Aus dem Institut für Agrar- und Ernährungswissenschaften
(Geschäftsführender Direktor: Prof. Dr. R. Jahn)

der Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. P. Wycisk)

der Martin-Luther-Universität Halle-Wittenberg

Transport freier und Dipeptid-gebundener Maillard-Reaktionsprodukte über die Epithelien von Darm und Niere

Dissertation

zur Erlangung des akademischen Grades
Doktor der Trophologie (Dr. troph.)

vorgelegt von

Diplom-Ernährungswissenschaftlerin Stefanie Geißler

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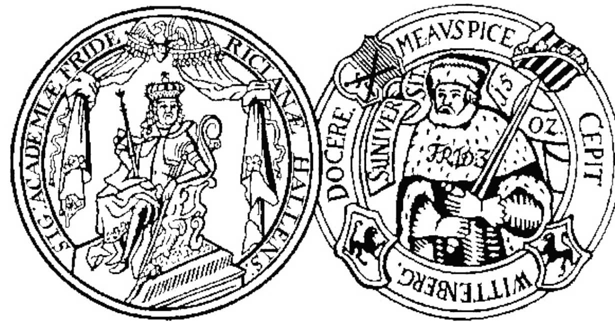

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Inhaltsverzeichnis

Abkürzungsverzeichnis .... **ii**

Tabellen- und Abbildungsverzeichnis .... **iii**

1 Einleitung **1**

1.1 Die Maillard-Reaktion und ihre Produkte 1

1.2 Die Protonen-abhängigen Peptidtransporter 6

1.3 Die Transportsysteme für kationische Aminosäuren 9

2 Zielstellung **12**

3 Übersicht über eingesetzte Methoden **14**

4 Originalarbeiten **16**

Studie 1: *Transport of free and peptide-bound glycated amino acids: synthesis, transepithelial flux at Caco-2 cell monolayers, and interaction with apical membrane transport proteins.* 17

Studie 2: *Transport of free and peptide-bound pyrraline at intestinal and renal epithelial cells.* 27

Studie 3: *Transport of the advanced glycation end products alanylpyrraline and pyrralylalanine by the human proton-coupled peptide transporter hPEPT1.* 34

Studie 4: *Synthesis and intestinal transport of the iron chelator maltosine in free and dipeptide form.* 39

5 Diskussion **47**

5.1 Der intestinale transepitheliale Transport 47

5.2 Die Interaktion mit intestinalen apikalen Transportsystemen 50

5.3 Die Interaktion mit renalen Transportsystemen und der renale transepitheliale Transport 56

5.4 Die ernährungsphysiologische und pharmazeutische Relevanz der Ergebnisse 57

6 Zusammenfassung **60**

7 Summary **62**

8 Literatur **64**

Abkürzungsverzeichnis

AGE	*advanced glycation end product*
Ala	Alanin, Alanyl-
Apy	Argpyrimidin, Argpyrimidyl-
$ATB^{0,+}$	Aminosäure-Transporter $B^{0,+}$
ATP	Adenosintriphosphat
CAA	kationische Aminosäuren
Caco-2	Zelllinie eines humanen Colonkarzinoms
CAT	kationischer Aminosäure-Transporter
CEL	Carboxyethyllysin, Carboxyethyllysyl-
CML	Carboxymethyllysin, Carboxymethyllysyl-
Fom	Formylin, Formylinyl-
FL	Fruktoselysin
Gly-Sar	Glycylsarkosin
HeLa	Zelllinie eines humanen Cervixkarzinoms
PEPT1/2	Protonen-abhängiger Peptidtransporter 1/2
HPLC	*High performance liquid chromatography*, Hochleistungsflüssigchromatographie
K_i	inhibitorische Konstante
Lys	Lysin, Lysyl-
Mal	Maltosin, Maltosinyl-
MG-H1	*methylglyoxal-derived hydroimidazolone 1*
MRP	Maillard-Reaktionsprodukt
NAA	neutrale Aminosäuren
OK	Zelllinie des proximalen Nierentubulus des Opossums
Pyrr	Pyrralin, Pyrralyl-
SKPT	Zelllinie des proximalen Nierentubulus einer spontan-hypertensiven Ratte
SLC	*solute carrier*
Spezies	h ≙ Mensch
TMD	Transmembrandomäne

Tabellen- und Abbildungsverzeichnis

Tabellen		Seite
Tab. 1.1	Vorkommen und Gehalt einiger Maillard-Reaktionsprodukte in Lebensmitteln	3
Tab. 1.2	Transportsysteme für kationische Aminosäuren	10
Tab. 5.1	Klassifizierung der freien und Dipeptid-gebundenen MRPs nach ihrer Affinität zum hPEPT1-Protein	51

Abbildungen		
Abb. 1.1	Strukturen der in der vorliegenden Arbeit untersuchten Maillard-Reaktionsprodukte	1
Abb. 3.1	Querschnitt durch eine Transwell®-Kammer: eine semipermeable Membran bewachsen mit einem Zellmonolayer	14
Abb. 5.1	Schematische Darstellung des Transportes freier und Dipeptid-gebundener Maillard-Reaktionsprodukte an intestinalen Epithelzellen	55

1 Einleitung

In Lebensmitteln kommt es im Verlauf von Erhitzungsprozessen wie Backen, Rösten, Grillen oder Braten, aber auch während der Lagerung zu einer nicht-enzymatischen Reaktion zwischen Aminosäuren und reduzierenden Zuckern, der sogenannten Maillard-Reaktion oder Glykierung. Es entstehen erwünschte Aroma- und Farbverbindungen (braune Pigmente), welche zur Schmackhaftigkeit der Lebensmittel beitragen. Diese Reaktion kann sich aber auch ernährungsphysiologisch nachteilig auf Lebensmittel auswirken (LEDL und SCHLEICHER 1990).

1.1 Die Maillard-Reaktion und ihre Produkte

Der französische Biochemiker LOUIS-CAMILLE MAILLARD beschrieb 1912 erstmals die nach ihm benannte Reaktion zwischen Aminen und reduzierenden Zuckern unter Bildung von Bräunungsprodukten, den sogenannten Melanoidinen, und flüchtigen, aromawirksamen Verbindungen (MAILLARD 1912). Diese nicht-enzymatischen Bräunungsprozesse sind komplexe, teils aufeinanderfolgende und teils parallel ablaufende Reaktionen mit einer Vielzahl an daraus hervorgehenden Maillard-Reaktionsprodukten (MRPs; Abbildung 1.1; Übersichten zur Maillard-Reaktion in: HODGE 1953, LEDL und SCHLEICHER 1990, FRIEDMANN 1996, HENLE 2005).

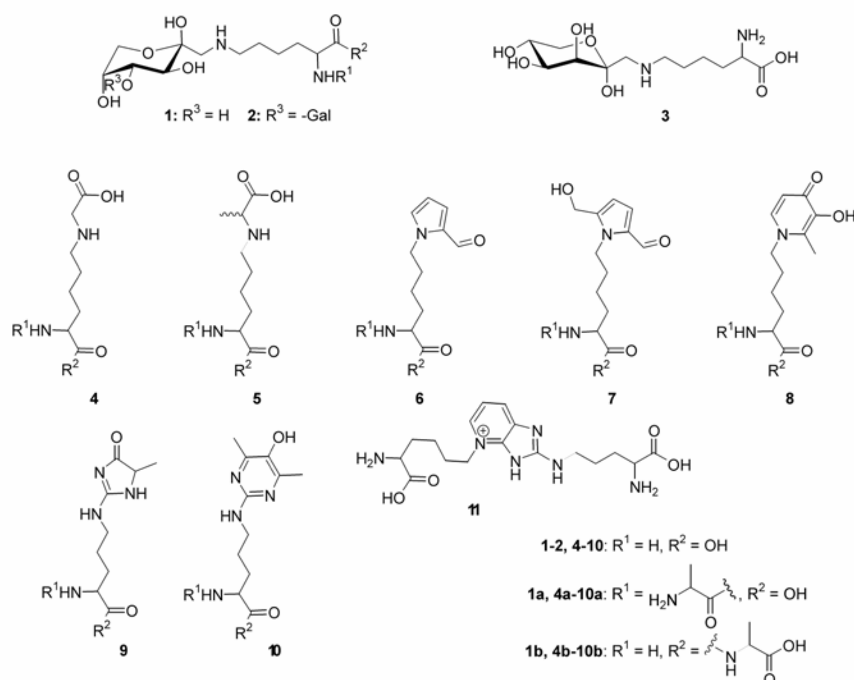

Abbildung 1.1. Strukturen der in der vorliegenden Arbeit untersuchten Maillard-Reaktionsprodukte (MRPs). Fruktoselysin (1), Laktuloselysin (2), Tagatoselysin (3), CML (4), CEL (5), Formylin (6), Pyrralin (7), Maltosin (8), MG-H1 (9), Argpyrimidin (10) und Pentosidin (11). a, Ala-MRP Dipeptid-Derivate; b, MRP-Ala Dipeptid-Derivate. (aus HELLWIG *et al.* 2011)

HODGE (1953) teilte die Maillard-Reaktion in die frühe, fortgeschrittene und späte Phase ein: In der **frühen Phase** reagiert die Aminogruppe von Aminosäuren (primär die ε-Aminogruppe von Lysin), Peptiden oder Proteinen als nukleophile Verbindung leicht mit der Carbonylfunktion reduzierender Zucker wie Glukose, Laktose und Galaktose, zu N-Glykosylaminen. Durch Eliminierungs- und Umlagerungsreaktionen entstehen über die Bildung einer instabilen *Schiff'schen* Base (Imin) die ersten stabilen Zwischenprodukte der Maillard-Reaktion, die Amadori-Produkte, entsprechend N^{ε}-(1-Desoxy-1-Fruktosyl)-lysin (Fruktoselysin; FL), N^{ε}-(1-Desoxy-1-laktulosyl)-lysin (Laktuloselysin) bzw. N^{ε}-(1-Desoxy-1-tagatosyl)-lysin (Tagatoselysin).

Die **fortgeschrittene Phase** ist charakterisiert durch Enolisierungs-, Umlagerungs- und Oxidationsprozesse der Amadori-Produkte. Es entstehen die hoch reaktiven kurzkettigen 1,2-Dicarbonylverbindungen, z.B. Glyoxal, Methylglyoxal und 3-Desoxyglucosulose.

Diese 1,2-Dicarbonylverbindungen reagieren in der **späten Phase** der Maillard-Reaktion mit der ε-Aminogruppe von Lysin oder der Guanidinofunktion von Arginin zu verschiedenen nieder- und hochmolekularen stabilen Endprodukten, den *advanced glycation end products* (AGEs). Hierbei entstehen Lysinderivate wie N^{ε}-(Carboxymethyl)-lysin (CML; AHMED *et al.* 1986), N^{ε}-(1-Carboxyethyl)-lysin (CEL; AHMED *et al.* 1997), 6-(2-Formyl-5-hydroxymethyl-1-pyrrolyl)-norleucin (Pyrralin, Pyrr; NAKAYAMA *et al.* 1980, HENLE und BACHMANN 1996) und 6-(2-Formyl-1-pyrrolyl)-norleucin (Formylin; Fom; HELLWIG und HENLE 2010). Durch die Reaktion der Lysin-Seitenkette mit dem während der Degradation von Disacchariden gebildeten Isomaltol entsteht das 6-(3-Hydroxy-4-oxo-2-methyl-4(1*H*)-1-pyridinyl)-L-norleucin (Maltosin, Mal; LEDL *et al.* 1989). Argininderivate wie N^{δ}-(5-Methyl-4-oxo-5-hydro-2-imidazolonyl)-ornithin (*methylglyoxal-derived hydroimidazolone*, MG-H1; HENLE *et al.* 1994a) und N^{δ}-(5-Hydroxy-4,6-dimethyl-2-pyrimidinyl)-ornithin (Argpyrimidin, Apy; SHIPANOVA *et al.* 1997) können aus der Reaktion von Arginin mit Methylglyoxal hervorgehen. Das Quervernetzungsprodukt Pentosidin, eine sogenannte *crosslink*-Aminosäure, resultiert aus der Reaktion von Pentosen mit Lysin und Arginin, kann aber auch aus Aldosen oder Amadori-Produkten gebildet werden (SELL und MONNIER 1989, DYER *et al.* 1991).

Die Maillard-Reaktion in Lebensmitteln

Ausgehend von einem gewöhnlichen Ernährungsverhalten beträgt in den westlichen Industrieländern die tägliche alimentäre Aufnahme von Amadori-Produkten 0,5-1,2 g (berechnet als Fruktoselysin; FL) und von AGEs 25-75 mg, hauptsächlich als Pyrralin und

CML (HENLE 2003)[1]. Genauere Informationen über das Vorkommen verschiedener MRPs in Lebensmitteln können Tabelle 1.1 entnommen werden.

Tabelle 1.1. Vorkommen und Gehalt einiger Maillard-Reaktionsprodukte in Lebensmitteln.

Maillard-Reaktionsprodukt	Lebensmittel	Konzentration (mg/kg Protein)	Referenzen
Formylin	Butterkeks Roggenbrotkruste	1,8-2,9 10-33	HELLWIG und HENLE 2011
Fruktoselysin[a]	Brot Schokolade Pasta UHT-Milch Kekse	40-50 314 ± 110 38 ± 4 2-7 60-80	ERBERSDOBLER und FAIST 2001
Maltosin	Milch, Weizenmehl	bis 100	HENLE *et al.* 1994b
Pentosidin	Brotkruste Laugenbrezel Röstkaffeepulver Backwaren	0,4-2,6 9,3-22,8 10,8-39,9 bis 12,7	HENLE *et al.* 1997
Pyrralin	Pasta, Weizenmehl, Milchpulver; Brotkruste, Zwieback, Kräcker	90-150 bis 3700	HENLE *et al.* 1994b RESMINI und PELLEGRINO 1994

[a] Konzentrationen in mg/100 g Lebensmittel angegeben (ERBERSDOBLER und FAIST 2001)
UHT-Milch, ultrahocherhitzte Milch

Aus ernährungsphysiologischer Sicht kann die Maillard-Reaktion zu einer Nährwertminderung des Lebensmittels führen (FRIEDMAN 1996): Die Modifizierung der Seitenkette von Lysin- und Argininresten führt zu einem Verlust dieser essentiellen bzw. semi-essentiellen Aminosäuren. Solch eine Modifizierung kann 10-75% der Lysin- und Arginin-Seitenketten eines Proteins betreffen und vermindert somit die biologische Wertigkeit von Proteinen (FINOT und MAGNENAT 1981). Auch die Bildung von *crosslink*-Aminosäuren, wie dem Pentosidin, resultiert in einem Verlust essentieller Aminosäuren.

Die Maillard-Reaktion in vivo

Es wird gegenwärtig kontrovers diskutiert, ob MRPs ein Risiko für die menschliche Gesundheit darstellen (SOMOZA 2005, AMES 2007, HENLE 2007): Die Maillard-Reaktion findet *in vivo* ebenso statt wie in Lebensmitteln. Bedingt durch die geringere Reaktionstemperatur von 37 °C läuft die Reaktion im Organismus im Vergleich zu Lebensmittelprozessen aber wesentlich langsamer ab. Die Reaktion von Zuckern mit Proteinen *in vivo* geht mit strukturellen und funktionellen Veränderungen dieser einher. Vor allem im Alter, aber

[1] Hierbei wird u.a. die Aufnahme von einem Liter Milch, 500 g Backwaren und 400 ml Kaffee angenommen, wobei der Erhitzungsgrad des Lebensmittels eine entscheidende Rolle spielt (HENLE 2003).

auch bei Diabetes mellitus, bedingt durch erhöhte Blutglukosespiegel, und bei Nierenerkrankungen, bedingt durch verminderte Ausscheidungsfunktion, ist die Plasma-Konzentration der AGEs erhöht (SCHWENGER *et al.* 2001). In diesem Zusammenhang wird von einer Beteiligung der Maillard-Reaktion am Alterungsprozess sowie an der Pathogenese und dem Fortgang der Urämie und des Diabetes mellitus[2] gesprochen (AHMED *et al.* 1997, KOSCHINSKY *et al.* 1997, HENLE und MIYATA 2003, AHMED *et al.* 2007). Unklar ist jedoch, ob die Akkumulation der AGEs *in vivo* ursächlich für diese Krankheiten ist oder nur eine Begleiterscheinung darstellt (AMES 2007). Die Aufnahme an AGEs in Form von hitzebehandelten Lebensmitteln kann dennoch zusätzlich einen erheblichen Beitrag zur AGE-Konzentration *in vivo* leisten (KOSCHINSKY *et al.* 1997, ERBERSDOBLER *et al.* 2001, HENLE 2003). Der Begriff „Glykotoxine" wurde daher für Nahrungs-AGEs geschaffen, um den Risikofaktor zu verdeutlichen (KOSCHINSKY *et al.* 1997). Es konnte nachgewiesen werden, dass diese Glykotoxine den oxidativen Stress erhöhen und inflammatorische Prozesse induzieren (VLASSARA *et al.* 2002, URIBARRI *et al.* 2007). Um die alimentär-bedingt erhöhte Konzentration an AGEs im Organismus zu vermindern, wird eine schonendere Lebensmittelzubereitung bezüglich der Temperatur und Zeit ebenso empfohlen, sowie eine generelle Senkung der Zufuhr an Nahrungs-AGEs (URIBARRI *et al.* 2010).

Während der Maillard-Reaktion entstehen außerdem Verbindungen, welche antinutritive und toxische Eigenschaften aufweisen: Pyrralin beeinflusst zum Beispiel proteolytische Enzyme und gilt als potentiell mutagen (OMURA *et al.* 1983, ÖSTE *et al.* 1987). Maltosin hingegen besitzt metallbindende Eigenschaften (SEIFERT *et al.* 2004) und beeinflusst nach alimentärer Aufnahme die Eisenabsorption bzw. -ausscheidung (REHNER und WALTER 1991). Andererseits können aus der Maillard-Reaktion auch Verbindungen mit antioxidativer, chemopräventiver oder antimutagener Wirkung hervorgehen, wie das Pronyllysin (LINDENMEIER *et al.* 2002).

AGEs können ebenso durch das Binden an Zelloberflächen-Rezeptoren, die sogenannten Rezeptoren für AGEs (RAGEs), pathophysiologisch wirken. RAGEs sind multiligande Rezeptoren der Immunglobulin-Superfamilie von Zelloberflächenmolekülen, welche auf der Oberfläche von Endothelzellen, Makrophagen, Phagozyten, Lymphozyten und vaskulären glatten Muskelzellen nachgewiesen wurden. AGE-RAGE-Interaktionen lösen Signalkaskaden aus, welche zu zellulär oxidativen Stress führen und die Transkription von

[2] Die Bestimmung des glykierten Hämoglobins (HbA_{1C}) wird bei Diabetes-Patienten als diagnostisches Maß des Krankheitsverlaufes bzw. der Insulineinstellung herangezogen (COHEN und CLEMENTS 1999).

proinflammatorischen und -thrombotischen Molekülen fördern (SCHMIDT *et al.* 1996, LINDSAY *et al.* 2009).

Studien zur Absorption von Maillard-Reaktionsprodukten

Die Metabolisierung, vor allem Absorptions- und Ausscheidungsmechanismen, alimentär aufgenommener bzw. *in vivo* gebildeter MRPs und somit ihr (patho)physiologischer Beitrag im Organismus ist Gegenstand vieler Forschungsarbeiten (ERBERSDOBLER und FAIST 2001, FÖRSTER *et al.* 2005, SOMOZA *et al.* 2006). Die Untersuchungen der Exkretion von zuvor verabreichten definierten Mengen an proteingebundenem FL an gesunden Probanden bzw. Versuchstieren ergaben minimale Konzentrationen von FL in Harn und Fäzes (LEE und ERBERSDOBLER 1994, FÖRSTER *et al.* 2005). Der ungewisse Verbleib der restlichen oral aufgenommenen Menge an FL (~95%) unterliegt möglicherweise einem enzymatischen Abbau (WIAME *et al.* 2002) oder wird durch die intestinale Flora abgebaut (ERBERSDOBLER *et al.* 1991, LEE und ERBERDOBLER 1994, ERBERSDOBLER und FAIST 2001). An gesunden Probanden durchgeführte Bilanzstudien mit Pyrralin zeigten dagegen eine nahezu vollständige renale Ausscheidung von Pyrralin nach alimentärer Zufuhr in Protein-gebundener Form (FÖRSTER und HENLE 2003, FÖRSTER *et al.* 2005). Pentosidin ist in seiner freien Form ähnlich bioverfügbar wie Pyrralin (FÖRSTER *et al.* 2005). Während die renale Exkretion von Pyrralin allerdings ausschließlich durch die alimentäre Zufuhr bestimmt wird, wird die Ausscheidung von Pentosidin von endogenen Faktoren (*in-vivo*-Bildung oder Protein-Abbau) mitbestimmt (FÖRSTER *et al.* 2005). Der Nachweis der MPRs im Urin in diesen Verdauungsstudien ist hinweisend für einen intestinalen Transport der alimentär aufgenommenen freien und Peptid-gebundenen MRPs. Bisher ist allerdings noch nicht bekannt, wie die freien und gebundenen MRPs die intestinale epitheliale Barriere überwinden.

Die intestinale epitheliale Barriere

Die Barrierefunktion intestinaler Epithelien ist entscheidend für die Bioverfügbarkeit sowohl von Mikro- und Makronährstoffen, als auch von pharmazeutischen Substanzen. Das Überwinden der intestinalen Barriere erfolgt entweder durch parazelluläre (passive) oder transzelluläre (passive oder aktive) Transportvorgänge (Übersicht in SUGANO *et al.* 2010). Der parazelluläre Transport wird durch die Schlussleisten (*tight junctions*) kontrolliert. Der passive transzelluläre Transport erfolgt bis zum Konzentrationsausgleich entweder mittels einfacher Diffusion (hydrophobe Substanzen) oder für hydrophilere Substanzen vermittelt durch spezifische Membran-Transportproteine (erleichterte Diffusion). Dagegen kann ein

aktiver transzellulärer Transportmechanismus Substanzen entgegen eines Konzentrationsgefälles transportieren. Dieser Transport verbraucht direkt oder indirekt Energie in Form von Adenosintriphosphat (ATP) und kann primärer, sekundärer oder tertiärer Natur sein. Bei einem primär aktiven Prozess ist der ATP-Verbrauch direkt an den Transport gekoppelt. Der dabei gebildete elektrochemische Gradient wird von einem sekundär aktiven Transportsystem genutzt, um zum Beispiel Substanzen im Kotransport mit Ionen zu transportieren. Dieser hierdurch entstandene Ionengradient ist wiederum treibende Kraft für einen tertiär aktiven Transportmechanismus. Schließlich können Substanzen auch mittels endo-, trans- oder potozytotischer Transportmechanismen transportiert werden.

Alimentär aufgenommene Proteine werden durch gastrische und pankreatische Proteasen sowie durch die an der Bürstensaummembran des Dünndarmepithels gebundenen Endo- und Exopeptidasen in Di- und Tripeptide bzw. bis in ihre kleinsten Einheiten, die Aminosäuren, hydrolysiert. Diese Hydrolyseprodukte werden folglich über den Protonen-abhängigen Peptidtransporter (PEPT) 1 bzw. verschiedene Aminosäure-Transporter intestinal aufgenommen. Aufgrund der Struktur der in den vorliegenden Studien eingeschlossenen MRPs – freie oder Dipeptid-gebundene glykierte Lysine oder Arginine – könnten der PEPT1 ebenso wie die Aminosäure-Transportsysteme für kationische Aminosäuren potentielle *Carrier*-Proteine für die intestinale zelluläre Aufnahme der MRPs darstellen. GRUNWALD *et al.* (2006) beschäftigten sich *in vitro* mit der Interaktion und dem intestinalen transepithelialen Transport freier MRPs und kamen zum dem Schluss, dass diese Substanzen weder mit intestinalen Peptid- oder Aminosäure-Transportern interagieren, noch intestinal transepithelial transportiert werden. Möglicherweise stellen aber Dipeptid-gebundene MRPs Substrate für die intestinalen Peptidtransporter und Aminosäure-Transporter kationischer Aminosäuren dar. Eine renale Rückresorption freier und Dipeptid-gebundener MRPs könnte ebenso durch renale Peptidtransporter oder Transportsysteme für kationische Aminosäuren vermittelt sein.

1.2 Die Protonen-abhängigen Peptidtransporter

Lange wurde angenommen, dass Aminosäuren ausschließlich in freier Form über intestinale Barrieren aufgenommen werden. Der Nachweis eines aktiven direkt sättigbaren Transportes von Gly-Gly und die anschließende intrazelluläre Hydrolyse gaben erste Hinweise auf die Existenz eines Peptidtransportsystems (NEWEY und SMYTH 1962). Das Ausbleiben eines Mangels essentieller Aminosäuren nach oraler Dipeptid-Zufuhr bei Patienten mit Defekten im Aminosäure-Transportsystem (Cystinurie, Hartnup-Syndrom)

lieferte weitere Hinweise darauf, dass Aminosäuren auch Peptid-gebunden intestinal aufgenommen werden (ASATOOR *et al.* 1970, HELLIER *et al.* 1972). Durch den Einsatz hydrolysestabiler Dipeptide, wie Gly-Sar und L-Carnosin (β-Alanyl-L-histindin) konnten erstmals Dipeptide in der Blutzirkulation nachgewiesen werden (ADDISON *et al.* 1972, ADDISON *et al.* 1973).

Mechanismus der Peptidtranslokation

Die Protonen-abhängigen Peptidtransporter (PEPT) sind tertiär aktive Transportsysteme, welche ihre Substanzen im Kotransport mit Protonen[3] transportieren (GANAPATHY und LEIBACH 1985). Dieser als treibende Kraft nach innen gerichtete elektrochemische Protonen-Gradient wird durch den apikal lokalisierten Na^+/H^+-Austauscher (NHE3) aufrechterhalten (MURER *et al.* 1976, BRANT *et al.* 1995, KENNEDY *et al.* 2002, THWAITES *et al.* 2002), welcher wiederum durch die basolaterale Na^+-K^+-ATPase angetrieben wird.

Es existieren zwei Isoformen des Protonen-abhängigen Peptidtransporters: PEPT1 und PEPT2. PEPT1 ist für die intestinale Aufnahme von Di- und Tripeptiden verantwortlich. Diese Substrate gelangen entgegen ihres Konzentrationsgefälles in die Zellen und werden akkumuliert, wobei Di- und Tripeptide durch zytosolische Peptidasen schnell hydrolysiert werden (GANAPATHY und LEIBACH 1985). Die hierbei entstandenen freien Aminosäuren werden entweder von der Zelle verwertet oder verlassen die Zellen *via* basolaterale Aminosäure-Transporter (DEVÉS und BOYD 1998). Hydrolyseresistente(re) Peptide gelangen vermutlich über einen bisher nur funktionell charakterisierten basolateralen Peptidtransporter auf die abluminale Zellseite (DYER *et al.* 1990, SAITO und INUI 1993, TERADA *et al.* 1999). Neben der Isoform PEPT2, wird auch PEPT1 in der Niere exprimiert. Beide Transporter sind für die renale Rückresorption von Di- und Tripeptiden verantwortlich.

Molekularer Aufbau der Peptidtransporter

PEPT1 und PEPT2 können aufgrund ihrer Struktur- und Funktionsähnlichkeit den Familien der Protonen-gekoppelten Oligopeptid-Transporter (POT; PAULSEN und SKURRAY 1994) oder den Peptidtransportern (PTR; STEINER *et al.* 1995) zugeordnet werden. Mittels Hydrophobizitätsanalysen wurden zwölf Transmembrandomänen (TMD) vorhergesagt (FEI *et al.* 1998), welche durch Epitop-Insertionsanalysen bestätigt worden sind (COVITZ *et al.* 1998).

[3] Der PEPT-vermittelte Substrattransport ist ein geordneter simultaner Prozess, bei dem zuerst das Proton bindet und dann das Substrat (MACKENZIE *et al.* 1996). Proton und Substrat werden in Abhängigkeit von der Ladung der Substrate und Position der Ladung im Substrat (Dipeptid) in unterschiedlichen stöchiometrischen Verhältnissen transportiert (MACKENZIE *et al.* 1996, CHEN *et al.* 1999, KOTTRA *et al.* 2002).

Neben einer großen, extrazellulär liegenden, hydrophilen Schleife und den zytoplasmatisch lokalisierten *N*- und *C*-Termini sind auch potentielle *N*-Glykosylierungs- und Phosphorylierungsstellen enthalten. Die Proteine des humanen PEPT1 (*solute carrier* (SLC) 15A1) und des humanen PEPT2 (SLC15A2) zeigen 50% Sequenzidentität und 70% -ähnlichkeit (Übersicht in FEI *et al.* 1998). Die TMD 1-9 entscheiden über den phänotypischen Charakter des *Carrier*-Proteins (DÖRING *et al.* 1996, DÖRING *et al.* 2002). Ortsgerichtete Mutagenesen führten zur Identifizierung von essentiellen Aminosäureresten für die PEPT1/2-Transportaktivität (TERADA *et al.* 1996, FEI *et al.* 1997, FEI *et al.* 1998).

