A Novel Inhibitor of the Mammalian Peptide Transporter PEPT1†

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ABSTRACT: This study was initiated to develop inhibitors of the intestinal H^+/peptide symporter. We provide evidence that the dipeptide derivative Lys[Z(NO_2)]-Pro is an effective competitive inhibitor of mammalian PEPT1 with an apparent binding affinity of 5−10 μM. Characterization of the interaction of Lys[Z(NO_2)]-Pro with the substrate binding domain of PEPT1 has been performed in (a) monolayer cultures of human Caco-2 cells expressing PEPT1, (b) transgenic *Pichia pastoris* cells expressing PEPT1, and (c) *Xenopus laevis* oocytes expressing PEPT1. By competitive uptake studies with radiolabeled dipeptides, HPLC analysis of Lys[Z(NO_2)]-Pro in cells, and electrophysiological techniques, we unequivocally show that Lys[Z(NO_2)]-Pro binds with high affinity to PEPT1, competes competitively with various dipeptides for uptake into cells, but is not transported itself. Lack of transport was substantiated by the absence of Lys[Z(NO_2)]-Pro in Caco-2 cell extracts as determined by HPLC analysis, and by the absence of any positive inward currents in oocytes when exposed to the inhibitor. The fact that Lys[Z(NO_2)]-Pro can bind to PEPT1 from the extracellular as well as the intracellular site was shown in the oocyte expression system by a strong inhibition of dipeptide-induced currents under voltage clamp conditions. Our findings serve as a starting point for the identification of the substrate binding domain in the PEPT1 protein as well as for studies on the physiological and pharmacological role of PEPT1.

The mammalian peptide transporter PEPT1 mediates the electrogenic uphill transport of dipeptides, tripeptides, and numerous peptidomimetics into intestinal epithelial cells. PEPT1 has been characterized in a number of studies with respect to expression, tissue localization, protein structure, and function (*1*−*7*). Whereas PEPT1 is recognized as the transporter that allows orally active peptidomimetics such as amino-β-lactam antibiotics to be absorbed very efficiently, its quantitative contribution to absorption of dietary amino acids in the peptide-bound form is still not known.

Although PEPT1 can transport a huge variety of different substrates, essentially nothing is known about the location and structure of the substrate binding domain within the PEPT1 protein. Available data are restricted to results obtained in experiments where chimeric mammalian peptide transporters have been derived from the intestinal and renal isoforms (*8*, *9*) or from preliminary site-directed mutagenesis studies (*10*).

The lack of knowledge about the contribution of PEPT1 to both the overall amino acid transport in intestine and the substrate binding site is mainly due to the unavailability of a specific high-affinity inhibitor for PEPT1. Recently, some potential inhibitors of PEPT1 have been described (*11*−*13*). Whereas Asp(OBzl)-Sar[1] (*11*) and Val-Lys(fluorescein) (*13*) were shown to inhibit dipeptide uptake in cell systems competitively, the conclusion that these agents are not transported was only circumstantial by demonstration of the absence of saturable transport kinetics. In the case of 4-aminomethylbenzoic acid (*12*), the reported affinity for PEPT1 was very low, i.e., almost 10 times lower than that of a normal dipeptide consisting of L-α-amino acids. In our study, we thoroughly characterized a novel high-affinity inhibitor for PEPT1 in three different biological systems.

EXPERIMENTAL PROCEDURES

Materials. The Caco-2 human colon carcinoma cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Culture media and supplements, fetal bovine serum, and the trypsin solution were purchased from Life Technologies, Inc. [*glycine*-1-^{14}C]-Gly-Sar (53 mCi/mmol) was obtained from Amersham International. Gly-Gln, Lys-Pro, and Gly-Sar were from Sigma (Deisenhofen, Germany); D-Phe-Ala was purchased from Bachem (Heidelberg, Germany). Custom synthesized

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Z(NO_2), 4-nitrobenzyloxycarbonyl; OBzl, benzyl ester.

