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Research paper

Synthesis and intestinal transport of the iron chelator maltosine in free and dipeptide form

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ABSTRACT

Maltosine, a 3-hydroxy-4-pyridinone derivative of lysine formed in the course of the advanced Maillard reaction, is an effective metal chelating agent. It therefore represents an interesting compound for the treatment of metal ion storage diseases. We synthesized 6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine (free maltosine) and its dipeptide derivatives alanylmaltosine (Ala-Mal) and maltosinylalanine (Mal-Ala) and examined the transepithelial flux of these compounds across Caco-2 cells and their interaction with membrane transporters. Transepithelial flux of maltosine was significantly higher when added as Ala-Mal and Mal-Ala than in free form. Assays at Caco-2 cells and at HeLa cells expressing the human peptide transporter (hPEPT)1 revealed that Ala-Mal and Mal-Ala show medium to high affinity to the system. Only free but not peptide-bound maltosine inhibited the uptake of L-[^{3}H]lysine in Caco-2 and OK cells. Maltosine dipeptides were transported by hPEPT1 across cell membranes and accumulated in hPEPT1-transfected HeLa cells. In electrophysiological measurements at hPEPT1-expressing *Xenopus laevis* oocytes, Ala-Mal and Mal-Ala induced significant inward directed currents. We conclude that Ala-Mal and Mal-Ala are transported by hPEPT1 into intestinal cells and then hydrolyzed to free maltosine and alanine. The results suggest that the oral bioavailability of maltosine can be increased significantly by applying this drug candidate in peptide-bound form.

1. Introduction

Iron is an essential mineral for many metabolic functions in the organism. It is stored in ferritin and hemosiderin and circulates in plasma associated with the iron transport protein transferrin [1–3]. An abnormal accumulation of iron in the body can be caused by primary overload (hemochromatosis), by diseases such as diabetes mellitus, by alcohol abuse or by multiple blood transfusions (for example during the treatment of β-thalassemia, secondary iron overload). It is essential to eliminate excess iron from the body to prevent dysfunctions of liver, heart and endocrine glands [3,4]. Patients are usually treated with iron chelators to remove iron *via* urine and faeces. Ideally, such iron chelators are characterized by (i) efficiency, (ii) specificity, (iii) low toxicity, (iv) low molecular weight and sufficient lipophilicity, (v) slow rate of metabolism and (vi) oral bioavailability [1,5]. Desferrioxamine is used during the treatment of β-thalassemia but it is orally inactive and shows serious toxic side effects [6,7]. Therefore, a new class of orally active chelators, the hydroxypyridinones, especially the 3-hydroxy-4-pyridinones (3,4-HP), has been developed, among them deferiprone (1,2-dimethyl-3-hydroxy-4-pyridinone) [8–10]. 3,4-HP derivatives show high affinities for iron, gallium and aluminium and are resistant to enzymatic hydrolysis [7].

The 3,4-HP-derivative named maltosine (6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine, Fig. 1) has been identified by Ledl and co-workers as a reaction product formed between the ε-amino group of lysine and carbonyl degradation products of oligosaccharides during the advanced Maillard reaction [11]. The Maillard reaction, also known as non-enzymatic glycosylation, occurs between reducing carbohydrates and lysine or arginine – as free amino acids or bound in peptides or proteins – during heating or storage of food. Some of the resulting compounds strongly bind metal ions [12–15]. Maltosine has been detected by amino acid analysis in heated milk and whey powders in concentrations of about 100 mg/kg of protein [16]. When Rehner and Walter examined the bioavailability of iron, copper and zinc in the presence of maltosine and other Maillard reaction compounds, they found that maltosine inhibited the intestinal iron uptake and increased

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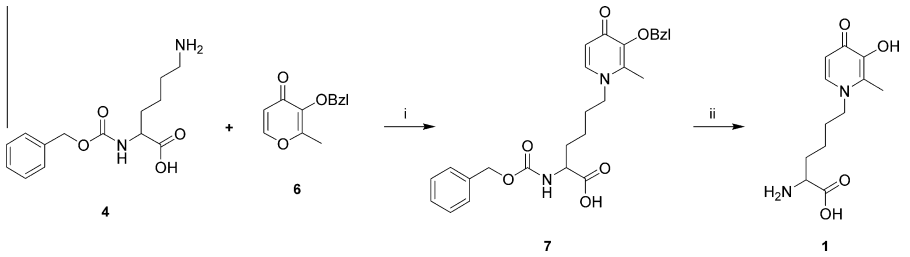

Fig. 1. Synthesis of 6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine (maltosine) as the free amino acid. (i) Ethanol/borate buffer, pH 10.0 1/1, 80 °C, 24 h. (ii) H_2, 10% Pd/C, ethanol, rt, 18 h. **4** Z-Lys-OH, **6** 3-benzyloxy-2-methyl-4-pyrone (benzyl maltol), **7** Z-Mal(Bzl)-OH, **1** maltosine.

