

**A rapid *in vitro* screening for delivery of peptide-derived
peptidase inhibitors as potential drug candidates via
epithelial peptide transporters**

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ABSTRACT

Targeting drugs or prodrugs to a specific enzyme by simultaneously targeting cell-membrane carriers for efficient transport should provide the highest bioavailability along with specificity at the site of action. The peptide transporters PEPT1 and PEPT2 are expressed in a variety of tissues, including the brush-border membranes of epithelial cells of the small intestine and kidney. The transporters accept a wide range of substrates and are therefore good targets for a transporter-mediated drug delivery. Here we report a screening procedure for peptidomimetic drug candidates combining two independent expression systems: (a) a competition assay in transgenic *Pichia pastoris* yeast cells expressing either mammalian PEPT1 or PEPT2 for identifying substrate interaction with the transporter binding site, and (b) a *Xenopus laevis* based oocyte expression of the peptide transporter for assessing electrogenic transport of drug candidates.

Based on the known oral availability and *in vivo* efficacy of the dipeptidyl peptidase IV (DPIV)-inhibitor isoleucine-thiazolidide and its peptide-like structure we first tested whether this compound is a substrate of epithelial peptide transporters. Additionally, a series of structurally related inhibitors was analyzed for transport. We identified various compounds that serve as substrates of the intestinal peptide transporter PEPT1. In contrast, none of these DPIV-inhibitors did show electrogenic transport by PEPT2 although a variety of the compounds displayed good affinities for competition in peptide uptake in PEPT2-expressing cells suggesting that they may serve as efficient inhibitors. In conclusion, we have applied an *in vitro* screening system that predicts efficient intestinal absorption of peptide-derived peptidase inhibitors via PEPT1 *in vivo*.

INTRODUCTION

The mammalian proton/peptide symporters PEPT1 and PEPT2 mediate the proton driven and membrane potential (V_m) dependent cellular uptake of dipeptides and tripeptides into a variety of epithelial tissues. PEPT1 and PEPT2 have been characterized with respect to expression, tissue localization and function (Covitz *et al.*, 1998; Fei *et al.*, 1998; Amidon and Lee, 1994; Adibi, 1997; Daniel and Herget, 1997; Leibach and Ganapathy, 1996; Nussberger *et al.*, 1997). It is known that in addition to di- and tripeptides both mammalian peptide transporters have the capability to accept a large number of peptidomimetic drugs such as β -lactam antibiotics, ACE inhibitors, selected peptidase inhibitors and prodrugs (Rubio-Aliaga and Daniel, 2002). PEPT1 possesses a high transport capacity, is expressed along the entire small intestine epithelium and is central for the known high oral availability of peptidomimetic drug substrates. The function of the high affinity type transporter PEPT2 in drug transport is mainly reabsorption of the compounds after glomerular filtration in the kidney. PEPT2 expression has also been shown in other tissues such as epithelium of the choroid plexus, lung, mammary gland and glia cells of the central nervous system. Recent studies have identified major differences in the structural requirements for binding and transport in substrates of PEPT1 and PEPT2. Whereas ω -aminofatty acids and amino acid-arylamides are recognized and transported by PEPT1 as high affinity substrates, PEPT2 does not accept ω -aminofatty acids and displays a wide range of affinities and different transport characteristics when studied with amino acid-arylamides (Borner *et al.*, 1998; Doring *et al.*, 1998a). However, modifications, like the introduction of a carbonyl group into the backbone of omega-aminofatty acids, can transform a substrate with very low affinity into a high affinity compound that is transported by PEPT2 electrogenically

(Theis *et al.*, 2002) suggesting that PEPT2 has more specific requirements for substrate recognition. This could also be important for a specific delivery of drugs such as DPIV or prolyl endopeptidase (PEP) inhibitors.

The therapeutic potential of inhibitors of post-proline cleaving enzymes has been the focus of recent pharmaceutical research (Rosenblum and Kozarich, 2003). Dipeptidyl peptidase IV (DPIV, EC 3.4.14.5), also known as CD26, is a membrane-associated peptidase found in numerous tissues. Significant DPIV-like activity is also detectable in plasma from humans and rodents (De Meester *et al.*, 1999). Special attention has recently been given to DPIV-inhibitors, which were found to act as efficient antidiabetic drug candidates in first human trials (Ahren *et al.*, 2002; Hoffmann *et al.*, 2001). Similar, inhibitors of the related enzyme prolyl endopeptidase (PEP, EC 3.4.21.26) have potential as drugs to treat neurodegenerative disorders (Rosenblum and Kozarich, 2003).

DPIV inhibitors with structures of or similar to di- and tripeptides offer the opportunity to target the mammalian peptide transporter PEPT1 for oral delivery which would result in an efficient intestinal permeability. The specific DPIV inhibitor isoleucine-thiazolidide (di-[2S,3S]-2-amino-3-methyl-pentanoic-1,3-thiazolidine fumarate, L-threo-isoleucine-thiazolidide, Ile-Thia) when given orally in humans resulted in a dose dependent decrease of plasma DPIV and a concomitant increase of active GLP-1 (Hoffmann *et al.*, 2001) suggesting its efficient intestinal absorption possibly by PEPT1. Since DPIV/CD26 possesses multiple functions, a central question associated with the emerging use of DPIV inhibitors is that of potential side effects such as immunosuppression or inhibition of DPIV-like enzymes which are partially intracellular located enzymes (Kubota *et al.*, 1992; Sedo and Malik, 2001).

Therefore, peptide transporters in peripheral tissues could be of importance in this respect by allowing DPIV or PEP inhibitors to be taken up into specific cells.

Here we have analyzed whether a) Ile-Thia based on its peptide-like structure is a substrate of the mammalian peptide transporters PEPT1 and PEPT2 and b) whether structurally related compounds can be identified for a targeted delivery and high oral availability. Since proline-containing tri- and tetrapeptide analogues are capable to inhibit DPIV and PEP, we also included such structures into the study.