Lokalisation der Peptidtransporter

PEPT1 ist primär an der apikalen Membran im Dünndarmepithel lokalisiert und in geringerem Maß im renalen proximalen Tubulus (S1-Segment), in der Leber, im Pankreas und in den Cholangiozyten des Gallengangs. PEPT2 ist an der apikalen Membran der epithelialen Zellen des proximalen Tubulus (S2- und S3-Segment), im Nervensystem, in der Lunge, im Herz und in der Milchdrüse lokalisiert (Übersichten zur Lokalisation von PEPT1 und PEPT2 in Organen und Geweben in: DANIEL und KOTTRA 2004, BRANDSCH *et al.* 2008). Jüngst konnten AGU *et al.* (2011) zeigen, dass beide Transportproteine funktionell im Nasenepithel exprimiert werden.

Substrate der Peptidtransporter und Substratspezifität

Die multispezifisch arbeitenden Peptidtransporter tolerieren neben den physiologischen Substraten auch eine Vielzahl strukturell modifizierter Aminosäuren, Peptide und Peptidmimetika ohne signifikante Änderung der Affinität und der Transportaktivität. PEPT1 und PEPT2 unterscheiden sich hierbei in ihrer Substratspezifität, -affinität und Transportkapazität: PEPT1 ist der *low-affinity/high-capacity* Transporter und PEPT2 der *high-affinity/low-capacity* Transporter (DANIEL *et al.* 1991, GANAPATHY *et al.* 1995).

Modifizierungen von Di- und Tripeptiden können zu Liganden führen, welche entweder an das PEPT-Protein binden und transportiert werden oder nicht-transportierte Inhibitoren darstellen (Übersicht zur Strukturanforderung an ein PEPT-Substrat bzw. -Inhibitor in BRANDSCH *et al.* 2008). Insbesondere führte in unterschiedlichen Studien eine Modifizierung der Seitenketten *N*- und *C*-terminaler Aminosäuren im Dipeptid mit hydrophoben Blockierungsgruppen zu einer gesteigerten Affinität zum PEPT-Protein (DANIEL *et al.* 1992, TATEOKA *et al.* 2001, KNÜTTER *et al.* 2004). Die Seitenketten können folglich mit den hydrophoben Bindungstaschen des *Carriers* in Wechselwirkung treten (DANIEL *et al.* 1992).

Ebenso konnte die Affinität zum PEPT1 durch das Derivatisieren der ε-Aminogruppe in Lys-Ala (Nle-Ala) erhöht werden (Brandsch *et al.* 1999). Hierbei wurde angenommen, dass durch die Blockierung der positiven Ladung der Lysin-Seitenkette auch die elektrostatischen Wechselwirkungen wegfallen, welche von der Bindungsdomäne im Transportprotein nicht toleriert werden (Brandsch *et al.* 1999). Aufgrund der Tatsache, dass die MRPs ebenfalls eine blockierte Seitenkette aufweisen, kann vermutet werden, dass die Dipeptid-gebundenen MRPs, aber auch die freien modifizierten Aminosäuren ebenso Liganden des PEPT1 sein können.

1.3 Die Transportsysteme für kationische Aminosäuren

Während ausschließlich PEPT1 die intestinale und sowohl PEPT1, als auch PEPT2 die renale Translokation von Di- und Tripeptiden vermittelt, existieren für die Aminosäuren multiple Transportsysteme mit überlappender Substratspezifität. Die in den vorliegenden Untersuchungen eingeschlossenen freien MRPs stellen modifizierte Lysin- und Arginin-Derivate dar, welche daher auch potentielle Substrate für Transportsysteme kationischer Aminosäuren sein könnten. Die vier für den Transport kationischer Aminosäuren verantwortlichen Systeme – $B^{0,+}$, y^+, $b^{0,+}$ und y^+L – unterscheiden sich in der Interaktion mit neutralen Aminosäuren und anorganischen Kationen. Tabelle 1.2 gibt eine Übersicht dieser Transportsysteme bezüglich der bevorzugten Substrate, Transportmechanismen und ihrer Gewebeexpression.

Die Transportsysteme y^+, $b^{0,+}$ und y^+L gehören zur SLC7 Familie, welcher zwei Unterfamilien angehören (Übersichten in: PALACÍN und KANAI 2004, VERREY *et al.* 2004): Die Mitglieder der Unterfamilie kationischer Aminosäure-Transporter (CAT-1 bis CAT-4) sind glykosylierte Transportproteine mit intrazellulär liegendem *N*- und *C*-Terminus, denen 14 TMD vorhergesagt sind. Der durch CAT-1 vermittelte Transport bezüglich kationischer und neutraler Aminosäuren entspricht dem des Systems y^+.

Den Glykoprotein-assoziierten Transportproteinen (gpaAT; z.B. $b^{0,+}$AT, y^+LAT1/2), welche die zweite Unterfamilie bilden, sind 12 putative TMD vorhergesagt. Die gpaATs bilden als leichte Untereinheit der heterodimeren Aminosäure-Transporter (HAT) ein Heterodimer mit einem Glykoprotein der SLC3 Familie (rBAT und 4F2hc; schwere Untereinheit). Beide Untereinheiten sind durch eine Disulfidbrücke verbunden und müssen koexprimiert werden, um die Plasmamembran zu erreichen und Transportaktivitäten ausüben zu können. rBAT interagiert mit $b^{0,+}$AT und die Aminosäure-Transportaktivität entspricht der des

Tabelle 1.2. Transportsysteme für kationische Aminosäuren.

Protein	System	humanes Gen	bevorzugte Substrate	Transportmechanismus		Gewebe/Organ	Referenzen[a]
apikale Membran							
CAT-1	y^+	SLC7A1	CAA; Inhibitor: *N*-Ethylmaleimid	CAA: Na^+-, pH-unabhängig NAA: Na^+-abhängig; K_m für CAA ~100 µM	Uniporter; sättigbar; *trans*-stimulierbar	ubiquitär, ausgenommen Leber	CHRISTENSEN *et al.* 1969 WHITE *et al.* 1982 KIM *et al.* 1991
rBAT/ $b^{0,+}$AT	$b^{0,+}$	SLC3A1/ SLC7A9	CAA, Cystin, größere NAA ohne Verzweigung am C_α- oder C_β-Atom	Na^+-unabhängig; K_m für L-Lys, L-Leu 100 µM	Aminosäure-Austauscher, *trans*-Stimulation	apikale Membran des proximalen Nierentubulus, Dünndarm; Leber, Pankreas, Gehirn, Plazenta	VAN WINKLE *et al.* 1988
$ATB^{0,+}$	$B^{0,+}$	SLC6A14	CAA, NAA mit Verzweigung am C_α- oder C_β-Atom, BCO Inhibitor: BCH	Na^+-abhängig Cl^--abhängig K_m für L-Lys 140 µM, L-Ala 35 µM	Kotransporter (2 Na^+, 1 Cl^-)	apikale Membran von Dünndarm, Dickdarm, Lunge, Brustdrüse	VAN WINKLE *et al.* 1985 SLOAN *et al.* 1999
basolaterale Membran							
4F2hc/ y^+LAT1	y^+L	SLC3A2/ SLC7A7	CAA, NAA	NAA: Na^+-abhängig, CAA: Na^+-unabhängig; K_m für L-Lys, L-Leu ~10 µM	Aminosäure-Austauscher, *trans*-Stimulation	proximale Nierentubulus, Dünndarm, Leukozyten, Plazenta, Lunge	DEVÉS *et al.* 1992 TORRENTS *et al.* 1998
4F2hc/ y^+LAT2	y^+L	SLC3A2/ SLC7A6	CAA, NAA	NAA: Na^+-abhängig CAA: Na^+-unabhängig K_m für L-Lys, L-Leu ~10 µM	Aminosäure-Austauscher	nicht-epitheliale und epitheliale Gewebe	BRÖER *et al.* 2000

CAA, kationische Aminosäuren; NAA, neutrale Aminosäuren; K_m, Michaelis-Konstante; BCH, 2-*endo*-Aminobicyclo[2,2,1]heptan-2-carbonsäure; 3-Amino-*endo*-bicyclo-[3,2,1]octan-3-carboxylsäure; [a] Übersichten in: DEVÉS und BOYD 1998, PALACÍN und KANAI 2004, VERREY *et al.* 2004, CLOSS *et al.* 2006

Systems $b^{0,+}$. 4F2hc dimerisiert mit y^+LAT1 oder y^+LAT2 und vermittelt die System y^+L-Aminosäure-Transportaktivität.

Die SLC3 Proteine sind Typ II Membran-Glykoproteine mit einem zytoplamatischen *N*-Terminus, einem einzelnen transmembranären Segment und einem glykosylierten extrazellulären *C*-Terminus (TEIXEIRA *et al.* 1987). Mutationen in $b^{0,+}$AT oder rBAT und y^+LAT1 führen zu Cystinurie (PRAS *et al.* 1995) bzw. zur Lysinurischen Proteinintoleranz (TORRENTS *et al.* 1998).

Das einzige Transportsystem, welches kationische Aminosäuren Na^+- (und Cl^--) abhängig transportiert, ist das System $B^{0,+}$. Das diesen Transport vermittelnde Protein, der humane Aminosäure-Transporter $B^{0,+}$ (hATB$^{0,+}$), ist ein Mitglied der Familie der Na^+/Cl^--abhängigen Neurotransmitter-Transporter (SLOAN *et al.* 1999). Dem Protein sind 12 TMD vorhergesagt. Die Substratspezifität des Systems $B^{0,+}$ ist ähnlich der des Systems $b^{0,+}$. System $B^{0,+}$ akzeptiert aber auch kleine sowie am α- und β-C-Atom verzweigte neutrale Aminosäuren (VAN WINKLE *et al.* 1985). Zudem weist es die höchsten Affinitäten für hydrophobe Aminosäuren auf (SLOAN *et al.* 1999).

2 Zielstellung

Alimentär aufgenommene Maillard-Reaktionsprodukte (MRPs) können ihre (patho)physiologischen Funktionen im Organismus nur ausüben, wenn sie entweder über die Enterozyten des Dünndarms gelangen oder an apikal lokalisierte Rezeptoren binden und somit eine Signalkaskade auslösen. Metabolische Studien beobachteten aber eine renale Ausscheidung von MRPs (Pyrralin, CML, FL) nach oraler Aufnahme dieser Produkte in freier und Protein-gebundener Form und implizieren daher einen stattgefundenen intestinalen Transport. Diese MRPs könnten sowohl parazellulär mittels einfacher Diffusion oder transzellulär durch Endozytose, Diffusion oder Transportprotein-vermittelt intestinal aufgenommen worden sein. Da die MRPs strukturell gesehen freie, Peptid- oder Protein-gebundene glykierte Aminosäuren sind, liegt die Vermutung nahe, dass diese demnach *via* intestinale Aminosäure- oder Peptidtransporter in den Organismus gelangen könnten und somit potentielle Substrate für diese Transportsysteme darstellen. Aus der Studie von GRUNWALD *et al.* (2006) ist jedoch bekannt, dass die freien MRPs N^{ε}-Carboxymethyllysin, N^{α}-Hippuryl-N^{ε}-carboxymethyllysin, N^{ε}-Fruktosyllysin und N^{α}-Hippuryl-N^{ε}-fruktosyllysin weder mit intestinalen Peptid- oder Aminosäure-Transportern interagieren, noch intestinal transepithelial transportiert werden.

Das Ziel der vorliegenden Arbeit war es daher, neue Informationen bezüglich der intestinalen Aufnahme und der renalen Ausscheidung von MRPs, durch Interaktion mit spezifischen Transportsystemen und dem dadurch möglicherweise vermittelten Transport der MRPs, zu gewinnen. Modifizierte Lysin- und Arginin-Derivate, welche während der Maillard-Reaktion entstehen, sollten in freier und insbesondere in Dipeptid-gebundener Form verwendet werden.

Die *Studie 1* gibt einen Überblick über den intestinalen transepithelialen Transport und das Interaktionsverhalten der freien und Dipeptid-gebundenen MRPs mit intestinalen Aminosäure- und Peptidtransportern. Dies könnte einen Ansatz zur Klärung der oralen Bioverfügbarkeit von MRPs liefern.

Untersuchungen im Rahmen der *Studie 1* zeigten, dass einige der MRPs in Dipeptid-gebundener Form mittel- bis hoch-affin mit dem PEPT1-Protein interagieren und intestinal als Dipeptid zellulär aufgenommen werden. PEPT1 kann daher als beteiligtes Transportsystem diskutiert werden. Detaillierte Untersuchungen bezüglich des tatsächlichen PEPT1-

vermittelten Transportes ausgewählter MRPs schlossen sich dem an. Zum Einen wurde in den *Studien 2* und *3* Pyrralin, als das quantitativ in Lebensmitteln dominierende AGE, eingesetzt. Zum Anderen sollte Maltosin, welches Komplexe mit Metallionen eingehen kann, in *Studie 4* Einsatz finden. Durch eine mögliche orale Bioverfügbarkeit könnte Maltosin pharmazeutische Relevanz bei der Therapie von Eisenspeichererkrankungen erlangen.

Um Aufschluss darüber zu geben, ob die Dipeptid-Derivate von Pyrralin und Maltosin nicht nur spezifisch an PEPT1 binden, sondern tatsächlich auch aktiv PEPT1-vermittelt zellulär aufgenommen werden, wurde PEPT1 in HeLa-Zellen sowie in *Xenopus laevis*-Oozyten heterolog exprimiert.

Nachdem gezeigt werden konnte, dass Pyrralin und Maltosin *in vitro* nach intestinaler zellulärer Aufnahme in Dipeptid-gebundener Form als freie Aminosäure die Zellen abluminal wieder verlassen (*Studie 1*), sollte schließlich untersucht werden, ob diese MRPs einer renalen Rückresorption unterliegen könnten oder mit dem Harn ausgeschieden werden. Hierzu wurden die Interaktion beider MRPs mit renalen Transportsystemen für L-Lysin und der renale transepitheliale Transport untersucht.

3 Übersicht über eingesetzte Methoden

In der vorliegenden Arbeit wurden die Untersuchungen zum intestinalen Transport der MRPs an Caco-2-Zellen, eine humane Kolonkarzinom-Zelllinie, durchgeführt. Caco-2-Zellen ähneln in ihren morphologischen und funktionellen Eigenschaften dem humanen Dünndarm (HIDALGO *et al.* 1989). Diese Zellen exprimieren an der apikalen Membran PEPT1 (GANAPATHY *et al.* 1995) sowie verschiedene Aminosäure-Transportsysteme, darunter die für die Translokation von L-Lysin verantwortlichen Systeme (THWAITES *et al.* 1996).

Die Untersuchungen zum renalen Transport der MRPs wurden an der epithelialen Zelllinie des proximalen Nierentubulus eines Opossums (OK-Zellen) durchgeführt. Diese Zellen exprimieren an der apikalen Membran Transportsysteme für kationische Aminosäuren (MORA *et al.* 1996), besitzen allerdings nicht den für die Niere typischen Peptidtransporter PEPT2. Daher wurde zur Untersuchung der Interaktion Dipeptid-gebundener MRPs zusätzlich die Zelllinie des proximalen Nierentubulus einer spontan-hypertensiven Ratte (SKPT-Zellen) verwendet, welche an der apikalen Membran PEPT2 exprimiert (BRANDSCH *et al.* 1995).

Die Interaktion mit spezifischen Transportsystemen wurde in Kompetitionsstudien in Anwesenheit einer fixen bzw. steigenden Konzentration des jeweiligen MRP untersucht. Die Bestimmung der Substratspezifitäten bzw. -affinitäten zum entsprechenden *Carrier*-Protein wurden an OK-, Caco-2- bzw. SKPT-Zellen durchgeführt. Das radioaktiv markierte und enzymatisch stabile Dipeptid [^{14}C]Gly-Sar diente hierbei als Referenzsubstanz für PEPT1 bzw. PEPT2 und L-[^{3}H]Lysin als Standardsubstrat für Transportsysteme basischer Aminosäuren. Die Analysen der Proben erfolgten mittels Flüssigszintillationsspektrometrie.

Mit Hilfe von Transwell®-Kammern (Abbildung 3.1) wurde an Caco-2- und OK-Zellen

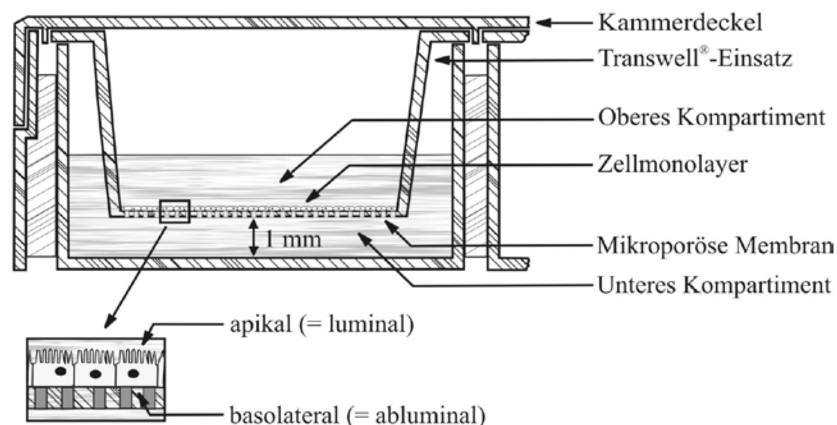

Abbildung 3.1. Querschnitt durch eine Transwell®-Kammer: eine semi-permeable Membran bewachsen mit einem Zellmonolayer.

der transepitheliale Transport (Flux) gemessen. Die Zellen wurden hierbei auf semi-permeablen Membranen kultiviert, die einen Transport von luminal nach abluminal ermöglichten. Die während der Versuche gewonnenen Proben wurden mittels Hochleistungs-flüssigchromatographie (*high performance liquid chromatography*; HPLC) analysiert.

Für die Untersuchungen des tatsächlichen direkten PEPT1-vermittelten Transportes der MRPs wurde der humane Peptidtransporter (hPEPT1) durch Injektion der hPEPT1-cRNA heterolog in *Xenopus laevis*-Oozyten exprimiert. Da der PEPT1-vermittelte Transport von Substanzen im Kotransport mit Protonen verläuft (GANAPATHY und LEIBACH 1985, DANIEL 2004), konnte dieser elektrogen verlaufende Prozess elektrophysiologisch durch die Anwendung der Zweielektroden-Spannungsklemmtechnik an den hPEPT1-exprimierenden *Xenopus laevis*-Oozyten gemessen werden. Um die Aufnahme und die intrazelluläre Akkumulation der MRPs nachzuweisen, wurde hPEPT1 ebenfalls heterolog in HeLa-Zellen, eine Zelllinie eines humanen Cervixkarzinoms, exprimiert. Diese Expression des Transportproteins erfolgte durch Transfektion der Zellen mit hPEPT1-cDNA. Der zelluläre Nachweis der MRPs erfolgte dabei mittels HPLC.

Die Synthesen der in dieser vorliegenden Arbeit verwendeten modifizierten freien und Dipeptid-gebundenen Aminosäuren, sowie die Analysen mittels HPLC wurden vom Diplom-Lebensmittelchemiker MICHAEL HELLWIG, Institut für Lebensmittelchemie der Technischen Universität Dresden, durchgeführt: Die Synthesen erfolgten auf der Basis von Standard-methoden. Die Dipeptide (Ala-MRP und MRP-Ala) wurden mit einer modifizierten Amino-säure sowohl in *N*-, als auch in *C*-terminaler Position synthetisiert, da die Aminosäure-Position im Dipeptid für deren inhibitorische Aktivität und Transport relevant ist (DANIEL 2004). Die Aminosäure Alanin wurde gewählt, da diese eine häufige in Nahrungsproteinen vorkommende Aminosäure ist. Verunreinigungen während der Herstellung durch andere Aminosäuren oder Dipeptide wurden durch anschließende Aminosäure-Analysen ausge-schlossen.

Weitere Details zu Material und Methodik sowie die Ergebnisse und Schlussfolgerungen der Untersuchungen finden sich in den Originalarbeiten.

4 Originalarbeiten

Studie 1:

Hellwig M., Geissler S., Matthes R., Peto A., Silow C., Brandsch M., Henle T., Transport of free and peptide-bound glycated amino acids: synthesis, transepithelial flux at Caco-2 cell monolayers, and interaction with apical membrane transport proteins. ***ChemBioChem.*** *2011, 12, 1270-1279.*

Studie 2:

Hellwig M., Geissler S., Peto A., Knütter I., Brandsch M., Henle T., Transport of free and peptide-bound pyrraline at intestinal and renal epithelial cells. ***J. Agric. Food Chem.*** *2009, 57, 6474-6480.*

Studie 3:

Geissler S., Hellwig M., Zwarg M., Markwardt F., Henle T., Brandsch M., Transport of the advanced glycation end products alanylpyrraline and pyrralylalanine by the human proton-coupled peptide transporter hPEPT1. ***J. Agric. Food Chem.*** *2010, 58, 2543-2547.*

Studie 4:

Geissler S., Hellwig M., Markwardt F., Henle T., Brandsch M., Synthesis and intestinal transport of the iron chelator maltosine in free and dipeptide form. ***Eur. J. Pharm. Biopharm.*** *2011, 78, 75-82.*

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Transport of Free and Peptide-Bound Glycated Amino Acids: Synthesis, Transepithelial Flux at Caco-2 Cell Monolayers, and Interaction with Apical Membrane Transport Proteins

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In glycation reactions, the side chains of protein-bound nucleophilic amino acids such as lysine and arginine are post-translationally modified to a variety of derivatives also known as Maillard reaction products (MRPs). Considerable amounts of MRPs are taken up in food. Here we have studied the interactions of free and dipeptide-bound MRPs with intestinal transport systems. Free and dipeptide-bound derivatives of N^6-(1-fructosyl)-lysine (FL), N^6-(carboxymethyl)lysine (CML), N^6-(1-carboxyethyl)-lysine (CEL), formyline, argpyrimidine, and methylglyoxal-derived hydroimidazolone 1 (MG-H1) were synthesized. The inhibition of L-[^{3}H]lysine and [^{14}C]glycylsarcosine uptakes was measured in Caco-2 cells which express the H^+/peptide transporter PEPT1 and lysine transport system(s). Glycated amino acids always displayed lower affinities than their unmodified analogues towards the L-[^{3}H]lysine transporter(s). In contrast, all glycated dipeptides except Ala-FL were medium- to high-affinity inhibitors of [^{14}C]Gly-Sar uptake. The transepithelial flux of the derivatives across Caco-2 cell monolayers was determined. Free amino acids and intact peptides derived from CML and CEL were translocated to very small extents. Application of peptide-bound MRPs, however, led to elevation (up to 80-fold) of the net flux and intracellular accumulation of glycated amino acids, which were hydrolyzed from the dipeptides inside the cells. We conclude 1) that free MRPs are not substrates for the intestinal lysine transporter(s), and 2) that dietary MRPs are absorbed into intestinal cells in the form of dipeptides, most likely by the peptide transporter PEPT1. After hydrolysis, hydrophobic glycated amino acids such as pyrraline, formyline, maltosine, and argpyrimidine undergo basolateral efflux, most likely by simple diffusion down their concentration gradients.

Introduction

Amino acids are targets for a variety of non-enzymatic chemical processes during food processing, such as oxidations or reactions with reducing sugars and their degradation products. This process is generally referred to as the "Maillard reaction" or "glycation".[1]

In the first stage of this process, lysine ε-amino groups react with reducing sugars such as glucose, lactose, and galactose to form the Amadori products N^6-(1-deoxy-1-fructosyl)lysine (fructoselysine, FL, **1**; Scheme 1), N^6-(1-deoxy-1-lactulosyl)lysine (lactuloselysine, **2**), and N^6-(1-deoxy-1-tagatosyl)lysine (tagatoselysine, **3**), respectively. Amadori products can degrade to highly reactive 1,2-dicarbonyl compounds, which again react with lysine ε-amino groups and arginine guanidino groups to form "advanced glycation end products" (AGEs). Lysine, for example, can be modified to N^6-(carboxymethyl)lysine (CML, **4**),[2] N^6-(1-carboxyethyl)lysine (CEL, **5**),[3] 6-(2-formyl-1-pyrrolyl)norleucine (formyline, Fom, **6**),[4] and 6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)norleucine (pyrraline, Pyrr, **7**).[5,6] 6-(3-Hydroxy-2-methyl-4-oxo-4(1*H*)-1-pyridinyl)-L-norleucine (maltosine, Mal, **8**) is formed during disaccharide degradation through reactions between lysine residues and isomaltol.[7] Reactions between arginine residues and methylglyoxal lead to N^5-(5-methyl-4-oxo-5-hydro-2-imidazolonyl)-L-ornithine (methylglyoxal-derived hydroimidazolone 1, MG-H1, **9**)[8] and N^5-(5-hydroxy-4,6-dimethyl-2-pyrimidinyl)-L-ornithine (argpyrimidine, Apy, **10**) as a fluorescent minor product.[9] Pentosidine (**11**) is an amino acid containing a lysine and an arginine residue.[10] In the late stages of the Maillard reaction, AGEs, proteins, sugars, and their degradation products react with one another to form colored high-molecular-weight networks, which is why the Maillard reaction is also termed "nonenzymatic browning".

Humans are exposed to these substances from heat-treated foods such as bread, cereals, cookies, and dairy products. The Amadori products, mainly those derived from glucose and di-

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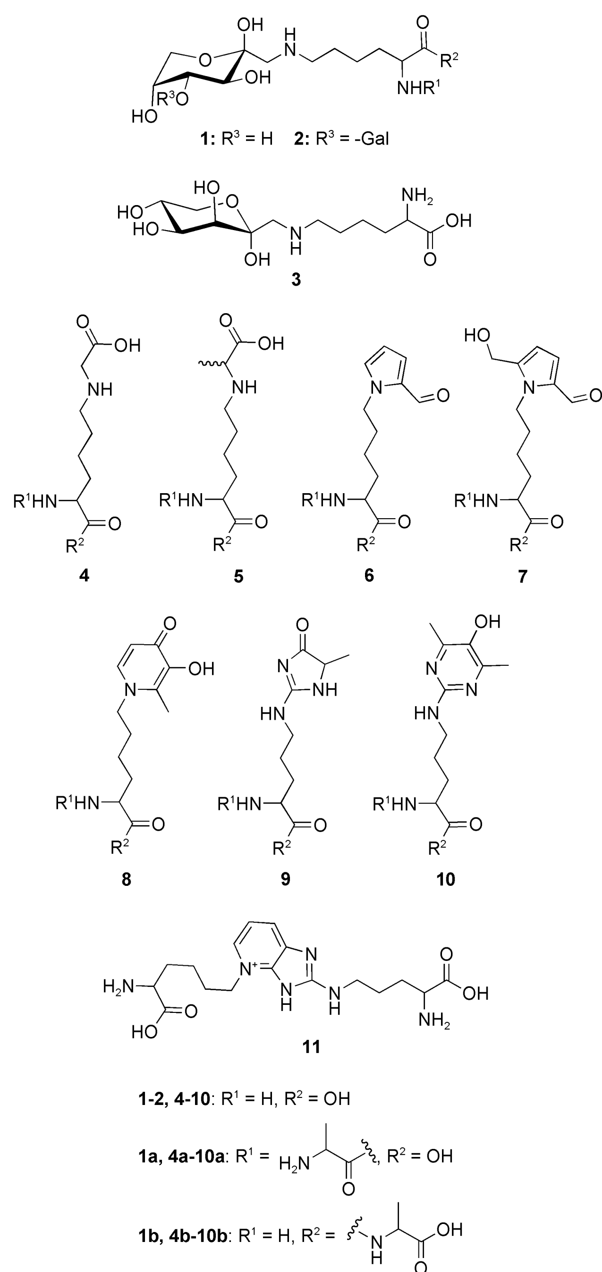

Scheme 1. Chemical structures of the investigated Maillard reaction products fructoselysine (**1**), lactuloselysine (**2**), tagatoselysine (**3**), CML (**4**), CEL (**5**), formyline (**6**), pyrraline (**7**), maltosine (**8**), MG-H1 (**9**), argpyrimidine (**10**), and pentosidine (**11**).

and oligosaccharides, are quantitatively the dominant Maillard reaction products (MRPs) in food. Amounts of between 0.5 and 1.2 g are ingested daily, together with 25 to 75 mg of AGEs (mainly **4** and **7**).[11] Recently, the daily CML intake from two standard diets used for clinical studies was determined to be 2.2–5.4 mg.[12] Because concentrations of other AGEs in most foods are not known, it is impossible to assess their daily intake.