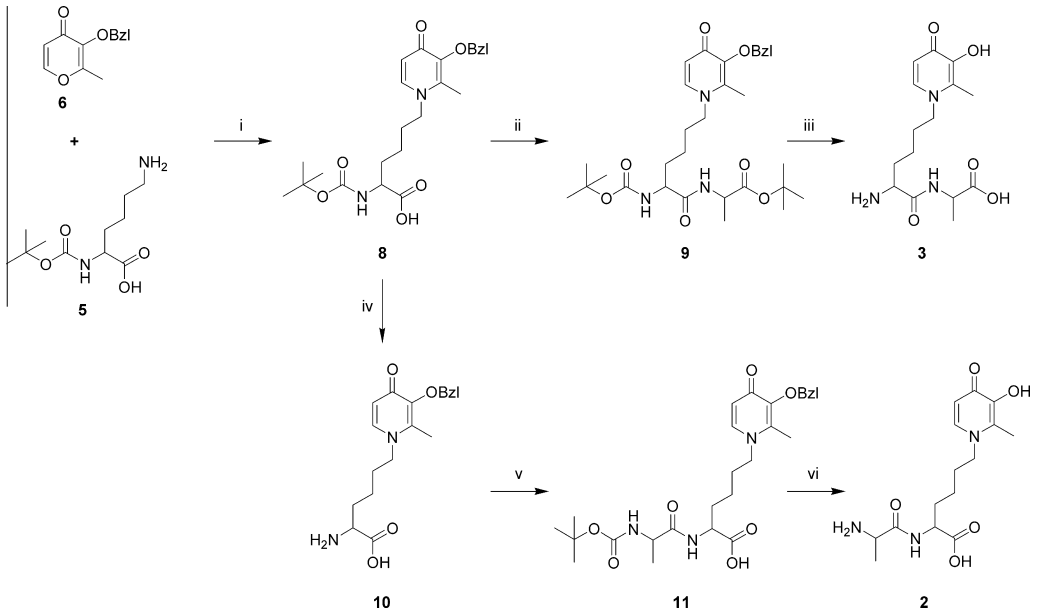

Fig. 2. Synthesis of peptide-bound 6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine (Mal-Ala and Ala-Mal). (i) Ethanol/water 1/1, pH 13, 80 °C, 18 h. (ii) DIPEA, TSTU, DCM, rt, 30 min, then H-Ala-OBu^t, DIPEA, rt, 30 min. (iii) H_2, 10% Pd/C, methanol, rt, 18 h, then 6 N HCl/THF 1/1, rt, 60 min. (iv) 10% HOAc, 70 °C, 4 h. (v) Boc-Ala-OSu, DIPEA, DCM, rt, 18 h. (vi) H_2, 10% Pd/C, methanol, rt, 18 h, then 6 N HCl/THF 1/1, rt, 60 min. **6** 3-benzyloxy-2-methyl-4-pyrone (benzyl maltol), **5** Boc-Lys-OH, **8** Boc-Mal(Bzl)-OH, **10** H-Mal(Bzl)-OH, **9** Boc-Mal(Bzl)-Ala-OBu^t, **11** Boc-Ala-Mal(Bzl)-OH, **2** Ala-Mal, **3** Mal-Ala.

the renal iron excretion [12]. Very recent results suggest that maltosine is even more effective than the commonly used iron chelator deferiprone [unpublished data, 17].

The purpose of the present investigation was to characterize the intestinal maltosine transport and to test the hypothesis that the intestinal maltosine absorption can be increased by employing peptide-bound maltosine, e.g. alanylmaltosine (Ala-Mal) and maltosinylalanine (Mal-Ala, Fig. 2). The compounds were synthesized and characterized spectroscopically. Their total transepithelial net flux across cell monolayers was examined. In competition assays, the interaction of the compounds with the intestinal and renal lysine transporter(s) and the intestinal human peptide transporter (hPEPT)1 was studied. Experiments at hPEPT1-transfected HeLa cells and electrophysiological measurements at *Xenopus laevis* oocytes expressing hPEPT1 revealed an active, uphill and specific transport of dipeptide-bound maltosine by the intestinal peptide transporter.

2. Materials and methods

2.1. Materials

HPLC-grade acetonitrile, deuterium oxide, formic acid, unlabeled Gly-Sar, Ala-Lys and L-lysine were purchased from Sigma-Aldrich (Steinheim, Germany). *O*-(*N*-Succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TSTU) and 1-heptanesulfonic acid (sodium salt) were obtained from Molekula (Taufkirchen, Germany). Boc-Ala-OSu, 3-hydroxy-2-methyl-4-pyrone (maltol) and Z-Lys-OH **4** (Fig. 1) from Fluka (Steinheim, Germany) were used. *N*,*N*-Diisopropylethylamine (DIPEA), sodium hydroxide and trisodium citrate dihydrate were from Merck (Darmstadt, Germany). Benzyl bromide was purchased from ABCR (Karlsruhe, Germany) and H-Ala-OBu^t monohydrochloride and Lys-Ala from Bachem (Bubendorf, Switzerland). Boc-Lys-OH **5** was obtained from IRIS Biotech (Martinsried, Germany). Hydrochloric acid and palladium on activated charcoal (Pd/C, 10% w/w) were from VWR International (Darmstadt, Germany). The synthesis of 3-benzyloxy-2-methyl-4-pyrone **6** (benzyl maltol) was performed by benzylation of maltol with benzyl bromide in acetone in the presence of potassium carbonate. All other chemicals were purchased from standard suppliers and were of the highest purity available.

The cell line Caco-2 and the epithelial cervical cancer cell line HeLa were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The renal cell line OK was provided by H. Daniel (Technische Universität, Munich, Germany). Cell culture media, supplements and trypsin were purchased from Invitrogen (Karlsruhe, Germany) or PAA (Cölbe, Germany), fetal bovine serum from Biochrom (Berlin, Germany).

[Glycine-1-^{14}C]glycylsarcosine (Gly-Sar; specific radioactivity 56 mCi/mmol) and L-[4,5-^{3}H]lysine monohydrochloride (specific radioactivity 99 Ci/mmol) were synthesized by GE Healthcare (Little Chalfont, UK). [^{14}C]Mannitol (specific radioactivity 53 mCi/mmol) was from Hartmann Analytic GmbH (Braunschweig, Germany) [18,19].

2.2. Methods

2.2.1. Thin layer chromatography (TLC)

TLC was performed on silica gel 60 plates (Merck, Darmstadt, Germany) using the solvent systems stated in the synthesis sections. Visualization was achieved by spraying the plates with a 0.1% solution of ninhydrin in ethanol acidified with 3% (v/v) of glacial acetic acid followed by heating until spots appeared. For the identification of target fractions after chromatographic separations, 1 μl of each fraction was spotted onto TLC plates and sprayed with the ninhydrin reagent. Silica gel plates were used except for completely deprotected maltosine derivatives, for which cellulose F plates (Merck, Darmstadt, Germany) were used.

2.2.2. High-pressure liquid chromatographic (HPLC)

HPLC analyses were performed using a high-pressure gradient system from Amersham Pharmacia Biotech (Uppsala, Sweden), consisting of a pump P-900 with an online degasser (Knauer, Berlin, Germany), a column oven, and a UV detector UV-900. Analytical separation of maltosine from the respective dipeptide analogues was achieved using a polymer-based RP-18-column (PLRP-S, 100 Å, 8 μm, 250 mm × 4.6 mm, Polymer Laboratories, Darmstadt, Germany). The column temperature was set to 30 °C, and UV detection was performed at 280 nm. The mobile phases were 5 mM sodium heptanesulfonate, pH 2.0 (solvent A), and a mixture of 50% of solvent A and 50% of acetonitrile (solvent B). A linear gradient from 4% to 70% B in 20 min was used for all measurements. The flow rate was 1 ml/min. External calibration was performed with the synthesized standards.

The samples collected in flux measurements (Caco-2 and OK cells) were diluted with HPLC solvent A and centrifuged before analysis (10,000 rpm, 15 min). The cell monolayers on polycarbonate filters cut out of the well inserts were thawed and refrozen three times and then diluted with solvent A. These samples were membrane filtered (0.45 μm) to remove cell debris. Then, 50 μl of the samples obtained from the cell culture experiments were injected.