We first determined the apparent affinity of Ile-Thia and the other test compounds for interaction with peptide transporters based on competition with the uptake of the radiolabelled dipeptide D-Phe-Ala in *Pichia pastoris* yeast cells expressing heterologously either human PEPT1 or PEPT2. To be able to differentiate between compounds that only interact with the substrate-binding site of the transporter and those that are electrogenically transported, we performed electrophysiological studies by recording inward currents induced by the compounds in *Xenopus laevis* oocytes expressing either of the peptide transporter forms. This procedure also permitted a comparison between the two transporters with respect to differences in substrate specificity for binding and transport.

MATERIAL AND METHODS

Materials

Glycyl-L-glutamine (Gly-Gln) was obtained from Sigma Chemie (Deisenhofen, Germany); D-phenylalanyl-alanine (D-Phe-Ala) was purchased from Bachem (Heidelberg, Germany). Custom-synthesized [^3H]D-Phe-Ala ([alanine-2,3- ^3H]D-Phe-Ala, specific radioactivity 40 Ci*mmol $^{-1}$) was obtained from Biotrend (Cologne, Germany), and collagenase A was obtained from Roche Molecular Biochemicals (Mannheim, Germany). All noncommercially available test compounds were synthesized by the Department of Medicinal Chemistry at Probiobdrug (Halle, Germany) according to known standard methods of peptide synthesis as briefly described by Schon et al. (1991) and Demuth et al. (1993).

Pichia pastoris strains and transport assays in yeast

Cultures of *P. pastoris* strains expressing human PEPT1 or PEPT2 were prepared as described previously (Doring et al., 1997; Doring et al., 1998b). Cells were centrifuged at 3000g for 10 min and formed into a pellet, washed twice with 100 mM potassium phosphate buffer (PPB; pH 6.5), and resuspended to 5×10^7 cells/20 μl of PPB. Uptake measurements were performed at 22 to 24°C using a rapid-filtration technique on 96-well filter plates (filter material HATF type, 0.45- μm pore size; Millipore Corporation, Eschborn, Germany). In brief, uptake was initiated by mixing 20 μl of the cell suspension with 30 μl of PPB containing 0.1 μCi corresponding to 50 nM (PEPT1) or 0.05 μCi corresponding to 25 nM (PEPT2) of the radiolabelled dipeptide D-Phe-Ala either with or without competitors (final concentration, 0 - 20 mM). After 15 min of incubation, the uptake was terminated by the addition of 200 μl of ice-cold PPB followed by filtration. The filters were washed four more times with

200 μ l of PPB. The radioactivity associated with the filter was directly measured by liquid-scintillation counting.

***Xenopus laevis* oocytes handling and cRNA injection**

Female *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Oocyte handling and transporter cRNA injection has been described previously (Boll *et al.*, 2002). Oocytes were injected with 15 nl sterile water (control), 15 nl rabbit PEPT1-cRNA (15 ng) or 15 nl rabbit PEPT2-cRNA (30 ng), respectively. The oocytes were kept in modified Barth solution (88 mM NaCl, 1 mM KCl, 0.8 mM MgSO₄, 0.4 mM CaCl₂, 0.3 mM Ca(NO₃)₂, 2.4 mM NaHCO₃ and 10 mM HEPES (pH 7.5)) at 18°C until further use (3 - 5 days after injection).

Two-electrode voltage clamp

Two-electrode voltage clamp experiments were performed as described previously (Kottra and Daniel, 2001). Briefly, the oocyte was placed in an open chamber and continuously superfused with modified Barth's solution or with solutions of Gly-Gln and/or substrates to be tested. Electrodes with a resistance between 1 and 10 M Ω were connected to a TEC-05 amplifier (NPI Electronic, Tamm, Germany). Oocytes were voltage-clamped at -60 mV, and current-voltage (I-V) relations were measured using short (100 ms) pulses separated by 200-ms pauses in the potential range -160 to +80 mV. I-V measurements were made immediately before and 20-30 s after substrate application when current flow reached steady state. The current evoked by PEPT1 or PEPT2 at a given V_m was calculated as the difference between the currents measured in the presence and the absence of substrate. Each substrate was tested against the maximal inward current elicited by 5 mM Gly-Gln, allowing for

a comparison of current recordings that are independent of the level of functional expression of various oocyte batches. Current traces were recorded during voltage clamping to -60 mV and substrates were perfused at concentrations of 5 mM for 20 sec. The electrophysiological measurement data are representative of at least two independent experiments with different oocyte batches.

Statistics

All calculations (linear as well as non-linear regression analyses) were performed using Prism software (GraphPad, San Diego, CA). At least two independent experiments with three replicates were carried out for each variable in the Yeast competition assays. Transport measurements in *Xenopus* oocytes were performed at least two times with oocytes from different oocyte batches. Data are presented as mean \pm S.E.M.

RESULTS

Pre-screening for selecting potential substrates

We have used a competition assay in *Pichia pastoris* yeast cells, expressing the human intestinal proton-coupled peptide transporter PEPT1, as a prescreening approach to separate rapidly compounds which do interact with the transporter from those that do not interfere with the transporter substrate binding domain. We have previously shown that the uptake of the tracer peptide [³H]D-Phe-Ala into PEPT1 expressing *Pichia pastoris* yeast cells is linear for at least 30 min of incubation, even at high substrate concentrations (Doring et al., 1997) and therefore have chosen an incubation time of 15 min for all uptake experiments. We screened a wide range of different dipeptides and structurally related compounds. As shown in figure 1, the compounds when provided at a concentration of 10 mM displayed quite different potencies for reducing the influx of the tracer [³H]D-Phe-Ala (50 nM) via PEPT1 into the yeast cells. Dipeptides and tripeptides, inter alia Lys-Phe (Fig. 2, No. 1) and Lys-Phe-Pro (Fig.2, No. 2) were able to reduce the uptake of [³H]D-Phe-Ala to 8.2 ± 1.8 and 10.3 ± 0.7 % of control levels, but also some of the DPIV-inhibitors such as Ile-Thia (Fig. 2, No. 11) and His-Thia (Fig. 2, No. 9) reduced tracer uptake to 19.9 ± 2.6 and 34.8 ± 1.4 % of that in controls.