The question of whether or not dietary AGEs play a causative role in the etiology of diseases such as diabetes and uremia is intensely debated.[13,14] Dietary AGEs are reported to enhance oxidative stress, to induce low-grade inflammation, and to promote atherosclerosis.[15] Many AGEs are strongly retained in end-stage renal disease.[16] Patients are encouraged to reduce their dietary AGE intakes in order to optimize their inflammatory status[17] and to prevent diseases. However, whether the accumulation of (dietary) AGEs in physiological fluids of patients detrimentally aggravates the diseases or whether it simply represents a side effect of uncontrolled sugar and carbonyl stress has not yet been shown.[18]

Food-borne AGEs can only affect physiological functions if they are absorbed from the diet. If it is assumed that MRPs, like other amino acids, are liberated from proteins, they arrive at the intestinal epithelial barrier bound in small peptides or, to a lesser extent, as the free amino acids.[19] Hypothetically, these products can cross the intestinal epithelium paracellularly by simple diffusion or transcellularly by diffusion, by endocytotic processes, or mediated by transport proteins. For the translocation of lysine and arginine through the apical membrane, enterocytes possess at least three different amino acid transporters: namely the systems $B^{0,+}$, $b^{0,+}$, and y^{+}.[20] Di- and tripeptides are transported by the proton-coupled peptide transporter 1 (PEPT1), which is driven by a transmembrane H^{+} gradient and catalyzes the cotransport of its substrates with H^{+} into intestinal and other cells (for a review, see ref. [21]). PEPT1 accepts many modified amino acids and dipeptides as substrates as long as the structural requirements for substrate binding and translocation are met.[22]

From the transit of immunoreactivity, Koschinsky et al.[13] estimated the absorption of AGEs in general to be about 10%, even though AGEs are a structurally quite heterogeneous group of substances (Scheme 1). We hypothesize that MRPs, due to their structural diversity, must interact quite differently with intestinal and renal amino acid and peptide transporters. Circumstantial evidence is provided by balance studies showing that less than 5% of ingested protein-bound fructoselysine (**1**) and pentosidine (**11**), but 50–100% of protein-bound pyrraline (**7**), appear in the urine.[23] In infants, the urinary excretion of CML (**4**) is dependent on the dietary intake.[24] The question that therefore arises is of how these compounds cross the intestinal barrier. Pyrraline (**7**) and maltosine (**8**) are actively transported by human PEPT1 in peptide form, but leave the cells as free **7** and **8** after peptidolysis.[25–27] Like fructoselysine (**1**) and CML (**4**),[28] free **7** and **8** are not transported through Caco-2 cells.

This study was directed towards characterizing the interaction of a broader range of glycated amino acids and dipeptides with membrane transport systems that might accept these types of compounds as substrates. Furthermore, the net transepithelial transport of these compounds across Caco-2 cell monolayers cultured on permeable filters in Transwell chambers was determined. Taken together, these techniques provide new information on the degree and mechanism of intestinal AGE absorption in vitro.

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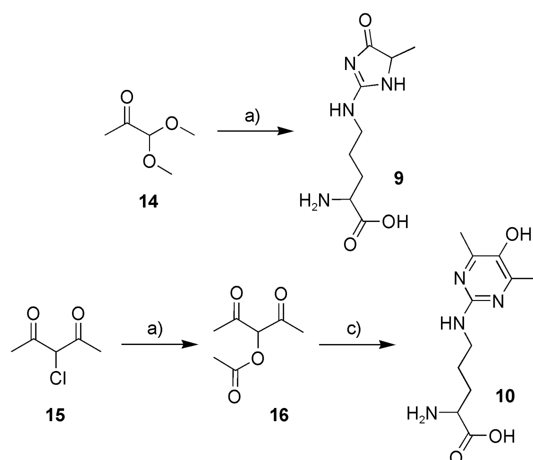
Results and Discussion

Synthesis and analysis of free and peptide-bound glycated amino acids

The Maillard reaction products investigated in this study are not commercially available, so sufficient amounts (30–200 mg) of ligands had to be synthesized. Dipeptides with the glycated amino acids both in the N-terminal and in the C-terminal positions were prepared, because the sequence of a peptide is crucial for its inhibitory and transport characteristics.[22,29] In this study, the synthesis of 12 glycated peptides is described for the first time. Moreover, the synthesis of MG-H1 (**9**, Scheme 2)



Scheme 2. Syntheses of N^5-(5-methyl-4-oxo-5-hydroimidazolon-2-yl)-L-ornithine (MG-H1, **9**) and N^5-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-L-ornithine (argpyrimidine, Apy, **10**). a) L-Arg, HCl (12 N), RT, 8 h. b) DMSO, NaOAc, RT, 3 h. c) L-Arg, HCl (12 N), RT, 20 h.

was significantly improved in terms of yield and ease of preparation by allowing unprotected arginine and the dimethylacetal **14** of methylglyoxal (which forms methylglyoxal in situ) to react in 12 M HCl. At arginine concentrations of about 10 mg mL^{-1}, compound **9** was obtained as the main product in one step in more than 40% molar yield. The synthesis could also be adapted for peptides incorporating **9**, but the incubation times were shortened in order to prevent peptide bond cleavage. We are currently investigating whether this can be applied as a general method for the synthesis of hydroimidazolones.

Another new strategy[30] was applied for the synthesis of argpyrimidine (**10**, Scheme 2). The 3-acetoxypentane-2,4-dione (**16**) precursor, however, was not synthesized from lead(IV) acetate,[30] but from 3-chloropentane-2,4-dione[31] (**15**), and was then incubated in 12 M HCl with unprotected arginine. Argpyrimidine (**10**) could be utilized as a "building block" for the synthesis of Ala-Apy and, after introduction of the Boc protecting group, for the synthesis of Apy-Ala. Taken together, the synthesis operations afforded all compounds in sufficient and partly unexpectedly high yields and purities as their formates, acetates, or hydrochlorides. Impurities, especially other amino acids or dipeptides, were absent, as verified by amino acid analysis (AAA).

The fluxes of UV-absorbing amino acids (**6**, **7**, **8**, **10**, **11**) and the corresponding dipeptides were measured by RP-HPLC after optimization of the gradients in terms of the best possible separation of the dipeptides from their constituent glycated amino acids. Other fluxes were measured by AAA with use of a lithium-based system with different gradient programs. The fluxes of **1 a** and **1 b**, however, additionally had to be measured with a sodium system, because fructoselysine (**1**) was not separated from the parent dipeptides in the lithium system.

Inhibition of L-[^{3}H]lysine uptake

The human intestinal cell line Caco-2 is a commonly used model for study of the intestinal transport of di- and tripeptides and amino acids.[32] Caco-2 cells express the PEPT1 and different amino acid transporters in their apical membranes. The three systems $B^{0,+}$, $b^{0,+}$, and y^{+},[20] responsible for the transport of lysine and arginine, are also possible candidates for the transport of MRPs of lysine and arginine, and so the interaction of free and peptide-bound MRPs with the L-lysine transporter(s) was examined. Radiolabeled L-[^{3}H]lysine was used as the reference substrate for the lysine transporter(s). In competition assays, inhibition of the uptake of L-[^{3}H]lysine by each compound was first investigated with a concentration of 10 mM to determine the substrate specificity. If the compounds were able to inhibit the transport by at least 40%, increasing concentrations of them were applied for inhibition to allow calculation of their IC_{50} values. These values were converted into inhibition constants (K_i) as described earlier.[33]

At a concentration of 10 mM, free and dipeptide-bound MRPs inhibited the transport of L-[^{3}H]lysine by 20–75% and 0–38%, respectively (Table 1). For strongly inhibiting compounds, K_i values between 0.32 mM for **7**[25] and 4.6 mM for **10** were calculated. The unlabeled reference amino acid L-lysine itself inhibited L-[^{3}H]lysine transport with a K_i value of 0.11 ± 0.01 mM (Table 1). Gly-Sar and the other tested unlabeled unmodified dipeptides showed no affinity towards the lysine transporter(s).

In conclusion, a minor part of the free MRPs, but not the dipeptide-bound derivatives, interacts with the transporter(s) for the amino acids L-lysine and L-arginine, either as substrates or as inhibitors. Pyrraline (**7**) was the only compound to inhibit L-[^{3}H]lysine transport similarly to L-lysine, and it can be considered a high-affinity ligand (substrate or inhibitor).

Inhibition of [^{14}C]Gly-Sar uptake

Unlike amino acid transport, the uptake of peptides from the gut lumen is mediated by a single transport protein—PEPT1—that tolerates many side-chain-modified peptides without losing affinity.[22] Therefore, the interactions of glycated amino acids and dipeptides with PEPT1 in Caco-2 cells were studied. The radiolabeled dipeptide [^{14}C]Gly-Sar, which is relatively resistant to intra- or extracellular enzymatic hydrolysis, served as the reference substrate. Competition experiments were first

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Table 1. Inhibition of L-[^{3}H]lysine (2 nM) and [^{14}C]Gly-Sar (10 μM) uptake in Caco-2 cells by Maillard reaction products and control substances. Uptakes were measured at pH 6.0 either for 5 min (L-[^{3}H]lysine) or for 10 min ([^{14}C]Gly-Sar) in the absence (control) or in the presence of the compounds, either at fixed concentrations (10 mM) for % uptake or at increasing concentrations (0.01–10 mM) of unlabeled compounds. K_i values were derived from competition curves. Data are means ± SEM. n = 3, 4.

Compound		L-[^{3}H]Lysine uptake		[^{14}C]Gly-Sar uptake	
		uptake [% of control]	K_i [mM]	uptake [% of control]	K_i [mM]
fructoselysine	**1**	41 ± 1	2.7 ± 0.1	32 ± 2[a]	8.7 ± 2.2[a]
Ala-FL	**1a**	100 ± 6	–	60 ± 1	> 10 (≈ 13)[b]
FL-Ala	**1b**	69 ± 11	–	36 ± 2	4.5 ± 0.2
lactuloselysine	**2**	80 ± 6	–	49 ± 2	8.7 ± 1.1
tagatoselysine	**3**	40 ± 1	3.1 ± 0.6	33 ± 3	3.5 ± 0.1
carboxymethyllysine	**4**	61 ± 1	–	93 ± 4	–
Ala-CML	**4a**	87 ± 3	–	9.9 ± 0.4	0.07 ± 0.01
CML-Ala	**4b**	94 ± 3	–	14 ± 1	0.9 ± 0.1
carboxyethyllysine	**5**	70 ± 4	–	90 ± 4	–
Ala-CEL	**5a**	97 ± 11	–	11 ± 1	0.22 ± 0.01
CEL-Ala	**5b**	96 ± 8	–	16 ± 1	1.1 ± 0.1
formyline	**6**	42 ± 1	2.2 ± 0.3	82 ± 2	–
Ala-Fom	**6a**	82 ± 5	–	10 ± 1	0.09 ± 0.01
Fom-Ala	**6b**	96 ± 7	–	9 ± 1	0.05 ± 0.01
pyrraline	**7**	25 ± 1[c]	0.32 ± 0.04[c]	77 ± 2[c]	> 10[c]
Ala-Pyrr	**7a**	66 ± 5[c]	> 10[c]	5.5 ± 0.4[c]	0.19 ± 0.01[c]
Pyrr-Ala	**7b**	78 ± 7[c]	> 10[c]	9.4 ± 0.2[c]	0.03 ± 0.01[c]
maltosine	**8**	48 ± 5	3.5 ± 0.4[d]	85 ± 2	> 10[d]
Ala-Mal	**8a**	95 ± 1	> 10[d]	17 ± 1	0.73 ± 0.05[d]
Mal-Ala	**8b**	92 ± 5	> 10[d]	10 ± 1	0.25 ± 0.02[d]
MG-H1	**9**	39 ± 4	1.6 ± 0.2	89 ± 1	–
Ala-(MG-H1)	**9a**	62 ± 11	–	17 ± 1	0.95 ± 0.02
(MG-H1)-Ala	**9b**	n.det.	–	n.det.	0.59 ± 0.05
argpyrimidine	**10**	55 ± 5	4.6 ± 1.1	39 ± 3	6.7 ± 0.6
Ala-Apy	**10a**	74 ± 2	–	13 ± 1	0.37 ± 0.04
Apy-Ala	**10b**	n.det.	–	n.det.	0.19 ± 0.03[e]
Gly-Sar		105 ± 5[c]	–	15 ± 1[c]	0.74 ± 0.01[c]
Lys		17 ± 2[c]	0.11 ± 0.01[c]	101 ± 4[c]	–
Arg		18 ± 1	0.027 ± 0.003	96 ± 3	–
Ala-Lys		85 ± 4	–	8.6 ± 0.2	0.23 ± 0.02
Lys-Ala		84 ± 6	–	13 ± 1	0.34 ± 0.02[f]
Ala-Arg	**12**	74 ± 3	–	12 ± 1	0.18 ± 0.01
Arg-Ala	**13**	65 ± 8	–	12 ± 1	0.29 ± 0.02

[a] Value from ref. [28]. [b] Extrapolated beyond measurement range. [c] Value from ref. [25]. [d] Value from ref. [27]. [e] n = 2. [f] Value from ref. [29]. n.det.: not determined.

performed at 10 mM concentrations of each compound to determine the substrate specificity. For ligands that inhibited [^{14}C]Gly-Sar uptake by at least 40%, competition assays were subsequently carried out with increasing concentrations of the compounds, to allow calculation of their K_i values. At a concentration of 10 mM, most of the free modified amino acids inhibited [^{14}C]Gly-Sar uptake only weakly, with the exceptions of fructoselysine (**1**), lactuloselysine (**2**), tagatoselysine (**3**), and argpyrimidine (**10**), for which K_i values between 3.5 and 8.7 mM were calculated (Table 1). According to Brandsch,[32] these compounds can be classified as medium- (**3**) and low-affinity ligands (**1**, **2**, **10**) for PEPT1. For purposes of comparison, L-lysine and L-arginine showed no affinity towards PEPT1.

The glycated dipeptides, however, inhibited [^{14}C]Gly-Sar uptake by 40 to 94%. K_i values between 34 μM (Pyrr-Ala) and > 10 mM (Ala-FL) were determined (Table 1). The affinities towards PEPT1 were strongly modulated, relative to those of the parent dipeptides, by the modifications. Peptides with pyrrole-modified side chains (**6a**/**6b**, **7a**/**7b**) displayed the highest affinities (34–190 μM). By Brandsch's classification,[32] Ala-CML, Ala-CEL, Mal-Ala, and both dipeptide derivatives of pyrraline, of formyline, and of argpyrimidine can be classified as high-affinity ligands (K_i < 0.5 mM). The other tested dipeptides can be considered medium-affinity ligands (0.5 mM < K_i < 5 mM), except for Ala-FL, which is a low-affinity compound. Gly-Sar represents a medium-affinity PEPT1 substrate with a K_i value of 0.74 ± 0.01 mM (Table 1), whereas the unmodified lysine and arginine dipeptides are high-affinity substrates with K_i values between 0.18 and 0.34 mM. It is the hydrophobic peptides that show high affinity, which agrees with recent findings about the affinity-enhancing effects of hydrophobic side chain modifications.[29,34] Interestingly, the alanyl peptides **4a** and **5a**, which each have a negative charge in the side chain, bind to the carrier with affinities five and 13 times higher, respectively, than the corresponding lysyl dipeptides **4b** and **5b**. This could possibly be the result of unequal distribution of charges in the binding region of PEPT1. In contrast, the alanyl dipeptides of all other tested compounds had lower affinities towards PEPT1 (1.6 to 5.6 times) than the corresponding lysyl dipeptides. This is also consistent with recent studies on model compounds in which bulky hydrophobic groups were introduced onto the lysyl side chains of Ala-Lys and Lys-Ala, thereby strongly enhancing the affinities of lysyl peptides.[29]

Transepithelial transport across intestinal epithelial cell monolayers

Interactions of compounds with transport systems do not necessarily mean that they are indeed transported. The molecular features necessary for uptake inhibition need not be the same as for translocation, so the inhibitors might be nontransported compounds with certain affinities towards the transporters. On the other hand, several other carriers at the intestinal epithelium can be responsible for the translocation of substances. Flux measurements were performed as in our previous studies;[25,27] [^{14}C]Gly-Sar and L-[^{3}H]lysine were again used as the reference substrates. The space marker [^{14}C]mannitol served as a reference compound for paracellular transport processes. Despite their relatively low inhibitions of L-[^{3}H]lysine uptake, all free amino acids were subjected to the transport experiment. No CML or CEL was found in the receiver compartment after 120 min (Table 2). The flux rates of other glycated amino acids ranged between 0.008 and 0.09% $cm^{-2}h^{-1}$ and were always lower than that of the space marker [^{14}C]mannitol (0.13 ± 0.03% $cm^{-2}h^{-1}$) and far lower than the flux of L-[^{3}H]lysine (6.86 ± 0.15% $cm^{-2}h^{-1}$). This argues against active transport of

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Table 2. Transepithelial flux of Maillard reaction products across Caco-2 cells and cellular uptake after 120 min. Fluxes were determined in the presence of the compound in question (1 mM), [14C]Gly-Sar (10 μM), L-[3H]lysine (2 nM), and [14C]mannitol (10 μM), at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Data are means ± SEM. $n=3$.

Compound		Flux of the dipeptide [% $cm^{-2}h^{-1}$]	Cellular uptake[a] [%]	Flux of the amino acid [% $cm^{-2}h^{-1}$]	Cellular uptake[a] [%]
fructoselysine	**1**	–	–	0.008 ± 0.003[b]	0.03 ± 0.01[b]
Ala-FL	**1a**	n.d.	n.d.	n.d.	n.d.
FL-Ala	**1b**	n.d.	n.d.	n.d.	n.d.
lactuloselysine	**2**	–	–	n.d.	n.d.
tagatoselysine	**3**	–	–	n.d.	n.d.
carboxymethyllysine	**4**	–	–	n.d.[b]	0.62 ± 0.02[b]
Ala-CML	**4a**	0.02 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	2.46 ± 0.08
CML-Ala	**4b**	0.02 ± 0.01	0.05 ± 0.01	0.17 ± 0.07	13 ± 2
carboxyethyllysine	**5**	–	–	n.d.	1.36 ± 0.07
Ala-CEL	**5a**	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	3.1 ± 1.5
CEL-Ala	**5b**	0.03 ± 0.01	0.17 ± 0.01	0.10 ± 0.05	12 ± 1
formyline	**6**	–	–	0.04 ± 0.01	0.08 ± 0.01
Ala-Fom	**6a**	n.d.	n.d.	3.37 ± 0.13	2.39 ± 0.08
Fom-Ala	**6b**	n.d.	n.d.	1.09 ± 0.16	0.86 ± 0.08
pyrraline	**7**	–	–	0.07 ± 0.02[c]	0.6 ± 0.1[c]
Ala-Pyrr	**7a**	n.d.[c]	n.d.[c]	1.06 ± 0.27[c]	8.8 ± 0.7[c]
Pyrr-Ala	**7b**	n.d.[c]	n.d.[c]	0.28 ± 0.08[c]	2.6 ± 0.2[c]
maltosine	**8**	–	–	0.02 ± 0.01[d]	0.07 ± 0.01[d]
Ala-Mal	**8a**	n.d.[d]	n.d.[d]	0.27 ± 0.08[d]	16 ± 2[d]
Mal-Ala	**8b**	n.d.[d]	n.d.[d]	0.16 ± 0.06[d]	2.7 ± 0.2[d]
MG-H1	**9**	–	–	0.09 ± 0.08	0.32 ± 0.04
Ala-(MG-H1)	**9a**	n.d.	n.d.	0.24 ± 0.11	10 ± 2
(MG-H1)-Ala	**9b**	n.d.	n.d.	0.22 ± 0.03	3.12 ± 0.17
argpyrimidine	**10**	–	–	0.01 ± 0.01	0.05 ± 0.02
Ala-Apy	**10a**	n.d.	n.d.	0.30 ± 0.02	1.79 ± 0.14
Apy-Ala	**10b**	n.d.	n.d.	0.14 ± 0.04	0.38 ± 0.03
pentosidine	**11**	–	–	0.03 ± 0.01	0.01 ± 0.01
[14C]Gly-Sar		2.79 ± 0.47	7.67 ± 0.16	–	–
[14C]mannitol		–	–	0.13 ± 0.03[c]	0.23 ± 0.02
L-[3H]Lys		–	–	6.86 ± 0.15	4.01 ± 0.24

[a] Total cellular uptake at the end of the experiment (120 min). [b] Value from ref. [28]. [c] Value from ref. [25]. [d] Value from ref. [27]. n.d.: not detectable.

glycated amino acids by any of the Caco-2 amino acid transport systems.

The transport studies were then performed with glycated dipeptides. Caco-2 cells express membrane-bound peptidases, so the dipeptides were partly hydrolyzed in the donor compartment during the flux measurement. The fluxes and cellular uptakes reported in Table 2 therefore have to be regarded as minimum values. All MRPs except for fructoselysine appeared inside the cells and in the receiver compartment, but only the dipeptides of CML and CEL could be recovered, to small extents, in intact form. When calculated for the intact dipeptides, the flux rates of **4a**/**4b** and **5a**/**5b** are lower than that of the space marker. However, all dipeptides were hydrolyzed very quickly inside the cells, and the MRPs passed into the receiver compartment in the form of amino acids. Therefore, the fluxes are also calculated for the amino acids cleaved from the dipeptides (Table 2). Amino acids from the donor compartment cannot interfere with this calculation because they are not transported. The net flux rates of glycated amino acids, when applied as dipeptides, are increased by up to 80-fold relative to the free glycated amino acids (e.g., formyline **6**, 0.04 % $cm^{-2}h^{-1}$; **6** from **6a**, 3.37 % $cm^{-2}h^{-1}$). Most flux rates were higher than the flux of the [14C]mannitol space marker. This means that glycated dipeptides are absorbed into the cells, most probably by PEPT1, and hydrolyzed to the free modified amino acids and alanine by intracellular peptidases. The amino acids reach the basolateral compartment either through the action of different amino acid transporters or possibly by simple diffusion.

Pronounced differences with regard to the tendencies of the glycated amino acids to leave the cells were found. The total proportions of glycated dipeptides and amino acids inside the cells and in the receiver compartment after 120 min are shown in Figure 1. More than 50 % of the glycated amino acids hydrolyzed from the dipeptides of CML, CEL, maltosine, and MG-H1 after absorption remain in the Caco-2 cells. This underlines the transport capacity of PEPT1 as the likely responsible carrier, capable even of transporting its substrates uphill against a concentration gradient.[21] The MRPs face the next barrier, the basolateral cell membrane, not as dipeptides, but as amino acids. Hydrophobic glycated amino acids such as **6**, **7**, and **10** can pass through the basolateral membrane more quickly than **4**, **5**, **8**, or **9**, which are strongly retained, if not trapped, inside the cells. We assume that the strong retention, especially of hydrophilic amino acids, inside the cells is due to the fact that they have to diffuse through the basolateral membrane, a process that is easier for hydrophobic amino acids. The hydrophobic amino acids permeate to the basolateral cell side to a greater extent if their side chain modifications lack the capacity to donate hydrogen bonds, as is the case for formyline **6**, but not for **7**, **8**, and **10**. No correlation between the affinity of a dipeptide towards PEPT1 and the actual transport was found.

Conclusions

Free glycated amino acids are not inhibitors of the lysine transport system(s), nor are they transported in significant amounts across cell monolayers. In contrast, several glycated dipeptides are high-affinity inhibitors of [14C]Gly-Sar uptake. In particular, the results for the carboxyalkylated peptides (**4a**/**4b** and **5a**/**5b**) show that not only side chain hydrophobization but also the introduction of hydrophilic and charged carboxyl groups can lead to strong inhibitors of [14C]Gly-Sar uptake. Those derivatives that show high rates of [14C]Gly-Sar uptake inhibition and high flux rates across Caco-2 monolayers are most likely substrates of the intestinal proton-coupled peptide transporter PEPT1. Depending on the kind of modification and the peptide sequence, glycated peptides can be transported by PEPT1 into the cells, where they are rapidly hydrolyzed. After addition of glycated dipeptides to the apical compartment, no dipeptide derivatives were detected in the basolateral compartment, but free glycated amino acids were found. In particular, hydropho-

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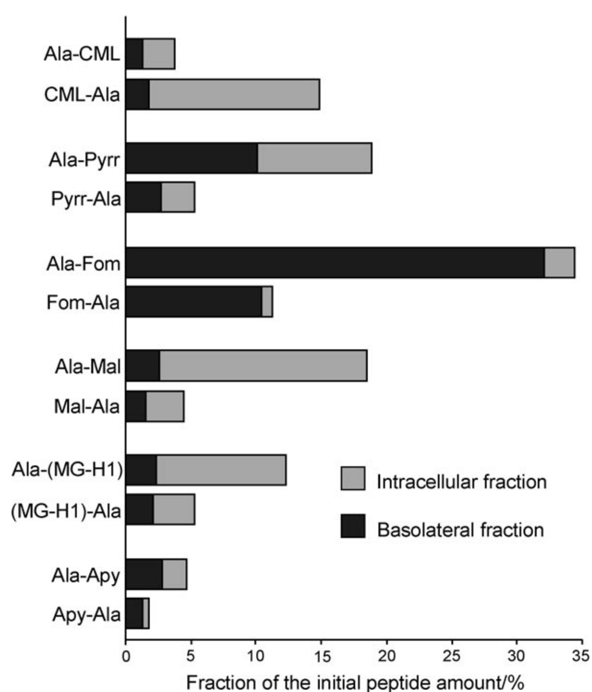

Figure 1. Total basolateral (■) and intracellular (■) proportions of glycated peptides and amino acids at 120 min after apical application of glycated peptides (1 mM). Molar amounts of glycated peptides and the corresponding glycated amino acids were summed, divided by the initial peptide amount, and expressed in %.

bic glycated amino acids formed in the later stages of the Maillard reaction can quickly permeate the basolateral cell membrane either by simple diffusion or through the action of amino acid transporters, whereas hydrophilic amino acids are released much more slowly. Uptake rates of different dipeptides of the same MRP differ by factors of up to 4. It is highly relevant whether MRPs are in the N- or the C-terminal position. These findings are of nutritional and physiological relevance and should be discussed as part of "risk assessment". The data show that free and dipeptide-bound Amadori products, which represent more than 90% of the MRPs detected in foods, are not taken up into cells in vitro. Others such as CML (**4**), CEL (**5**), and MG-H1 (**9**) are strongly retained inside the cells. Further studies should show whether these products are released very slowly or are irretrievably trapped inside epithelial cells until desquamation. In particular, few hydrophobic MRPs such as pyrraline (**7**), maltosine (**8**), formyline (**6**), or argpyrimidine (**10**) are transported through the cells. Because dietary pyrraline (**7**), but not fructoselysine (**1**), is to a large extent excreted in the urine, and because **7**, but not **1**, is absorbable in its peptide form,[23] it can be assumed that maltosine (**8**), formyline (**6**), and argpyrimidine (**10**) can also be absorbed after the digestion of glycated food proteins. Further research should focus on hepatic metabolism and renal handling. Digestibility studies with proteins modified by the Maillard reaction are required in order to provide information as to what extent MRPs appear in absorbable peptide forms, and whether they are hydrolyzed by luminal or membrane-bound peptidases. The longer these MRPs are peptide-bound during intestinal digestion and the more hydrophobic they are, the higher is the chance of their appearance in the circulation.

Experimental Section

Materials: Boc-Ala-OSu, L-arginine, di-*tert*-butyl dicarbonate, glyoxylic acid monohydrate, and 1,1-dimethoxyacetone were obtained from Fluka. Pd on activated charcoal (Pd/C, 10%, *w/w*), 3-chloropentane-2,4-dione, glycylsarcosine (Gly-Sar), and Ala-Lys were purchased from Sigma–Aldrich. Boc-Ala-Lys, Boc-Lys-Ala, Boc-Ala-Arg, and Fmoc-Arg-Ala were from IRIS Biotech (Martinsried, Germany). Microcrystalline cellulose (particle size 20–160 μm), pyruvic acid, and *N*,*N*-diisopropyethylamine (DIPEA) from Merck were used. Ala-O*t*Bu and Lys-Ala were purchased from Bachem (Bubendorf, Switzerland) and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TSTU) from Molekula (Taufkirchen, Germany). The following substances were synthesized by literature methods: 3-deoxyglucosone[6] (3-DG), fructoselysine[35] (**1**), 3-deoxypentosone[4] (3-DPs), lactuloselysine[4] (**2**), formyline[4] (**6**), and pentosidine[36] (**11**). The water used for the preparation of buffers and solutions was obtained by use of a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All other chemicals were purchased from standard suppliers and were of the highest purity available.