2.2.3. Mass spectroscopy, nuclear magnetic resonance spectrometry and elemental analyses

For mass spectroscopic analyses, a PerSeptive Biosystems Mariner time-of-flight mass spectrometry instrument equipped with an electrospray ionization source (ESI-TOF-MS, Applied Biosystems, Stafford, TX) working in the positive mode was used. Calibration of the mass scale was established using a mixture of bradykinin, angiotensin I and neurotensin. After appropriate dilution of the samples with 1% formic acid in 50% aqueous methanol, the sample was injected at a flow rate of 5 μl/min into the ESI source by a syringe pump. ^{1}H NMR spectra were recorded on a Bruker DRX 500 (Rheinstetten, Germany) at 500 MHz. Deuterium oxide was used as the solvent. Proton chemical shifts are given relative to the internal HOD signal (4.70 ppm). Elemental analyses were performed on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy).

2.2.4. Synthesis of 6-(3-hydroxy-4-oxo-2-methyl-4(1H)-pyridin-1-yl)-L-norleucine (maltosine)

Z-Lys-OH **4** (1.13 g, 4.0 mmol) was dissolved in 0.2 M sodium borate buffer, pH 10.0 (200 ml), and benzyl maltol **6** (1.60 g, 7.4 mmol) in 10 ml of ethanol was added. The mixture was incubated at 70 °C for 24 h. After cooling, the pH value was adjusted to 7.0 and the solution was extracted with diethyl ether (3 × 100 ml), which was discarded. The aqueous phase was then acidified to pH 1.0 and extracted with ethyl acetate (3 × 100 ml). The same extraction was repeated at pH 2.0. The combined extracts were evaporated to dryness using a rotary evaporator. The crude product Z-Mal(Bzl)-OH **7** was dissolved in ethanol (100 ml) and hydrogenated in the presence of Pd/C (99.5 mg) by stirring under H_2 atmosphere at 20 °C and atmospheric pressure for 18 h. The catalyst was then filtered off and ethanol was removed *in vacuo*. The residue was dissolved in 0.1 N sodium citrate buffer, pH 3.0 (30 ml), and the pH adjusted to 3.0. The solution was applied to a column (1.5 × 48 cm) of strongly acidic cation exchange resin DOWEX 50 WX-8 (200–400 mesh; Acros, Geel, Belgium) previously equilibrated with 250 ml each of 6 N HCl, water, 1 N NaOH, water and 0.1 N sodium citrate buffer, pH 3.0. Maltosine **1** was eluted by gravity with 0.3 N sodium citrate buffer, pH 5.35, at a flow rate of 0.35 ml/min. Fractions of 10 ml were collected, and the presence of **1** was monitored by the spotting test and analytical HPLC as described earlier. Fractions containing **1** (generally between 200 and 350 ml) were combined and desalted as follows: The pH of the unified fractions was adjusted to 1.8 and the solution was loaded onto a column (2.5 × 15 cm) of DOWEX 50 WX-8 (200–400 mesh) equilibrated with 250 ml each of 6 N HCl and water. Citrate and sodium ions were eluted with water and 1 N HCl, respectively (each 250 ml) [20]. Maltosine was eluted with 4 N HCl (250 ml), and the eluate was evaporated *in vacuo* until the smell of hydrochloric acid had become imperceptible. Finally, the residue was lyophilized to give an amorphous light yellow powder of maltosine, which was stored at −20 °C.

Maltosine data: ESI-MS, $[M+H]^+$ *m/z* 255.1; ^{1}H NMR, δ [ppm]: 1.40 (m, 2H, Lys-H4); 1.78–1.91 (m, 4H, Lys-H3, Lys-H5); 2.49 (s, 3H, Mal-CH_3); 3.89 (t, 1H, J = 6.3 Hz, Lys-H2); 4.26 (dd, 2H, J = 6.3 Hz and 9.0 Hz, Lys-H6); 7.03 (d, 1H, J = 7.0 Hz, Mal-H5); 7.95 (d, 1H, J = 7.0 Hz, Mal-H6). Elemental analysis: $C_{12}H_{18}N_2O_4$ (MW = 254.28) requires C 56.68%, H 7.13%, N 11.02%, C/N = 5.14; found, C 41.98%, H 6.99%, N 8.23%, C/N = 5.10; content = 74.7%, based on nitrogen. Yield = 874.5 mg (63.9%).

2.2.5. Synthesis and isolation of 6-(3-hydroxy-4-oxo-2-methyl-4(1H)-pyridin-1-yl)-L-norleucyl-L-alanine (Mal-Ala)

Boc-Lys-OH **5** (2.7 g, 11.0 mmol) and benzyl maltol **6** (2.6 g, 12.0 mmol) were dissolved in 50 ml of 50% aqueous ethanol, and conc. sodium hydroxide was added until pH was 13.0. The solution was heated under reflux at 80 °C for 18 h. After cooling, the ethanol was evaporated under reduced pressure, and 25 ml of water was added. The pH value was adjusted to 7.0 and the solution was extracted with diethyl ether (3 × 50 ml). The aqueous phase was acidified until turbidity (pH = 5–6) and extracted with ethyl acetate (3 × 50 ml). Acidification and extraction were repeated until the appearance of the turbidity had ceased (2–3 times). The organic phases were combined and evaporated to dryness. The residue was purified by flash column chromatography (FC) on 50 g of silica gel (particle size 0.063–0.200 mm; Merck, Darmstadt, Germany) using methanol as the eluent. Fractions of 10 ml were collected. Spotting of the fractions revealed that Boc-Mal(Bzl)-OH **8** eluted between 60 and 180 ml. The combined fractions were filtered and evaporated to near dryness. The residue crystallized from methanol and the crystals were dried *in vacuo* yielding 2.58 g of Boc-Mal(Bzl)-OH **8**.