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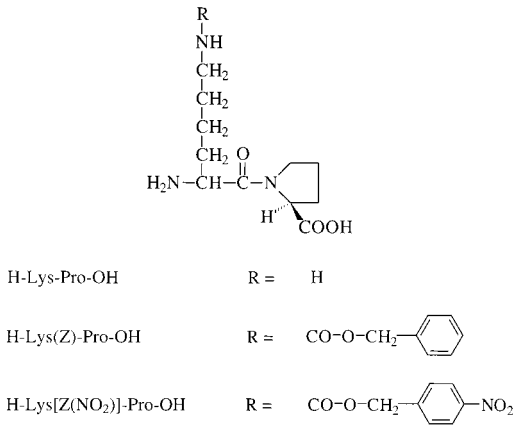

FIGURE 1: Structural formulas of the tested inhibitors.

[*alanine*-2,3-^{3}H]-D-Phe-Ala (specific radioactivity of 40 Ci/mmol) was obtained from Biotrend (Cologne, Germany).

Synthesis of Lys-Pro Derivatives. The dipeptide derivatives H-Lys(Z)-Pro-OH and H-Lys[Z(NO_2)]-Pro-OH (Figure 1) were synthesized by coupling the *N*-hydroxysuccinimide ester of Boc-Lys(Z)-OH and Boc-Lys[Z(NO_2)]-OH, respectively, with L-proline. The crude products were purified by flash chromatography on silica gel. After removal of the N^{α}-Boc group by treatment with concentrated formic acid at room temperature, the resulting N^{ϵ}-protected dipeptides were characterized as formiates. Purity was assessed by TLC, reversed phase HPLC, and mass spectrometry.

Caco-2 Cell Culture and Uptake Assessments. The Caco-2 human colon carcinoma cell line was routinely cultured (passages 20−46) with minimum essential medium supplemented with 10% fetal bovine serum, a 1% nonessential amino acid solution, and gentamicin (45 μg/mL) (*14*, *15*). For most experiments, the cells were seeded in 35 mm disposable Petri dishes (Becton Dickinson) at a density of 0.8×10^6 cells per dish. The uptake assessments were performed on the sixth to seventh day after confluence. Caco-2 cells were also cultured on permeable polycarbonate Transwell cell culture inserts (24.5 mm diameter, 3 μm pore size, Costar GmbH, Bodenheim, Germany) (*16*). Subcultures were started at a cell density of 43 000 cells/cm^2 and cultured for 17−23 days. Uptake of [^{14}C]Gly-Sar in cells cultured on plastic dishes was assessed as described previously (*14*, *15*). The uptake buffer (1 mL) contained 25 mM Mes/Tris (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 10 μM [^{14}C]Gly-Sar, and increasing concentrations of unlabeled inhibitors. After incubation for 10 min, the cells were quickly washed, solubilized, and prepared for liquid scintillation spectrometry. Transepithelial flux of compounds across cells cultured on permeable filters was measured as described previously (*16*). Uptake was started by adding buffer (pH 6.0, 1.5 mL) containing the test compounds to the donor side. At time intervals of 10−120 min, samples were taken from the receiver compartment. After 2 h, the filters were washed, cut out of the plastic insert, and prepared for HPLC.

HPLC Analysis. Test compounds in extracellular uptake medium, samples in flux studies, and samples in cells were quantified according to the laboratory standard HPLC (LaChrom, Merck-Hitachi) with a diode array detector and a LiChrosphere 100RP-18 column (125-4, 5 μm). The eluent was a 30% acetonitrile/0.1% trifluoroacetic acid mixture in water. UV detection was carried out at 220 nm. The injection volume was 20 μL and the flow rate 0.8 mL/min.