Kinetics of transport of Ile-Thia and Thia-derivatives

We next determined inhibition constants of D-Phe-Ala uptake via PEPT1 by the DPIV-inhibitor Ile-Thia. D-Phe-Ala uptake was assessed at a concentration of 10 μ M in the presence of increasing Ile-Thia concentrations (0 - 20 mM) at an external pH of 6.5. Inhibition of D-Phe-Ala uptake by Ile-Thia followed typical first order competition kinetics with an apparent EC₅₀ value of 510 ± 94 μ M (Fig. 3A). This

apparent affinity is in the similar range as the EC_{50} value of $164 \pm 18 \mu\text{M}$ of the dipeptide Gly-Gln (Fig. 3A). This demonstration that an amino acid coupled via its carboxy group to a thiazolidide function is recognized by PEPT1 led to the question whether di- or tripeptides with a thiazolidide-group replacing the free carboxy group are also potential PEPT1 substrates. The dipeptide-derivative Glu-Gly-Thia competed dose dependently with D-Phe-Ala for uptake by PEPT1 but with a considerably lower affinity than Ile-Thia ($EC_{50} = 3.23 \pm 0.05 \text{ mM}$) (Fig. 3B). Pro-Ile-Thia exhibited almost no affinity for the PEPT1 transporters as indicated by extrapolated EC_{50} values greater than 30 mM (Fig. 3B). In case of the tripeptide-derivatives Gly-Pro-Ile-Thia and Pro-Pro-Ile-Thia affinity for PEPT1 was completely abolished (Fig. 3B). Since concentration-dependent competition with a dipeptide for binding at the substrate binding site of PEPT1 does not establish that the thiazolidide derivatives are indeed transported, we employed the two-electrode voltage clamp technique in *X. laevis* oocytes expressing PEPT1. As shown in figure 3C, perfusion of oocytes with 5 mM Gly-Gln induced an PEPT1-mediated inward current of 700 nA at a V_m of -60 mV and in the same oocyte, 5 mM Ile-Thia induced a current of around 200 nA. In water-injected controls neither Gly-Gln nor Ile-Thia induced any inward currents (Fig. 3C). In contrast to Ile-Thia, all dipeptide- and tripeptide-derivatives (Glu-Gly-Thia, Pro-Ile-Thia, Gly-Pro-Ile-Thia and Pro-Pro-Ile-Thia) failed to induce any inward currents at substrate concentrations of 5 mM (data not shown).

This establishes that although Glu-Gly-Thia possesses considerable affinity for interaction with the substrate binding domain of PEPT1, it is an inhibitor whereas Ile-Thia is an electrogenically transported substrate.

Transport properties of radiolabelled Ile-Thia

To demonstrate by independent methods that amino acid thiazolidide-derivatives such as Ile-Thia are in fact taken up into the cell by PEPT1, we used radio-tracer flux studies in *P. pastoris* cells with [^{14}C]L-Ile-Thia as a representative substrate to assess its transport characteristics by PEPT1. As shown in figure 4A, labeled Ile-Thia was taken up into the yeast cells expressing PEPT1 in a concentration dependent manner following Michaelis-Menten kinetics. An apparent K_t value of $684 \pm 80 \mu\text{M}$ was determined using nonlinear regression (Prism, GraphPad). Moreover, uptake of ^{14}C -Ile-Thia was inhibited by classical substrates of PEPT1 such as Gly-Gln, D-Phe-Ala and Cefadroxil (Fig 4B). The free amino acid isoleucine failed to inhibit uptake of Ile-Thia. These results confirm that Ile-Thia is a PEPT1-substrate with a substrate affinity similar to normal dipeptides.

As PEPT2 has a similar but not identical substrate specificity as PEPT1, we also determined the interaction of the various DPIV-inhibitors with the high affinity peptide transporter expressed in *P. pastoris* cells and in *X. laevis* oocytes. Ile-Thia displayed in the competition assay with D-Phe-Ala as a substrate a fairly low apparent EC_{50} value of $530 \pm 40 \mu\text{M}$. The dipeptide derivative Pro-Ile-Thia possessed a EC_{50} value of $1.50 \pm 0.01 \text{ mM}$, whereas Val-Thia showed with a EC_{50} value of $60 \pm 17 \mu\text{M}$ strong inhibitory potency. However, none of the tested compounds was able to induce substrate-evoked inward currents in oocytes expressing PEPT2 when compared to the dipeptide glycyl-glutamine, even at hyperpolarized membrane potentials of -120mV demonstrating that PEPT2 does not transport these compounds, although they are inhibitors with lower affinities.

Role of the amino acid residue in amino acid-thiazolidide derivatives on transport by PEPT1

Comparative analysis of transport of a series of compounds with a C-terminal thiazolidide-function and various N-terminal amino acid residues established that all of the tested aminoacyl-thiazolidides interacted with the peptide transporter PEPT1 in *P. pastoris* cells. The apparent EC₅₀ values obtained for the different substrates are summarized in table 1. Similar to normal dipeptides, hydrophobic N-terminal amino acid residue such as isoleucine, leucine or valine increase the substrate affinity to PEPT1 when compared to compounds with more hydrophilic properties like glutamate. This effect was also observed for their interaction with PEPT2. Val-Thia exhibited with an apparent EC₅₀ value of $60 \pm 17 \mu\text{M}$ a very high affinity for inhibition of D-Phe-Ala uptake, whereas Glu-Thia displayed a very low affinity, represented by an EC₅₀ value of $4.36 \pm 1.51 \text{ mM}$. Despite the high affinity of Val-Thia we did not observe electrogenic transport of this compound in oocytes expressing PEPT2. Perfusion of the other Thia-derivatives led also not to a depolarization of the oocyte membrane potential (table 1) whereas oocytes expressing PEPT1 responded to perfusion of Val-Thia with an inward current as high as that of the reference dipeptide Gly-Gln. Additionally, Ile-Thia, Leu-Thia and Glu-Thia also displayed similar PEPT1-mediated transport currents (table 1).