A solution of **8** (750 mg, 1.7 mmol), TSTU (610.5 mg, 2.0 mmol) and DIPEA (862.5 μl, 5.1 mmol) in 10 ml of dichloromethane (DCM) was stirred for 30 min at 20 °C. Then, H-Ala-OBu^t × HCl (460 mg, 2.5 mmol) and DIPEA (426 μl, 2.5 mmol) were added and the solution was stirred for 30 min. DCM was distilled off

and the residue transferred to a separatory funnel with diethyl ether (50 ml). The organic phase was extracted with 1 N HCl (2 × 50 ml), 5% $NaHCO_3$ (2 × 50 ml) and water (2 × 50 ml), dried (Na_2SO_4), filtered, evaporated to dryness and purified by FC on 50 g of silica gel using a mixture of ethyl acetate and methanol (9/1, v/v) as the eluent. Fractions between 300 and 570 ml showing an R_f value of 0.15 on TLC using the same solvent were combined, filtered and evaporated to dryness. Remaining Boc-Mal(Bzl)-Ala-OBu^t **9** was taken up in methanol (40 ml) and hydrogenated in the presence of Pd/C (100 mg) as described earlier. The catalyst was removed by filtration, methanol was evaporated *in vacuo* and the residue was dissolved in a mixture of tetrahydrofuran and 6 N HCl (40 ml, 1/1, v/v) and stirred for 1 h at 20 °C [21]. After the evaporation of the solvents, the residue was taken up in 0.1 N sodium citrate buffer, pH 3.0 (40 ml), and the pH adjusted to 3.0. Ion-exchange chromatography (IEC) was performed as described for **1**, except that 0.3 N sodium citrate buffer, pH 5.25, was used for the elution. Mal-Ala **3** usually eluted between 200 and 450 ml of the elution buffer. The combined fractions were processed as described previously.

Mal-Ala data: ESI-MS, $[M + H]^+$ *m/z* 326.2; ^{1}H NMR, δ [ppm]: 1.30 (d, 3H, *J* = 7.3 Hz, Ala-CH_3); 1.36 (m, 2H, Lys-H4); 1.74–1.85 (m, 4H, Lys-H3, Lys-H5); 2.48 (s, 3H, Mal-CH_3); 3.86 (dd, 1H, *J* = 5.9 Hz and 7.5 Hz, Lys-H2) 4.26 (m, 3H, Ala-H2, Lys-H6); 7.01 (d, 1H, *J* = 7.0 Hz, Mal-H5); 7.92 (d, 1H, *J* = 7.0 Hz, Mal-H6). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36) requires C 55.37%, H 7.13%, N 12.91%, C/N = 4.29; found, C 41.05%, H 6.44%, N 9.53%, C/N = 4.31; content = 73.8%, based on nitrogen. Yield = 175.3 mg (12.9%).

2.2.6. Synthesis and isolation of L-alanyl-6-(3-hydroxy-4-oxo-2-methyl-4(1H)-pyridin-1-yl)-L-norleucine (Ala-Mal)

Boc-Mal(Bzl)-OH **8** (978.4 mg, 2.2 mmol) was added to 1000 ml of 10% aqueous acetic acid and stirred for 4 h at 70 °C. TLC (methanol) revealed that the starting material had disappeared. Acetic acid was removed *in vacuo* and the residue was purified by FC on 100 g of silica gel using methanol as the eluent. The fractions between 260 and 750 ml were combined, filtered and evaporated to dryness yielding 720 mg of white H-Mal(Bzl)-OH **10**.

H-Mal(Bzl)-OH data: ESI-MS, $[M + H]^+$ *m/z* 345.2; ^{1}H NMR, δ [ppm]: 1.25 (m, 2H, Lys-H4); 1.56/1.75 (2 m, 4H, Lys-H3, Lys-H5); 1.96 (s, 3H, Mal-CH_3); 3.57 (t, 1H, *J* = 6.2 Hz, Lys-H2); 3.87 (dd, 2H, *J* = 6.8 Hz and 8.2 Hz, Lys-H6); 4.93 (s, 2H, O-CH_2-Ph); 6.46 (d, 1H, *J* = 7.4 Hz, Mal-H5); 7.30 (m, 5H, Phenyl-H); 7.61 (d, 1H, *J* = 7.4 Hz, Mal-H6).

Seven hundred and twenty milligrams of **10** was dissolved in DCM (20 ml) and DIPEA (1.068 ml, 6.3 mmol). After the addition of Boc-Ala-OSu (897.5 mg, 3.1 mmol), the turbid solution was stirred overnight. DCM was distilled off and the residue taken up in ethyl acetate (100 ml). The suspension was extracted with 1 N HCl (2 × 50 ml). The pH value of the combined aqueous phases was adjusted to 1.0 and the solution was extracted with ethyl acetate (2 × 100 ml). All organic phases were combined and evaporated to dryness. The residue was purified by FC on 50 g of silica gel using a mixture of ethyl acetate and methanol (1/1, v/v) as the eluent. Fractions showing an R_f value of 0.37 on TLC using the same solvent (90–300 ml) were combined, filtered and evaporated to dryness. Removal of the protecting groups from the intermediate product, Boc-Ala-Mal(Bzl)-OH **11**, and IEC were performed exactly as described for **3**. Ala-Mal **2** usually eluted between 200 and 450 ml of the elution buffer.

Ala-Mal data: ESI-MS, $[M + H]^+$ *m/z* 326.2; ^{1}H NMR, δ [ppm]: 1.34 (m, 2H, Lys-H4); 1.43 (d, 3H, *J* = 7.3 Hz, Ala-CH_3); 1.67–1.82 (m, 4H, Lys-H3, Lys-H5); 2.48 (s, 3H, Mal-CH_3); 3.99 (q, 1H, *J* = 7.1 Hz, Ala-CH); 4,23 (m, 3H, Lys-H2, Lys-H6); 6.99 (d, 1H, *J* = 6.8 Hz, Mal-H5); 7.93 (d, 1H, *J* = 6.2 Hz, Mal-H6). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36) requires C 55.37%, H 7.13%, N 12.91%, C/N = 4.29; found, C 41.23%, H 6.20%, N 9.67%, C/N = 4.31; content = 74.9%, based on nitrogen. Yield = 108.3 mg (6.2%).

2.2.7. Cell culture

Caco-2 cells were routinely cultured in 75-cm^2 culture flasks with minimum essential medium supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml) and amino acid solution (1%) at 37 °C in a humidified atmosphere containing 5% CO_2 [18,22]. Cells at 80% confluence were released by trypsinization. For the uptake experiments, Caco-2 cells were seeded in 35-mm disposable Petri dishes (Sarstedt, Nümbrecht, Germany) at a density of 0.8 × 10^6 cells per dish. The uptake measurements were performed on the seventh day after seeding. Protein content per dish was determined with Pierce® 660 nm Protein Assay (Thermo Fisher Scientific, Schwerte, Germany). For the flux experiments, Caco-2 cells were cultured on permeable polycarbonate Transwell® cell culture inserts (diameter 24 mm, pore size 3 μm, Costar GmbH, Bodenheim, Germany) with a cell density of 0.2 × 10^6 cells/filter for 21 days [18]. The lower (receiver) compartment contained 2.6 ml medium and the upper (donor) compartment 1.5 ml medium. The transepithelial electrical resistance was measured at day 21 using a Millicell ERS (Millipore Intertech, Bedford, USA).

