a concentration of 20 mM, all five dipeptides/mimetics were able to induce consistent and reproducible inward currents in *SLC36A1* cRNA-injected oocytes (Figure 1). For Gly-Gly and Gly-Sar, a concentration-dependent ability to induce currents was shown at three different concentrations. The dipeptides Gly-Ala, Gly-Pro and Gly-Phe did, however, not induce significant changes in the current at 20 or 100 mM. The specificity of the currents induced by Gly-Gly and Gly-Sar in the SLC36A1-expressing oocytes was verified by applying the dipeptides to water-injected oocytes, where no induction of current was observed (upper trace in Figure 2).

The currents induced by Gly-Gly and Gly-Sar in SLC36A1 cRNA-injected oocytes are not due to amino acid impurities

The most convenient way of negating the possibility that Gly-Gly, Gly-Sar and Gly-Gly mimetics are substrates for SLC36A1 would be to attribute the signal measured in *SLC36A1* cRNA-injected oocytes to amino acid impurities, that is, our observations could be merely the results of free amino acids present in the chemicals used. Hence, two experiments were conducted to compare the current induced by increasing concentrations of Gly-Gly or Gly-Sar to the current induced by free Gly

Figure 2

Comparison of SLC36A1-mediated current evoked by Gly-Gly and Gly-Sar to current evoked by Gly. Representative traces showing inward current obtained by application of increasing concentrations of (A) Gly-Gly or (B) Gly-Sar to the current evoked by increasing concentrations of Gly. Upper traces: water injected oocyte; lower traces: SLC36A1 cRNA injected oocyte. Experiments on at least eight different oocytes showed similar results.

(Figure 2; eight individual experiments showed similar results). As shown in Figure 2A, 50 mM Gly-Gly induced approximately the same current as 10 mM Gly. If the responses induced by Gly-Gly are solely due to free Gly, the amount of Gly in the Gly-Gly batch should therefore be approximately 20%. From Figure 2A, it is evident that even if the Gly content was 1 or 10%, this would not be a sufficient amount of Gly to cause the current observed for 100 mM Gly-Gly. In the case of Gly-Sar, previous investigations have shown that the Gly-Sar batch used did not contain detectable amounts of Sar and only 0.1% Gly (Frolund *et al.*, 2010). The results depicted in Figure 2B show that 0.1% Gly, that is, 0.1 mM Gly, is not a high enough concentration to evoke the inward current observed for 100 mM Gly-Sar. In fact, the results in Figure 2B show that the amount of residual amino acids in the Gly-Sar batch should be as high as 5–10%, if the observed responses were solely due to the presence of amino acids. Collectively, the inward currents evoked by application of Gly-Gly or Gly-Sar cannot be due to amino acid impurities,

Figure 3

Concentration-dependent SLC36A1-mediated current induced by Gly-Gly and Gly-Sar in *Xenopus laevis* oocytes. Currents were analysed using Eq. (1) giving K_m values of (A) 21 ± 2.2 mM for Gly-Gly and (B) 49 ± 9 mM for Gly-Sar. Each data point represents mean $\pm$ SEM for $n = 6$ –12 different oocytes.

and consequently, both are due to Gly-Gly and Gly-Sar being translocated via SLC36A1.

SLC36A1-mediated inward currents induced by Gly-Gly, Gly-Sar and Gly-Gly mimetics are concentration-dependent and saturable

To further investigate the interaction of Gly-Gly mimetics with SLC36A1, the concentration-dependent uptake was measured in SLC36A1 cRNA-injected oocytes. In Figure 3, concentration-dependent uptake curves for Gly-Gly and Gly-Sar are presented. It is evident that both Gly-Gly and Gly-Sar are able to induce currents, which are saturable with increasing concentrations, and that the inward currents induced may be described using Michaelis–Menten kinetics, indicating a 1:1 binding to a single carrier system. In addition, the concentration-dependence of the ability to induce inward currents was studied for Pro, Gly, GABA, APA, AEG and ALA. The K_m values obtained and the structure of the compounds are given in Table 1. The compounds may, based on their affinity for SLC36A1, be divided into three groups. One group,

Figure 4

Inhibition of apical L-[³H]-Pro uptake by Gly-Gly and Gly-Sar in Caco-2 cells. IC₅₀ values were estimated using Eq. (2) to be 4.6 mM (logIC₅₀ of 0.66 ± 0.04) and 57 mM (logIC₅₀ of 1.76 ± 0.17) for Gly-Gly and Gly-Sar, respectively. Each data point represents the mean ± SEM of single measurements in three different cell passages.