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell culture media, supplements, and trypsin solution were purchased from Invitrogen or PAA (Cölbe, Germany). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]Gly-Sar (specific radioactivity 56 mCi mmol^{-1}) and L-[4,5-^{3}H]lysine monohydrochloride (specific radioactivity 99 Ci mmol^{-1}) were synthesized by GE Healthcare. [^{14}C]Mannitol (specific radioactivity 53 mCi mmol^{-1}) was obtained from Hartmann Analytic (Braunschweig, Germany).

Thin layer chromatography: TLC was performed on Merck silica gel 60 plates with dichloromethane/methanol/25% aqueous NH_3 (2:2:1, *v/v/v*) as the mobile phase. Visualization was achieved by spraying the plates with a solution of ninhydrin in ethanol (0.1%) acidified with glacial acetic acid (3%, *v/v*) followed by heating until the appearance of spots. TLC plates were also used for the spotting test to identify target fractions after chromatographic separations. Each fraction (1 μL) was spotted onto the TLC plate and sprayed either with the ninhydrin reagent or with a solution of triphenyltetrazolium chloride (TTC, 1%) in NaOH (1 N).[4,35]

High-pressure liquid chromatography: All analytical HPLC analyses were performed with an Äkta 10XT high-pressure gradient system (Amersham Pharmacia Biotech, Uppsala, Sweden), consisting of a P-900 pump with an online degasser (Knauer, Berlin, Germany), a column oven, and a UV-900 UV detector. All separations were performed with a stainless steel column (150×4.6 mm) filled with Eurospher-100 RP-18 material of 5 μm particle size with an integrated guard column (5×4 mm) of the same material at a column temperature of 30 °C. The injection volume was 50 μL. A previously published solvent and gradient system permitted the separation of **6** from both its peptides; the measurements were performed at a wavelength of 293 nm.[4]

For the analyses of **10**, **11**, and the peptides **10a**/**10b**, the eluent was ammonium formate (pH 4.0, 10 mM), to which heptafluorobutyric acid (HFBA, 650 μL L^{-1}) was added (solvent A, final pH: 3.5). Solvent B consisted of a mixture of ammonium formate (pH 4.0, 50 mM, 200 mL) and methanol (800 mL), to which HFBA (650 μL L^{-1}) was added. A linear gradient from 3 to 40% B in 20 min and then to 80% B in 3 min was applied at a flow rate of

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1.0 mL min^{-1}. Argpyrimidine and its dipeptides were quantified with use of a fluorescence detector (F-1050, Merck Hitachi) at excitation and emission wavelengths of 320 and 380 nm, respectively. For pentosidine, the wavelengths were set at 335 and 385 nm, respectively. External calibration was performed with the synthesized standards.

Amino acid analysis (AAA): Flux measurements were performed with an amino acid analyzer (S 433, Sykam, Fürstenfeldbruck, Germany) and a PEEK column (150×4.6 mm) filled with the cation exchange resin LCA K07/Li (particle size, 7 μm). Lithium buffers were purchased ready for use from Sykam and employed for different gradient programs according to the manufacturer's instructions. The lithium system was also used for the determination of the purities of synthesized substances, which were injected at a concentration of 40 μg mL^{-1}. Flux samples of fructoselysine dipeptides additionally had to be analyzed with the Alpha Plus amino acid analyzer (LKB Biochrom, Cambridge, UK) and a PEEK column (190×4.6 mm) filled with a cation-exchange resin (particle size, 5 μm, K. Grüning, Olching, Germany). The conditions are available from the literature.[37] With both systems, post-column derivatization with ninhydrin was applied, and VIS detection was performed with integrated two-channel photometers working simultaneously at 440 nm and 570 nm. External calibration was performed with the synthesized standards. The injection volume was 50 μL.

Nuclear magnetic resonance spectroscopy, mass spectrometry, and elemental analysis (EA): Proton spectra were recorded with a Bruker DRX 500 instrument (Rheinstetten, Germany) at 500 MHz in D_2O as the solvent. Chemical shifts are given in parts per million (ppm) relative to the internal HOD signal (4.70 ppm). For ESI-MS, a PerSeptive Biosystems Mariner time-of-flight mass spectrometer fitted with an electrospray ionization source (ESI-TOF-MS, Applied Biosystems) working in the positive mode was used. Calibration of the mass scale was established with a mixture of bradykinin, angiotensin I, and neurotensin. After appropriate dilution with formic acid (1%) in aqueous acetonitrile (50%), the samples were injected into the ESI source by syringe pump at a flow rate of 5 μL min^{-1}. EA data were obtained with a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy). Elemental analysis was used to calculate the product contents of the preparations. The percentage of nitrogen in the preparation was divided by the theoretical percentage of nitrogen of the target substance and the content was expressed in per cent by weight. All data relating to the characterization of synthesis products are given in the Supporting Information.

Synthesis of ligands—general procedures: Purification of ligands was performed by ion-exchange chromatography (IEC) with the strongly acidic cation exchange resin DOWEX 50 WX-8 (200–400 mesh) unless otherwise stated. Before use, the resin was activated with three times its volume both of HCl (6 N) and of water. When the resin was to be used in the H^+ form, the material was placed in a suitable Econo glass column (BioRad, Munich, Germany) and equilibrated with three times its volume of HCl (0.01 N). When it was to be used in the Na^+ form, the activated resin was rinsed with three times its volume both of NaOH (1 N) and of water, placed in a suitable column, and equilibrated with three times its volume of sodium citrate buffer (pH 3.00, 0.1 N). When the resin was required in its pyridinium (Py^+) form, it was rinsed with three times its volume both of aqueous pyridine (2 M) and of water, placed in a suitable column, and equilibrated with three times its volume of pyridinium formate buffer (pH 3.00, 0.1 N). The synthesis mixtures were dissolved in equilibration buffer (30 mL) and applied to the column after adjustment of the pH to 3.0. After rinsing of the resin with a small volume of equilibration buffer, the products were eluted by gravity with the elution buffers stated below at flow rates of 0.2–0.4 mL min^{-1}. Fractions (10 mL) were collected with a fraction collector (RediFrac, Amersham Pharmacia Biotech) and the presence of the product was monitored by spotting of the fractions (1 μL) on TLC plates and spraying with the appropriate reagent(s). Fractions containing the target product were repeatedly concentrated to dryness and taken up in water until the smell of pyridine or HCl had become imperceptible. Combined fractions containing sodium citrate buffer were first desalted as described earlier.[27] All products were stored at −20 °C.

Ala-FL (1 a) and FL-Ala (1 b): Boc-Ala-Lys (507.3 mg, 1.6 mmol), and D-glucose (1.73 g, 9.6 mmol) were dissolved in dry methanol (90 mL) and heated under reflux for 4 h. The methanol was then evaporated in vacuo and the residue was taken up in water (150 mL). The pH was adjusted to 2.0 and the solution was transferred to a column (2.5×20 cm) filled with the cation exchanger Lewatit S100, previously equilibrated with HCl (6 M) and water (each 250 mL). The column was washed with water (300 mL) to remove the sugar, and the product was eluted with NH_3 (2 M, 300 mL) after overnight incubation.[35] Ammonia was removed with the aid of a rotary evaporator and the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. Compound **1 a** was eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 230–340 mL), as revealed by the spotting test (TTC and ninhydrin). The product was dissolved in methanol (2–3 mL) and precipitated in ice-cold butanone as described in the literature[4,35] to yield **1 a** as a white solid (384.7 mg, 53.2%).

For compound **1 b**, Boc-Lys-Ala (506 mg, 1.59 mmol) and D-glucose (1.72 g, 9.6 mmol) were dissolved in water (50 mL). After freeze-drying, the lyophilizate was incubated for 4 h at 70 °C in a sand bath in a drying oven. The synthesis mixture was worked up as described for **1 a**. Compound **1 b** was eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 350–550 mL) and was isolated as a white solid after precipitation in ice-cold butanone (309.3 mg, 49.4%).

Tagatoselysine (3): Boc-Lys-OH (621.7 mg, 2.5 mmol) and D-galactose (2.71 g, 15.1 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF)/methanol (3:7, *v*/*v*, 100 mL) and heated under reflux for 2 h. The solvents were then evaporated in vacuo with repeated addition of water. Removal of the Boc protecting group and the excess of the sugar were performed by IEC as described for **1 a**. Ammonia was removed with the aid of a rotary evaporator and the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. The spotting test (ninhydrin, TTC) revealed that **3** eluted with pyridinium acetate buffer (pH 4.35, 0.4 N, 160–250 mL). The product was precipitated in ice-cold butanone as described above, to yield **3** as a white solid (420.6 mg, 43.4%).

CML (4), Ala-CML (4 a), CML-Ala (4 b), CEL (5), Ala-CEL (5 a), and CEL-Ala (5 b): These syntheses were performed by reductive alkylation of lysine derivatives with α-keto acids.[28] The amounts of reactants given below were dissolved in water (30 mL) and the pH of the solutions was adjusted to 8.75 with NaOH (1 M) prior to the addition of Pd catalyst. The mixture was hydrogenated at room temperature (RT) and atmospheric pressure for 24 h. During the syntheses of **5**, **5 a**, and **5 b**, the H_2 was renewed and hydrogenation was continued for further 24 h. The catalyst was then filtered off. Boc-CML and Boc-CEL were dissolved in HCl (3 M) to concentrations of 10–20 mg mL^{-1} and heated under reflux for 3 h to remove the Boc protecting group. The protected peptides were dissolved in aqueous acetic acid (10%, 1000 mL) and heated under reflux for 4 h at 70 °C.[6] After the removal of the acids with the aid of a rotary evaporator, the products were subjected to IEC on a column (1.5×50 cm) in the H^+ form. For the elution, a step gradient of ascending HCl concentrations (1, 1.5, 2 M HCl, each 300 to 600 mL) was applied. The spotting test (ninhydrin) showed that the products were well resolved from their educts and usually eluted with 1.5–2 M HCl. After the evaporation of HCl, the products were

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lyophilized to yield the hydrochlorides as slightly yellow amorphous powders.

CML (4): Boc-Lys (1000 mg, 4.1 mmol), glyoxylic acid (480 mg, 5.2 mmol), Pd/C (50 mg). Yield 517 mg (46.4 %).

Ala-CML (4 a): Boc-Ala-Lys (500 mg, 1.6 mmol), glyoxylic acid (187 mg, 2.0 mmol), Pd/C (38 mg). Yield 369 mg (60.7 %).

CML-Ala (4 b): Boc-Lys-Ala (500.3 mg, 1.6 mmol), glyoxylic acid (710.8 mg, 7.5 mmol), Pd/C (50.6 mg). Yield 183.4 mg (31.2 %).

CEL (5): Boc-Lys (1243 mg, 5.1 mmol), pyruvic acid (586 mg, 6.5 mmol), Pd/C (84.0 mg). Yield 980 mg (61.5 %).

Ala-CEL (5 a): Boc-Ala-Lys (500.0 mg, 1.6 mmol), pyruvic acid (357 mg, 4.0 mmol), Pd/C (34 mg). Yield 430.0 mg (66.9 %).

CEL-Ala (5 b): Boc-Ala-Lys (500 mg, 1.6 mmol), pyruvic acid (695 mg, 7.7 mmol), Pd/C (35 mg). Yield 377 mg (58.4 %).

Ala-Fom (6 a) and Fom-Ala (6 b): For **6 a**, Boc-Ala-Lys (486 mg, 1.5 mmol) and 3-DPs (1000 mg) were dissolved in water (5.6 mL). The solution was mixed with cellulose (4.5 g), and after lyophilization the mixture was incubated at 70 °C in a drying oven. The brown cake was extracted with water (3×100 mL). The pH of the combined aqueous phases was adjusted to 1.0, and the solution was extracted with ethyl acetate (3×100 mL). The pH of the solution was adjusted to 4.5, and the extraction was repeated (3×100 mL ethyl acetate). The combined organic layers were evaporated to dryness with the aid of a rotary evaporator. Boc-protected intermediates were deprotected as described above for carboxyalkylated peptides. After evaporation of acetic acid, the residue was subjected to IEC on a column (1.5×20 cm) in the Py^+ form. Elution was performed first with pyridinium formate buffer (pH 3.75, 0.3 N, 250 mL) and then with pyridinium formate buffer (pH 4.05, 0.3 N). Compound **6 a** eluted between 20 and 140 mL of the second buffer. After evaporation, the residue was precipitated in butanone as described above, to yield **6 a** as a light yellow powder (73.4 mg, 15.2 %).

The synthesis of Fom-Ala (**6 b**) was performed in the same way, starting from Boc-Lys-Ala (452.7 mg, 1.4 mmol) and 3-DPs (750 mg) dissolved in water (4.8 mL) and deposited on cellulose (3.8 g). During IEC, compound **6 b** eluted with 15–200 mL of the second elution buffer. Precipitation in butanone provided **6 b** as a light yellow powder (56.8 mg, 12.4 %).

MG-H1 (9), Ala-(MG-H1) (9 a), and (MG-H1)-Ala (9 b): L-Arginine (1.009 g, 5.8 mmol) and 1,1-dimethoxyacetone (**14**, 678 μL, 5.7 mmol) were dissolved in HCl (12 M, 110 mL). After the system had been stirred at RT for 8 h, water (200 mL) was added and the solution was concentrated to dryness in vacuo. The dried residue was taken up in water (150 mL), and the pH was adjusted to 2.0. The solution was transferred to a column (2.5×20 cm) filled with the cation exchanger Lewatit S100, previously equilibrated with HCl (6 M) and water (each 250 mL). The column was washed with water (300 mL) to remove uncharged byproducts, and the product was eluted immediately with HCl (4 M, 300 mL). After evaporation of the acid, the residue was subjected to IEC on a column (1.5×50 cm) in the Na^+ form. Elution was first performed with sodium citrate buffer (pH 4.50, 0.2 N, 300 mL) and then with sodium citrate buffer (pH 5.28, 0.3 N). MG-H1 (**9**) eluted with the second buffer (180–330 mL). After desalting and lyophilization, off-white amorphous **9** was obtained (794.4 mg, 41.5 %).

Ala-(MG-H1) (**9 a**) was prepared accordingly, from a mixture of **12** (78.5 mg, 0.23 mmol) and **14** (28.0 μL, 0.23 mmol) in HCl (12 M, 9 mL), which was stirred at RT for 4 h. After the removal of byproducts as described for **9**, the residue was subjected to IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium acetate buffer (pH 4.35, 0.4 N, 400 mL), and then with pyridinium acetate buffer (pH 5.00, 0.4 N). Compound **9 a** eluted with the second buffer (140–200 mL). After evaporation of the buffer, the residue was lyophilized, to yield **9 a** as a light brownish powder (24.7 mg, 27.2 %).

(MG-H1)-Ala (**9 b**) was prepared accordingly, from a mixture of **13** (81.6 mg, 0.23 mmol) and **14** (26.6 μL, 0.22 mmol) in HCl (12 M, 9 mL), which was stirred at RT for 4 h. During IEC, **9 b** eluted with the second elution buffer (270–400 mL). After the removal of the buffer, the product was lyophilized, to yield **9 b** as a light yellow powder (27.1 mg, 31.9 %).

Argpyrimidine (10): 3-Chloropentane-2,4-dione (**15**, 1.68 mL, 14.9 mmol) was dissolved in DMSO (25 mL), and anhydrous sodium acetate (2.44 g, 29.7 mmol) was added.[31] After the mixture had been stirred for 3 h at RT, water was added (200 mL). The mixture was extracted with diethyl ether (5×100 mL) after cooling. The extracts were dried ($MgSO_4$) and concentrated to dryness. The remaining brownish oil was subjected to flash chromatography (FC) on silica gel (20 g) with petroleum ether (40–60 °C)/ethyl acetate (8:2, *v/v*). Target fractions of 3-acetoxypentane-2,4-dione **16** eluted between 80–170 mL as revealed by the spotting test (TTC). The slightly red oil that remained after evaporation of the solvents was immediately added to a solution of L-arginine (1.047 g, 6.0 mmol) in HCl (12 M, 35 mL).[30] A second portion of **16** was added after 3 h and the mixture was stirred at RT for 20 h. The solution was then diluted with water (200 mL) and extracted with diethyl ether (3×100 mL). The aqueous phase was concentrated to dryness and the residue was subjected to FC on silica gel (30 g) with methanol/ethyl acetate (2:1, *v/v*).[9] TLC of the fractions revealed that **10** eluted between 60–180 mL ($R_f = 0.77$). After evaporation of the solvents, argpyrimidine was isolated by IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium formate buffer (pH 4.05, 0.3 N, 200 mL), and then with pyridinium acetate buffer (pH 4.35, 0.4 N). Compound **10** eluted with the second buffer (380–600 mL). After buffer removal, the residue was precipitated in butanone as described for **1 a**, to yield **10** as a white powder (468.0 mg, 29.7 %).

Ala-Apy (10 a) and Apy-Ala (10 b): Boc-Ala-OSu (840.9 mg, 2.94 mmol), compound **10** (199.1 mg, 0.78 mmol), and DIPEA (330 μL, 1.9 mmol) were dissolved in a mixture of DCM (20 mL) and methanol (10 mL). After overnight stirring at RT, the solvents were removed under reduced pressure. The residue was dissolved in $NaHCO_3$ solution (5 %, 50 mL) and extracted with ethyl acetate (3×50 mL). The pH was adjusted to 1.0 with HCl (6 M), and the aqueous phase was extracted with ethyl acetate (3×100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated to dryness. The protected derivative was dissolved in HCl (6 M)/tetrahydrofuran (1:1, *v/v*, 30 mL) and stirred at RT for 1 h.[38] The solvents were removed with the aid of a rotary evaporator. Compound **10 a** was then isolated by IEC on a column (1.5×50 cm) in the Py^+ form. Elution was performed first with pyridinium acetate buffer (pH 4.35, 0.4 N, 300 mL) and then with pyridinium acetate buffer (pH 5.00, 0.4 N). Compound **10 a** eluted with the second buffer (440–650 mL). After the removal of the buffer, the residue was lyophilized, to yield **10 a** as a white powder (53.3 mg, 17.9 %).

For **10 b**, compound **10** (162.7 mg, 0.64 mmol) was dissolved at 2 °C in a mixture of tetrahydrofuran (10 mL) and Na_2CO_3 solution (0.5 M, 10 mL). Di-*tert*-butyl dicarbonate (560 mg, 1.28 mmol) was then added slowly. The cooling bath was removed and the mixture was stirred for 2 h at RT. After removal of the solvents, the residue was partitioned between water (30 mL) and ethyl acetate (10 mL). The pH was adjusted to 2.4 and the aqueous phase was extracted with ethyl acetate (3×30 mL). The combined organic layers were extracted with water (2×20 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The residue, consisting of Boc-argpyrimidine, was taken up in DCM (5 mL), and DIPEA (329 μL, 1.9 mmol) and TSTU (232 mg, 0.77 mmol) were added. The mixture was

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stirred at RT for 20 min. A portion of Ala-O*t*Bu hydrochloride (261.0 mg, 1.4 mmol) in DCM (5 mL) was then added, and the solution was stirred for 30 min at RT. DCM was removed and the residue was dissolved in diethyl ether (100 mL) and extracted with HCl (1 M, 2×50 mL), $NaHCO_3$ solution (5%, 2×50 mL), and water (2×50 mL). The organic layer was concentrated under reduced pressure and the residue was deprotected and isolated as described for **10a**. Compound **10b** eluted with the second elution buffer (630–880 mL). After the removal of the buffer, the residue was lyophilized, to yield **10b** as a white powder (32.7 mg, 11.9%).

Ala-Arg (12) and Arg-Ala (13): Boc-Ala-Arg (509.7 mg, 1.4 mmol) was deprotected as described for **10a**. The dried residue was subjected to IEC on a column (1.5×50 cm) in the Na^+ form. The column was first rinsed with sodium citrate buffer (pH 5.35, 0.3 N, 400 mL), and then with sodium citrate buffer (pH 6.00, 0.3 N, 400 mL). The product was then eluted with sodium citrate buffer (pH 6.00, 0.5 N, 300 mL). After desalting, off-white amorphous **12** was obtained (452.6 mg, 92.7%).

For **13**, Fmoc-Arg-Ala (704.8 mg, 1.5 mmol) was dissolved in DMF/methanol/morpholine (72:8:20, *v*/*v*/*v*, 100 mL). The mixture was stirred at RT for 1 h and then concentrated to dryness. The residue was partitioned between water (100 mL) and diethyl ether (50 mL). The aqueous phase was extracted with diethyl ether (2×50 mL) and then concentrated to dryness. The residue was subjected to IEC on a column (1.5×50 cm) in the Na^+ form. After rinsing of the column with sodium citrate buffer (pH 6.00, 0.3 N, 500 mL), the product was eluted with sodium citrate buffer (pH 6.00, 0.5 N). TLC showed a chromatographically pure fraction eluting between 180–310 mL ($R_f = 0.27$). After desalting, off-white amorphous **13** was obtained (270.4 mg, 49.4%).

Cell culture: Caco-2 cells were routinely cultured in culture flasks (75 cm^2) with minimum essential medium supplemented with fetal bovine serum (10%), gentamicin (50 $\mu g\,mL^{-1}$), and nonessential amino acid solution (1%) at 37 °C under a humidified atmosphere [CO_2 (5%), O_2 (95%)].[25,28,33] Cultures with a confluence of 80% were treated for 5 min with Dulbecco's phosphate-buffered saline followed by a 2 min incubation with trypsin solution. For uptake experiments, the 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 by a Pierce Protein Assay (660 nm, Thermo Fisher Scientific) by the manufacturer's protocol.

For the flux measurements, Caco-2 cells were cultured on permeable polycarbonate Transwell cell culture inserts (diameter 24 mm, pore size 3 μm, Costar, Bodenheim, Germany) with a cell density of 0.2×10^6 cells per filter for 21 days.[25,28] The lower (receiver) compartment contained medium (2.6 mL) and the upper (donor) compartment medium (1.5 mL). The transepithelial electrical resistance was measured at day 21 with a Millicell ERS (Millipore Intertech).

Transport studies: Uptake of [^{14}C]Gly-Sar and L-[^{3}H]lysine into Caco-2 cells cultured on plastic dishes was measured at RT as described earlier.[25,28,33] The uptake buffer contained Mes/Tris (pH 6.0, 25 mM), NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), glucose (5 mM), [^{14}C]Gly-Sar (10 μM), or L-[^{3}H]lysine (2 nM), together with unlabeled compounds at increasing concentrations (0–10 mM). After incubation either for 10 min ([^{14}C]Gly-Sar uptake) or for 5 min (L-[^{3}H]lysine uptake), the cells were quickly washed four times, dissolved in Igepal CA-630 buffer, and prepared for liquid scintillation spectrometry. The nonsaturable component of [^{14}C]Gly-Sar and L-[^{3}H]lysine uptake (diffusion, adherent radioactivity) determined by measuring the uptake of the labeled compound in the presence of the unlabeled compound [Gly-Sar (50 mM) or L-lysine (20 mM)] represented 8% and 21% of the total uptake, respectively. This value was used during nonlinear regression analysis of inhibition constants.[25]

Transepithelial flux experiments at Caco-2 cell monolayers were performed at day 21 after seeding at 37 °C in a shaking table incubator.[25,28,33] In brief, after washing of the inserts with buffer [Hepes/Tris (pH 7.5, 25 mM), NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), glucose (5 mM)], uptake was started by addition of uptake buffer (pH 6.0, 1.5 mL) containing compounds (1 mM) to the donor side. After 10, 30, 60, and 120 min, samples (200 μL) were taken from the receiver compartment (2.6 mL) and replaced with fresh buffer (pH 7.5). Samples were stored at −20 °C until analysis by HPLC. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic inserts and stored in TCA solution (10%, 1 mL), which was frozen and defrosted three times. Before HPLC and AAA, the samples were diluted appropriately with the solvent A and loading buffers, respectively.

Data analysis: Results are given as means ±SEM ($n=4$ to 9). IC_{50} values (that is, concentration of unlabeled compounds necessary to inhibit 50% of [^{14}C]Gly-Sar or L-[^{3}H]lysine carrier-mediated uptake) were determined by nonlinear regression by using the logistic Equation (1) for an asymmetric sigmoid (allosteric Hill kinetics):

$$Y = \mathrm{Min} + \frac{\mathrm{Max}-\mathrm{Min}}{1+(X/\mathrm{IC}_{50})^P} \quad (1)$$

where Max is the initial Y value, Min the final Y value, and the power P represents Hill's coefficient (SigmaPlot program, Systat, Erkrath, Germany), and converted into inhibition constants (K_i).[33] Flux data were calculated after correction for the removed amounts by linear regression of appearance in the receiver well versus time.

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[1] T. Henle, *Amino Acids* **2005**, *29*, 313 – 322; b) F. Ledl, E. Schleicher, *Angew. Chem.* **1990**, *102*, 597 – 626; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 565 – 594.
[2] M. U. Ahmed, S. R. Thorpe, J. W. Baynes, *J. Biol. Chem.* **1986**, *261*, 4889 – 4894.
[3] M. U. Ahmed, E. Brinkmann Frye, T. P. Degenhardt, S. R. Thorpe, J. W. Baynes, *Biochem. J.* **1997**, *324*, 565 – 570.
[4] M. Hellwig, T. Henle, *Eur. Food Res. Technol.* **2010**, *230*, 903 – 914.
[5] T. Nakayama, F. Hayase, H. Kato, *Agric. Biol. Chem.* **1980**, *44*, 1201 – 1202.
[6] T. Henle, A. Bachmann, *Z. Lebensm.-Unters. Forsch.* **1996**, *202*, 72 – 74.
[7] F. Ledl, H. Osiander, O. Pachmayr, T. Severin, *Z. Lebensm.-Unters. Forsch.* **1989**, *188*, 207 – 211.
[8] T. Henle, A. W. Walter, R. Haeßner, H. Klostermeyer, *Z. Lebensm.-Unters. Forsch.* **1994**, *199*, 55 – 58.

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[9] I. N. Shipanova, M. A. Glomb, R. H. Nagaraj, *Arch. Biochem. Biophys.* **1997**, *344*, 29 – 36.

[10] a) D. R. Sell, V. M. Monnier, *J. Biol. Chem.* **1989**, *264*, 21 597 – 21 602; b) S. J. Cho, G. Roman, F. Yeboah, Y. Konishi, *Curr. Med. Chem.* **2007**, *14*, 1653 – 1671.

[11] T. Henle, *Kidney Int.* **2003**, *63*, S145-S147.

[12] I. Birlouez-Aragon, G. Saavedra, F. J. Tessier, A. Galinier, L. Ait-Aimeur, F. Lacoste, C. N. Niamba, N. Alt, V. Somoza, J. M. Lecerf, *Am. J. Clin. Nutr.* **2010**, *91*, 1220 – 1226.

[13] T. Koschinsky, C.-J. He, T. Mitsuhashi, R. Bucala, C. Liu, C. Bünting, K. Heitmann, H. Vlassara, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6474 – 6479.

[14] a) T. Henle, *Mol. Nutr. Food Res.* **2007**, *51*, 1075 – 1078; b) N. Ahmed, P. J. Thornalley, *Diabetes Obes. Metab.* **2007**, *9*, 233 – 245.

[15] K. Šebeková, V. Somoza, *Mol. Nutr. Food Res.* **2007**, *51*, 1079 – 1084.

[16] G. Cohen, G. Glorieux, P. Thornalley, E. Schepers, N. Meert, J. Jankowski, V. Jankowski, A. Argiles, B. Anderstam, P. Brunet, C. Cerini, L. Dou, R. Deppisch, B. Marescau, Z. Massy, A. Perna, J. Raupachova, M. Rodriguez, B. Stegmayr, R. Vanholder, W. H. Hörl, *Nephrol. Dial. Transplant.* **2007**, *22*, 3381 – 3390.

[17] J. Uribarri, S. Woodruff, S. Goodman, W. Cai, X. Chen, R. Pyzik, A. Yong, G. E. Striker, H. Vlassara, *J. Am. Diet. Assoc.* **2010**, *110*, 911 – 916.

[18] J. M. Ames, *Mol. Nutr. Food Res.* **2007**, *51*, 1085 – 1090.

[19] S. A. Adibi, D. W. Mercer, *J. Clin. Invest.* **1973**, *52*, 1586 – 1594.

[20] R. Devés, C. A. R. Boyd, *Physiol. Rev.* **1998**, *78*, 487 – 545.

[21] H. Daniel, *Annu. Rev. Physiol.* **2004**, *66*, 361 – 384.