OK cells were cultured in Dulbecco's modified Eagle's medium (D-MEM)/F12 nutrient mixture (1:1, v/v) supplemented with 10% fetal bovine serum, penicillin–streptomycin (1%) and glutamine (1%). Cells were seeded in 35-mm disposable Petri dishes at a density of 0.8 × 10^6 cells per dish. The uptake measurements were performed on the seventh day after seeding. OK cells were also cultured in Transwell® chambers (diameter 24 mm, pore size 0.4 μm, Costar GmbH, Bodenheim, Germany) with a seeding cell density of 0.4 × 10^6 cells/filter and a culture period of 21 days [18].

Culture medium for HeLa cells was D-MEM (with Glutamax; 4500 mg/l glucose) supplemented with 10% fetal bovine serum [23]. For uptake experiments, cells were seeded in 24-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of 0.75 × 10^6 cells per well [23].

2.2.8. Transport experiments

Uptake of [^{14}C]Gly-Sar in Caco-2 cells cultured on plastic dishes was measured at room temperature as described earlier [18,22]. The uptake buffer 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 unlabeled compounds at increasing concentrations (0–10 mM). After incubation for 10 min, the cells were quickly washed four times, dissolved and prepared for liquid scintillation spectrometry. The non-saturable component of [^{14}C]Gly-Sar uptake (diffusion, adherent radioactivity) determined by measuring the uptake of [^{14}C]Gly-Sar in the presence of 50 mM unlabeled Gly-Sar represented 8.4% of the total uptake. This value was taken into account during non-linear regression analysis of inhibition constants.

Uptake of L-[^{3}H]lysine in Caco-2 and OK cells cultured on plastic dishes was measured in the absence or presence of unlabeled compounds for 5 min. The non-saturable component of L-[^{3}H]lysine uptake determined by measuring the uptake of L-[^{3}H]lysine in the presence of 20 mM unlabeled L-lysine represented 21% (Caco-2) and 8% (OK) of the total uptake [18].

Transepithelial flux of maltosine, Ala-Mal, Mal-Ala, [^{14}C]Gly-Sar or [^{14}C]mannitol across Caco-2 cell monolayers and the flux of maltosine and [^{14}C]mannitol across OK cell monolayers was measured as described previously [18,23] at day 21 after seeding at 37 °C in a shaking table incubator. After washing the inserts with buffer (25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose), uptake was started

by adding uptake buffer (pH 6.0, 1.5 ml) containing the test compound (1 mM) to the donor side. After 10, 30, 60 and 120 min, 200 μl samples were taken from the receiver compartment and replaced with fresh buffer (pH 7.5). Samples were stored until analysis by HPLC measurements. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic insert, placed in 1 ml 10% TCA solution and frozen.

2.2.9. Heterologous expression of hPEPT1 in HeLa cells and uptake measurements

hPEPT1 was heterologously expressed in HeLa cells using the pcDNA3-hPEPT1 cDNA construct (1 μg/well) and Turbofect (1.5 μl/well; Fermentas, St. Leon-Rot, Germany) according to manufacturers' protocols [23]. The transfection was done 1 h post-seeding in 24-well plates. The uptake of 20 μM [^{14}C]Gly-Sar was measured in the absence or presence of Gly-Sar, maltosine, Ala-Mal or Mal-Ala (0–10 mM) 20–24 h post-transfection. The uptake buffer contained 25 mM Mes/Tris (pH 6.0), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose [23]. After incubation for 10 min, the monolayers were washed, solubilized and prepared for liquid scintillation spectrometry. The non-saturable component of [^{14}C]Gly-Sar uptake represented 3.7% of the total uptake [23].

In another type of experiment, HeLa cells transfected with the empty vector (control) or pcDNA3-hPEPT1, respectively, were incubated with unlabeled Ala-Mal or Mal-Ala (1 mM) for 30 min at pH 6.0 and 37 °C. After incubation, the cells were quickly washed four times with ice-cold uptake buffer and 500 μl aqua bidest was added. After freezing and thawing the cell layers three times, the cell suspension was transferred to 1.5-ml reaction tubes, homogenized with a 25-gauge needle and centrifuged in a tabletop centrifuge at 13,000 rpm for 45 min. After second centrifugation, the supernatants were transferred to HPLC vials [23].

2.2.10. Xenopus laevis oocytes expressing hPEPT1 and electrophysiology

hPEPT1-cRNA was synthesized and purified as described previously [24]. Oocytes were surgically removed from the *X. laevis* frogs and prepared as described by Riedel and co-workers [25]. Then, 23 nl hPEPT1-cRNA solution (1.1 μg/μl) or water were injected per oocyte. Oocytes were maintained at 19 °C in modified Barth's medium (5 mM HEPES/NaOH (pH 7.4), 84 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 1 mM $CaCl_2$, 10,000 U/ml penicillin and 10 mg/ml streptomycin). Four days post-injection, the electrophysiological measurements were performed. Oocytes were placed in a flow-through chamber and continuously superfused (75 μl/s) with oocyte Ringer (ORi) buffer (10 mM Mes/Tris (pH 6.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM KCl) in the absence or presence of control dipeptides (Gly-Sar, Ala-Lys, Lys-Ala) and test compounds (maltosine, Ala-Mal and Mal-Ala), respectively [24,25]. Oocytes were voltage clamped at a membrane potential of −60 mV.

2.2.11. Data analysis

Experiments were done in duplicate or triplicate, and each experiment was repeated two to three times. Results are given as mean ± SE. The concentrations of the unlabeled compound necessary to inhibit 50% of the carrier-mediated uptake of [^{14}C]Gly-Sar or L-[^{3}H]lysine (IC_{50} values) were determined by non-linear regression using the logistical equation for an asymmetric sigmoid (allosteric Hill kinetics, SigmaPlot program, Systat, Erkrath, Germany). They were converted into inhibitory constants (K_i values) according to the formula $K_i = IC_{50}/(1 + [S]/K_t)$, where [S] is the tracer concentration of [^{14}C]Gly-Sar (10 or 20 μM) or L-[^{3}H]lysine (2 nM), respectively, and K_t is the Michaelis constant of Gly-Sar or L-lysine uptake, respectively.

Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver compartment *vs.* time.

Oocyte data were analyzed using the Superpatch 2000 program (Julius-Bernstein-Institute of Physiology, SP-Analyzer by T. Böhm, Halle, Germany). Statistical values of oocyte experiments are expressed as mean ± SE from measurements of 5–9 oocytes each from two batches of oocyte preparation.