with the highest affinity, consists of the standard substrates Pro and GABA, with K_m values of 1.8 ± 0.17 mM and 2.4 ± 0.28 mM, respectively. The second group contains test compounds with K_m values that are approximately a factor of 10 higher; included in this group is the standard substrate Gly ($K_m = 11 \pm 1.0$ mM) and the dipeptidomimetics APA ($K_m = 12 \pm 1.6$ mM) and ALA ($K_m = 15 \pm 1.6$ mM), as well as the dipeptide Gly-Gly ($K_m = 21 \pm 2.2$ mM). Finally, the group with the lowest estimated affinity consisted of AEG and Gly-Sar with K_m values of 37 ± 2.2 mM and 49 ± 9 mM, respectively. Thus, Gly-Gly and Gly-Gly mimetics were unequivocally shown to be substrates for SLC36A1.

Apical Gly-Sar uptake via SLC36A1 and SLC15A1 in Caco-2 cells

To investigate if the identification of Gly-Gly, Gly-Sar and Gly-Gly mimetics as novel SLC36A1-substrates also results in relevant measurable SLC36A1-transport in intestinal cells, we measured the uptake of Pro and Gly-Sar into Caco-2 cells. First, the ability of Gly-Gly and Gly-Sar to inhibit the apical uptake of L-[³H]-Pro was investigated. Gly-Gly and Gly-Sar both inhibited L-[³H]-Pro uptake in a concentration-dependent manner with IC₅₀ values of 4.6 mM (logIC₅₀ of 0.66 ± 0.04, measured in three different cell passages) and 57 mM (logIC₅₀ of 1.76 ± 0.17, measured in three different cell passages), respectively (Figure 4). The apical uptake of [¹⁴C]Gly-Sar was then investigated in order to estimate the relative contribution of SLC36A1 and SLC15A1 to the total Gly-Sar uptake. The apical uptake of Gly-Sar was measured at two different apical concentrations of Gly-Sar, that is, 1 mM and

Figure 5

Inhibition of apical [¹⁴C]Gly-Sar uptake in Caco-2 cells by 5-HTP and Bip-Pro. Uptake of (A) 1 mM or (B) 30 mM Gly-Sar measured in the absence or presence of 10 mM 5-HTP (+ 5-HTP), 0.5 mM Bip-Pro (+ Bip-Pro) or 10 mM 5-HTP and 0.5 mM Bip-Pro (+ 5-HTP + Bip-Pro). Each column represents mean ± SEM measured in triplicate in three different cell passages. ** Denotes $P < 0.01$ from uptake measured in the absence of inhibitor.

30 mM (Figure 5). The apical uptake of 1 mM Gly-Sar was significantly inhibited by 0.5 mM Bip-Pro to 21% of the control uptake ($P < 0.01$, measured in triplicate in three different cell passages). The SLC36A1-inhibitor 5-HTP did not influence the apical uptake of 1 mM Gly-Sar. The uptake of 1 mM Gly-Sar in the presence of both 5-HTP and Bip-Pro was reduced to 14% of the control uptake ($P < 0.01$, measured in triplicate in three different cell passages). Investigating the apical Gly-Sar uptake using a higher Gly-Sar concentration (30 mM) showed a different pattern. The apical uptake of 30 mM Gly-Sar was significantly inhibited by 10 mM 5-HTP to 80% of the uptake in the absence of inhibitor ($P < 0.01$, measured in triplicates in three different cell passages). Likewise, 0.5 mM Bip-Pro inhibited the uptake of 30 mM Gly-Sar to 78% of the control

uptake ($P < 0.01$, measured in triplicate in three different cell passages). Thus, the uptake of 30 mM Gly-Sar has a component that is mediated by both SLC15A1 and SLC36A1. The inhibitory abilities of 5-HTP and Bip-Pro were additive, since the uptake of 30 mM Gly-Sar in the presence of both was 52% of the control uptake ($P < 0.01$, measured in triplicate in three different cell passages; Figure 5B). The uptake of Gly-Sar at an apical concentration of 1 mM was thus only inhibited by the SLC15A1 inhibitor Bip-Pro, whereas the apical uptake of 30 mM Gly-Sar was inhibited by both the SLC36A1 inhibitor 5-HTP and the SLC15A1 inhibitor Bip-Pro.