Pichia pastoris Strains and Transport Assays in Yeast. Cultures of *P. pastoris* strains expressing PEPT1 were prepared as described previously (*17*). Cells were pelleted at 3000*g* for 10 min, washed, and resuspended to a density of 5×10^7 cells per 20 μL of potassium phosphate buffer (PPB). Uptake assessments were performed at 22−24 °C by using a rapid filtration technique on 96-well filter plates (HATF type, 0.45 μm pore size; Millipore, Eschborn, Germany). In brief, uptake was initiated by mixing 20 μL of the cell suspension with 30 μL of PPB containing 0.05 μCi of [^{3}H]-D-Phe-Ala either with or without competitors. After 20 min, uptake was terminated and the amount of radioactivity associated with the filter was measured.

Studies in Xenopus laevis Oocytes Expressing PEPT1. Expression, treatment, and handling of *X. laevis* oocytes has been described previously (*18*). Individual oocytes were administered 30 nL of RNA solution containing 30 ng of PEPT1 cRNA. All electrophysiological measurements were performed after incubation of oocytes for 3 days in a buffer composed of 88 mM NaCl, 1 mM KCl, 0.82 mM $CaCl_2$, 0.41 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, and 10 mM Mes/Tris at pH 6.5 (modified Barth solution).

Electrophysiology. The two-electrode voltage clamp technique was used to characterize responses in inward current (*I*) to substrate addition. Steady-state currents were measured in the absence and presence of Gly-Gln and/or Lys-Pro, Lys(Z)-Pro, or Lys[Z(NO_2)]-Pro with the membrane potential in oocytes clamped to −60 mV. In a series of experiments, oocytes expressing PEPT1 were administered either 25 nL of H_2O, 25 nL of 100 mM D-Phe-Ala, or 25 nL of 100 mM Lys[Z(NO_2)]-Pro. After incubation for 30 min, current responses were measured during perfusion with a 10 mM Gly-Gln solution.

Calculations and Statistics. All data are given as the mean ± the standard error of three to four independent experiments. The significance of differences between the uptake rates and calculated constants was determined by a nonpaired *u* test. IC_{50} values (i.e., concentration of the unlabeled compound necessary to inhibit 50% of radiolabeled dipeptide carrier-mediated uptake) were determined by nonlinear regression using the logistical equation for an asymmetric sigmoid (allosteric Hill kinetics): $y = \text{Min} + (\text{Max} - \text{Min})/[1 + (X/IC_{50})^{-P}]$, where Max is the initial *Y* value, Min the final *Y* value, and the power *P* represents the Hill coefficient. Inhibition constants (K_i) were calculated from IC_{50} values according to the method published by Cheng and Prusoff (*19*).

RESULTS AND DISCUSSION

Inhibition of Gly-Sar Uptake in Caco-2 Cells and D-Phe-Ala Uptake in Yeast. Uptake of Gly-Sar into confluent monolayers of Caco-2 cells is mediated by the H^+/peptide symport system PEPT1 (*14*). In the presence of an inwardly directed H^+ gradient, we observed a concentration-dependent inhibition of [^{14}C]Gly-Sar uptake by Lys-Pro and its derivatives Lys(Z)-Pro and Lys[Z(NO_2)]-Pro (Figure 2A). Both Lys-Pro derivatives were recognized by PEPT1 in Caco-2 cells with apparent K_i values of 33 ± 1 and 10 ± 1 μM, respectively. These values are considerably lower than that