3. Results and discussion

3.1. Synthesis and analysis of maltosine and the respective dipeptides

The synthesis of maltosine as the free amino acid was based on a standard technique for the synthesis of N-substituted 3-hydroxy-4-pyridinone derivatives [26]. Lysine as a polyfunctional amino acid was utilized as the N-α-Z-protected derivative **4** (Fig. 1), which resulted in high extraction yields during synthesis and allowed the deprotection of both protecting groups in one step. For the syntheses of dipeptide-bound maltosine derivatives, we initially sought to start from the unmodified dipeptides (e.g., Boc-Ala-Lys-OH) as performed similarly in earlier works [18], but as the yields were quite low, we developed the multistep syntheses depicted in Fig. 2. For the synthesis of Mal-Ala, Boc-Mal(Bzl)-OH was activated *in situ* to the succinimide active ester and coupled directly with the C-protected C-terminal amino acid (step ii in Fig. 2) [27]. This procedure can be employed in the syntheses of further dipeptides beyond the one presented here. Short-chain peptides with maltosine in positions other than the N-terminus can be synthesized by the same methods using H-Mal(Bzl)-OH as a building block as performed for Ala-Mal. Ion-exchange chromatography was applied for the purification of the compounds to be applied to cells yielding the substances as their hydrochlorides in high purity as revealed by the C/N quotient. Due to structural similarity to furosine, a previously published ion-exchange system [20] could easily be transferred to maltosine and its derivatives. The metal chelating properties of 3-hydroxy-4-pyridinones required the application during all synthesis steps of ultra-pure water purified by an ion-exchange resin. Moreover, stainless steel columns could not be utilized for HPLC analysis because of remarkable peak broadening. Therefore, a polymer-based column was used, which also permitted the separation of maltosine from both peptides investigated.

3.2. Transepithelial transport across Caco-2 and OK cell monolayers

To measure the total net transepithelial flux of maltosine and its dipeptide derivatives Ala-Mal and Mal-Ala across intestinal epithelial cells, Caco-2 cells were cultured on permeable filter membranes for 21 days. Compounds (1 mM) were added to the apical side and samples were taken after 10, 30, 60 and 120 min for HPLC analysis. When maltosine was added in free form to the apical side, a transepithelial flux rate even lower than that of the space marker [^{14}C]mannitol was observed (0.02 ± 0.01%/cm^2 × h *vs.* 0.13 ± 0.03%/cm^2 × h, Fig. 3). Within the cells, only 0.07% of the maltosine amount added to the apical side was found after 2 h (Fig. 3, inset). We then added the maltosine containing dipeptides to the apical side of cells. Neither Ala-Mal nor Mal-Ala could be detected in the intracellular and basolateral compartments. However, analyzing the samples for free maltosine, much higher amounts were measured in both compartments compared to the experiments in which free maltosine had been added to the cells. For Ala-Mal, a maltosine flux of 0.27 ± 0.08%/cm^2 × h was determined. In the case of Mal-Ala, the flux rate of maltosine was 0.16 ± 0.06%/cm^2 × h.

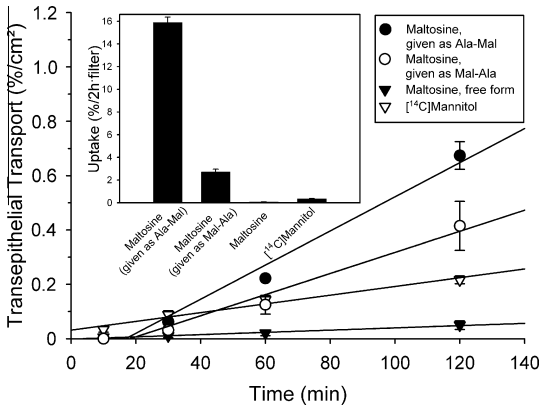

Fig. 3. Transepithelial flux of maltosine, Ala-Mal, Mal-Ala (all 1 mM) and [^{14}C]mannitol (10 μM) across Caco-2 cell monolayers. Flux was determined at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Inset: Uptake of the compounds into the cells grown on filter membranes within 2 h. $n = 3$.

Also within the cells, free maltosine – but not Ala-Mal and Mal-Ala – was found after 2 h (15.9% and 2.7%, respectively, Fig. 3, inset).

These data show that the transepithelial flux of maltosine is 14-fold and 8-fold higher when maltosine is added as Ala-Mal or Mal-Ala, respectively, compared to maltosine in free form. Intracellular Ala-Mal or Mal-Ala taken up from the incubation buffer is almost completely hydrolyzed to maltosine and alanine by enzymes. Free maltosine then permeates the basolateral cell membrane by simple diffusion down its concentration gradient and possibly by the action of basolateral amino acid transporters.

Considering these flux experiments as a model for *in vivo* absorption, we speculate that under physiological conditions only free maltosine would appear in the blood. To test whether free maltosine could be reabsorbed at the renal epithelium, we studied the transepithelial maltosine transport across monolayers of a renal cell line, the OK cells. Maltosine (1 mM) was transported across the cells from the apical to the basolateral side at a flux rate of $0.97 \pm 0.19\%/cm^2 \times h$. Again, this flux rate was lower than that of the space marker [^{14}C]mannitol at these cells ($1.19 \pm 0.08\%/cm^2 \times h$). Only $0.44 \pm 0.02\%$ of maltosine was found inside the cell monolayers after 2 h of incubation. The data allow the speculation that the reabsorption of maltosine at renal cells is very low and that under physiological conditions maltosine would mainly be excreted *via* the urine. This hypothesis has to be tested *in vivo*.

3.3. Interaction with peptide and cationic amino acid transporter(s)

Based on the chemical structure (Figs. 1 and 2), it can be hypothesized that maltosine might be a substrate for intestinal amino acid transporters for cationic amino acids. Ala-Mal and Mal-Ala, on the other hand, might represent substrates for the intestinal proton-coupled transporter for di- and tripeptides, PEPT1. In competition assays using the enzymatically stable and radiolabeled dipeptide [^{14}C]Gly-Sar as substrate for PEPT1 and L-[^{3}H]lysine as prototype substrate for cationic amino acid transporters, we tested whether these compounds interact with the respective membrane transporters. From the curves shown in Figs. 4A and 5, IC_{50} values for maltosine, Ala-Mal, Mal-Ala and reference substrates were calculated and converted into K_i values. Ala-Mal and Mal-Ala inhibited the [^{14}C]Gly-Sar uptake into Caco-2 cells with K_i values of 0.73 ± 0.05 mM and 0.25 ± 0.02 mM, respectively (Fig. 4A and Table 1). Maltosine showed only slight inhibition of the [^{14}C]Gly-Sar uptake (Fig. 4A) but inhibited the L-[^{3}H]lysine transport with a K_i value of 3.5 ± 0.4 mM (Table 2 and Fig. 5). Neither Ala-Mal nor Mal-Ala inhibited the uptake of L-[^{3}H]lysine in significant amounts (Fig. 5).