Selective recognition of isoleucine-thiazolidide stereoisomers by PEPT1 and PEPT2

Because of the second asymmetric carbon center at carbon position three along the side chain of isoleucine there are four possible configurations of isoleucine-thiazolidide. We therefore examined if the various Ile-Thia stereoisomers display different transport characteristics for PEPT1 and PEPT2. In case of PEPT1, L-allo-

Ile-Thia possessed an up to 40-fold higher affinity than the corresponding D-enantiomer (Fig. 5 and table 2). However, L-allo-Ile-Thia displayed with an EC_{50} value of $130 \pm 31 \mu M$ a 4-fold higher affinity than its diastereomer L-threo-Ile-Thia ($510 \pm 60 \mu M$). D-allo-Ile-Thia exhibited almost 5-fold lower affinity than its diastereomer D-threo-Ile-Thia (Fig. 5, table 2). Similarly, all L-isoleucine containing enantiomers displayed 3-4-fold higher affinities compared to the D-isomers for binding to PEPT2 and substrates with allo-configuration also showed the highest affinities. Interestingly, D-allo-Ile-Thia was found to be selective for PEPT2. With this compound we observed a 30-fold higher affinity to PEPT2 compared to PEPT1.

DISCUSSION

The *P. pastoris* expression system has been established to study the substrate specificity of PEPT1 and PEPT2 and was shown to possess phenotypically all transport functions of peptide transporters in other expression systems (Doring et al. 1997, Doring et. al 1998b). The transgenic yeast cells can be grown in large quantities with high expression levels of the transporters. We have used this system before in combination with the *X. laevis* system for defining the template for substrate recognition by the peptide transporters (Theis et al. 2002; Knutter et al. 2004). Here we describe that the combination of these two peptide transporter expression systems may also be useful for predicting the oral availability of peptide-derived peptidase inhibitors as a new class of potential drug candidates.

The peptide transporter PEPT1 and PEPT2 have proven to be relevant targets for drug delivery due to their uniquely broad substrate specificity and predominant expression in brush-border membranes of epithelial cells of the small intestine, lung, choroid plexus and kidney (Rubio-Aliaga and Daniel, 2002). It was shown in different cell systems, expressing the peptide transporter either heterologously or endogenously, that β -lactam antibiotics, ACE inhibitors and selected peptidase inhibitors are high affinity substrates of both transporters. In case of PEPT1 these substrates also do show an good oral availability (Rubio-Aliaga and Daniel, 2002). Moreover, poorly absorbed drugs can be turned into rapidly and efficiently transported compounds by rendering them into PEPT-substrates, as shown for example for the antiviral nucleoside acyclovir by esterification with L-valine (Ganapathy et al., 1998; Lycke et al., 2003).

Based on this knowledge, we have screened a variety of DPIV- and PEP-inhibitors for their transport by mammalian peptide transporters to assess whether they could be orally available by efficient uptake via PEPT1 or delivered via PEPT2 to the lung epithelium or for renal reabsorption that could alter the compounds pharmacokinetics. By uptake inhibition experiments with D-Phe-Ala as a substrate we demonstrate that Ile-Thia and other amino acids coupled via the carboxy group to a thiazolidide function do interfere with the substrate binding domains of PEPT1 and PEPT2 with high affinities. That the thiazolidide derivatives are recognized by the transporters is not necessarily surprising as they match at least partially the template for structural requirements in substrates which consist of (i) a free N-terminus or instead a weakly basic group in α -position at the N-terminus, (ii) an extended planar backbone, not longer than four methylene groups, with a correctly positioned backbone carbonyl group and finally (iii) an ionic function at the C-terminus (Daniel and Kottra, 2004; Brandsch *et al.*, 2004; Bailey *et al.*, 2000; Doring *et al.*, 1998a). Although a carboxyl or C-terminal ionic group is missing in the amino acid thiazolidide derivatives, other compounds lacking this function such as alanyl aryl amides were also shown to be transported by PEPT1 (Borner *et al.*, 1998). Very recently Gebauer *et al.* (Gebauer *et al.*, 2003) reasoned by means of three-dimensional quantitative structure-activity relationship analysis based on a series of dipeptide substrates, that an ionized C-terminal group can be replaced by a ring system with a high electronic density without a loss in substrate affinity. This may explain the good affinity of the aminoacyl-thiazolidides and is obviously also sufficient to allow transport of the compounds, at least in case of PEPT1.

However, when di- and tripeptides were coupled with a thiazolidide function no transport was observed, although some of the compounds retained reasonable

affinities for binding. That the tripeptide derivatives are not transported is also in accordance with other findings on substrate specificity and template requirements (Terada *et al.*, 2000; Chen *et al.*, 2002). However, the dipeptide derivatives that by coupling to the thiazolidide moiety mimic a tripeptide structure also lacked electrogenic transport while displaying high affinities. This can currently not be explained. This finding addresses the important question of whether transport predictions made on basis of competition studies are valid. Template predictions available are so far based on substrate affinities as derived from competition experiments in different expression systems. They thereby do allow a fair description of substrate requirements for binding to PEPT1 or PEPT2 but not necessarily for transport. We here demonstrate by combining competition assays and electrophysiological measurements that most of the DPIV-inhibitors despite of their good substrate affinity can not be transported. However, the aminoacyl-thiazolidides are transported substrates of PEPT1. Using radiolabelled Ile-Thia, we also demonstrate that hPEPT1 in *Pichia pastoris* cells allows efficient Ile-Thia uptake and that this influx is inhibited by dipeptides and aminocephalosporines. That Ile-Thia may be a PEPT1-substrate was suggested by uptake inhibition experiments in Caco-2 cells with glycyl-sarcosine as a substrate of the endogenous PEPT1 protein but with lower affinity as found here (Brandsch *et al.*, 1999). This difference, however, may be explained in part by the different experimental conditions such as cell system (yeast and oocyte *versus* Caco-2), the tracer used (D-Phe-Ala *versus* Gly-Sar) and membrane potential. In particular the latter is known to affect substrate affinity significantly and therefore studies carried out under voltage clamp conditions as provided here in oocytes allow a more defined determination of substrate affinity.