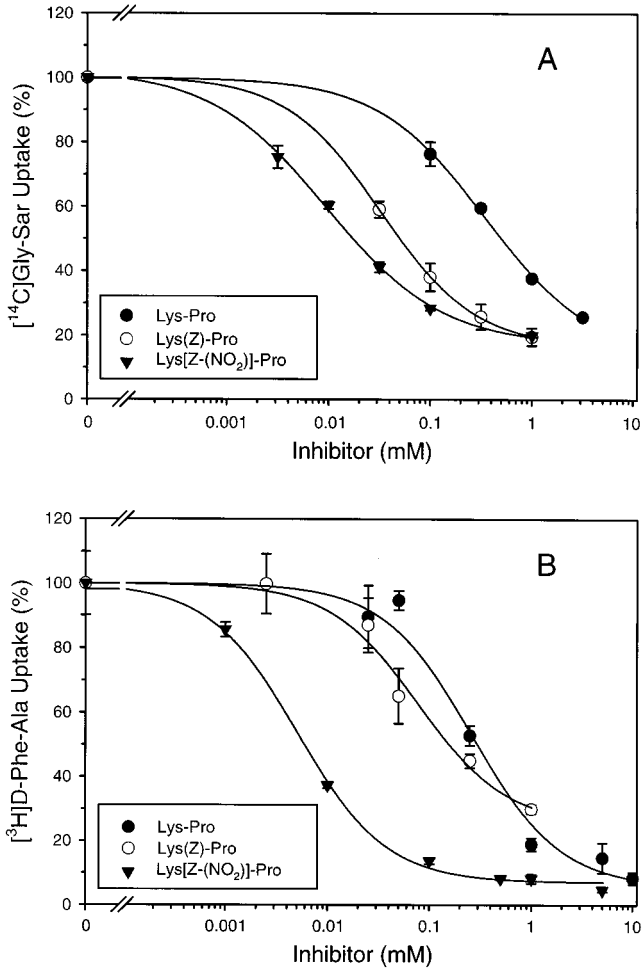

FIGURE 2: Inhibition of dipeptide transport via PEPT1 by Lys-Pro derivatives (0−10 mM). (A) Inhibition of [^{14}C]Gly-Sar uptake into Caco-2 cells (10 μM, pH 6.0, 10 min). The rate of uptake measured in the absence of inhibitors [237 ± 12 pmol (10 min)$^{-1}$ (mg of protein)$^{-1}$] was taken to be 100% (n = 4). (B) Inhibition of [^{3}H]-D-Phe-Ala uptake into *P. pastoris* cells expressing PEPT1 (2 μCi/mL, pH 6.5, 20 min). The rate of uptake measured in the absence of inhibitors [381 ± 29 pmol (20 min)$^{-1}$ OD^{-1}] was taken to be 100% (n = 3).

of the parent dipeptide Lys-Pro (K_i = 350 ± 90 μM). Therefore, this particular side chain modification increases the affinity of Lys-Pro derivatives for PEPT1 by a factor of 11 in the case of Lys(Z)-Pro and 35 in the case of Lys-[Z(NO$_2$)]-Pro. To demonstrate that the Lys-Pro derivatives also interact with the substrate binding site of PEPT1 when expressed heterologously, we performed competition studies in *P. pastoris* cells. Uptake of [^{3}H]-D-Phe-Ala was dose-dependently inhibited by Lys-Pro, Lys(Z)-Pro, and Lys-[Z(NO$_2$)]-Pro (Figure 2B) with apparent K_i values of 263 ± 60, 78 ± 18, and 5.1 ± 0.4 μM, respectively. These affinity constants were very similar to those obtained in Caco-2 cells. In two recent studies, we have shown that PEPT1 preferentially recognizes and transports the trans conformation of dipeptides (*15*, *20*). The trans contents of Lys-Pro, Lys(Z)-Pro, and Lys[Z(NO$_2$)]-Pro, however, measured by high-performance capillary electrophoresis (HPCE) were almost identical, 64 ± 5, 61 ± 3, and 67 ± 3%, respectively (data not shown). Therefore, different affinities of the compounds cannot be explained by different ratios of peptide bond conformers. On the basis of the specificity for the trans conformers of proline-containing peptides, the "true" affinity