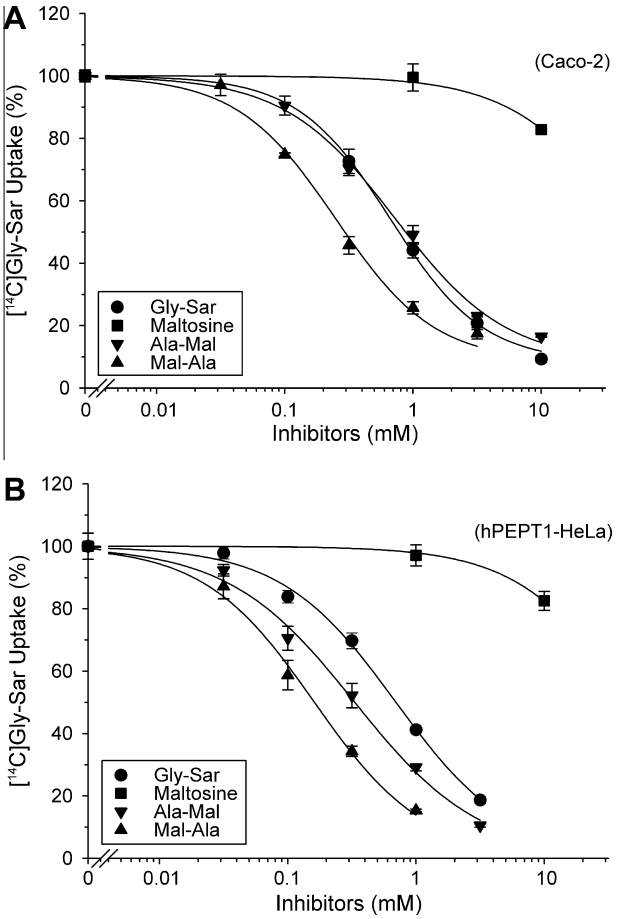

Fig. 4. Inhibition of [^{14}C]Gly-Sar uptake into Caco-2 and hPEPT1-HeLa cells. (A) Uptake of 10 μM [^{14}C]Gly-Sar into Caco-2 cells and (B) uptake of 20 μM [^{14}C]Gly-Sar into HeLa cells was measured for 10 min at pH 6.0 in the absence (control) or presence of increasing concentrations of maltosine, Ala-Mal, Mal-Ala or Gly-Sar. $n = 3–4$.

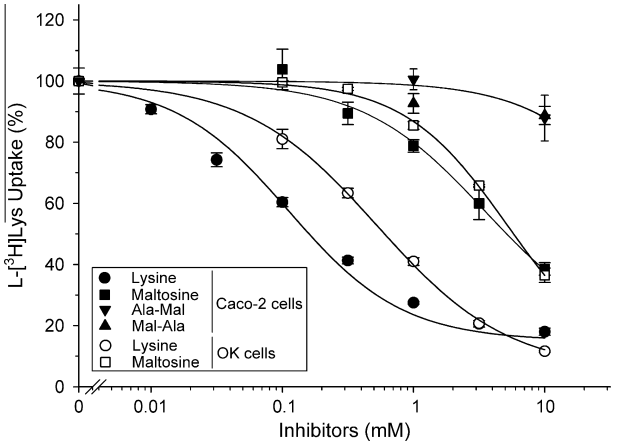

Fig. 5. Inhibition of L-[^{3}H]lysine uptake into Caco-2 and OK cells. Uptake of 2 nM L-[^{3}H]lysine was measured for 5 min at pH 6.0 in the absence (control) or presence of increasing concentrations of maltosine, Ala-Mal, Mal-Ala and L-lysine. $n = 3–4$.

According to our classification [28], Ala-Mal and Mal-Ala can be considered as medium- and high-affinity ligands for hPEPT1, whereas maltosine showed no affinity. The side-chain modification (lysine to maltosine in dipeptides) has only minor consequences for the binding affinity to hPEPT1 since the K_i values of Ala-Mal and Mal-Ala are comparable to those of Ala-Lys and Lys-Ala (K_i:

Table 1
Inhibition constants (K_i) of Gly-Sar, maltosine, Ala-Mal and Mal-Ala at Caco-2 cells and hPEPT1-transfected HeLa cells. Uptake of [^{14}C]Gly-Sar (10 μM at Caco-2 and 20 μM at HeLa cells) was measured at pH 6.0 for 10 min at increasing concentrations of unlabeled maltosine and its dipeptide derivatives. K_i values were derived from the competition curves shown in Fig. 4. n = 3–4.

Compound	K_i (mM)	
	hPEPT1 Caco-2	hPEPT1 HeLa
Gly-Sar	0.74 ± 0.01	0.64 ± 0.02
Maltosine	>10	>10
Ala-Mal	0.73 ± 0.05	0.33 ± 0.03
Mal-Ala	0.25 ± 0.02	0.16 ± 0.01

Table 2
Inhibition constants (K_i) of L-lysine, maltosine, Ala-Mal and Mal-Ala at Caco-2 and OK cells. Uptake of 2 nM L-[^{3}H]lysine was measured at pH 6.0 for 5 min at increasing concentrations of unlabeled lysine, maltosine and its dipeptide derivatives. K_i values were derived from the competition curves shown in Fig. 5. n = 3–4.

Compound	K_i (mM)	
	Caco-2	OK
L-Lysine	0.11 ± 0.01	0.51 ± 0.09
Maltosine	3.5 ± 0.4	5.3 ± 0.2
Ala-Mal	>10	n.d.
Mal-Ala	>10	n.d.

n.d., not determined.

Ala-Lys 0.21 ± 0.02 mM, Lys-Ala 0.34 ± 0.02 mM, [29]). For comparison, the unlabeled reference substrate Gly-Sar represents a medium-affinity substrate for hPEPT1 (K_i = 0.74 ± 0.01 mM). The apparent K_i value for inhibition of L-[^{3}H]lysine uptake by L-lysine itself is 0.11 ± 0.01 mM and thereby much lower than that of maltosine (Table 2).