Electrogenic transport was in our studies also observed for Leu-Thia, Val-Thia and Glu-Thia. Val-Thia exhibited highest transport currents and is also an effective DPIP-inhibitor. The amino acid thiazolidide derivatives tested do show the known stereospecificity of the peptide transporters (Li *et al.*, 1998) with all L-isomers displaying higher affinities as the corresponding D-isomers. This was most pronounced when comparing L-allo-Ile-Thia with D-allo-Ile-Thia with a difference in affinity of more than 40-fold.

The inhibitory effects of various di- and tripeptides as well as aminoacyl-thiazolidides and –pyrrolidides on DPIP *in vitro* is well known (Demuth and Heins, 1995; Rahfeld *et al.*, 1991). However, recently the inhibition of DPIP was also observed *in vivo* after oral administration of Ile-Thia in diabetic animals and patients (Hoffmann *et al.*, 2001; Pospisilik *et al.*, 2002b; Pospisilik *et al.*, 2002a). *In vivo* studies in rats, dogs and monkeys using the DPIP inhibitors Ile-Thia and its allo-stereoisomer have recently shown that these compounds are well absorbed, as more than 80% of the administered dose was found in plasma (Beconi *et al.*, 2003). Our findings suggest that this high oral availability of Ile-Thia *in vivo* and that of the similar derivatives is based on their efficient uptake via PEPT1 across the apical membrane into the intestinal epithelial cell. A recent study employing rat jejunum has shown a close correlation between the intestinal permeability of identified PEPT1 substrates and the PEPT1 protein expression level (Naruhashi *et al.*, 2002). Although a paracellular permeation of the DP-IV inhibitors may also contribute to intestinal absorption, their good affinity and the carrier-mediated transport shown here argue for a significant contribution of PEPT1 to overall absorption of the compounds.

Regarding PEPT2, we demonstrate that this protein does not allow Ile-Thia and Thia-derivatives to be transported which may be important in view of possible unwanted side effects by uptake of the inhibitors into extraintestinal tissues. Finally, there is a clear selectivity of aminoacyl-thiazolidine derivatives for PEPT1 over related tri- and tetrapeptide derivatives, which further helps discriminating DPIV- and PEP-activity inhibition by such compounds.

In conclusion, we provide direct evidence, that the transport of the DPIV-inhibitor isoleucine-thiazolidide occurs by the intestinal peptide transporters PEPT1 with all the characteristics known such as membrane potential dependency, affinity pattern and stereospecificity. Efficient handling of Ile-Thia by PEPT1 does have pharmacological implications for treatment of diabetes based on the Ile-Thia induced specific inhibition of the dipeptidyl peptidase DPIV/CD26 that in turn increases the circulating levels of the insulinotropic gut hormones GLP-1 and GIP. Moreover, our expression systems for PEPT1 and PEPT2 in *Pichia pastoris* cells in combination with electrophysiological recordings in *Xenopus laevis* oocytes proved to be valid in rapidly screening a large numbers of potential drug candidates and to easily distinguish that solely bind to the transporters from those that are also transported.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Structures of the dipeptide Gly-Gln and the peptidomimetic Thia-derivatives Ile-Thia and Gly-Glu-Thia

Figure 2: Pre-screening for selecting potential substrates

Uptake of [^3H]D-Phe-Ala (2 $\mu\text{Ci/ml}$, 50nM) into *Pichia pastoris* yeast cells expressing human PEPT1 was measured at pH 6.5 in the presence of 10 mM of competitor after a 15-min incubation and was normalized against the optical density (OD) of cells. Uptake measured in the absence of competitors was taken as 100%. Values represent the mean of two different uptakes. 01 Lys-Phe, 02 Lys-Phe-Pro, 03 Pro-Pro-Ile-Thia, 04 Gly-Pro-Ile-Thia, 05 Ile-Pro-Ile-Thia, 06 H-Asn-Pyrr, 07 H-Asn-Thia, 08 H-His-Pyrr, 09 H-His-Thia, 10 H-Ile-Pyrr, 11 H-Ile-Thia, 12 H-Asp-Pyrr, 13 H-Asp-Thia, 14 H-Glu-Pyrr, 15 H-Glu-Thia, 16 H-Glu(NHOH)-Pyrr, 17 H-Glu(NHO-Bz)-Pyrr, 18 H-Asp(NHOH)-Pyrr, 19 H-Asp(NHO-Bz)-Pyrr, 20 Boc-Asn-Pyrr, 21 Boc-Asn-Thia, 22 Boc-His(Boc)-Pyrr, 23 Boc-His(Boc)-Thia, 24 Boc-Ile-Pyrr, 25 Boc-Ile-Thia, 26 Boc-Asp-Pyrr, 27 Boc-Asp-Thia, 28 Boc-Glu-Pyrr, 29 Boc-Glu-Thia, 30 Boc-Glu(NHOH)-Pyrr, 31 Boc-Glu(NHO-Bz)-Pyrr, 32 Boc-Asp(NHOH)-Pyrr, 33 Boc-Asp(NHO-Bz)-Pyrr

Figure 3: Kinetics of transport of Ile-Thia and Thia-derivatives

Inhibition of [^3H]D-Phe-Ala (2 $\mu\text{Ci/ml}$, 50nM) uptake into *Pichia pastoris* cells expressing PEPT1 in the absence and presence of increasing concentrations (0 - 20 mM) of inhibitor was measured after a 15 min incubation. The rate of uptake measured in the absence of inhibitors ($381 \pm 29 \text{ pmol} \cdot 15 \text{ min}^{-1} \cdot \text{OD}^{-1}$) was taken to be 100%. **A** Inhibition kinetics of Ile-Thia (squares) and Gly-Gln (rhombus) **B** Effect of an elongated aminoterminal residue on inhibition kinetics, Glu-Gly-Thia (squares),

Pro-Ile-Thia (triangles), Gly-Pro-Ile-Thia (filled rhombus), Pro-Pro-Ile-Thia (circles) and Gly-Gln (open rhombus) **C** Representative current trace of a PEPT1 expressing oocyte showing current responses following the successive addition of Gly-Gln and Ile-Thia at a concentration of 5 mM in standard perfusion buffer solution (pH 6.5). The upper line represents a current trace of a equally treated water-injected control oocyte.