Discussion and conclusions

The present study demonstrates for the first time that the proton-coupled amino acid transporter SLC36A1 is a transporter of a real dipeptide, that is, Gly-Gly, and furthermore transports Gly-Sar and other Gly-Gly mimetics. Gly-Gly, ALA and APA are substrates of γ -glutamyl transferase and have, in some animal models, been used as anticonvulsant compounds (Samuels *et al.*, 1983). APA is a GABA_A agonist and a weak GABA_B antagonist (Bowery and Brown, 1974; Muhyaddin *et al.*, 1982). ALA is used for photodynamic diagnosis in patients with prostate cancer and for the treatment of bladder cancer (Waidelich *et al.*, 2001; Zaak *et al.*, 2008). The findings presented here add to the recent identification of a substrate overlap between the pharmacologically relevant proton-coupled amino acid and di-/tripeptide transporters, SLC36A1 and SLC15A1. Intestinal absorption of drugs via SLC36A1 may thus be affected by Gly-Gly present in the diet or by other drugs that are SLC36A1 substrates.

SLC36A1 is a transporter of Gly-Gly and Gly-Sar

Recently, we reported that SLC36A1 is able to transport the dipeptidomimetic anti-cancer drug ALA (Frolund *et al.*, 2010). Moreover, preliminary data indicated that the standard SLC15A1 substrate Gly-Sar could also be a substrate for SLC36A1. We therefore hypothesized that SLC36A1 might also be able to transport simple dipeptides such as Gly-Gly and Gly-Sar. A series of five Gly-Gly mimetic compounds were selected and their translocation via SLC36A1 was investigated in *X. laevis* oocytes. All five test compounds were translocated via SLC36A1 resulting in K_m values of 12–49 mM, whereas Gly-Ala, Gly-Pro and Gly-Phe were not translocated at the concentrations investigated. The next step in establishing SLC36A1 as a transporter of Gly-Gly mimetics was to evaluate if these affinities could result in

uptake that was measurable and physiologically relevant. We therefore investigated the uptake of the dipeptide Gly-Sar into an intestinal cell line, that is, Caco-2 cell monolayers. We chose to use Gly-Sar because in the present study, it was shown to be the dipeptide with the poorest affinity for SLC36A1, it is a standard substrate for SLC15A1 and it is metabolically stable in Caco-2 cells (Addison *et al.*, 1972). The apical uptake of Gly-Sar in Caco-2 cells was studied at two different concentrations and in the presence of inhibitors of both SLC15A1 and SLC36A1. At a Gly-Sar concentration of 1 mM, which is close to the affinity for SLC15A1, we were not able to inhibit the uptake with 5-HTP, whereas Bip-Pro inhibited the uptake to approximately 20% of the control uptake. Thus, at 1 mM Gly-Sar, SLC36A1 does not play a significant role in the total uptake of Gly-Sar. With 30 mM Gly-Sar, which is closer to its affinity for SLC36A1, and a concentration where SLC15A1 should be saturated, we found that SLC36A1 had a pronounced impact on the total uptake of Gly-Sar; both 5-HTP and Bip-Pro reduced the uptake of Gly-Sar by approximately 20%. More importantly, the effect of the two inhibitors was additive; the presence of both 5-HTP and Bip-Pro reduced the Gly-Sar uptake by approximately 50%. Our findings are quite close to the estimates presented in the Methods section (see Data analysis), which estimated a Bip-Pro mediated inhibition of the uptake of 1 mM Gly-Sar to approximately 16% of the control uptake, and a reduction of the uptake of 30 mM Gly-Sar to 65%, 81% and 43% in the presence of 5-HTP, Bip-Pro or 5-HTP and Bip-Pro, respectively. This indicates that Gly-Sar is transported across the apical membrane of Caco-2 cell monolayers via SLC15A1 and SLC36A1 only. Furthermore, at 30 mM, SLC36A1 and SLC15A1 seem equally important in mediating the apical uptake of Gly-Sar in confluent Caco-2 cell monolayers. Recently, we found that Gly-Sar was able to inhibit the apical uptake of Pro in Caco-2 cells and the uptake of ALA in COS-7 cells transiently transfected with SLC36A1 (Frolund *et al.*, 2010). Therefore, collectively, there is strong experimental evidence that Gly-Sar is a substrate for SLC36A1, and that Gly-Sar, and presumably also Gly-Gly, at physiologically relevant concentrations, may interact with SLC36A1 and thus might affect the uptake of drugs such as ALA, vigabatrin (Abbot *et al.*, 2006) and gaboxadol (Larsen *et al.*, 2009).