[22] a) P. D. Bailey, C. A. Boyd, J. R. Bronk, I. D. Collier, D. Meredith, K. M. Morgan, C. S. Temple, *Angew. Chem.* **2000**, *112*, 515 – 518; *Angew. Chem. Int. Ed.* **2000**, *39*, 505 – 508; b) C. U. Nielsen, R. Andersen, B. Brodin, S. Frokjaer, M. E. Taub, B. Steffansen, *J. Controlled Release* **2001**, *76*, 129 – 138; c) T. Terada, K. Inui, *Curr. Drug Metab.* **2004**, *5*, 85 – 94; d) M. Brandsch, I. Knütter, F. H. Leibach, *Eur. J. Pharm. Sci.* **2004**, *21*, 53 – 60; e) I. Rubio-Aliaga, H. Daniel, *Xenobiotica* **2008**, *38*, 1022 – 1042; f) B. S. Vig, T. R. Stouch, J. K. Timoszyk, Y. Quan, D. A. Wall, R. L. Smith, T. N. Faria, *J. Med. Chem.* **2006**, *49*, 3636 – 3644.

[23] A. Förster, Y. Kühne, T. Henle, *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 474 – 481.

[24] K. Šebeková, G. Saavedra, C. Zumpe, V. Somoza, K. Klenoviscová, I. Birlouez-Aragon, *Ann. N.Y. Acad. Sci.* **2008**, *1126*, 177 – 180.

[25] M. Hellwig, S. Geissler, A. Peto, I. Knütter, M. Brandsch, T. Henle, *J. Agric. Food Chem.* **2009**, *57*, 6474 – 6480.

[26] S. Geissler, M. Hellwig, M. Zwarg, F. Markwardt, T. Henle, M. Brandsch, *J. Agric. Food Chem.* **2010**, *58*, 2543 – 2547.

[27] S. Geissler, M. Hellwig, F. Markwardt, T. Henle, M. Brandsch, *Eur. J. Pharm. Biopharm.* **2011**, *78*, 75 – 82.

[28] S. Grunwald, R. Krause, M. Bruch, T. Henle, M. Brandsch, *Br. J. Nutr.* **2006**, *95*, 1221 – 1228.

[29] I. Knütter, B. Hartrodt, S. Theis, M. Foltz, M. Rastetter, H. Daniel, K. Neubert, M. Brandsch, *Eur. J. Pharm. Sci.* **2004**, *21*, 61 – 67.

[30] N. Sreejayan, X. P. Yang, K. Palanichamy, K. Dolence, J. Ren, *Eur. J. Pharmacol.* **2008**, *593*, 30 – 35.

[31] L. Valgimigli, G. Brigati, G. F. Pedulli, G. A. DiLabio, M. Mastragostino, C. Arbizzani, D. A. Pratt, *Chem. Eur. J.* **2003**, *9*, 4997 – 5010.

[32] M. Brandsch, *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 887 – 905.

[33] I. Knütter, G. Kottra, W. Fischer, H. Daniel, M. Brandsch, *Drug Metab. Dispos.* **2009**, *37*, 143 – 149.

[34] R. Tateoka, H. Abe, S. Miyauchi, S. Shuto, A. Matsuda, M. Kobayashi, K. Miyazaki, N. Kamo, *Bioconjugate Chem.* **2001**, *12*, 485 – 492.

[35] R. Krause, K. Knoll, T. Henle, *Eur. Food Res. Technol.* **2003**, *216*, 277 – 283.

[36] T. Henle, U. Schwarzenbolz, H. Klostermeyer, *Z. Lebensm.-Unters. Forsch.* **1997**, *204*, 95 – 98.

[37] T. Henle, H. Walter, I. Krause, H. Klostermeyer, *Int. Dairy J.* **1991**, *1*, 125 – 135.

[38] M. A. Glomb, C. Pfahler, *J. Biol. Chem.* **2001**, *276*, 41638 – 41647.

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ARTICLE

Transport of Free and Peptide-Bound Pyrraline at Intestinal and Renal Epithelial Cells

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Pyrraline is a quantitatively dominating glycation compound of the advanced Maillard reaction in foods and can be found in urine after consumption of pyrraline-containing food items. The purpose of this study was to investigate the transport of pyrraline and its dipeptide derivatives alanylpyrraline (Ala-Pyrr) and pyrralylalanine (Pyrr-Ala) at intestinal and renal cell lines. Pyrraline inhibited the L-[^{3}H]lysine uptake with IC_{50} values of 0.3 mM (Caco-2 cells) and 3.5 mM (OK cells), respectively, but not the uptake of [^{14}C]Gly-Sar (Caco-2 and SKPT cells). In contrast, Ala-Pyrr strongly inhibited the uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells with IC_{50} values of 0.19 and 0.017 mM, respectively. Pyrr-Ala inhibited the carrier-mediated uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells by 50% at concentrations of 0.03 and 0.008 mM, respectively. The transepithelial flux of peptide-bound pyrraline across Caco-2 cell monolayers was up to 15-fold higher compared to the flux of free pyrraline. We conclude that free pyrraline is not a substrate for the intestinal lysine transporter and that the absorption of dietary pyrraline occurs most likely in the form of dipeptides rather than as the free amino acid.

KEYWORDS: Glycation; Maillard reaction; AGE; pyrraline; membrane transport; intestine; kidney; absorption

INTRODUCTION

The Maillard reaction, often also termed nonenzymatic browning or glycation, is of utmost importance for the formation of flavor and color during thermal processing of food and furthermore influences the nutritional quality of stored foodstuffs. The reaction mainly occurs between reducing carbohydrates and their degradation products and the ε-amino group of lysine, the N-termini of proteins, and the guanidino group of arginine (*1*). In addition to the Amadori compounds (e.g., N^{ε}-fructosyllysine and N^{ε}-lactulosyllysine), which are predominantly formed in the early course of the reaction cascade, several structures from later stages of the reaction called "advanced glycation end products" (AGEs) have been identified in foods (*2*), among them pyrraline (6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin), which is generated during the reaction of 3-deoxy-D-*erythro*-hexos-2-ulose (3-deoxyglucosulose, 3-DG) with the ε-amino group of lysine (*3*). Pyrraline was first isolated by Nakayama et al. (*4*) and was shown to have low mutagenic activity (*5*) and antinutritional properties due to the inhibition of intestinal peptidases (*6*). Pyrraline, as quantified in food by amino acid analysis (*7*), can be found predominantly on sites of high thermal impact and low moisture content, like in bread crust, rusk, or crackers, in which its content may range up to 3680 mg per kg of protein. Other important sources are dried foodstuffs like milk and whey powder (*7*) and pasta products (*8*) with concentrations varying between 90 and 150 mg per kg of protein. The daily intake of AGEs (mainly pyrraline and CML) has been estimated to be 25−75 mg (*2*).

It is currently under debate whether dietary AGEs represent a risk to human health (*9*). Dietary AGEs are reported to promote oxidative stress and to aggravate the sequelae of diabetes and uremia (*10*). A reduced intake of AGEs from food is advised in order to prevent age-related diseases like diabetes (*11*, *12*), often presuming general absorption of AGEs from the diet with a consequential increase of inflammatory markers reflecting a low-grade systemic inflammation (*11*, *13*). On the other hand, no significant proof could yet be given if the accumulation in vivo of AGEs in the course of the diseases mentioned is a causative or concomitant effect (*14*).

To elicit effects within the human body, however, absorption in effective amounts across the intestinal epithelial barrier is required. In contrast to their relevance in conventional nutrition and their possible pathophysiological role, quite little is known about the "metabolic fate" of AGEs. A balance study with 18 healthy volunteers showed individual absorption and elimination properties of individual AGEs (*15*, *16*). After the consumption of a test meal containing N^{ε}-fructosyllysine, pyrraline, and pentosidine within a food matrix, between 50% and 100% of the administered protein-bound pyrraline was found as the free amino acid in the urine together with 60% of pentosidine administered as the free amino acid, while only less than 5% of

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the initially protein-bound pentosidine and N^{ε}-fructosyllysine were recovered.

Hypothetically, the compounds can cross the intestinal epithelial barrier via the paracellular route (simple diffusion) or transcellularly by diffusion, endocytotic processes, or mediation by transport proteins. Possible candidates for carrier-mediated transport are the naturally occurring carriers for amino acids, sugars, or peptides. We have shown recently that N^{ε}-carboxymethyllysine, N^{α}-hippuryl-N^{ε}-fructosyllysine, N^{α}-hippuryl-N^{ε}-carboxymethyllysine, and N^{ε}-fructosyllysine are neither transported by PEPT1 nor transported by carriers for neutral amino acids (*17*). The low transepithelial flux from the apical to the basolateral side of Caco-2 cell monolayers measured for these compounds occurred most likely by simple diffusion.

The situation might be very different for dipeptide derivatives of AGEs. In this study, we investigated whether pyrraline or the dipeptide derivatives alanylpyrraline (Ala-Pyrr) and pyrralyl-alanine (Pyrr-Ala) are potential substrates for lysine or peptide transporters. It has been established in recent years that at enterocytes three different transporters of the solute carrier (SLC) superfamily are able to translocate lysine across the apical membrane into the cells, namely, the systems $B^{0,+}$, $b^{0,+}$ and y^{+}. Di- and tripeptides are transported by PEPT1. This transporter is driven by a transmembrane H^{+} gradient and catalyzes the cotransport of its substrates with H^{+} into intestinal and other cells (for a review see ref *18*). PEPT1 accepts many amino acid derivatives and modified dipeptides as substrates as long as the structural requirements for substrate binding and translocation are met (for a review see ref *19*).

To study the intestinal and renal transport, pyrraline and its alanyl dipeptide derivatives were synthesized and characterized spectroscopically. Alanine was chosen as “partner” for pyrraline because it represents a common hydrophobic amino acid in food proteins. Furthermore, alanine-containing dipeptides generally are used for investigating structural requirements of peptide carriers (*20*). In competition assays versus radiolabeled lysine and Gly-Sar, their interaction with the carriers responsible for the uptake of cationic amino acids and dipeptides was determined in Caco-2, SKPT, and OK cells. Moreover, we measured the total transepithelial net flux across monolayers of the human colon cell line Caco-2 and the renal cell line OK.

MATERIALS AND METHODS

Materials. N^{α}-*tert*-Butoxycarbonyl-L-lysine (N^{α}-Boc-lysine) was obtained from Fluka (Steinheim, Germany). N^{ε}-*tert*-Butoxycarbonyl-L-alanyl-L-lysine (Boc-Ala-Lys) and N^{ε}-*tert*-butoxycarbonyl-L-lysyl-L-alanine (Boc-Lys-Ala) were purchased from Iris Biotech (Martinsried, Germany). 3-Deoxyglucosulose (3-DG) was prepared according to Henle and Bachmann (*3*). HPLC gradient grade acetonitrile was from VWR Prolabo (Leuven, Belgium) and sodium 1-heptanesulfonate from Alfa Aesar (Karlsruhe, Germany). Microcrystalline cellulose (particle size 20−160 μm) from Merck (Darmstadt, Germany) was used. DOWEX AG 50W-X8 ion-exchange resin (100−200 mesh) was from Acros (Geel, Belgium). The water used for the preparation of buffers and solutions was obtained using a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany). All other chemicals were purchased from standard suppliers and were of the highest purity available.

The human colon carcinoma cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The renal cell line SKPT-0193 Cl.2, established from isolated cells of rat proximal tubules, was provided by U. Hopfer (Case Western Reserve University, Cleveland, OH; cf. ref *21*). The opossum kidney cell line OK was provided by H. Daniel (Molecular Nutrition Unit, Technical University of Munich, Germany). Cell culture media, supplements, and trypsin solution were purchased from Life Technologies, Inc. (Karlsruhe, Germany) or PAA (Pasching, Austria). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]glycylsarcosine (specific radioactivity 56 mCi/mmol) and L-[4,5-^{3}H]lysine monohydrochloride (specific radioactivity 99 Ci/mmol) were obtained from GE Healthcare (Little Chalfont, U.K.). [^{14}C]Mannitol (specific radioactivity 53 mCi/mmol) was obtained from Hartmann Analytic GmbH (Braunschweig, Germany).

High Pressure Liquid Chromatography (HPLC) Analysis of Pyrraline and Pyrraline Containing Dipeptides. All analytical 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 pyrraline and 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). An amount of 50 μL of the samples obtained from the cell culture experiments was injected. The column temperature was set to 30 °C, and UV detection was performed at 297 nm. The mobile phase consisted of 5 mM sodium heptanesulfonate, pH 2.0 (solvent A), and acetonitrile (solvent B). A linear gradient from 2% to 27% B in 23 min was used for the measurements of pyrraline and Ala-Pyrr containing samples (gradient A). The gradient for Pyrr-Ala samples was from 7% to 18.5% B in 35 min (gradient B). The flow rate was 1.0 mL/min. External calibration was performed with the synthesized standards.

The samples from the flux measurements were treated as follows: an amount of 50 μL of samples from the apical compartment was diluted with 450 μL of solvent A, and an amount of 50 μL of samples from the basolateral compartment was mixed with 100 μL of solvent A. The membrane samples were thawed and refrozen three times in order to completely release the cellular contents. A total of 300 μL of this solution was then added to 300 μL of solvent A.

Mass Spectrometry. For MS 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% acetonitrile, the sample was injected at a flow rate of 5 μL/min into the ESI source by a syringe pump. Spray tip potential, nozzle potential, quadrupole rf voltage, and detector voltage were set at 4812.3, 80, 1000, and 2400 V, respectively.

Nuclear Magnetic Resonance Spectrometry (NMR). ^{1}H NMR spectra were recorded on a Bruker DRX 500 instrument (Reinstetten, 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 Analysis. Elemental analysis data were obtained on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy).

Synthesis and Isolation of 6-(2-Formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin (Pyrraline). The synthesis method described by Henle and Bachmann (*3*) was followed with modified isolation of the synthesis product. Then 534 mg (2.2 mmol) of Boc-Lys-OH and 1400 mg (8.7 mmol) of 3-deoxyglucosulose (3-DG) were dissolved in 6.5 mL of 0.1 N sodium acetate buffer, pH 5.0, and the pH was adjusted to 5.0 with acetic acid. The solution was mixed with 5.2 g of cellulose powder and incubated for 4 h at 70 °C in a drying oven after lyophilization. The brown powder was then extracted three times with 100 mL portions of water. The pooled extracts were concentrated to 50 mL in vacuo at 40 °C. Isolation of crude Boc-pyrraline was performed by setting the pH value to 1.0 with sulfuric acid and extracting at least 4 times each with 50 mL of diethyl ether. Black precipitates, which might appear, were always dissolved with dilute sodium hydroxide and united with the aqueous phase, the pH of which was adjusted to 1.0 with sulfuric acid. The combined organic layers were evaporated to dryness and dissolved in 1000 mL of 10% acetic acid. The solution was heated under reflux at 70 °C for 4 h in order to hydrolyze the Boc protecting group. Acetic acid was then removed by rotary evaporation. The brown residue was dissolved in 15 mL of 0.1 M pyridine/formic acid buffer, pH 3.0, and the pH adjusted to 3.0 with formic acid. The separation of pyrraline from byproduct was achieved by semipreparative ion-exchange chromatography (*4*, *22*) using a column (1.5 cm × 48 cm) of DOWEX 50 WX-8 (100−200 mesh) previously

equilibrated with 250 mL of 6 N hydrochloric acid, 250 mL of water, 250 mL of 2 N aqueous pyridine, 250 mL of water, and 250 mL of 0.1 M pyridine/formic acid buffer, pH 3.0. Pyrraline was eluted with 0.3 M pyridine/formic acid buffer, pH 3.75, at a flow rate of 0.35 mL/min. Fractions of 10 mL were collected using a fraction collector (RediFrac, Pharmacia Biotech, Uppsala, Sweden), and the presence of the product was first monitored by spotting 1 μL of the fractions on TLC plates and spraying with 0.1% ninhydrin in ethanol. Selected fractions were then diluted and analyzed by HPLC using the appropriate gradient systems described above. Pyrraline was found to elute between 130 and 250 mL. The fractions were combined, repeatedly evaporated in vacuo, and taken up in water until the smell of pyridine had become imperceptible. Finally, the residue was dissolved in a small volume of water; the solution was filtered and lyophilized to give an amorphous light-brown powder of pyrraline, which was stored at −20 °C.

Pyrraline: ESI-MS, positive mode, $[M + H]^+$ m/z 255.1; ^1H NMR (500 MHz, D_2O), δ [ppm] 1.29 (2H, m, Lys-H4), 1.66 (2H, m, Lys-H5), 1.76 (2H, m, Lys-H3), 3.60 (1H, t, Lys-H2), 4.20 (2H, t, Lys-H6), 4.57 (2H, s, 5-$C\underline{H}_2OH$), 6.25 (1H, d, $J = 4.1$ Hz, pyrrolyl-H4), 7.02 (1H, d, $J =$ 4.2 Hz, pyrrolyl-H3), 9.23 (1H, s, 2-CHO). Elemental analysis: $C_{12}H_{18}N_2O_4$ (MW = 254.28), calcd, C 56.68%, H 7.13%, N 11.02%; found, C 55.08%, H 8.02%, N 10.66%; content = 96.7%, based on nitrogen. Yield = 119 mg (molar yield = 23.0%).

Synthesis and Isolation of Peptide-Bound Pyrraline as L-Alanyl-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin (Ala-Pyrr) and 6-(2-Formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucyl-L-alanine (Pyrr-Ala). The method described above was adapted. Then 495 mg (1.6 mmol) of the Boc-protected dipeptides and 1.08 g (6.7 mmol) of 3-DG were dissolved in 4.7 mL of 0.1 N sodium acetate buffer, pH 5.0, and the pH was adjusted to 5.0 with acetic acid. After the solutions were mixed with 3.8 g of cellulose powder, the incubation and the extraction of the crude reaction product were like for pyrraline. After the first extraction step, however, the pH of the aqueous phase was adjusted to pH 4.5 and additionally extracted four times with ethyl acetate. Deprotection and separation were performed as above. Under these conditions, the modified dipeptides eluted between 400 and 650 mL.

Ala-Pyrr: ESI-MS, positive mode, $[M + H]^+$ m/z 326.2; ^1H NMR (500 MHz, D_2O), δ [ppm] 1.24 (2H, m, Lys-H4), 1.43 (3H, d, Ala-CH_3), 1.61 (2H, m, Lys-H5), 1.70 (2H, m, Lys-H3), 3.97 (1H, qd, Ala-H2), 4.03 (1H, dd, Lys-H2), 4.17 (2H, t, Lys-H6), 4.57 (2H, s, 5-$C\underline{H}_2OH$), 6.25 (1H, d, $J = 4.1$ Hz, pyrrolyl-H4), 7.01 (1H, d, J = 4.1 Hz, pyrrolyl-H3), 9.22 (1H, s, 2-CHO). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36), calcd, C 55.37%, H 7.13%, N 12.91%; found, C 52.44%, H 6.42%, N 11.40%; content = 88.3%, based on nitrogen. Yield = 248 mg (molar yield = 42.1%).

Pyrr-Ala: ESI-MS, positive mode, $[M + H]^+$ m/z 326.2; ^1H NMR (500 MHz, D_2O), δ [ppm] 1.24 (3H, d, Ala-CH_3), 1.32 (2H, m, Lys-H4), 1.66 (2H, m, Lys-H5), 1.79 (2H, m, Lys-H3), 3.85 (1H, t, Lys-H2), 4.03 (1H, qd, Ala-H2), 4.21 (2H, t, Lys-H6), 4.57 (2H, s, 5-$C\underline{H}_2OH$), 6.25 (1H, d, $J = 4.1$ Hz, pyrrolyl-H4), 7.03 (1H, d, $J = 4.2$ Hz, pyrrolyl-H3), 9.23 (1H, s, 2-CHO). Elemental analysis: $C_{15}H_{23}N_3O_5$ (MW = 325.36), calcd, C 55.37%, H 7.13%, N 12.91%; found, C 55.06%, H 6.88%, N 12.09%; content = 93.6%, based on nitrogen. Yield = 156 mg (molar yield = 28.0%).

Cell Culture. Caco-2 cells (passage 35−99) were routinely cultured in 75 cm^2 culture flasks with minimum essential medium supplemented with 10% fetal bovine serum, gentamicin (50 μg/mL), and 1% nonessential amino acid solution at 37 °C in a humidified atmosphere with 5% CO_2 (*17*, *23*−*25*). Subconfluent cultures (90% of confluence) were treated 5 min with Dulbecco's phosphate-buffered saline followed by a 2 min incubation with trypsin solution. For most experiments, the 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 according to the Bradford method. Caco-2 cells were also 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 (*17*, *23*, *24*). The lower (receiver) compartment contained 2.6 mL of medium and the upper (donor) compartment 1.5 mL of medium. The transepithelial electrical resistance was measured at day 21 using a Millicell ERS (Millipore Intertech, Bedford, MA). Caco-2 cell monolayers reached a transepithelial electric resistance of 619 ± 23 Ω cm^2.

OK cells (passage 39−66) were cultured in Dulbecco's modified Eagle's medium/F12 nutrient mixture (1:1, v/v) supplemented with fetal bovine serum (10%, w/v), penicillin−streptomycin (1%), and glutamine (1%). OK cells were seeded in 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.

Culture medium for SKPT cells (passage 58−89) was Dulbecco's modified Eagle's medium/F12 nutrient mixture (1:1, v/v) supplemented with fetal bovine serum (10%, w/v), gentamicin (50 μg/mL), epidermal growth factor (10 ng/mL), insulin (4 μg/mL), dexamethasone (5 μg/mL), and apo-transferrin (5 μg/mL). SKPT cells were seeded in Petri dishes at a density of 0.8×10^6 cells per dish. The uptake measurements were performed on the fourth day after seeding (*21*, *25*).

Transport Studies. Uptake of [^{14}C]Gly-Sar in Caco-2 and SKPT cells cultured on plastic dishes was measured at room temperature as described earlier (*17*, *21*, *23*, *25*). 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, [^{14}C]Gly-Sar, and unlabeled compounds at increasing concentrations. After incubation for 10 min, the cells were quickly washed four times, dissolved in Igepal CA-630 buffer, and prepared for liquid scintillation spectrometry. The nonsaturable 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 (Caco-2) or 20 mM (SKPT) unlabeled Gly-Sar represented 8.4% and 8.9% of the total uptake, respectively. This value was taken into account during nonlinear regression analysis of inhibition constants.

Uptake of L-[^3H]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 nonsaturable component of L-[^3H]lysine uptake (diffusion, adherent radioactivity) determined by measuring the uptake of L-[^3H]-lysine in the presence of 20 mM unlabeled L-lysine represented 21% (Caco-2) and 8% (OK) of the total uptake.

Transepithelial flux of pyrraline, Ala-Pyrr, and Pyrr-Ala across Caco-2 and OK cell monolayers was measured as follows (*17*, *23*, *24*). All experiments were performed at day 21 after seeding at 37 °C in a shaking water bath. 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 compounds (1 mM) to the donor side. At time intervals of 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. After 2 h, the filters were quickly washed four times with ice-cold uptake buffer, cut out of the plastic insert, stored in 1 mL of 10% TCA solution, and frozen.

Data Analysis. Experiments were done in duplicate or triplicate, and each experiment was repeated two to three times. Results are given as mean values ± SEM. IC_{50} values (i.e., concentration of unlabeled compounds necessary to inhibit 50% of [^{14}C]Gly-Sar or l-[^3H]lysine carrier-mediated uptake) were determined by nonlinear regression. Flux data were calculated after correction for the amount taken out by linear regression of appearance in the receiver well vs time.

RESULTS AND DISCUSSION

Synthesis and Analysis of Free and Peptide-Bound Pyrraline. In two previous studies, we provided evidence that nearly the complete amount of peptide-bound pyrraline administered with heated foods such as bakery products or milk can be found in the urine of healthy volunteers within 24 h (*15*, *16*). For a renal excretion, the release of pyrraline from food proteins during gastrointestinal digestion is necessary. Absorption from the chyme then requires transport of compounds across the intestinal epithelium, which in the case of amino acids and peptides can be effected by different amino acid transporters but also by the di- and tripeptide transporter PEPT1 (*18*). Since pyrraline is a known inhibitor of intestinal peptidases (*6*), it should quite likely

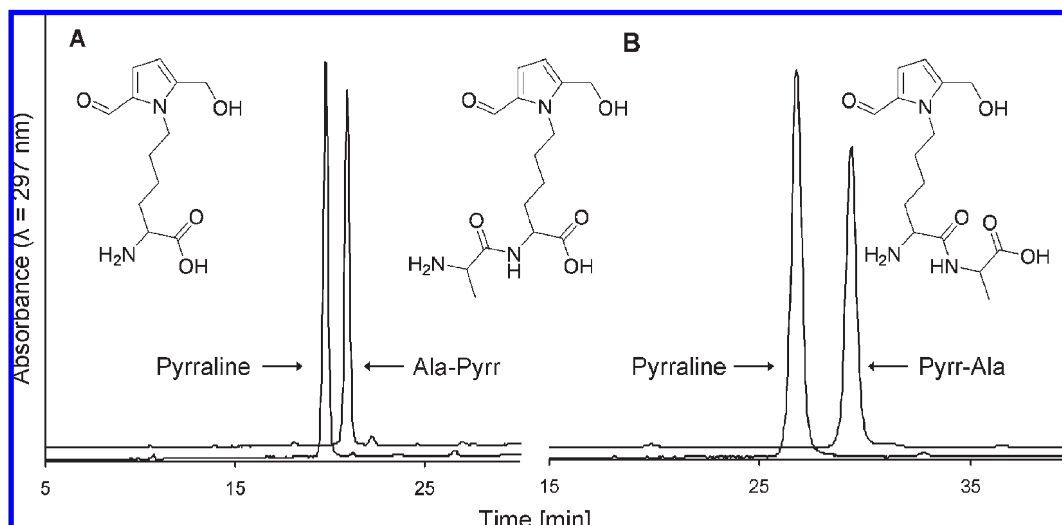

Figure 1. Structures of pyrraline and its dipeptide derivatives and HPLC chromatograms showing the purity and separation of pyrraline and Ala-Pyrr (**A**) and pyrraline and Pyrr-Ala (**B**), respectively, using two different gradient systems.

accumulate bound in small peptides. As a continuation of our studies on the nutritional physiology of early and advanced glycation compounds (*17*), we wanted to investigate how pyrraline permeates the intestinal epithelial barrier. Attention was not only paid to the free amino acid but also to peptide-bound pyrraline as well. The position of a modified amino acid in a dipeptide can be decisive for its inhibitory and transport characteristics (*18*). We therefore modified the lysyl residues in both the dipeptides Ala-Lys and Lys-Ala to pyrraline. As a prerequisite for our studies, sufficient amounts of free and peptide-bound pyrraline were needed. The synthesis protocol of Henle and Bachmann (*3*), which affords high pyrraline yields, was followed and the resulting pyrraline further purified by ion-exchange chromatography after the extraction of Boc-pyrraline from the acidified reaction mixture. The method was adapted to the protected dipeptides Boc-Ala-Lys and Boc-Lys-Ala, which were commercially available, resulting in substantial amounts of the pyrraline-modified dipeptides for the first time. **Figure 1** shows the structures and the chromatographic purity of the synthesized compounds. The identity of the compounds was checked by mass spectrometry. Results of NMR were in agreement with published data (*3*).

The analytical separation of pyrraline from its dipeptide derivatives was achieved by RP-HPLC using a polymer-based column and heptanesulfonic acid as an ion-pairing reagent (**Figure 1**). As low as 0.5 nmol/mL pyrraline and 0.8 nmol/mL Ala-Pyrr could be quantified with gradient A and 1.0 nmol/mL pyrraline and 1.3 nmol/mL Pyrr-Ala with gradient B. The intra-assay coefficient of variation was lower than 5%. The limits of quantification represent about 0.1% of the concentration applied to the apical membrane at the beginning of the flux measurements, thus enabling evaluation of quite small transport phenomena.