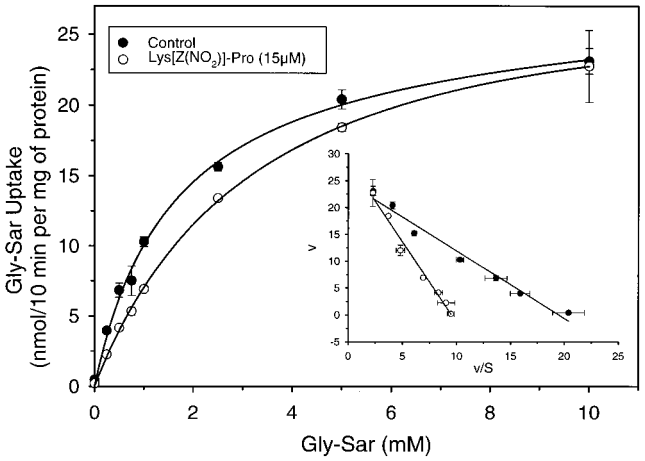

FIGURE 3: Effect of Lys[Z(NO$_2$)]-Pro on the saturation kinetics of Gly-Sar uptake into Caco-2 cells. The rate of uptake of Gly-Sar (0.01−10 mM) was measured at pH 6.0 for 10 min in confluent monolayer cultures. The results represent saturable uptake rate values after correction for the nonsaturable component. If not shown, error bars are smaller than the symbols. The inset shows Eadie−Hofstee transformations of the data (n = 4).

of Lys[Z(NO$_2$)]-Pro is consequently ~3−7 μM. Because of this very high affinity constant, Lys[Z(NO$_2$)]-Pro was chosen for further experiments. As an important prerequisite for any inhibitor use in biological systems, Lys[Z(NO$_2$)]-Pro was found to be highly resistant against enzymatic hydrolysis. Over an incubation period of 30 min at 37 °C in the Caco-2 cell monolayer studies, the compound remained intact to >97% (data not shown). Interestingly, Lys[Z(NO$_2$)]-Pro has been described as a dipeptidyl peptidase IV inhibitor involved in the modulation of the immune response and in wound healing processes (*21*, *22*).

We next investigated the kinetics of inhibition of Gly-Sar uptake in Caco-2 cells caused by Lys[Z(NO$_2$)]-Pro. Gly-Sar uptake was assessed over the concentration range of 0.01−10 mM in the absence or presence of Lys[Z(NO$_2$)]-Pro at a concentration of 15 μM. Figure 3 shows the relationship between the uptake rates and the Gly-Sar concentration. Under both conditions, the Eadie−Hofstee transformations of the saturation kinetics were linear (r^2 > 0.98), indicating that a single transport system is responsible for Gly-Sar uptake. In the absence of the inhibitor, the Michaelis−Menten constant, K_t, for Gly-Sar was 1.3 ± 0.1 mM and the maximal velocity, V_{max}, was 24.4 ± 1.1 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$, in agreement with previously reported values (*14*). The corresponding kinetic constants obtained in the presence of Lys[Z(NO$_2$)]-Pro were as follows: K_t = 3.0 ± 0.1 mM and V_{max} = 28.7 ± 1.3 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$. Hence, the presence of the inhibitor at a concentration close to its K_i value increased the K_t value for Gly-Sar by ~2-fold with the V_{max} not altered significantly. This suggested that Lys[Z(NO$_2$)]-Pro inhibits PEPT1-mediated Gly-Sar uptake into Caco-2 cells in a competitive manner. We also determined the inhibition constant (K_i) for Lys[Z(NO$_2$)]-Pro by measuring the rate of Gly-Sar uptake at two different Gly-Sar concentrations (50 and 500 μM) in the presence of increasing concentrations of Lys[Z(NO$_2$)]-Pro (from 0 to 0.1 mM). The results are presented as Dixon plots in Figure 4 and reveal linearity at both Gly-Sar concentrations with lines intersecting above the abscissa in the fourth quadrant, as expected for a competitive inhibitor.