Figure 4: Transport properties of radiolabelled Ile-Thia

A. Concentration dependent uptake of [^{14}C]L-Ile-Thia (0.01 - 10 mM) into *P. pastoris* cells expressing PEPT1. Uptake of [^{14}C]L-Ile-Thia was measured after 15 min of incubation in potassium phosphate buffer at pH 6.5. Data represent total uptake rates not corrected for a diffusional component. The small linear fraction of uptake was determined from the uptake of [^{14}C]L-Ile-Thia measured in the presence of excess Gly-Gln and was subtracted from total uptake rates prior to the calculation of the kinetic parameters. **B.** Inhibition of [^{14}C]L-Ile-Thia uptake by PEPT1 substrates into *P. pastoris* cells expressing PEPT1. The [^{14}C]L-Ile-Thia (1 $\mu\text{Ci/ml}$, 50 nM) uptake was measured at pH 6.5 in the presence of 10 mM nonradiolabelled D-phenylalanyl-alanine (D-Phe-Ala), glycyl-glutamine (Gly-Gln), cefadroxil and isoleucine after a 15-min incubation and was normalized against the optical density (OD) of cells. Uptake measured in the absence of competitors was taken as 100%.

Figure 5: Selective recognition of isoleucine-thiazolidide stereoisomers by PEPT1

Uptake of [^3H]D-Phe-Ala (2 $\mu\text{Ci/ml}$, 50 nM) into *P. pastoris* cells expressing PEPT1 as measured at pH 6.5 in the absence or presence of increasing concentrations (0 - 20 mM) of Ile-Thia stereoisomers after 15-min incubation. Uptake measured in the

absence of competitors ($420 \pm 35 \text{ pmol} \cdot 15 \text{ min}^{-1} \cdot \text{OD}^{-1}$) was taken as 100%. L-threo-Ile-Thia (open circles), D-threo-Ile-Thia (filled circles), L-allo-Ile-Thia (open triangles) and D-allo-Ile-Thia (filled triangles)

TABLES

Table 1: Apparent inhibition constants and PEPT1 as well as PEPT2 mediated currents of various thiazolidide-derivatives with varying N-terminal amino acid residues.

The concentration dependence of the different thiazolidide derivatives for inhibition of uptake of D-Phe-Ala in PEPT1 and PEPT2 expressing *P. pastoris* yeast cells was measured after 15 min incubation in presence of varying concentrations (0 - 20 mM) of competitor at pH 6.5. Apparent EC₅₀ values ± S.E. were calculated by non-linear regression analysis. Values represent the mean of at least two different uptakes. In the last two columns is shown the ability of the Thia-derivatives to serve as substrates for either PEPT1 or PEPT2. This was determined by applying the TEVC technique with *X. laevis* oocytes expressing the respective transporter. Data represent the normalized currents of the selected compounds (5 mM) relative to the glycyl-glutamine (5 mM) evoked currents at pH 6.5 at a membrane potential of -60 mV. I_{5 mM} values of compounds not significantly higher than background values in water-injected oocytes (p > 0.05, students paired t-test) are given as 0.

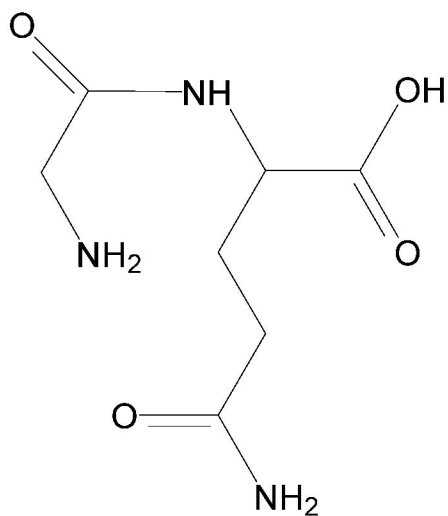
	apparent EC ₅₀ value (mM)		%I (I _{GQ 5 mM} = 100%)	
test compound	PEPT1	PEPT2	PEPT1	PEPT2
isoleucine-thiazolidide	0.51 ± 0.09	0.53 ± 0.04	55 ± 4	0
leucine-thiazolidide	0.34 ± 0.07	0.18 ± 0.01	40 ± 9	0
valine-thiazolidide	0.10 ± 0.01	0.06 ± 0.02	100 ± 12	0
histidine-thiazolidide	0.57 ± 0.05	-	-	-
glutamate-thiazolidide	1.17 ± 0.12	4.36 ± 1.51	20 ± 4	0
asparagine-thiazolidide	0.50 ± 0.03	-	-	-

Table 2: Apparent inhibition constants of various isoleucine-thiazolidide enantiomers.