It is worth noting that in the SLC15 family, where SLC15A1 (hPEPT1) is the most prominent member, SLC15A3 and SLC15A4 are peptide/histidine transporters (Botka *et al.*, 2000). There are thus other examples of transporters having both peptide and amino acid substrates; however, the

peptide/histidine transporters have never received much attention with regard to the absorption of drugs or peptides from the intestine (Nielsen and Brodin, 2003; Daniel, 2004; Hu *et al.*, 2008). At present, we can only speculate about the role of SLC36A1 in *in vivo* intestinal absorption of Gly-Gly in humans; further experiments are needed in order to resolve the role of SLC15A1, SLC36A1 and other transporters in oral absorption of Gly-Gly.

The results obtained from the study of SLC36A1-mediated transport of dipeptides such as Gly-Gly and Gly-Sar could potentially be biased by the fact that the constitutive amino acid themselves are substrates for SLC36A1. We found that Gly has a K_m value of 11 mM in SLC36A1-expressing *X. laevis* oocytes, whereas in Caco-2 cells, it has been shown that Gly and Sar have K_i values of 9.9 and 1.8 mM, respectively (Larsen *et al.*, 2008). Thus, if the constitutive amino acids were present in large amounts or the dipeptides were degraded during contact with cells, a false conclusion would be reached. For Gly-Sar, we know that the batch used does not contain detectable amounts of Sar and only 0.1% Gly (Frolund *et al.*, 2010); therefore, 100 mM Gly-Sar would yield 0.1 mM free Gly. This is an insufficient amount of Gly to cause the inward currents observed; thus, free amino acid are unlikely to cause false interpretations of the experimental data. In the two-electrode voltage clamp system, the oocyte is continuously perfused with the compound under investigation, for example, the dipeptide, and therefore, degradation products, if any, resulting from extracellular hydrolytic enzymes are flushed away. Furthermore, we have shown that dipeptidomimetics such as ALA and AEG, which lack an amide bond and hence are not susceptible to hydrolysis, are transported via SLC36A1. Therefore, our findings, that Gly-Gly and its mimetics are substrates for SLC36A1, are not the result of the presence of free amino acids.

Structure–activity relations for SLC36A1

The results from present study also add to the current understanding of the substrate–translocation relationship for SLC36A1. The amino acid substrates for SLC36A1 originating from metabolism of proteins from the food are Pro, Gly, Ala and taurine, having K_m values in the range of 2–10 mM (Thwaites *et al.*, 1994; 1995b; Boll *et al.*, 2002, Anderson *et al.*, 2009). The K_m values for Pro (1.8 ± 0.17 mM) and Gly (11 ± 1.0 mM) found in the present study are thus in good agreement with previous findings (Boll *et al.*, 2003; Foltz *et al.*, 2005). In this study, we investigated the effect of systematically changing the structure of Gly-Gly, focusing on the basic bioisosters of the amide bond,

on its affinity for SLC36A1; Gly-Gly is a substrate for SLC36A1, with an affinity that is only slightly poorer (a twofold change; K_m of Gly-Gly is 21 ± 2.2 mM) than that of the amino acid substrate Gly. The affinity of the ketomethylene dipeptidomimetic ALA was similar to that of Gly, that is, 15 ± 1.6 mM, corresponding well with our previous findings in Caco-2 cells ($IC_{50} = 11.3$ mM) (Frolund *et al.*, 2010) and the work of Anderson *et al.* (2010). Interestingly, we found that elimination of the ketone in ALA, leading to the δ -amino acid APA, did not change its affinity for SLC36A1 significantly ($K_m = 12 \pm 1.6$ mM). This is somewhat contradictory to the findings by Boll and colleagues, who reported a K_m value of 38 mM for APA (Boll *et al.*, 2003). They categorized APA as a non-substrate of murine slc36a1, based on its inability to inhibit a slc36a1-mediated Gly uptake and produce slc36a1-mediated inward current (Boll *et al.*, 2003). In the present study, APA induced higher currents and has a higher affinity; therefore, we consider APA to be a substrate for SLC36A1. Perhaps, minor functional differences between the murine and human SLC36A1 may account for these observations. Furthermore, alterations of the amide bond in Gly-Gly to either the methyleneamino dipeptidomimetic AEG or the N-methylated dipeptide Gly-Sar leads to additional reductions in affinity (37 ± 2.2 and 49 ± 9 mM for AEG and Gly-Sar, respectively). These affinity values are rather low; however, both compounds may still be categorized as substrates for SLC36A1. This is supported by the results in Figure 5, showing that at a physiologically relevant concentration of Gly-Sar (30 mM), its uptake into Caco-2 cells is mediated equally by SLC15A1 and SLC36A1. Thus, simple dipeptidomimetics of Gly-Gly are substrates for SLC36A1. Changing the C-terminal amino acid from Gly to Ala, Pro or Phe as in Gly-Ala, Gly-Pro and Gly-Phe completely abolished translocation via SLC36A1. The acceptability of the amid bond modifications thus requires that the L-configured C-terminal amino acid only has a –H in the side chain. Whether this is also the case for D-configured amino acids or the same is true for X_{aa} -Gly dipeptides remains to be investigated. Moreover, the tripeptide Gly-Sar-Sar is not translocated via SLC36A1 (Frolund *et al.*, 2010). It is thus unlikely that all dipeptides are substrates for SLC36A1, and Gly-Sar might structurally define the size limit of peptide transport via SLC36A1.