Interaction with Proton-Coupled Transporters for Di- and Tripeptides. We first investigated whether pyrraline, Ala-Pyrr, or Pyrr-Ala interact with the intestinal or renal H^+/peptide cotransporters PEPT1 and PEPT2. As a labeled reference substrate, we used [^{14}C]Gly-Sar (glycylsarcosine). Gly-Sar is used as reference dipeptide because it is relatively stable against intra- and intercellular enzymatic hydrolysis. Ala-Pyrr and Pyrr-Ala at a concentration of 10 mM inhibited the [^{14}C]Gly-Sar uptake into Caco-2 cells expressing PEPT1 by 94% and 91%, respectively (**Table 1**). In SKPT cells, which express the high-affinity isoform PEPT2 and, therefore, serve as standard model for PEPT2

Table 1. Inhibition of [^{14}C]Gly-Sar Uptake (10 μM) by Pyrraline, Ala-Pyrr, Pyrr-Ala, Gly-Sar, and L-Lysine in Caco-2 and SKPT Cells[a]

	Caco-2, [^{14}C]Gly-Sar uptake		SKPT, [^{14}C]Gly-Sar uptake	
compd	uptake (%)	IC_{50} (mM)	uptake (%)	IC_{50} (mM)
control	100 ± 8		100 ± 6	
Gly-Sar	15 ± 1	0.74 ± 0.01	9.4 ± 0.3	0.11 ± 0.01
L-lysine	101 ± 4		102 ± 3	
pyrraline	77 ± 2	>10 (~48)[b]	96 ± 3	>10 (~53)[b]
Ala-Pyrr	5.5 ± 0.4	0.19 ± 0.01	3.3 ± 0.6	0.017 ± 0.001
Pyrr-Ala	9.4 ± 0.2	0.03 ± 0.01	9.5 ± 1.2	0.008 ± 0.001

[a] Uptake was measured at pH 6.0 for 10 min in the absence (control) or presence of inhibitors at fixed concentrations (10 mM for Caco-2, 2 mM for SKPT) for % uptake or at increasing concentrations of unlabeled inhibitors for determination of IC_{50} values. Data are mean values ± SE (n=4). [b] IC_{50} values extrapolated beyond measurement range because of limited solubility of compounds or low inhibition.

studies, both compounds at a concentration of 2 mM inhibited [^{14}C]Gly-Sar uptake by 97% and 90%, respectively (**Table 1**). Pyrraline showed only a slight inhibition of the [^{14}C]Gly-Sar uptake in both cell lines (**Table 1**). We next performed competition experiments using increasing concentrations of the three Maillard reaction products to determine IC_{50} values for [^{14}C]-Gly-Sar uptake inhibition (**Figure 2A** and **Figure 2B**). For Ala-Pyrr, IC_{50} values of 0.19 ± 0.01 mM at Caco-2 cells (PEPT1) and 0.017 ± 0.001 mM at SKPT (PEPT2) were obtained. For Pyrr-Ala, the IC_{50} values were 0.03 ± 0.01 mM (PEPT1) and 0.008 ± 0.001 mM (PEPT2, **Table 1**). These values qualify both dipeptide derivatives according to our classification as high affinity ligands for PEPT1 as well as for PEPT2 (*19*). In contrast, pyrraline showed no affinity for the two peptide transporters (**Table 1**). For comparison, we also measured the interaction of L-lysine and the dipeptide Gly-Sar with PEPT1 and PEPT2. Whereas the amino acid L-lysine showed no affinity to PEPT1 and PEPT2, Gly-Sar represents a medium affinity PEPT1 and PEPT2 substrate with IC_{50} values of 0.74 ± 0.01 and 0.11 ± 0.01 mM, respectively (**Table 1**, **Figure 2**). We conclude that peptide-bound but not free pyrraline interacts with H^+/peptide cotransporters.

Interaction with L-Lysine Transporters. Transporters for L-lysine are possible candidates for transport of free pyrraline. Therefore, we studied whether pyrraline or its derivatives interfere with lysine uptake at Caco-2 and OK cells. OK cells do not express PEPT2. They are, however, an often used cell model for

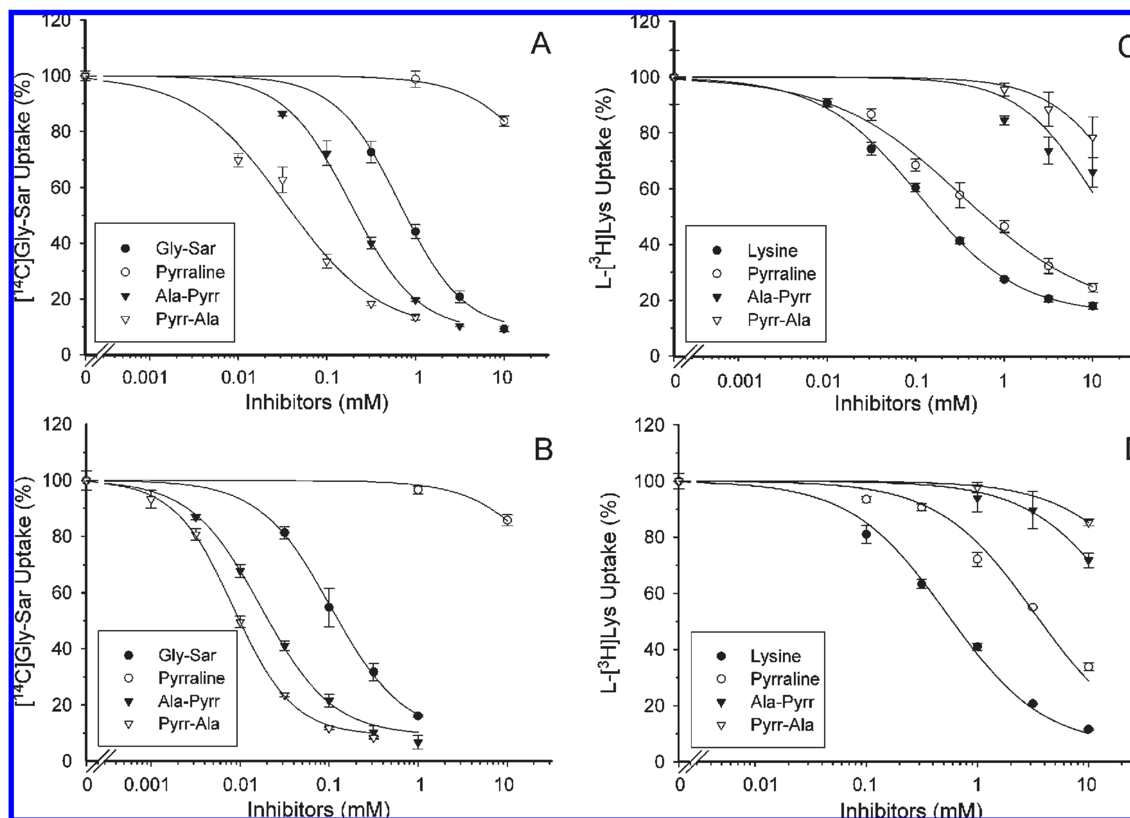

Figure 2. Inhibition of [^{14}C]Gly-Sar and L-[^{3}H]lysine uptake into Caco-2, OK, and SKPT cells by pyrraline, Ala-Pyrr, Pyrr-Ala, Gly-Sar, and L-lysine. Uptake of 10 μM [^{14}C]Gly-Sar was measured for 10 min in Caco-2 (**A**) and SKPT cells (**B**) at pH 6.0 in the absence (control) or presence of increasing concentrations of the compounds. Uptake of 2 nM L-[^{3}H]lysine was measured for 5 min in Caco-2 (**C**) and OK cells (**D**) at pH 6.0 in the absence (control) or presence of increasing concentrations of the compounds. Data are mean values $\pm$ SE, n = 3−4.

renal amino acid transport studies. Ala-Pyrr and Pyrr-Ala at a concentration of 10 mM only weakly inhibited the uptake of L-[^{3}H]lysine into Caco-2 and OK cells (**Table 2**). A stronger inhibition by 75% in Caco-2 cells and by 67% in OK cells was observed with free pyrraline. We determined IC_{50} values of 0.32 $\pm$ 0.04 mM (Caco-2) and 3.5 $\pm$ 0.2 mM (OK) for free pyrraline (**Table 2**, **Figure 2**). For comparison, we also measured the inhibition of L-[^{3}H]lysine uptake by unlabeled Gly-Sar and L-lysine. L-Lysine inhibited the uptake of L-[^{3}H]lysine with an IC_{50} value of 0.11 $\pm$ 0.01 mM (Caco-2) and 0.51 $\pm$ 0.09 mM (OK), whereas Gly-Sar showed no affinity to the amino acid transporter (**Table 2**, **Figure 2**). We then studied the stability of the derivatives in uptake buffer. When Ala-Pyrr and Pyrr-Ala were added to Caco-2 cells for 10 min at a concentration of 1 mM, 9.4 $\pm$ 0.3% Ala-Pyrr and 2.1 $\pm$ 0.3% Pyrr-Ala were hydrolyzed to pyrraline. Therefore, the moderate inhibition of L-[^{3}H]lysine uptake by Ala-Pyrr and Pyrr-Ala can easily be explained by free pyrraline originating from the dipeptides during incubation together with its high affinity toward the L-lysine transporter(s). We conclude that free pyrraline but not the dipeptide derivatives interacts with amino acid transporters for lysine, either as a substrate or as an inhibitor.

Transepithelial Flux of Pyrraline, Ala-Pyrr, and Pyrr-Ala. Inhibition of uptake of a transporter reference substrate does not necessarily mean that the interacting inhibitors are transported themselves. They might represent nontransported compounds with a certain affinity to the transporters. To investigate whether pyrraline and its dipeptide derivatives Ala-Pyrr and Pyrr-Ala show significant transepithelial transport, we determined their total net transepithelial flux across Caco-2 and OK cells by HPLC analysis. These experiments were done at pH 6.0 over 2 h. At the end of the flux measurements, the filters supporting the monolayers were cut out of the inserts and also analyzed.

Table 2. Inhibition of L-[^{3}H]Lysine Uptake (2 nM) by Pyrraline, Ala-Pyrr, Pyrr-Ala, L-Lysine, and Gly-Sar in Caco-2 and OK Cells[a]

	Caco-2, L-[^{3}H]lysine uptake		OK, L-[^{3}H]lysine uptake	
compd	uptake (%)	IC_{50} (mM)	uptake (%)	IC_{50} (mM)
control	100 $\pm$ 6		100 $\pm$ 4	
L-lysine	17 $\pm$ 2	0.11 $\pm$ 0.01	13 $\pm$ 1	0.51 $\pm$ 0.09
Gly-Sar	105 $\pm$ 5		104 $\pm$ 2	
pyrraline	25 $\pm$ 1	0.32 $\pm$ 0.04	33 $\pm$ 1	3.5 $\pm$ 0.2
Ala-Pyrr	66 $\pm$ 5	>10 (~11)[b]	72 $\pm$ 3	>10 (~24)[b]
Pyrr-Ala	78 $\pm$ 7	>10 (~30)[b]	93 $\pm$ 2	>10 (~54)[b]

[a] Uptake was measured at pH 6.0 for 5 min in the absence (control) or presence of inhibitors at a fixed concentration (10 mM) for % uptake or at increasing concentrations of unlabeled inhibitors for determination of IC_{50} values. Data are mean values $\pm$ SE (n = 4). [b] IC_{50} values extrapolated beyond measurement range because of limited solubility of compounds or low inhibition.

The results are as follows: When added at a concentration of 1 mM to the luminal (apical) side, neither Ala-Pyrr nor Pyrr-Ala was found at the basolateral (abluminal) side or in the cells. In both compartments, the substrate amounts were below the detection limit. Small amounts of free pyrraline, also added to the apical side at a concentration of 1 mM, could be detected both in the cells and in the basolateral compartment, but the flux rate was even lower than that of the space marker [^{14}C]mannitol (0.07 $\pm$ 0.02%/cm^2 h compared to 0.13 $\pm$ 0.03%/cm^2 h; **Figure 3**). The intracellular amount after 2 h was 0.59% of the total pyrraline amount applied (**Figure 3**, inset). From these data we conclude that Ala-Pyrr and Pyrr-Ala are not transported across

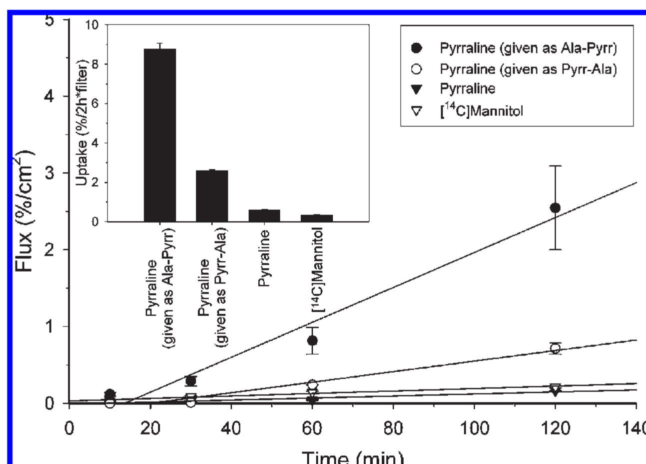

Figure 3. Transepithelial flux of pyrraline, Ala-Pyrr, Pyrr-Ala, and [^{14}C]-mannitol across Caco-2 cells. Flux was determined in the presence of 1 mM compounds and 10 μM [^{14}C]mannitol, respectively, at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Data are mean values ± SE, *n* = 3.

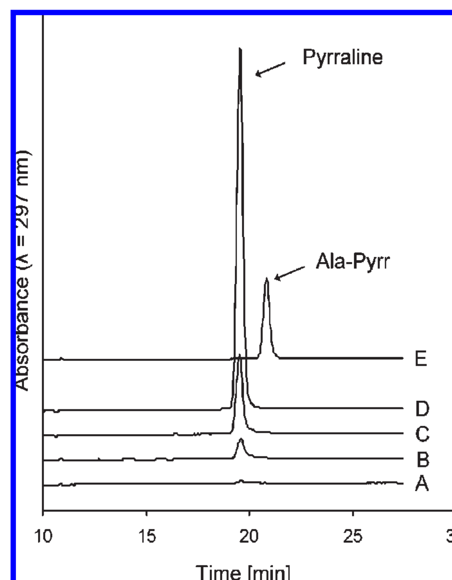

Figure 4. Appearance of pyrraline in the basolateral compartment during the flux measurement of Ala-Pyrr at Caco-2 cells 10 min (**A**), 30 min (**B**), 60 min (**C**), and 120 min (**D**) after addition of 1 mM Ala-Pyrr to the apical compartment. The intact dipeptide Ala-Pyrr (**E**, standard chromatogram) could not be detected.

Caco-2 cells in intact form. The transport of free pyrraline, even though it interacts with lysine transporting amino acid transporters, is neglectable.

The situation turned out to be completely different when we analyzed free basolateral and intracellular pyrraline concentration in samples where Ala-Pyrr and Pyrr-Ala (1 mM) had been added to the luminal compartment for 2 h. When given in the form of dipeptides, pyrraline appeared in the basolateral compartment (**Figure 4**), and high flux rates of pyrraline were obtained. In case of Ala-Pyrr, a pyrraline flux rate of 1.06 ± 0.27%/(cm^2 h) was observed (**Figure 3**). When 1 mM Pyrr-Ala was added to the cells, basolateral pyrraline was detected with a rate of 0.28 ± 0.08%/(cm^2 h) (**Figure 3**). In both cases, the flux rates were higher than the flux of the space marker [^{14}C]mannitol. Within the cells only pyrraline but not the peptide form was found (8.8% and 2.6%, respectively, **Figure 3**, inset).

We conclude that Ala-Pyrr and Pyrr-Ala, but not free pyrraline, are taken up into intestinal cells across the apical membrane by the peptide transporter PEPT1. Once inside the cells, Ala-Pyrr and Pyrr-Ala are hydrolyzed to free pyrraline and alanine. In the presence of a pH gradient, PEPT1 is able to transport its substrates uphill against a concentration gradient (*19*). Therefore, a pyrraline gradient across the basolateral membrane exists and the basolateral efflux of free pyrraline via simple diffusion will be driven by this gradient.

In order to evaluate a possible renal reabsorption of pyrraline from primary urine, we also measured the transepithelial flux of 1 mM pyrraline across OK cell monolayers. The lysine derivative was transported across the OK cells with a flux rate of 1.37 ± 0.23%/(cm^2 h), which is slightly but not significantly higher than the flux of [^{14}C]mannitol at these cells (1.19 ± 0.08%/(cm^2 h)). Uptake of pyrraline after 2 h was only 0.40 ± 0.05%. Hence, after intestinal absorption in the form of di- or tripeptides, which are degraded to the free amino acids, pyrraline is only poorly reabsorbed in the kidneys and should be excreted via the urine. This finding can easily explain the high excretion rates found in our previous investigations (*15*, *16*).

In conclusion, our study shows that the transepithelial transport of intact Ala-Pyrr, Pyrr-Ala and the flux of free pyrraline across Caco-2 cell monolayers is very low. However, the transepithelial transport of pyrraline at Caco-2 cells is increased up to 15-fold when it is given in the form of dipeptides. Since pyrraline is prone to exist in such peptide form due to its peptidase-inhibitory potential (*6*), its transport is most likely of high nutritional and physiological relevance. In the form of di- or tripeptides, it becomes a substrate for PEPT1 and PEPT2 and is accumulated in the epithelial cells, where the peptides are hydrolyzed very quickly producing high concentrations of free pyrraline. Pyrraline leaves the cell across the basolateral cell membrane by simple diffusion driven by its own gradient or mediated by putative basolateral peptide transporters (*19*). No further barrier exists between this compartment and the bloodstream.

In addition, the study demonstrates the importance of using protein- or peptide-bound substrates instead of free amino acids for the thorough characterization of their intestinal absorption. The transport characteristics of an amino acid such as pyrraline are obviously modulated by the incompleteness of intestinal digestion, whether intrinsic or induced by the antiproteolytic capabilities of the modified amino acids themselves. Further research is therefore needed to assess the impact of protein glycation on their intestinal digestibility. Studies concerning the epithelial transport of other free and peptide-bound Maillard reaction products are currently underway. Furthermore, the results so far show only interaction of Ala-Pyrr and Pyrr-Ala with protein-coupled peptide transporters. Future studies with carrier proteins expressed heterologously will be performed to characterize the specific translocation steps in detail.

ABBREVIATIONS USED

Caco, carcinoma colon; SKPT, spontaneous hypertensive rat kidney proximale tubule; OK, opossum kidney; 3-DG, 3-deoxyglucosulose; AGE, advanced glycation end product; Ala-Pyrr, alanylpyrraline; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; Pyrr-Ala, pyrralylalanine; TLC, thin layer chromatography.

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LITERATURE CITED

(1) Henle, T. Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids* **2005**, *29*, 313–322.
(2) Henle, T. AGEs in foods: Do they play a role in uremia?. *Kidney Int.* **2003**, *63*, S145–S147.
(3) Henle, T.; Bachmann, A. Synthesis of pyrraline reference material. *Z. Lebensm.-Unters. Forsch.* **1996**, *202*, 72–74.
(4) Nakayama, T.; Hayase, F.; Kato, H. Formation of ε-(2-formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine in the Maillard reaction between D-glucose and L-lysine. *Agric. Biol. Chem.* **1980**, *44*, 1201–1202.
(5) Omura, H.; Jahan, N.; Shinohara, K.; Murakami, H. Formation of mutagens by the Maillard reaction. *ACS Symp. Ser.* **1983**, *215*, 537–563.
(6) Öste, R. E.; Miller, R.; Sjöström, H.; Noren, O. Effect of Maillard reaction products on protein digestion. Studies on pure compounds. *J. Agric. Food Chem.* **1987**, *35*, 938–942.
(7) Henle, T.; Walter, A. W.; Klostermeyer, H. Simultaneous Determination of Protein-Bound Maillard Products by Ion-Exchange Chromatography and Photodiode Array Detection. In *Maillard Reactions in Chemistry, Food, and Health*; Labuza, T. P., Ed.; The Royal Society of Chemistry: Cambridge, U.K., 1994; pp 195–200.
(8) Resmini, P.; Pellegrino, L. Occurrence of protein-bound lysylpyrrolaldehyde in dried pasta. *Cereal Chem.* **1994**, *71*, 254–262.
(9) Henle, T. Dietary advanced glycation end products. A risk to human health? A call for an interdisciplinary debate. *Mol. Nutr. Food Res.* **2007**, *51*, 1075–1078.
(10) Sebekova, K.; Somoza, V. Dietary advanced glycation endproducts (AGEs) and their health effects—PRO. *Mol. Nutr. Food Res.* **2007**, *51*, 1079–1084.
(11) Koschinsky, T.; He, C.-J.; Mitsuhashi, T.; Bucala, R.; Liu, C.; Bünting, C.; Heitmann, K.; Vlassara, H. Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6474–6479.
(12) Uribarri, J.; Cai, W.; Peppa, M.; Goodman, S.; Ferrucci, L.; Striker, G.; Vlassara, H. Circulating glycotoxins and dietary advanced glycation endproducts: Two links to inflammatory response, oxidative stress, and aging. *J. Gerontol., Ser. A* **2007**, *62*, 427–433.
(13) Vlassara, H.; Cai, W.; Crandall, J.; Goldberg, T.; Oberstein, R.; Dardaine, V.; Peppa, M.; Rayfield, E. J. Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15596–15601.
(14) Ames, J. M. Evidence against dietary advanced glycation endproducts being a risk to human health. *Mol. Nutr. Food Res.* **2007**, *51*, 1085–1090.
(15) Förster, A.; Henle, T. Glycation in food and metabolic transit of dietary AGEs: studies on the urinary excretion of pyrraline. *Biochem. Soc. Trans.* **2003**, *31*, 1383–1385.
(16) Förster, A.; Kühne, Y.; Henle, T. Studies on absorption and elimination of dietary Maillard reaction products. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 474–481.
(17) Grunwald, S.; Krause, R.; Bruch, M.; Henle, T.; Brandsch, M. Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems. *Br. J. Nutr.* **2006**, *95*, 1221–1228.
(18) Daniel, H. Molecular and integrative physiology of intestinal peptide transport. *Annu. Rev. Physiol.* **2004**, *66*, 361–384.
(19) Brandsch, M.; Knütter, I.; Bosse-Doenecke, E. Pharmaceutical and pharmacological importance of peptide transporters. *J. Pharm. Pharmacol.* **2008**, *60*, 543–585.
(20) Börner, V.; Fei, Y. J.; Hartrodt, B.; Ganapathy, V.; Leibach, F. H.; Neubert, K.; Brandsch, M. Transport of amino acid aryl amides by the intestinal H^+/peptide cotransport system, PEPT1. *Eur. J. Biochem.* **1998**, *255*, 698–702.
(21) Brandsch, M.; Brandsch, C.; Prasad, P. D.; Ganapathy, V.; Hopfer, U.; Leibach, F. H. Identification of a renal cell line that constitutively expresses the kidney-specific high-affinity H^+/peptide cotransporter. *FASEB J.* **1995**, *9*, 1489–1496.
(22) Krause, R.; Knoll, K.; Henle, T. Studies on the formation of furosine and pyridosine during acid hydrolysis of different Amadori products of lysine. *Eur. Food Res. Technol.* **2003**, *216*, 277–283.
(23) Bretschneider, B.; Brandsch, M.; Neubert, R. Intestinal transport of β-lactam antibiotics: analysis of the affinity at the H^+/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. *Pharm. Res.* **1999**, *16*, 55–61.
(24) Metzner, L.; Kalbitz, J.; Brandsch, M. Transport of pharmacologically active proline derivatives by the human proton-coupled amino acid transporter hPAT1. *J. Pharmacol. Exp. Ther.* **2004**, *309*, 28–35.
(25) Neumann, J.; Bruch, M.; Gebauer, S.; Brandsch, M. Transport of the phosphonodipeptide alafosfalin by the H^+/peptide cotransporters PEPT1 and PEPT2 in intestinal and renal epithelial cells. *Eur. J. Biochem.* **2004**, *271*, 2012–2017.

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Transport of the Advanced Glycation End Products Alanylpyrraline and Pyrralylalanine by the Human Proton-Coupled Peptide Transporter hPEPT1

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The glycation compound pyrraline, which originates from the advanced Maillard reaction, appears in urine after consumption of pyrraline-containing food. We hypothesized that the absorption of pyrraline occurs in the form of dipeptides rather than the free amino acid. The human intestinal peptide transporter hPEPT1 was transiently expressed in HeLa cells. In hPEPT1-transfected cells but not in cells transfected with empty vector, the uptake of [^{14}C]glycylsarcosine was strongly inhibited by alanylpyrraline (Ala-Pyrr) and pyrralylalanine (Pyrr-Ala). Free pyrraline did not inhibit peptide uptake. In *Xenopus laevis* oocytes expressing human PEPT1, both Ala-Pyrr and Pyrr-Ala generated significant inward directed currents. In a third approach, uptake of the dipeptides into hPEPT1-transfected HeLa cells was analyzed by HPLC. Ala-Pyrr and Pyrr-Ala were taken up by hPEPT1-expressing cells at a 4- to 7-fold higher rate than by HeLa cells transfected with the empty vector. We conclude that pyrraline containing dipeptides are transported by hPEPT1 in an electrogenic manner into intestinal cells.

KEYWORDS: AGE; Maillard reaction; intestine; membrane transport; PEPT1; pyrraline; glycation

INTRODUCTION

Advanced glycation end products (AGEs) originate by the so-called Maillard reaction during thermal processing of food and during food storage (*1*). This reaction ("glycation") occurs between reducing carbohydrates or their degradation products and the ε-amino group of lysine, or the N-termini of proteins or the guanidino group of arginine. Whether dietary AGEs represent a risk to human health is currently under intense discussion (*2*−*5*). The daily intake of AGEs has been estimated to 25−75 mg (*6*). So far, however, no evidence has been brought forward showing that specific diseases result from AGE effects in a causative manner (*7*).

One of the quantitatively most dominating glycation compounds in food is the AGE pyrraline (6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-L-norleucin) (*8*, *9*). Free or peptide-bound pyrraline is generated during the reaction of 3-deoxy-D-*erythro*-hexos-2-ulose (3-deoxyglucosulose, 3-DG) with the ε-amino group of free lysine or lysine residues, respectively (*10*, *11*). Pyrraline is found predominantly on sites of high thermal impact and low moisture content, like in bread crust, rusk, or crackers, in which the pyrraline content may reach 3.7 g per kg of protein. In milk powder and whey powder (*12*) and pasta products (*13*) pyrraline concentrations vary between 90 and 150 mg per kg of protein.

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After intake of a pyrraline containing diet, significant amounts of free pyrraline appear in urine (*14*, *15*) raising the question how pyrraline can cross the intestinal epithelial barrier protecting the *milieu interieur*. In general, free or peptide/protein-bound AGEs from diet could cross the intestinal epithelium by simple diffusion (paracellularly or transcellularly), by endocytotic processes or mediated by transport proteins. Studying this question at intestinal Caco-2 cells cultured as monolayers on permeable filters, we observed recently that, in contrast to free pyrraline, the peptide derivatives alanylpyrraline (Ala-Pyrr) and pyrralylalanine (Pyrr-Ala) are taken up into the cells and then hydrolyzed intracellularly to alanine and free pyrraline (*16*). Only pyrraline entered the basolateral compartments of the Transwell chambers.

We hypothesized that peptide transporters located at the apical membrane of enterocytes might be responsible for the uptake of the dipeptide derivatives into the cells. Indeed, the uptake of the prototype reference dipeptide [^{14}C]glycylsarcosine ([^{14}C]Gly-Sar) into Caco-2 cells could be inhibited by excess amounts of Ala-Pyrr and Pyrr-Ala but not by free pyrraline. This result suggests competition between the pyrraline-containing dipeptides and Gly-Sar during specific transmembrane transport into the epithelial cells. Actual transport of pyrraline in any form could not be shown. The membrane transporter mainly responsible for uptake of small peptides originating from intestinal protein digestion is the H^+-coupled peptide transporter 1 (PEPT1) (*17*−*19*). This transporter protein accepts most physiologically occurring di- and

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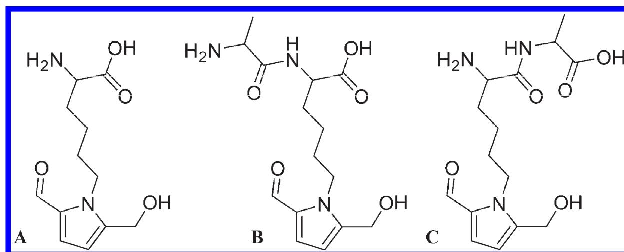

Figure 1. Chemical structures of pyrraline (**A**), Ala-Pyrr (**B**) and Pyrr-Ala (**C**).

tripeptides as substrates. Amino acids and oligopeptides larger than tripeptides are excluded. Interestingly, PEPT1 tolerates a great number of structural modifications of peptide derived substrates without significant alterations in transport capabilities (*17*, *18*). It has been shown that certain amino acid derivatives are recognized and transported by PEPT1. Several studies have demonstrated that dipeptide cores conjugated with different residues in their side chains are transported into intestinal cells. Other peptidomimetics transported by PEPT1 are δ-aminolevulinic acid and alafosfalin (*18*). The system also accepts many peptidomimetic drugs and prodrugs such as β-lactam-antibiotics or valacyclovir as substrates (*18*, *19*). For a PEPT1 substrate with high affinity we presently consider the following structural features as essential: (a) L-amino acids, (b) an acidic or hydrophobic function at the C-terminus, (c) a weakly basic group in α-position at the N-terminus, (d) a ketomethylene or acid amide bond and (e) when present, *trans* conformation of the peptide bond (*18*). Compounds serving as substrate for PEPT1 usually display apparent affinity constants (K_i, K_t) between 50 μM and 10 mM (*18*, *19*).