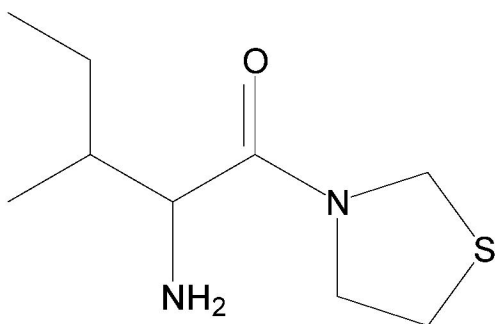
The concentration dependence of different Ile-Thia enantiomers for inhibition of uptake of radiolabelled D-Phe-Ala in PEPT1 and PEPT2 expressing *P. pastoris* yeast cells was measured after 15 min incubation in presence of varying concentrations (0 - 20 mM) of competitor at pH 6.5. Apparent EC₅₀-values ± S.E. were calculated from EC₅₀ values derived by non-linear regression analysis. Values represent the mean of at least two different uptakes.

compound	apparent EC ₅₀ -value (mM)	
	PEPT1	PEPT2
L-threo-Ile-Thia	0.51 ± 0.09	0.53 ± 0.04
D-threo-Ile-Thia	1.16 ± 0.32	2.21 ± 0.27
L-allo-Ile-Thia	0.13 ± 0.07	0.04 ± 0,01
D-allo-Ile-Thia	5.57 ± 0.81	0.18 ± 0.06