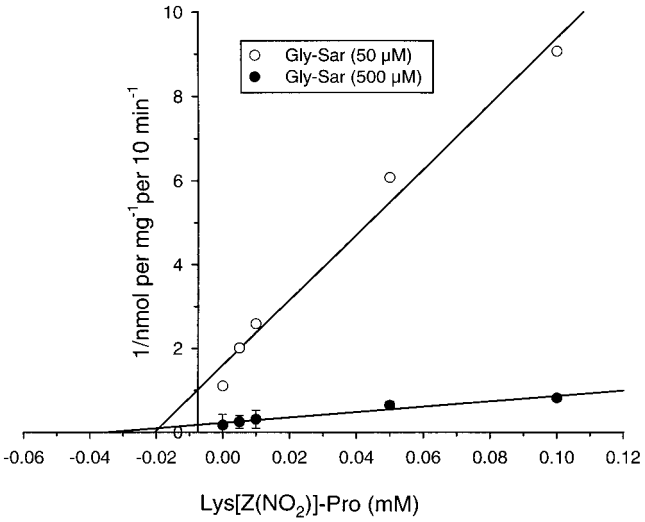

FIGURE 4: Determination of the inhibition constant with a Dixon plot. The rate of uptake of [^{14}C]Gly-Sar (10 μM) was measured at pH 6.0 for 10 min. The diffusional component of 11%, measured in the presence of an excess of Gly-Sar (50 mM), was subtracted from the total rate of uptake to calculate the mediated rate of uptake ($n = 4$).

A K_i value of 19 μM was calculated from the point of intersection.

As a first approach to assess whether Lys[Z(NO_2)]-Pro is transported into the Caco-2 cells, we performed a series of flux experiments. Employing HPLC analysis of cell extracts and the medium in the receiver compartment from Caco-2 cells apically exposed to Lys[Z(NO_2)]-Pro (5 mM), no uptake of the compound into Caco-2 cells was measurable and the transepithelial flux of Lys[Z(NO_2)]-Pro across the cell monolayers was 5-fold lower than that of the hydrophilic nontransported space marker mannitol (data not shown). It is important to note that Lys[Z(NO_2)]-Pro did not change unspecifically the mannitol flux of 0.1% h^{-1} cm^{-2} across Caco-2 monolayers.

Interaction of Lys-Pro and Its Derivatives with PEPT1 Expressed in Oocytes. We employed the two-electrode voltage clamp technique in *Xenopus* oocytes expressing PEPT1 to assess transport and transport inhibition by Lys-Pro and the two side chain-protected derivatives Lys(Z)-Pro and Lys[Z(NO_2)]-Pro. As shown in Figure 5 (right panel), Lys-Pro induces inward currents by electrogenic transport via PEPT1 that are identical to those of the reference dipeptide Gly-Gln of 215 nA at a holding potential of -60 mV. In contrast, Lys(Z)-Pro failed to cause any response in current when applied at a concentration of 1 mM but inhibited Gly-Gln-evoked currents by ~50%. When 1 mM Lys[Z(NO_2)]-Pro was applied, again no measurable inward current was obtained. However, perfusion of the same oocyte with increasing concentrations (from 0.01 to 2.5 mM) of Lys[Z(NO_2)]-Pro reduced the Gly-Gln currents dose-dependently to 10% of its initial value (Figure 5, left panel). Lys[Z(NO_2)]-Pro therefore competes efficiently with dipeptides at the binding site of PEPT1, but is itself obviously not transported electrogenically. Moreover, this experiment shows that inhibition of peptide transport caused by Lys[Z(NO_2)]-Pro is fully reversible as Gly-Gln-induced currents returned to the initial values when the inhibitor was washed out. When steady-state inward currents of 0.05, 0.1, 0.5, and 2.5 mM Gly-Gln were measured in the presence of