In conclusion, the present study identifies Gly-Gly and Gly-Gly mimetics as substrates for SLC36A1. This indicates that SLC36A1 may act as a physiologically relevant transporter of Gly-Gly, and that Gly-Gly originating from the diet may influence the absorption of drugs via SLC36A1. The

present findings thus add to the current understanding of substrate transport via SLC36A1.

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Conflict of interest

The authors state no conflict of interest.

References

- Abbot EL, Grenade DS, Kennedy DJ, Gatfield KM, Thwaites DT (2006). Vigabatrin transport across the human intestinal epithelial (Caco-2) brush-border membrane is via the H⁺-coupled amino-acid transporter hPAT1. *Br J Pharmacol* 147: 298–306.
- Addison JM, Burston D, Matthews DM (1972). Evidence for active transport of the dipeptide glycylsarcosine by hamster jejunum in vitro. *Clin Sci* 43: 907–911.
- Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition. *Br J Pharmacol* 153 (Suppl. 2): S1–S209.
- Anderson CM, Howard A, Walters JR, Ganapathy V, Thwaites DT (2009). Taurine uptake across the human intestinal brush-border membrane is via two transporters: H⁽⁺⁾-coupled PAT1 (SLC36A1) and Na⁽⁺⁾- and Cl⁽⁻⁾-dependent TauT (SLC6A6). *J Physiol* 587: 731–744.
- Anderson CM, Jevons M, Thangaraju M, Edwards N, Conlon NJ, Woods S *et al.* (2010). Transport of the photodynamic therapy agent 5-aminolevulinic acid by distinct H⁺-coupled nutrient carriers co-expressed in the small intestine. *J Pharmacol Exp Ther* 332: 220–228.
- Boll M, Foltz M, Rubio-Aliaga I, Kottra G, Daniel H (2002). Functional characterization of two novel mammalian electrogenic proton-dependent amino acid cotransporters. *J Biol Chem* 277: 22966–22973.
- Boll M, Foltz M, Anderson CM, Oechsler C, Kottra G, Thwaites DT *et al.* (2003). Substrate recognition by the mammalian proton-dependent amino acid transporter PAT1. *Mol Membr Biol* 20: 261–269.
- Botka CW, Wittig TW, Graul RC, Nielsen CU, Higaka K, Amidon GL *et al.* (2000). Human proton/oligopeptide transporter (POT) genes: identification of putative human genes using bioinformatics. *AAPS PharmSciTech* 2: E16.
- Bowery NG, Brown DA (1974). Depolarizing actions of gamma-aminobutyric acid and related compounds on rat superior cervical ganglia in-vitro. *Br J Pharmacol* 50: 205–218.
- Brandsch M, Knutter I, Leibach FH (2004). The intestinal H⁺/peptide symporter PEPT1: structure–affinity relationships. *Eur J Pharm Sci* 21: 53–60.
- Chen Z, Fei YJ, Anderson CM, Wake KA, Miyauchi S, Huang W *et al.* (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.
- Daniel H (2004). Molecular and integrative physiology of intestinal peptide transport. *Annu Rev Physiol* 66: 361–384.
- Doring F, Walter J, Will J, Focking M, Boll M, Amasheh S *et al.* (1998). Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. *J Clin Invest* 101: 2761–2767.
- Foltz M, Mertl M, Dietz V, Boll M, Kottra G, Daniel H (2005). Kinetics of bidirectional H⁺ and substrate transport by the proton-dependent amino acid symporter PAT1. *Biochem J* 386: 607–616.
- Frolund S, Cutillas O, Larsen M, Brodin B, Nielsen CU (2010). Delta-aminolevulinic acid is a substrate for SLC36A1 (hPAT1). *Br J Pharmacol* 159: 1339–1353.
- Gebauer S, Knutter I, Hartrodt B, Brandsch M, Neubert K, Thondorf I (2003). Three-dimensional quantitative structure–activity relationship analyses of peptide substrates of the mammalian H⁺/peptide cotransporter PEPT1. *J Med Chem* 46: 5725–5734.
- Hu YG, Smith DE, Ma K, Jappard D, Thomas W, Hillgren KM (2008). Targeted disruption of peptide transporter *Pept1* gene in mice significantly reduces dipeptide absorption in intestine. *Mol Pharm* 5: 1122–1130.
- Jensen TN, Nielsen J, Frederiksen K, Ebert B (2006). Molecular cloning and pharmacological characterization of serotonin 5-HT(3A) receptor subtype in dog. *Eur J Pharmacol* 538: 23–31.
- Knutter I, Hartrodt B, Toth G, Keresztes A, Kottra G, Mrestani-Klaus C *et al.* (2007). Synthesis and characterization of a new and radiolabeled high-affinity substrate for H⁺/peptide cotransporters. *FEBS J* 274: 5905–5914.
- Larsen M, Larsen BB, Frolund B, Nielsen CU (2008). Transport of amino acids and GABA analogues via the human proton-coupled amino acid transporter, hPAT1: characterization of conditions for affinity and transport experiments in Caco-2 cells. *Eur J Pharm Sci* 35: 86–95.
- Larsen M, Holm R, Jensen KG, Brodin B, Nielsen CU (2009). Intestinal gaboxadol absorption via PAT1 (SLC36A1): modified absorption in vivo following co-administration of L-tryptophan. *Br J Pharmacol* 157: 1380–1389.
- Liman ER, Tytgat J, Hess P (1992). Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron* 9: 861–871.