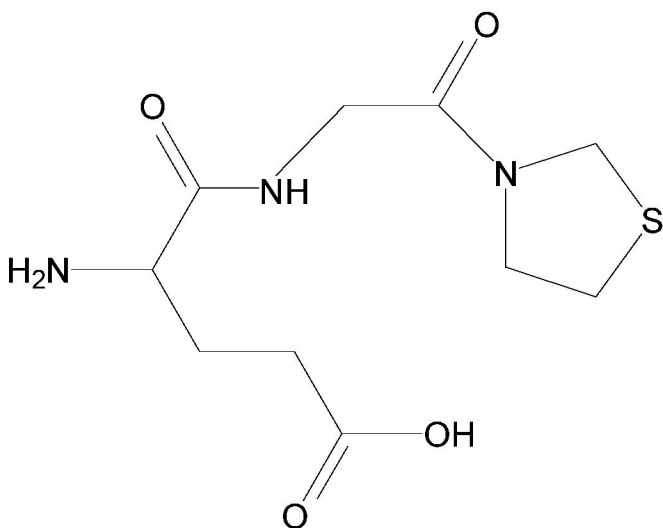
figure 1



Glycyl-glutamine



Isoleucine-thiazolidide



Glutamyl-glycine-thiazolidide

figure 2

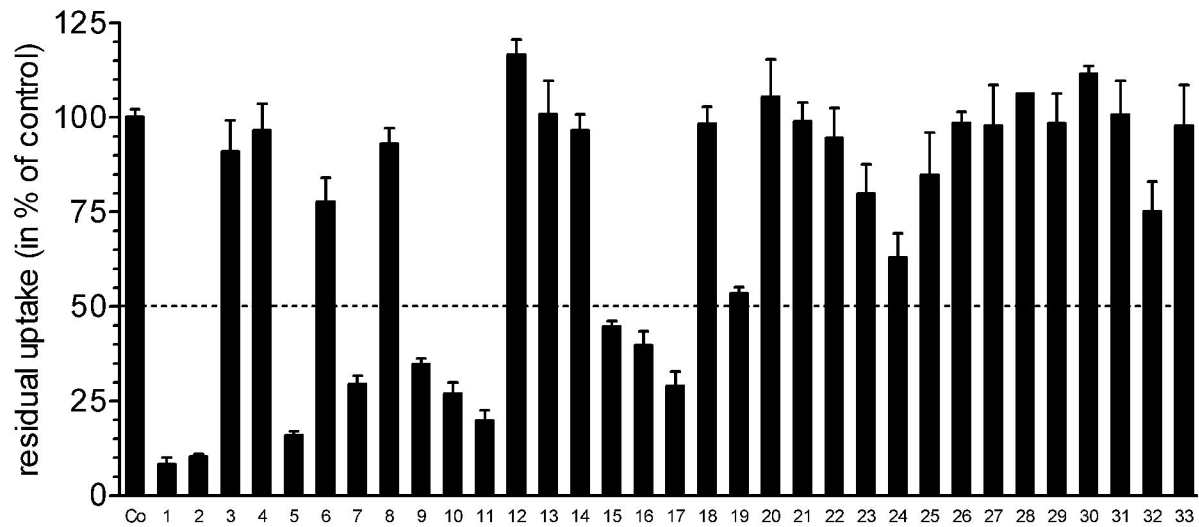


figure 3

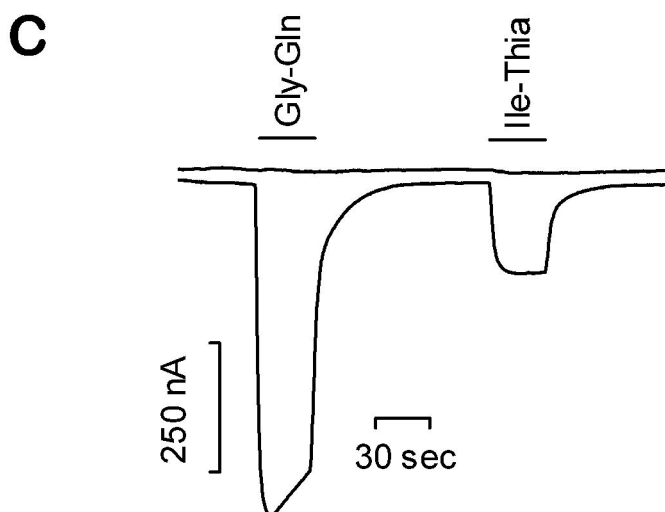
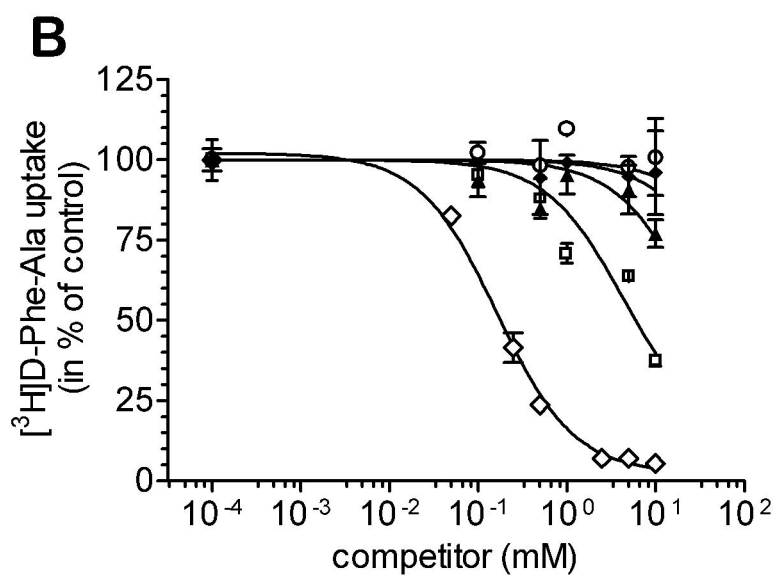
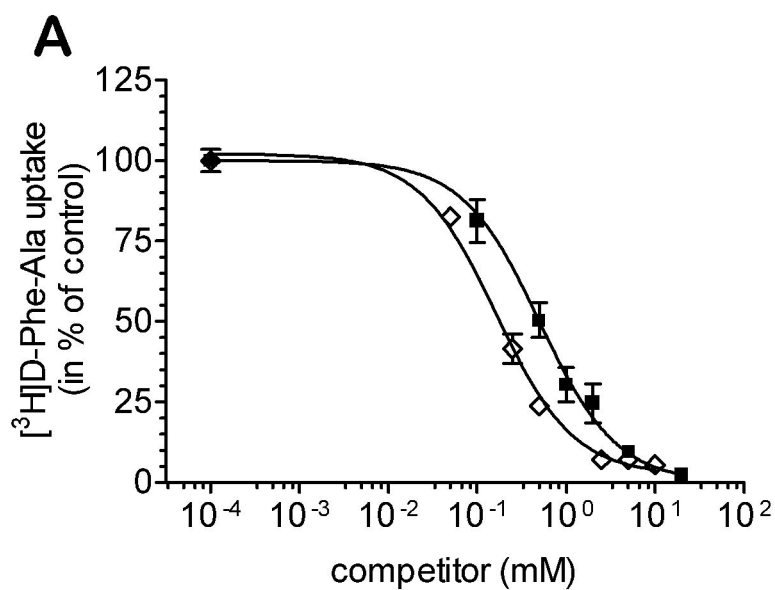
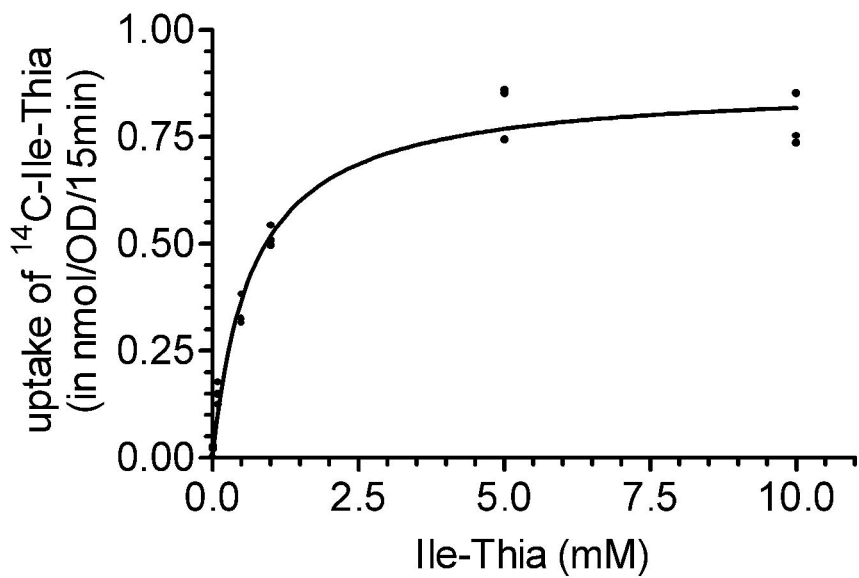


figure 4

A



B

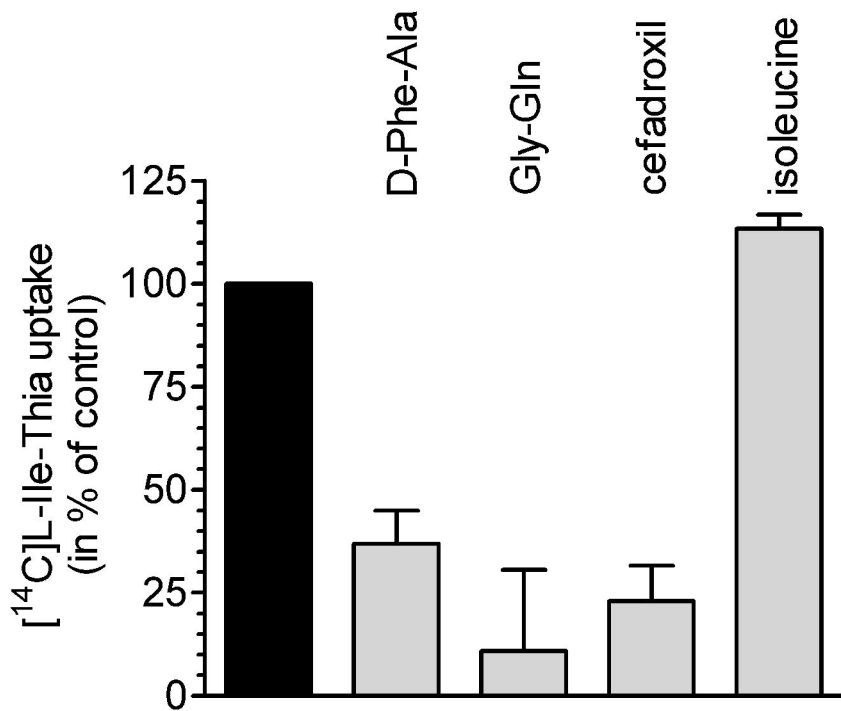


figure 5