At OK cells, maltosine inhibited the L-[^{3}H]lysine uptake with a K_i value of 5.3 ± 0.2 mM. For comparison, a K_i value of 0.51 ± 0.09 mM was calculated for L-lysine (Table 2 and Fig. 5). Hence, although maltosine interacts with the transporter(s) for cationic amino acids at Caco-2 and OK cells, the affinity to the system(s) is very low. Taken together with the result that at both cell lines, the transepithelial flux of free maltosine is much lower than that of the space marker mannitol, it can be concluded that flux of free maltosine is negligible.

3.4. Interaction with hPEPT1 heterologously expressed in HeLa cells

HeLa cells were transfected with the empty vector pcDNA3 (as control) and with pcDNA3-hPEPT1. Ala-Mal and Mal-Ala inhibited the transport of the reference peptide [^{14}C]Gly-Sar into hPEPT1-transfected HeLa cells with K_i values of 0.33 ± 0.03 mM and 0.16 ± 0.01 mM, respectively (Fig. 4B and Table 1). These results confirm the direct interaction of Ala-Mal and Mal-Ala with the human intestinal peptide transporter hPEPT1.

3.5. Electrophysiological measurements at Xenopus laevis oocytes expressing hPEPT1

The results described so far demonstrate (i) the specific interaction of the maltosine dipeptides with the intestinal hPEPT1 and (ii) a significant transport rate of maltosine dipeptides across Caco-2 cell monolayers. However, the above data do not conclusively demonstrate that the maltosine dipeptides are actually transported by hPEPT1. Therefore, the two-electrode voltage-clamp technique at *X. laevis* oocytes expressing hPEPT1 was employed. Gly-Sar was used as control substrate. At a concentration of 5 mM, Ala-Mal and Mal-Ala generated significant inward directed currents of 159 ± 6 nA and 205 ± 6 nA, respectively (Fig. 6). Gly-Sar (5 mM and 10 mM) induced inward currents of 539 ± 37 nA and 644 ± 15 nA, respectively. Ala-Lys and Lys-Ala (5 mM) generated currents of 625 ± 29 nA and 962 ± 50 nA, respectively. Neither glycine nor free maltosine generated inward directed currents. None of the compounds generated currents at water-injected oocytes (Fig. 6).

These data show that Ala-Mal and Mal-Ala are indeed transported by hPEPT1. The currents were lower than those generated by Gly-Sar and other prototype dipeptides but they are significant [19,22]. The moderate currents are most likely caused by the hydrophobic modifications of the side chains (lysine to maltosine) [19,29,30]. A positive correlation between affinity and inward current reflecting actual substrate translocation could not have been expected: The reduced transport rate of Ala-Mal and Mal-Ala compared to the unmodified dipeptides is very consistent with the results obtained for side-chain modified Lys-Ala and Ala-Lys derivatives [29]. Inward currents elicited by these substances were strongly reduced when compared to the unmodified peptide. We assume that space-demanding hydrophobic peptide side chains can impede the actual transport, i.e. the conformational change of loaded hPEPT1. It appears that binding of the substrate is not the rate-limiting step for hPEPT1-mediated translocations. This view is strongly supported by the fact that the competitive hPEPT1 inhibitors developed previously by side-chain modification of dipeptides even though they display affinities much higher than those of PEPT1 substrates with K_i values as low as 2 μM do not elicit any currents [19,29].

3.6. Accumulation in hPEPT1-HeLa cells

In a second approach to investigate the translocation of maltosine dipeptides across cell membranes, transfected HeLa cells were incubated for 30 min with 1 mM Ala-Mal and Mal-Ala, respectively. Intracellular contents of the compounds were analyzed by HPLC. Neither Ala-Mal nor Mal-Ala but very high amounts of free maltosine were found within the cells. The uptake was 10- and 16-fold higher in hPEPT1-transfected HeLa cells than in mock cells transfected with the empty vector (Fig. 7).

We conclude from these data that Ala-Mal and Mal-Ala are transported by hPEPT1 into intestinal cells. Inside the cells, the dipeptides are hydrolyzed by intracellular dipeptidases to free maltosine and alanine. Permeation of free maltosine into Caco-2 and OK cells is low and probably not mediated by transport proteins. From the viewpoint of nutritional physiology, it can be concluded that maltosine, a processing-induced food constituent that

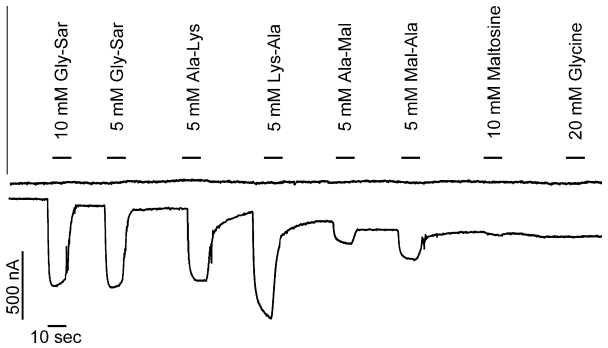

Fig. 6. hPEPT1-dependent currents at *Xenopus laevis* oocytes. Currents induced by Ala-Mal, Mal-Ala, maltosine and the reference compounds Gly-Sar, Ala-Lys, Lys-Ala and glycine in water- (upper trace) and hPEPT1-cRNA-injected oocytes (lower trace). Currents were recorded at pH 6.5 by two-electrode voltage clamp at a membrane potential of −60 mV.

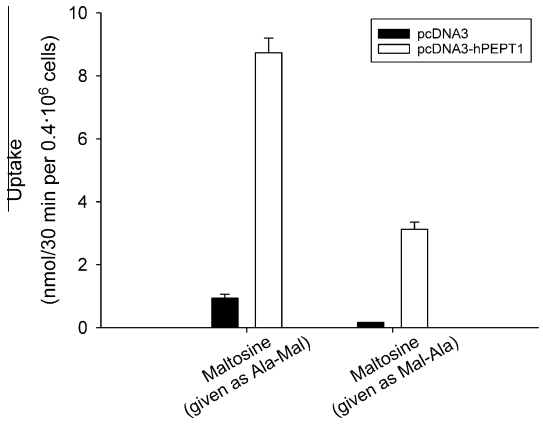

Fig. 7. Uptake of Ala-Mal and Mal-Ala (1 mM) into HeLa cells transfected with pcDNA3 or pcDNA3-hPEPT1. Uptake was measured for 30 min at pH 6.0 and at 37 °C. The cell contents were analyzed by RP-HPLC. $n = 5$.

is able to form complexes with iron and other metals like zinc, is absorbed from the diet when bound in dipeptides. More importantly, peptide-bound 3-hydroxy-4-pyridinones might serve as powerful prodrugs for the treatment of iron storage diseases combining very efficient metal-complexing properties of 3-hydroxy-4-pyridinones with the oral availability in dipeptide form.

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