The purpose of this study was to investigate whether the uptake of Ala-Pyrr and Pyrr-Ala into intestinal cells is catalyzed by the proton-coupled peptide transporter. We employed two techniques that specifically allow measurement of substrate transport by PEPT1, namely, the two-electrode voltage-clamp technique at *Xenopus laevis* oocytes expressing human PEPT1 and the transfection of PEPT1-negative human host cells with hPEPT1-cDNA.

MATERIALS AND METHODS

Materials. The epithelial cervical cancer cell line HeLa was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell culture media, supplements and trypsin solution were purchased from Life Technologies, Inc. (Karlsruhe, Germany) or PAA (Pasching, Austria). Fetal bovine serum was from Biochrom (Berlin, Germany). [Glycine-1-^{14}C]glycylsarcosine (Gly-Sar; specific radioactivity 56 mCi/mmol) was custom synthesized by GE Healthcare (Little Chalfont, U.K.). Gly-Sar and Ala-Lys were ordered from Sigma-Aldrich (Deisenhofen, Germany). 3-Deoxyglucosulose (3-DG) was synthesized according to Henle and Bachmann (*10*). For the synthesis of pyrraline, Ala-Pyrr and Pyrr-Ala (for structures see **Figure 1**), we followed our protocol described previously (*16*). Briefly, Boc-Lys-OH or the protected dipeptides Boc-Ala-Lys-OH and Boc-Lys-Ala-OH were incubated in dry state with a 4-fold molar excess of 3-DG for 4 h at 70 °C in a drying oven. After extraction of the crude reaction product, the Boc protecting group was cleaved and the deprotected pyrraline derivatives were finally purified by semipreparative ion exchange chromatography yielding the substances with an overall content of about 90% with residual formate as assessed by nuclear magnetic resonance spectroscopy. The absence of impurities, especially other amino acids or dipeptides, was verified by amino acid analysis and mass spectroscopy (data not shown). Spectroscopic data were in accordance with those published previously (*16*).

Cell Culture. HeLa cells were routinely cultured in 75 cm^2 culture flasks with D-MEM (with Glutamax; 4500 mg/L glucose) supplemented with 10% fetal bovine serum and gentamicin (50 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2 (*20*). Subconfluent cultures (80% of confluence) were treated for 5 min with Dulbecco's phosphate-buffered saline and trypsinized with 1% trypsin solution for 2 min. 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 (*20*).

Heterologous Expression of hPEPT1 in HeLa Cells and Uptake Measurements. The cDNA of human PEPT1 was cloned into pcDNA3 using the pBluescript constructs as a template for the PCR and *Xho*I and *Bam*HI as restriction sites (*20*). The resulting pcDNA3-hPEPT1 construct was confirmed by sequencing. Human PEPT1 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. The transfection was done 1 h post seeding in 24-well plates, and 20−24 h post transfection the uptake of [^{14}C]Gly-Sar (20 μM, pH 6.0) in the absence or presence of increasing concentrations of Gly-Sar, pyrraline, Ala-Pyrr or Pyrr-Ala was measured at room temperature. 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 (*20*). After incubation for 10 min the monolayers were quickly washed four times with ice-cold uptake buffer, solubilized and prepared for liquid scintillation spectrometry. The nonsaturable 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 only 3.7% of the total uptake. This value was taken into account during nonlinear regression analysis of inhibition constants (*20*).

Transfected HeLa cells were also used for uptake studies with the unlabeled compounds. HeLa cells transfected with the empty vector pcDNA3 (control) or pcDNA3-hPEPT1, respectively, were incubated with unlabeled pyrraline, Ala-Pyrr or Pyrr-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 of aqua bidest. was added. After freezing and thawing the cell layers three times, the cell suspension was transferred in 1.5 mL reaction tubes, homogenized with a 25 gauge needle and centrifuged in a table top centrifuge at 13000 rpm for 45 min. The supernatant was centrifuged again at 13000 rpm for 45 min and prepared for HPLC measurements (*20*).

High Pressure Liquid Chromatography (HPLC). All analytical HPLC analyses of pyrraline and pyrraline-containing dipeptides 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. The mobile phases consisted of 5 mM sodium heptanesulfonate, pH 2.0 (solvent A) and a mixture of 50% solvent A in acetonitrile (solvent B). Separations were performed at a flow rate of 1 mL/min 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 297 nm. A linear gradient from 4 to 54% B in 23 min was used for the measurements of pyrraline and Ala-Pyrr containing samples (gradient A). The gradient for Pyrr-Ala samples was from 14 to 37% B in 35 min (gradient B). External calibration was performed with the synthesized standards. 300 μL of the supernatants were added to 300 μL of solvent A. The mixture was centrifuged at 10 000 rpm for 15 min and 50 μL were injected.

***Xenopus laevis* Oocytes Expressing hPEPT1 and Electrophysiology.** The *Xenopus laevis* oocyte expression vector pNKS was kindly provided by Prof. G. Schmalzing (RWTH, Aachen, Germany). This vector contains the 5′ and 3′ UTRs of the *X. laevis* oocyte β-globin gene. To clone the transporter's cDNA into pNKS, *Aat*II and *Xba*I restriction sites were introduced at the 5′ and 3′ end, respectively, by PCR. As template the pBluescript-hPEPT1 vector was used. After restriction enzyme digestion the PCR product was ligated into the digested pNKS vector. The insertion of the correct cDNA was verified by sequencing. The pNKS-hPEPT1 construct served as template for cRNA synthesis. After linearizing the plasmids with *Not*I, cRNAs were synthesized using the mMESSAGE mMACHINE SP6 kit (Ambion, Huntingdon, U.K.). The cRNAs were purified with the MEGAclear kit (Ambion) and the concentration was determined by UV absorbance at 260 nm. The cRNAs were stored at −80 °C (*21*).

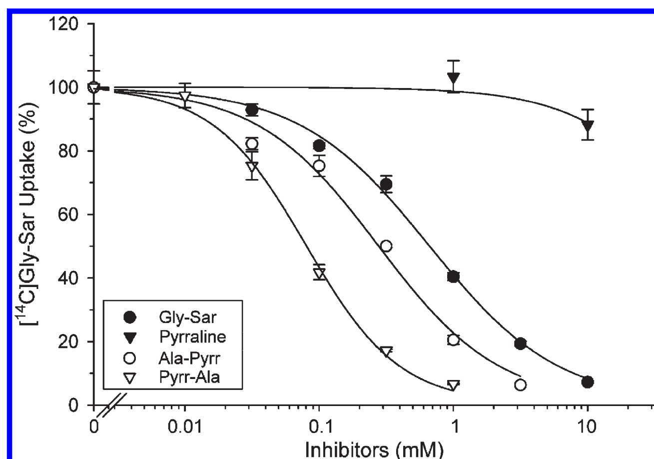

Figure 2. Inhibition of [^{14}C]Gly-Sar uptake into hPEPT1-transfected HeLa cells. Uptake of 20 μM [^{14}C]Gly-Sar was measured for 10 min at pH 6.0 in the presence of increasing concentrations of pyrraline, Ala-Pyrr, Pyrr-Ala and Gly-Sar. n = 3−4.

Oocytes were surgically removed from anesthetized *X. laevis* frogs, dissected and defolliculated as described by Riedel and co-workers (*22*). Tricaine methane sulfonate (Sigma) was used for anesthesia. The removed oocytes were separated by collagenase treatment (2 mg/mL) for 2 h. Healthy-looking oocytes (stages V−VI) were manually selected, and 23 nL (1.1 μg/μL) of cRNA solution of hPEPT1 was injected per oocyte. Water injected oocytes were used as control. Injected oocytes were maintained at 19 °C in modified Barth's medium (5 mM HEPES/NaOH pH 7.4, 100 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 000 U/mL penicillin and 10 mg/mL streptomycin). Five 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 Ala-Pyrr and Pyrr-Ala at a concentration of 3 mM. Quick and reproducible solution exchanges were achieved using a small tubelike chamber (0.1 mL) combined with fast superfusion (*21*−*24*). Microelectrodes with resistances between 0.8 and 1.4 MΩ were made of borosilicate glass and filled with 3 M KCl. Whole-cell currents were recorded and filtered at 100 Hz using a two-electrode voltage-clamp amplifier (OC-725C, Hamden, CT) and sampled at 85 Hz. Oocytes were voltage clamped at a membrane potential of −60 mV.

Data Analysis. Results are given as mean ± SEM. The concentration of the unlabeled compound necessary to inhibit 50% of [^{14}C]Gly-Sar carrier-mediated uptake were determined by nonlinear regression using the logistical equation for an asymmetric sigmoid (allosteric Hill kinetics): $Y = \text{Min} + (\text{Max} - \text{Min})/(1 + (X/\text{IC}_{50})^P)$ where Max is the initial Y value, Min the final Y value and the power P represents Hill's coefficient (SigmaPlot program, Systat, Erkrath, Germany) (*20*). Inhibitory constants (K_i) were calculated from the IC_{50} values according to the method developed by Cheng and Prusoff (*25*). Log $p_{O/W}$ values were calculated using ChemDraw Pro 5.0 (Cambridgesoft, Cambridge, MA).

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 ± SEM from measurements of 7−9 oocytes each from two batches of oocyte preparation. Currents induced by application of substances were calculated as the difference of the currents measured in the presence and absence of these substances.

RESULTS AND DISCUSSION

Inhibition of [^{14}C]Gly-Sar Uptake into HeLa Cells Transfected with hPEPT1. In a previous study (*16*) we observed that Ala-Pyrr and Pyrr-Ala are able to inhibit the uptake of [^{14}C]Gly-Sar into Caco-2 cells with high affinity. To show unequivocally that this inhibition occurs at the PEPT1 protein, in the present study measurements at the cloned human PEPT1 were performed. After heterologous expression of hPEPT1 in HeLa cells, inhibition of peptide uptake by increasing concentrations of pyrraline and its dipeptide derivatives was studied (**Figure 2**). From the inhibition curves, IC_{50} values, i.e. the inhibitor concentration necessary to inhibit the carrier-mediated [^{14}C]Gly-Sar uptake by 50%, were calculated and converted into K_i values. Ala-Pyrr and Pyrr-Ala inhibited the [^{14}C]Gly-Sar uptake with K_i values of 0.27 ± 0.02 mM and 0.08 ± 0.01 mM, respectively, whereas pyrraline showed no affinity to hPEPT1 (**Table 1**). According to our classification (*18*, *19*) Ala-Pyrr and Pyrr-Ala can be considered as high affinity ligands (inhibitors or substrates) of human PEPT1. The K_i values are very similar to those obtained at Caco-2 cells (**Table 1**) (*16*). For comparison, the enzymatically stable reference dipeptide Gly-Sar displayed a medium affinity with a K_i value of 0.64 ± 0.02 mM. Nontransfected HeLa cells do not possess endogenous peptide transport activity. Hence, from these results we conclude that the inhibitory effect of Ala-Pyr and Pyrr-Ala is due to direct interaction with the hPEPT1 protein.

Table 1. Inhibition Constants (K_i) of Gly-Sar, Ala-Pyrr, Pyrr-Ala and Pyrraline at hPEPT1-Transfected HeLa Cells and at Caco-2 Cells Expressing hPEPT1 Constitutively[a]

	K_i (mM)	
compound	hPEPT1 HeLa	hPEPT1 Caco-2[b]
Gly-Sar	0.64 ± 0.02	0.74 ± 0.01
pyrraline	>10	>10
Ala-Pyrr	0.27 ± 0.02	0.19 ± 0.01
Pyrr-Ala	0.08 ± 0.01	0.03 ± 0.01

[a] Uptake of [^{14}C]Gly-Sar was measured at pH 6.0 for 10 min at increasing concentrations of unlabeled pyrraline and its dipeptide derivatives. K_i values were derived from the competition curves shown in **Figure 2**. n = 4. [b] Hellwig et al. (*16*).

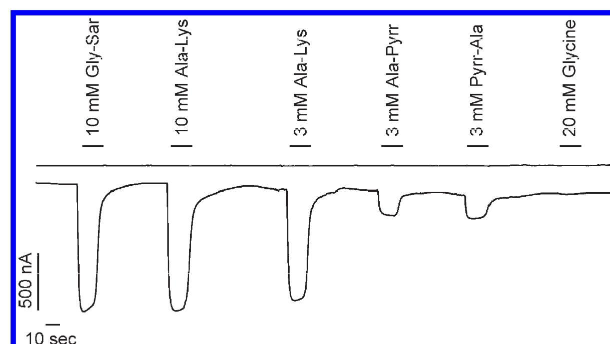

Figure 3. hPEPT1-mediated inward currents at hPEPT1-cRNA injected *Xenopus laevis* oocytes. Currents induced by Ala-Pyrr, Pyrr-Ala, Ala-Lys, Gly-Sar and glycine in water- (upper trace) and hPEPT1-cRNA-injected oocytes (lower trace). Currents were recorded at pH 6.5 by two-electrode voltage-clamp at a membrane potential of −60 mV.

Inward Directed Currents Generated by Ala-Pyrr and Pyrr-Ala at *X. laevis* Oocytes Expressing hPEPT1. Inhibition of [^{14}C]Gly-Sar uptake does not necessarily mean that Ala-Pyrr and Pyrr-Ala themselves are transported by hPEPT1. Using the two-electrode voltage-clamp technique we investigated whether Ala-Pyrr and Pyrr-Ala generate inward directed currents at *X. laevis* oocytes expressing hPEPT1. Such currents at *X. laevis* oocytes occur when a substrate is cotransported by PEPT1 with H^+ in an electrogenic manner. **Figure 3** shows that Ala-Pyrr and Pyrr-Ala (3 mM) generated inward currents of 272 ± 14 nA and 234 ± 12 nA, respectively. Gly-Sar (10 mM) and Ala-Lys (3 mM and 10 mM) generated inward currents of 1033 ± 68 nA, 818 ± 46 nA and 983 ± 44 nA, respectively. Glycine (20 mM, control), which is not transported by PEPT1, showed no currents. Also no currents were observed for either of the test compounds in water-injected oocytes (**Figure 3**).

The currents generated by these pyrraline-containing dipeptides are smaller than those triggered by Gly-Sar or Ala-Lys (**Figure 3**) or by other native dipeptides (*26*) but they are significant. We consider currents as significant when they are (i) transporter specific, i.e., when no signals are obtained using the same substrate concentration in control oocytes, (ii) at least 5% of the currents elicited by reference substrates (dipeptides) and (iii) statistically significantly different from zero (*27*). All three criteria are met. We conclude therefore that Ala-Pyrr and Pyrr-Ala are actively transported by the human PEPT1 in an electrogenic manner, i.e., an H^+-symport. Translocation of substrates by PEPT1 comprises at least the following steps: (i) binding of H^+ and the substrate, (ii) conformational change of the resulting complex, (iii) release of the substrate and H^+ into the cell, and (iv) conformational change of the protein to the initial stage the binding site facing outward. The rate-limiting step of the translocation is still not known. With the exception of the last step, the return of the empty carrier, different PEPT1 substrates might affect these steps differently depending on their specific chemical properties. Modification of lysine to pyrraline leads to an increase in hydrophobicity. The hydrophobicity as expressed by the log $p_{O/W}$ value of Ala-Pyrr and Pyrr-Ala ($\log p_{O/W} = -1.11$) is in the range of the side-chain modified peptides Ala-Lys(Ac) and Ala-Lys(Bu) (*26*) ($\log p_{O/W} = -1.88$ and -0.81, respectively). In most cases, the affinity of such dipeptides to PEPT1 equals or surpasses that of the unmodified peptides (*16*, *26*). The reduced transport rate of Ala-Pyrr and Pyrr-Ala compared to the unmodified dipeptides is very consistent with the results obtained for side-chain modified Lys-Ala and Ala-Lys or Orn-Ala and Ala-Orn derivatives. Inward currents elicited by these substances were reduced by almost 50% as compared to the unmodified peptide (*26*). Space-demanding hydrophobic peptide side-chains can impede the actual transport, i.e. the conformational change of loaded PEPT1. It appears therefore that binding of the substrate is not the rate-limiting step for PEPT1 mediated translocations. This view is strongly supported by the fact that the competitive PEPT1 inhibitors developed previously by side-chain modification of dipeptides (*18*, *26*), even though they display with K_i values as low as 2 μM affinities much higher than those of PEPT1 substrates, do not elicit any currents. Nonetheless, the currents of Pyrr-Ala and Ala-Pyrr are still slightly lower than expected. Hypothetically, the formyl group of pyrraline could react, e.g., with lysine residues to form Schiff's bases. Such bonds might be able to temporarily stabilize the carrier/substrate complex and delay translocation, thereby reducing the currents measured.

hPEPT1-Mediated Transport of Ala-Pyrr and Pyrr-Ala into Transfected HeLa Cells. To establish PEPT1-mediated uptake of pyrraline dipeptides unequivocally, we studied their transport in a second, independent and direct approach. Unlabeled pyrraline, Ala-Pyrr and Pyrr-Ala (1 mM, 30 min) were added to HeLa cells transfected with the empty vector pcDNA3 (control) or with pcDNA3-hPEPT1. Intracellular contents were analyzed by HPLC. The uptake of free pyrraline shows no difference between HeLa cells expressing hPEPT1 and mock cells (**Figure 4**). In contrast, when added as dipeptide, the concentration of intracellular pyrraline is 4- to 7-fold higher in hPEPT1-transfected HeLa cells than in cells transfected with the empty vector. Neither Ala-Pyrr nor Pyrr-Ala could be detected in the cells. We conclude that Ala-Pyrr and Pyrr-Ala are indeed transported by hPEPT1 into the cells. Inside the cells, both dipeptides are hydrolyzed by intracellular peptidases to free pyrraline and alanine.

Transport by hPEPT1 could explain the high oral absorption rates of certain AGEs observed *in vivo* (*14*, *15*). In these studies, healthy volunteers were first asked to renounce processed food

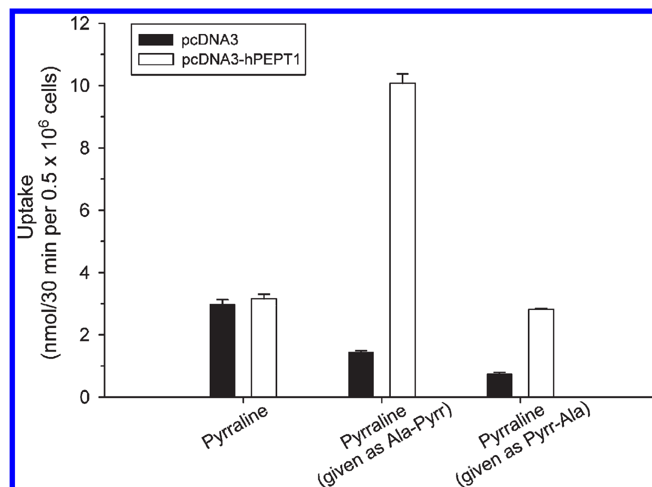

Figure 4. Uptake of pyrraline and its dipeptide derivatives into HeLa cells transfected with pcDNA3 (empty vector) or pcDNA3-hPEPT1. Uptake of pyrraline, Ala-Pyrr and Pyrr-Ala (1 mM) was measured for 30 min at pH 6.0 and 37 °C. The cell contents were analyzed by RP-HPLC. $n = 3-5$.

for 2 days. Then, one single meal with protein-bound pyrraline, which had been formed during processing, was administered. Urine of the volunteers was collected and analyzed for pyrraline and other glycation compounds throughout the study. 50−100% of the administered pyrraline were excreted *via* the urine, providing circumstantial evidence for (i) the ability of intestinal proteases and peptidases to hydrolyze glycated proteins into absorbable peptides and/or amino acids, and (ii) the actual transport. Other glycation compounds did not show such high excretion rates.

In order to demonstrate that pyrraline can indeed be released from glycated proteins, β-casein glycated with 3-DG was subjected to a two-step simulated gastrointestinal digestion. In a preliminary study, the digested sample was analyzed by HPLC−UV−ESI-MS showing that most of the pyrraline is peptide bound. Specific peptides like Ser-Pyrr and Val-Pyrr could be detected. From what we know about the structural requirements of PEPT1 substrates (*18*), we believe that all possible pyrraline dipeptides, probably with the exception of Pro-pyrraline, are transported.

Under physiological conditions, at the intestinal microclimate pH of 6.5 to 6.8 (*17*), hPEPT1 will transport such peptides even uphill against a concentration gradient and accumulate them in enterocytes where they are hydrolyzed. It should be noted that the peptide transporter hPEPT1 is also expressed in the kidney where it is located in the apical membrane of the epithelial cells of renal tubule. At this epithelium, hPEPT1 mediates the reabsorption of its substrates from the primary filtrate into the blood (for review see refs *17*−*19*). Even though our data imply that also the renal PEPT1 would be able to reabsorb Ala-Pyrr and Pyrr-Ala from the primary filtrate, it is very doubtful that these compounds ever appear in the blood and hence in the urine in intact form. Transport of Ala-Pyrr and Pyrr-Ala by renal hPEPT1 (or hPEPT2 (*17*, *19*)) is therefore probably irrelevant under physiological conditions.

In summary, this study established for the first time the transport of an advanced glycation end product by a peptide transporter. PEPT1 is most likely responsible for the intestinal absorption of pyrraline dipeptides after food intake. This finding necessitates further, intensified studies on potentially harmful effects of glycation products in the human body. Moreover, with Ala-Pyrr and Pyrr-Ala we identified new peptide transporter substrates.

ABBREVIATIONS USED

Ala-Pyrr, alanylpyrraline; Pyrr-Ala, pyrralylalanine; Gly-Sar, glycylsarcosine; PEPT1, proton-coupled peptide transporter 1; AGE, advanced glycation end product; HPLC, high pressure liquid chromatography; 3-DG, 3-deoxyglucosulose.

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LITERATURE CITED

(1) Henle, T. Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids* **2005**, *29*, 313–322.

(2) Henle, T. Dietary advanced glycation end products − a risk to human health? A call for an interdisciplinary debate. *Mol. Nutr. Food Res.* **2007**, *51*, 1075–1078.

(3) Koschinsky, T.; He, C.-J.; Mitsuhashi, T.; Bucala, R.; Liu, C.; Buenting, C.; Heitmann, K.; Vlassara, H. Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6474–6479.

(4) Uribarri, J.; Cai, W.; Peppa, M.; Goodman, S.; Ferrucci, L.; Striker, G.; Vlassara, H. Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *J. Gerontol., Ser. A* **2007**, *62*, 427–433.

(5) Vlassara, H.; Cai, W.; Crandall, J.; Goldberg, T.; Oberstein, R.; Dardaine, V.; Peppa, M.; Rayfield, E. J. Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15596–15601.

(6) Henle, T. AGEs in foods: Do they play a role in uremia? *Kidney Int.* **2003**, *63*, S145–7.

(7) Ames, J. M. Evidence against dietary advanced glycation endproducts being a risk to human health. *Mol. Nutr. Food Res.* **2007**, *51*, 1085–1090.

(8) Shinohara, K.; Jahan, N.; Tanaka, M.; Yamamoto, K.; Wu, R. T.; Murakami, H.; Omura, H. Formation of mutagens by aminocarbonyl reactions. *Mutat. Res.* **1983**, *122*, 279–286.

(9) Omura, H.; Jahan, N.; Shinohara, K.; Murakami, H. Formation of mutagens by the Maillard reaction. *ACS Symp. Ser.* **1983**, *215*, 537–563.

(10) Henle, T.; Bachmann, A. Synthesis of pyrraline reference material. *Z. Lebensm.-Unters. Forsch.* **1996**, *202*, 72–74.

(11) Nakayama, T.; Hayase, F.; Kato, H. Formation of ε-(2-formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine in the Maillard reaction between D-glucose and L-lysine. *Agric. Biol. Chem.* **1980**, *44*, 1201–1202.

(12) Henle, T.; Walter, A. W.; Klostermeyer, H. Simultaneous determination of protein-bound Maillard products by ion-exchange chromatography and photodiode array detection. In *Maillard Reactions in Chemistry, Food, and Health*; Labuza, T. P., Ed.; The Royal Society of Chemistry: Cambridge, U.K., 1994; pp 195−200.

(13) Resmini, P.; Pellegrino, L. Occurrence of protein-bound lysylpyrrolaldehyde in dried pasta. *Cereal Chem.* **1994**, *71*, 254–262.

(14) Förster, A.; Henle, T. Glycation in food and metabolic transit of dietary AGEs (advanced glycation end-products): studies on the urinary excretion of pyrraline. *Biochem. Soc. Trans.* **2003**, *31*, 1383–1385.

(15) Förster, A.; Kühne, Y.; Henle, T. Studies on absorption and elimination of dietary maillard reaction products. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 474–481.

(16) Hellwig, M.; Geissler, S.; Peto, A.; Knütter, I.; Brandsch, M.; Henle, T. Transport of free and peptide-bound pyrraline at intestinal and renal epithelial cells. *J. Agric. Food Chem.* **2009**, *57*, 6474–6480.

(17) Daniel, H. Molecular and integrative physiology of intestinal peptide transport. *Annu. Rev. Physiol.* **2004**, *66*, 361–384.

(18) Brandsch, M.; Knütter, I.; Leibach, F. H. The intestinal H^+/peptide symporter PEPT1: structure-affinity relationships. *Eur. J. Pharm. Sci.* **2004**, *21*, 53–60.

(19) Brandsch, M. Transport of drugs by proton-coupled peptide transporters: pearls and pitfalls. *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 887–905.

(20) Knütter, I.; Kottra, G.; Fischer, W.; Daniel, H.; Brandsch, M. High-affinity interaction of sartans with H^+/peptide transporters. *Drug Metab. Dispos.* **2009**, *37*, 143–149.

(21) Dorn, M.; Jaehme, M.; Weiwad, M.; Markwardt, F.; Rudolph, R.; Brandsch, M.; Bosse-Doenecke, E. The role of N-glycosylation in transport function and surface targeting of the human solute carrier PAT1. *FEBS Lett.* **2009**, *583*, 1631–1636.

(22) Riedel, T.; Lozinsky, I.; Schmalzing, G.; Markwardt, F. Kinetics of P2X7 receptor-operated single channels currents. *Biophys. J.* **2007**, *92*, 2377–2391.

(23) Bretschneider, F.; Markwardt, F. Drug-dependent ion channel gating by application of concentration jumps using U-tube technique. *Methods Enzymol.* **1999**, *294*, 180–189.

(24) Klapperstück, M.; Büttner, C.; Böhm, T.; Schmalzing, G.; Markwardt, F. Characteristics of P2X7 receptors from human B lymphocytes expressed in *Xenopus* oocytes. *Biochim. Biophys. Acta* **2000**, *1467*, 444–456.

(25) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(26) Knütter, I.; Hartrodt, B.; Theis, S.; Foltz, M.; Rastetter, M.; Daniel, H.; Neubert, K.; Brandsch, M. Analysis of the transport properties of side chain modified dipeptides at the mammalian peptide transporter PEPT1. *Eur. J. Pharm. Sci.* **2004**, *21*, 61–67.

(27) Knütter, I.; Wollesky, C.; Kottra, G.; Hahn, M. G.; Fischer, W.; Zebisch, K.; Neubert, R. H.; Daniel, H.; Brandsch, M. Transport of angiotensin-converting enzyme inhibitors by H^+/peptide transporters revisited. *J. Pharmacol. Exp. Ther.* **2008**, *327*, 432–441.

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Research paper

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

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Iron chelator
3-Hydroxy-4-pyridinone
Prodrug

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.