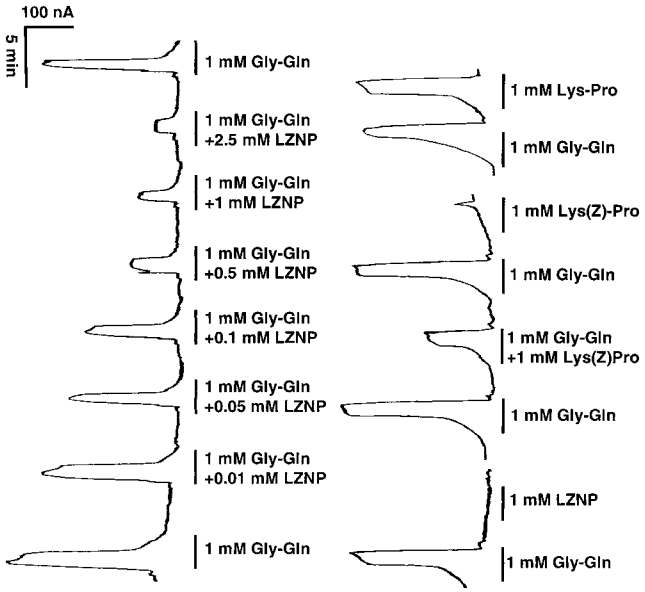

FIGURE 5: Recordings of substrate-induced inward currents in *X. laevis* oocytes expressing PEPT1. Typical recordings ($n = 3-4$) of inward currents in oocytes expressing PEPT1 in the presence of 1 mM Lys-Pro, Lys(Z)-Pro, Lys[Z(NO_2)]-Pro (LZNP), or Gly-Gln alone or with the indicated concentrations of Lys[Z(NO_2)]-Pro (from 0.01 to 2.5 mM). Oocytes were clamped to -60 mV and perfused in a modified Barth solution at pH 6.5. Control oocytes (water injected) did not show any response in inward current to the addition of either Gly-Gln or Lys[Z(NO_2)]-Pro.

increasing concentrations of Lys[Z(NO_2)]-Pro, the results confirmed that Lys[Z(NO_2)]-Pro is a competitive inhibitor of electrogenic peptide transport. The apparent K_i value for inhibition of Gly-Gln-induced currents by Lys[Z(NO_2)]-Pro was 29 $\pm$ 4 μM (data not shown).

We next determined whether Lys[Z(NO_2)]-Pro can inhibit PEPT1 also when applied to the cytosolic (trans) site. For this purpose, oocytes expressing PEPT1 were first perfused with 10 mM Gly-Gln and maximal steady-state inward currents were registered. Then oocytes were administered either 25 nL of H_2O, a 100 mM solution of D-Phe-Ala, or 100 mM Lys[Z(NO_2)]-Pro and incubated for 30 min prior to a second measurement of Gly-Gln-induced inward currents. Injection of Lys[Z(NO_2)]-Pro significantly decreased the Gly-Gln current by 56 $\pm$ 5% (data not shown). This result strongly suggests that Lys[Z(NO_2)]-Pro can effectively bind also to the internal substrate binding domain of PEPT1.

In conclusion, adding the Z group to the ϵ-amino group of the Lys residue turns Lys-Pro from a normal transported substrate into a nontransported derivative with significantly higher affinity. Addition of the NO_2 function to the hydrophobic ring moiety enhances the affinity for binding of Lys[Z(NO_2)]-Pro to PEPT1 further while maintaining its inability for electrogenic cotransport. The concomitant conformational change necessary for translocation of the loaded carrier seems to be prevented by spatial hindrance or by hydrophobic interaction of the ring system with amino acid residues in the binding pocket of the carrier protein. Lys[Z(NO_2)]-Pro represents the first specific competitive inhibitor of PEPT1 with high affinity and a reversible mode of action, with both the external and the internal substrate binding site. Employing this compound should allow new insights into the physiological as well as pharmacological role of PEPT1. Moreover, our findings may serve as a

starting point for synthesis of even more effective transport inhibitors that may help to identify the substrate binding domain within the PEPT1 protein.

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