- 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.
- Metzner L, Dorn M, Markwardt F, Brandsch M (2009). The orally active antihyperglycemic drug beta-guanidinopropionic acid is transported by the human proton-coupled amino acid transporter hPAT1. *Mol Pharm* 6: 1006–1011.
- Muhyaddin M, Roberts PJ, Woodruff GN (1982). Pre-synaptic gamma-aminobutyric acid receptors in the rat anococcygeus muscle and their antagonism by 5-aminovaleric acid. *Br J Pharmacol* 77: 163–168.
- Nielsen CU, Brodin B (2003). Di/tri-peptide transporters as drug delivery targets: regulation of transport under physiological and patho-physiological conditions. *Curr Drug Targets* 4: 373–388.
- Nielsen CU, Amstrup J, Steffansen B, Frokjaer S, Brodin B (2001). Epidermal growth factor (EGF) inhibits glycylsarcosine (Gly-Sar) transport and hPepT1 expression in a human intestinal cell line. *Am J Physiol Gastrointest Liver Physiol* 281: G191–G199.
- Nielsen CU, Amstrup J, Steffansen B, Frokjaer S, Brodin B (2003). Epidermal growth factor and insulin short-term increase hPepT1-mediated glycylsarcosine uptake in Caco-2 cells. *Acta Physiol Scand* 178: 139–148.
- Samuels S, Fish I, Schwartz SA (1983). Anti-convulsant activity of glycylglycine and delta-aminovaleric acid – evidence for glutamine exchange in amino-acid-transport. *J Neurochem* 40: 1063–1068.
- Thwaites DT, McEwan GT, Cook MJ, Hirst BH, Simmons NL (1993). H(+)-coupled (Na(+)-independent) proline transport in human intestinal (Caco-2) epithelial cell monolayers. *FEBS Lett* 333: 78–82.
- 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, 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, McEwan GT, Simmons NL (1995b). 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, Basterfield L, McCleave PM, Carter SM, Simmons NL (2000). Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers. *Br J Pharmacol* 129: 457–464.
- Waidelich R, Stepp H, Baumgartner R, Weninger E, Hofstetter A, Kriegmair M (2001). Clinical experience with 5-aminolevulinic acid and photodynamic therapy for refractory superficial bladder cancer. *J Urol* 165: 1904–1907.
- Zaak D, Sroka R, Khoder W, Adam C, Tritschler S, Karl A *et al.* (2008). Photodynamic diagnosis of prostate cancer using 5-aminolevulinic acid – first clinical experiences. *Urology* 72: 345–348.