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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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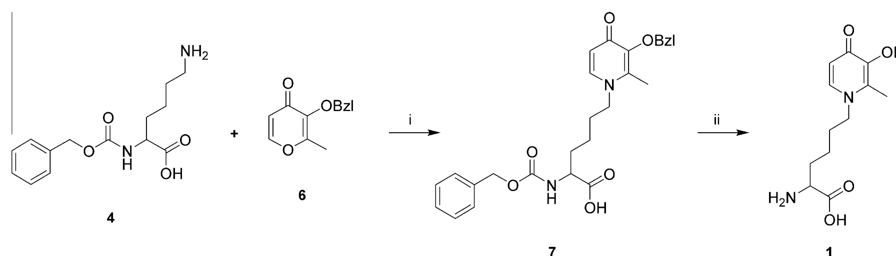

Fig. 1. Synthesis of 6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine (maltosine) as the free amino acid. (i) Ethanol/borate buffer, pH 10.0 1/1, 80 °C, 24 h. (ii) H_2, 10% Pd/C, ethanol, rt, 18 h. **4** Z-Lys-OH, **6** 3-benzyloxy-2-methyl-4-pyrone (benzyl maltol), **7** Z-Mal(Bzl)-OH,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

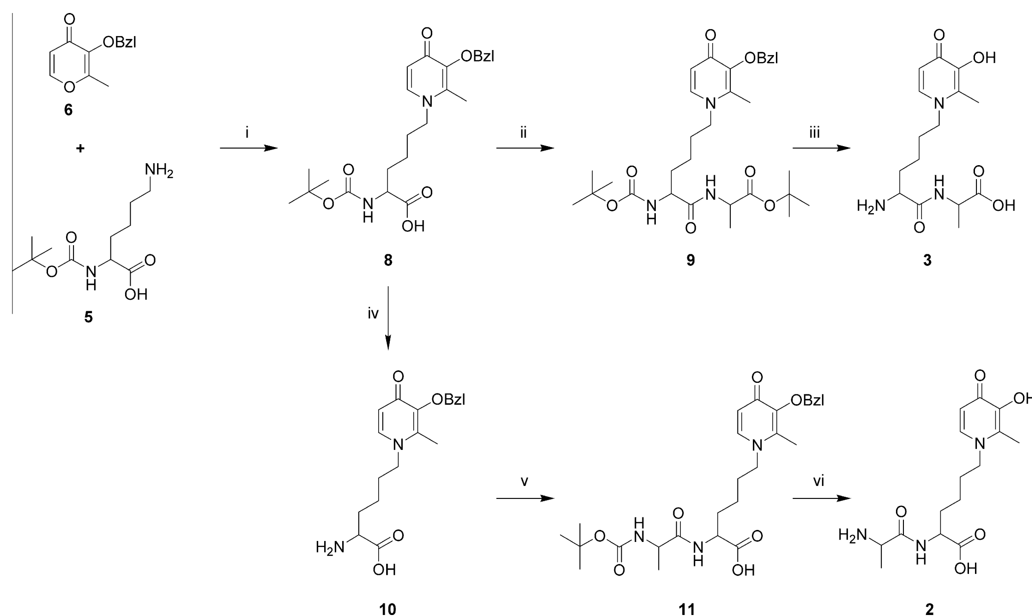

Fig. 2. Synthesis of peptide-bound 6-(3-hydroxy-4-oxo-2-methyl-4(1*H*)-pyridin-1-yl)-L-norleucine (Mal-Ala and Ala-Mal). (i) Ethanol/water 1/1, pH 13, 80 °C, 18 h. (ii) DIPEA, TSTU, DCM, rt, 30 min, then H-Ala-OBu^t, DIPEA, rt, 30 min. (iii) H_2, 10% Pd/C, methanol, rt, 18 h, then 6 N HCl/THF 1/1, rt, 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; ^1H 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; ^1H 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; ^1H 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-[^3H]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-[^3H]lysine uptake determined by measuring the uptake of L-[^3H]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 ($0.02 \pm 0.01\%/cm^2 \times h$ *vs.* $0.13 \pm 0.03\%/cm^2 \times h$, 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 $0.27 \pm 0.08\%/cm^2 \times h$ was determined. In the case of Mal-Ala, the flux rate of maltosine was $0.16 \pm 0.06\%/cm^2 \times h$.

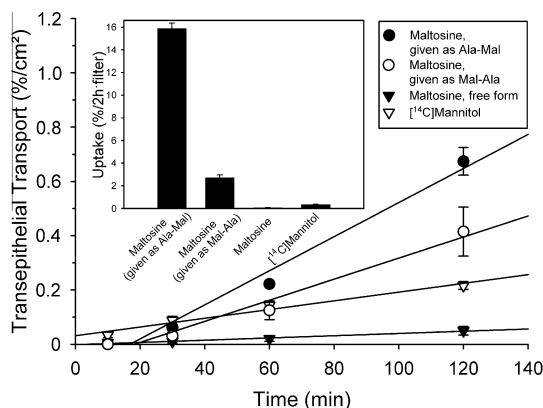

Fig. 3. Transepithelial flux of maltosine, Ala-Mal, Mal-Ala (all 1 mM) and [^{14}C]mannitol (10 μM) across Caco-2 cell monolayers. Flux was determined at pH 6.0 (apical) and pH 7.5 (basolateral) over 2 h. Inset: Uptake of the compounds into the cells grown on filter membranes within 2 h. n = 3.

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 ± 0.19%/cm^2 × h. Again, this flux rate was lower than that of the space marker [^{14}C]mannitol at these cells (1.19 ± 0.08%/cm^2 × h). Only 0.44 ± 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).

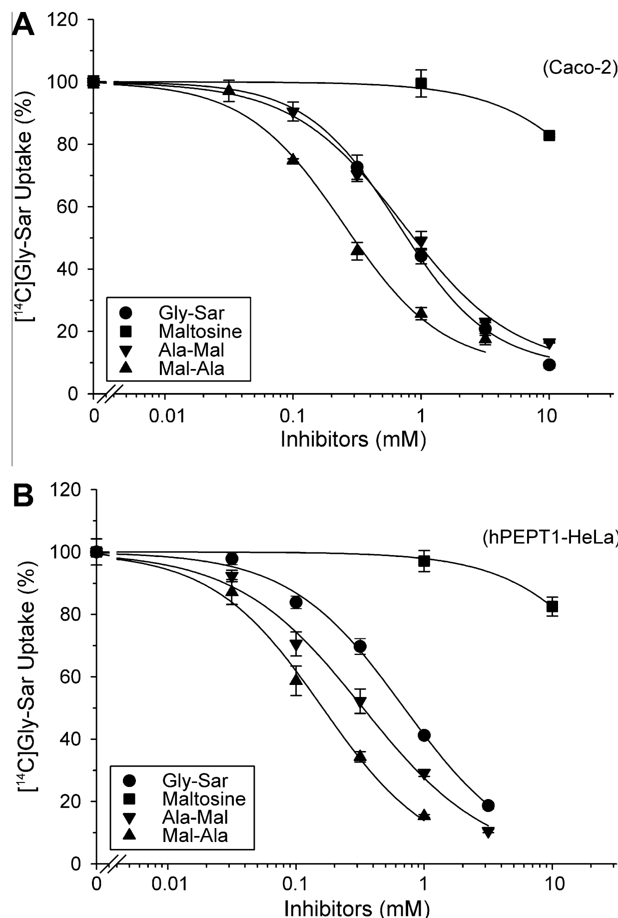

Fig. 4. Inhibition of [^{14}C]Gly-Sar uptake into Caco-2 and hPEPT1-HeLa cells. (A) Uptake of 10 μM [^{14}C]Gly-Sar into Caco-2 cells and (B) uptake of 20 μM [^{14}C]Gly-Sar into HeLa cells was measured for 10 min at pH 6.0 in the absence (control) or presence of increasing concentrations of maltosine,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

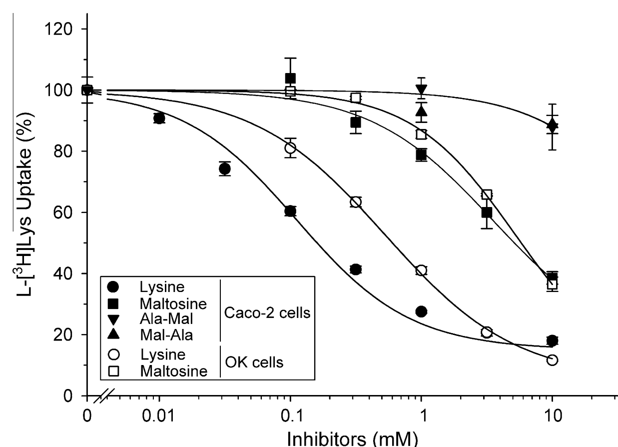

Fig. 5. Inhibition of L-[^{3}H]lysine uptake into Caco-2 and OK cells. Uptake of 2 nM L-[^{3}H]lysine was measured for 5 min at pH 6.0 in the absence (control) or presence of increasing concentrations of maltosine, Ala-Mal, Mal-Ala and L-lysine. n = 3–4.

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

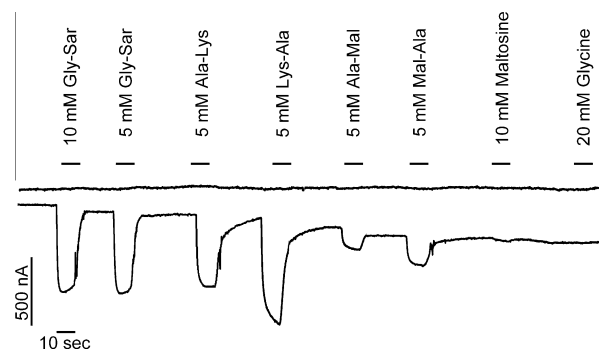

Fig. 6. hPEPT1-dependent currents at *Xenopus laevis* oocytes. Currents induced by Ala-Mal, Mal-Ala, maltosine and the reference compounds Gly-Sar, Ala-Lys, Lys-Ala and glycine in water- (upper trace) and hPEPT1-cRNA-injected oocytes (lower trace). Currents were recorded at pH 6.5 by two-electrode voltage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

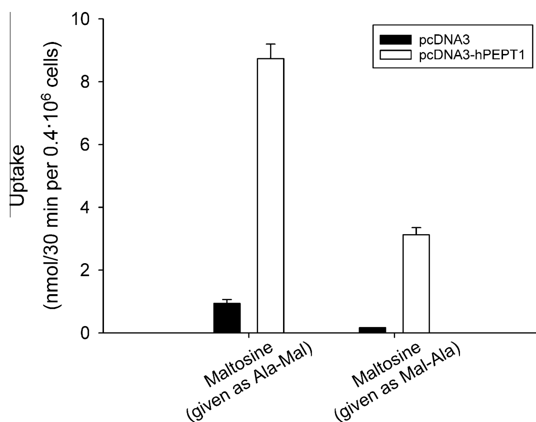

Fig. 7. Uptake of Ala-Mal and Mal-Ala (1 mM) into HeLa cells transfected with pcDNA3 or pcDNA3-hPEPT1. Uptake was measured for 30 min at pH 6.0 and at 37 °C. The cell contents were analyzed by RP-HPLC. *n* = 5.

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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References

[1] G. Faa, G. Crisponi, Iron chelating agents in clinical practice, Coord. Chem. Rev. 184 (1999) 291–310.
[2] M.A. Santos, Recent developments on 3-hydroxy-4-pyridinones with respect to their clinical applications: mono and combined ligand approaches, Coord. Chem. Rev. 252 (2008) 1213–1224.
[3] A. Shander, M.D. Cappellini, L.T. Goodnough, Iron overload and toxicity: the hidden risk of multiple blood transfusions, Vox Sang. 97 (2009) 185–197.
[4] A. Cao, R. Galanello, Beta-thalassemia, Genet. Med. 12 (2010) 61–76.
[5] G. Crisponi, M. Remelli, Iron chelating agents for the treatment of iron overload, Coord. Chem. Rev. 252 (2007) 1225–1240.
[6] N.F. Olivieri, J.R. Buncic, E. Chew, T. Gallant, R.V. Harrison, N. Keenan, W. Logan, D. Mitchell, G. Ricci, B. Skarf, et al., Visual and auditory neurotoxicity in patients receiving subcutaneous deferoxamine infusions, N. Engl. J. Med. 314 (1986) 869–873.
[7] M.A. Santos, M. Gil, L. Gano, S. Chaves, Bifunctional 3-hydroxy-4-pyridinone derivatives as potential pharmaceuticals: synthesis, complexation with Fe(III), Al(III) and Ga(III) and in vivo evaluation with ^{67}Ga, J. Biol. Inorg. Chem. 10 (2005) 564–580.
[8] G.J. Kontoghiorghes, New orally active iron chelators, Lancet 1 (1985) 817.
[9] G.J. Kontoghiorghes, M.A. Aldouri, L. Sheppard, A.V. Hoffbrand, 1,2-Dimethyl-3-hydroxypyrid-4-one, an orally active chelator for treatment of iron overload, Lancet 1 (1987) 1294–1295.
[10] G.J. Kontoghiorghes, M.A. Aldouri, A.V. Hoffbrand, J. Barr, B. Wonke, T. Kourouclaris, L. Sheppard, Effective chelation of iron in beta thalassaemia with the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one, Br. Med. J. 295 (1987) 1509–1512.
[11] F. Ledl, H. Osiander, O. Pachmayr, T. Severin, Formation of maltosine, a product of the Maillard reaction with a pyridone structure, Z. Lebensm. Unters. Forsch. A 188 (1989) 207–211.
[12] G. Rehner, T. Walter, Effect of Maillard products and lysinoalanine on the bioavailability of iron, copper and zinc, Z. Ernährwiss. 30 (1991) 50–55.
[13] J. O'Brien, P.A. Morrissey, Metal ion complexation by products of the Maillard reaction, Food Chem. 58 (1997) 17–27.
[14] L.D. Stegink, J.B. Freeman, L. Den Besten, L.J. Filer, Maillard reaction products in parenteral nutrition, Prog. Food Nutr. Sci. 5 (1981) 265–278.
[15] S. Seifert, R. Krause, K. Gloe, T. Henle, Studies on the metal complexation of the peptide-bound Maillard reaction products N^{ε}-fructoselysine and N^{ε}-carboxymethyllysine, J. Agric. Food Chem. 52 (2004) 2347–2350.
[16] T. Henle, A.W. Walter, H. Klostermeyer, Simultaneous determination of protein-bound Maillard products by ion-exchange chromatography and photodiode array detection, in: T.P. Labuza (Ed.), Maillard Reactions in Chemistry, Food, and Health, The Royal Society of Chemistry, Cambridge, UK, 1994, pp. 195–200.
[17] S. Seifert, Synthese und Komplexbildungseigenschaften ausgewählter Maillard-Reaktionsprodukte, PhD thesis, TU Dresden, 2008.
[18] M. Hellwig, S. Geissler, A. Peto, I. Knütter, M. Brandsch, T. Henle, Transport of free and peptide-bound pyrraline at intestinal and renal epithelial cells, J. Agric. Food Chem. 57 (2009) 6474–6480.
[19] S. Geissler, M. Hellwig, M. Zwarg, F. Markwardt, T. Henle, M. Brandsch, Transport of the advanced glycation end products alanylpyrraline and pyrralylalanine by the human proton-coupled peptide transporter hPEPT1, J. Agric. Food Chem. 58 (2010) 2543–2547.
[20] T. Henle, A.W. Walter, H. Klostermeyer, A simple method for the preparation of furosine and pyridosine reference material, Z. Lebensm. Unters. Forsch. 198 (1994) 66–67.
[21] M.A. Glomb, C. Pfahler, Amides are novel protein modifications formed by physiological sugars, J. Biol. Chem. 276 (2001) 41638–41647.
[22] I. Knütter, C. Wollesky, G. Kottra, M.G. Hahn, W. Fischer, K. Zebisch, R.H. Neubert, H. Daniel, M. Brandsch, Transport of angiotensin-converting enzyme inhibitors by H^+/peptide transporters revisited, J. Pharmacol. Exp. Ther. 327 (2008) 432–441.
[23] I. Knütter, G. Kottra, W. Fischer, H. Daniel, M. Brandsch, High-affinity interaction of sartans with H^+/peptide transporters, Drug Metab. Dispos. 37 (2009) 143–149.
[24] M. Dorn, M. Jaehme, M. Weiwad, F. Markwardt, R. Rudolph, M. Brandsch, E. Bosse-Doenecke, The role of N-glycosylation in transport function and surface targeting of the human solute carrier PAT1, FEBS Lett. 583 (2009) 1631–1636.
[25] T. Riedel, I. Lozinsky, G. Schmalzing, F. Markwardt, Kinetics of P2X7 receptor-operated single channels currents, Biophys. J. 92 (2007) 2377–2391.
[26] P.S. Dobbin, R.C. Hider, A.D. Hall, P.D. Taylor, P. Sarpong, J.B. Porter, G. Xiao, D. van der Helm, Synthesis, physicochemical properties, and biological evaluation of N-substituted 2-alkyl-3-hydroxy-4(1*H*)-pyridinones: orally active iron chelators with clinical potential, J. Med. Chem. 36 (1993) 2448–2458.
[27] W. Bannwarth, R. Knorr, Formation of carboxamides with N,N,N′,N′-tetramethyl (succinimido) uronium tetrafluoroborate in aqueous/organic solvent systems, Tetrahedron Lett. 32 (1991) 1157–1160.
[28] M. Brandsch, Transport of drugs by proton-coupled peptide transporters: pearls and pitfalls, Expert Opin. Drug Metab. Toxicol. 5 (2009) 887–905.
[29] I. Knütter, B. Hartrodt, S. Theis, M. Foltz, M. Rastetter, H. Daniel, K. Neubert, M. Brandsch, Analysis of the transport properties of side chain modified dipeptides at the mammalian peptide transporter PEPT1, Eur. J. Pharm. Sci. 21 (2004) 61–67.
[30] R. Tateoka, H. Abe, S. Miyauchi, S. Shuto, A. Matsuda, M. Kobayashi, K. Miyazaki, N. Kamo, Significance of substrate hydrophobicity for recognition by an oligopeptide transporter (PEPT1), Bioconjugate Chem. 12 (2001) 485–492.

5 Diskussion

Die metabolischen Studien zu alimentär aufgenommenen isolierten Maillard-Reaktionsprodukten (MRPs) oder MRP-reichen Lebensmitteln beschäftigen sich hauptsächlich mit der Untersuchung von Amadori-Produkten (Fruktoselysin) und den AGEs CML und Pyrralin (ERBERSDOBLER und FAIST 2001, FÖRSTER und HENLE 2003, FÖRSTER *et al.* 2005, SOMOZA *et al.* 2006). Durch die in diesen Studien gezeigten renalen Ausscheidungen der MRPs kann indirekt auf einen intestinal stattfindenden Transportprozess geschlossen werden, welcher parazellulär durch einfache Diffusion oder transzellulär durch Diffusion, Endozytose oder durch Transportproteine vermittelt sein kann. Da die MRPs ihrer Struktur nach freie, Peptid- oder Protein-gebundene modifizierte Aminosäuren (Lysin oder Arginin) sind, könnte die intestinale Absorption Dipeptid-gebundener MRPs durch Peptidtransporter und die der freien MRPs durch verschiedene Aminosäure-Transportsysteme vermittelt sein. Falls die MRPs die Nieren erreichen, könnten die MRPs entweder wieder ausgeschieden oder *via* Aminosäure- oder Peptidtransporter reabsorbiert werden. Dies wäre nicht nur für exogen zugeführte, sondern auch für endogen gebildete MRPs relevant.

Für die vorliegenden Untersuchungen sollten daher MRPs (Amadori-Produkte und AGEs) in freier Form, aber vor allem als Dipeptid-Derivate (Ala-MRP und MRP-Ala) hinsichtlich ihres intestinalen, sowie renalen Transportes untersucht und ein mögliches, den Transport vermittelndes *Carrier*-Protein identifiziert werden. Aufgrund der strukturellen Vielfalt der MRPs ist ein unterschiedliches Interaktionsverhalten mit den intestinalen und renalen Transportsystemen zu erwarten.

5.1 Der intestinale transepitheliale Transport

Die Untersuchungen des intestinalen transepitheliale Transportes (Flux) an Caco-2-Zellen können Aufschluss darüber geben, ob die Testsubstanzen intrazellulär aufgenommen und akkumuliert werden und möglicherweise die Zellen auf der abluminalen Seite wieder verlassen. Dies wiederum ist ausschlaggebend für die orale Bioverfügbarkeit von Substanzen.

Zunächst wurden die Caco-2-Zellen auf der apikalen Zellseite mit den freien, modifizierten Aminosäuren inkubiert (*Studie 1*). Die ermittelten Fluxe der getesteten freien MRPs betrugen zwischen 0,008 und 0,09 %/cm²×h, wobei diese unterhalb dem Flux des *space markers*

[^{14}C]Mannitol[1] (0,13 %/cm²×h) und weit unter dem Flux der Kontroll-Aminosäure L-[^{3}H]Lysin (6,86 %/cm²×h) lagen. Die zelluläre Akkumulation der glykierten Aminosäuren entsprachen weniger als 1% der Ausgangskonzentration (*Studie 1*: HELLWIG *et al.* 2011, Tabelle 2). Folglich resultiert der transepitheliale Transport der modifizierten Aminosäuren möglicherweise aus einer einfachen Diffusion entlang ihres Konzentrationsgradienten.

Nach der Inkubation der Caco-2-Zellen mit den Dipeptid-gebundenen glykierten Aminosäuren konnten die intakten Dipeptid-Derivate nur von CEL und CML intrazellulär wie basolateral nachgewiesen werden. Analysen der Proben der anderen Dipeptid-gebundenen MRPs zeigten in den Zellen und im basolateralen Kompartiment die jeweiligen freien Aminosäuren, für welche Fluxwerte von 0,06-3,37 %/cm²×h ermittelt wurden (*Studie 1*: HELLWIG *et al.* 2011, Tabelle 2). Diese Ergebnisse weisen darauf hin, dass die modifizierten Dipeptide in die Zellen gelangen und dort schnell durch Dipeptidasen in die jeweiligen freien MRP sowie Alanin hydrolysiert werden. Eine Ausnahme bildet hierbei FL, welches offensichtlich auch nicht als Ala-FL bzw. FL-Ala in die Zellen aufgenommen werden kann. Die intrazelluläre Detektion der Dipeptid-Derivate von CEL und CML war wahrscheinlich durch eine höhere Stabilität gegenüber enzymatischer Hydrolyse möglich. Die freien MRPs könnten anschließend durch entsprechende basolaterale Aminosäure-Transporter (DEVÉS und BOYD 1998) oder durch einfache Diffusion entlang des Konzentrationsgefälles auf die abluminale Zellseite gelangen. Hierbei stellt die basolaterale Zellseite für die hydrophoberen[2] modifizierten Aminosäuren (Argpyrimidin, Formylin, Pyrralin) eine geringere Barriere dar, als für die hydrophileren, wie CEL, CML, MG-H1 und Maltosin, da letztere stärker retendiert wurden (*Studie 1*: HELLWIG *et al.* 2011, Tabelle 2, Abbildung 1).

Die im Rahmen der *Studie 1* ermittelten Daten zeigen, dass es für die Charakterisierung der intestinalen Absorption bedeutsam ist, Substanzen in Peptid-gebundener statt in freier Form zu verwenden: Der intestinale transepitheliale Transport Dipeptid-gebundener glykierter Aminosäuren ist bis 80fach höher, als der der freien glykierten Aminosäuren. Im Vergleich zum [^{14}C]Mannitol liegen die gemessenen Fluxe meist höher, verglichen mit dem Kontroll-Dipeptid [^{14}C]Gly-Sar sind die Fluxe aber deutlich niedriger (2,79 %/cm²×h; *Studie 1*: HELLWIG *et al.* 2011, Tabelle 2). Dieser verminderte Transport glykierter Dipeptide könnte durch die von der Modifizierung der Dipeptide ausgehenden sterischen Behinderung des

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[1] Der *space marker* Mannitol ist eine standardmäßig eingesetzte Kontrollsubstanz in Fluxexperimenten und dient als Marker für parazelluläre Transportprozesse.

[2] Die Hydrophobizität einer Substanz wird durch den Oktanol-Wasser-Verteilungskoeffizient, dem $\log P_{o/w}$-Wert, ausgedrückt. Dieser $\log P_{o/w}$-Wert ist ein Maß für die Lipophilie einer Substanz: $\log P_{o/w}$-Werte für Arg-pyrimidin 0,21; Formylin 0,06; Pyrralin -0,46; MG-H1 -0,75; CEL -0,88; CML -1,37; Maltosin -1,94 (persönliche Mitteilung von MICHAEL HELLWIG: Werte berechnet mittels ChemDraw Pro 5.0 (Cambridgesoft, Cambridge, MA)).

Transportes bedingt sein. Bei einem Vergleich der Alanyl- und der Lysyl-Dipeptid-Derivate der gleichen glykierten Aminosäure zeigte sich, dass der Flux der Dipeptid-Derivate[3] mit *C*-terminaler Seitenketten-Modifizierung im Vergleich zum *N*-terminal modifizierten Derivat bis zu vierfach höher lag. Die Position der modifizierten Seitenkette der Dipeptid-gebundenen MRPs scheint demnach relevant für den transepithelialen Transport zu sein.

Diese *in vitro* Ergebnisse könnten hinweisend für die orale Bioverfügbarkeit von den *in vivo* ermittelten Daten sein: In den Studien von FÖRSTER *et al.* (2003, 2005) zeigte sich, dass Pyrralin nach alimentärer Zufuhr Pyrralin-haltiger Lebensmittel nahezu vollständig mit dem Harn wieder ausgeschieden wird, ohne weiteren metabolischen Einfluss im Organismus zu unterliegen. Dieser Befund stimmt mit den Ergebnissen der *Studie 1* überein: Dadurch, dass Pyrralin *in vitro* in Dipeptid-gebundener Form intestinal transepithelial transportiert wurde, kann vermutet werden, dass auch in den Arbeiten von FÖRSTER *et al.* (2003, 2005) Pyrralin in gleicher Weise transportiert wurde. Durch das inhibitorische Potential von Pyrralin gegenüber Verdauungsenzymen könnte Pyrralin zusätzlich die eigene Absorption in Dipeptid-gebundener Form in dem Sinne fördern, als das Dipeptid-gebundenes Pyrralin weniger hydrolysiert und somit als Dipeptid der Absorption zur Verfügung steht (ÖSTE *et al.* 1987). Ebenso wurden die *in vivo* Daten bezüglich des FL mit den in *Studie 1* ermittelten Daten bestätigt: Oral aufgenommenes Casein-gebundenes oder freies FL wird über den Harn bzw. Fäzes zu maximal 5% wieder ausgeschieden (ERBERSDOBLER *et al.* 1991, LEE und ERBERSDOBLER 1994, FÖRSTER und HENLE 2005). Ferner diffundierte freies FL in Experimenten an Darmsegmenten von Ratten nur passiv, im Gegensatz zum nicht-modifizierten Lysin (ERBERSDOBLER *et al.* 1981). In Übereinstimmung mit diesen Daten, konnte ebenso der in *Studie 1* gezeigte intestinale Transport von freiem FL nur auf eine einfache Diffusion zurückgeführt werden. Die intestinale Absorption von FL kann im Gegensatz zu Pyrralin offensichtlich auch nicht in Dipeptid-gebundener Form erhöht werden. Wiederrum kann über einen intestinal stattgefundenen Transport von Maltosin *in vivo* nur spekuliert werden. In einer Tierversuchsstudie mit Ratten von REHNER und WALTER (1991) zeigte sich, dass Maltosin, bedingt durch dessen komplexbildende Eigenschaft, nach oraler Aufnahme zu einer Erhöhung der renalen Ausscheidung von Eisenionen geführt hat und somit auch intestinal aufgenommen worden sein müsste. Die intestinale Absorption des Maltosins könnte hierbei in Dipeptid-gebundener Form stattgefunden haben.

[3] Im eigentlichen Sinne ist dieser Flux der der jeweiligen glykierten Aminosäure, welche nach intestinaler Aufnahme als Dipeptid-Derivat intrazellulär durch Hydrolyse freigesetzt wurde. Einfachheit halber wird aber von dem Flux der Dipeptid-Derivate gesprochen.

5.2 *Die Interaktion mit intestinalen apikalen Transportsystemen*

Die Untersuchungen bezüglich des intestinalen transepithelialen Transportes zeigen, dass der Transport der freien MRPs vernachlässigbar ist und der niedrige Flux vermutlich aus einer einfachen Diffusion resultiert. In Kompetitionsstudien an Caco-2-Zellen sollte die Interaktion der freien und Dipeptid-gebundenen MRPs mit den intestinalen Transportsystemen für kationische Aminosäuren bzw. Di- und Tripeptiden untersucht werden.

Hoch-affine Liganden eines Transportsystems können entweder Substrate oder Inhibitoren von Transportsystemen darstellen. Letzteres wäre vor allem aus ernährungsphysiologischer Sicht relevant. Alimentär aufgenommene freie MRPs, welche *in vitro* transepithelial nicht transportiert werden, könnten zum Beispiel mit nicht-modifizierten Aminosäuren aus der Nahrung um die gleichen Bindungsstellen konkurrieren und so die Bioverfügbarkeit essentieller Aminosäuren beeinflussen.

Die hier untersuchten freien modifizierten Aminosäuren inhibierten die L-[^{3}H]Lysin-Aufnahme in Caco-2-Zellen auf bis zu 60% der Kontrolle. Die aus der konzentrationsabhängigen Inhibierung des L-[^{3}H]Lysin-Transportes ermittelten Affinitätskonstanten (K_i[4]) lagen im niedrig affinen Bereich (1,6-4,6 mM). Einzig Pyrralin interagierte mit dem Transportsystem mit einer Affinität ähnlich der des L-Lysins (0,32 mM *vs.* 0,11 mM; *Studie 1*: HELLWIG *et al.* 2011, Tabelle 1). Pyrralin ist wahrscheinlich ein hoch-affiner, intestinal nicht-transportierter, Inhibitor intestinaler Transportsysteme basischer Aminosäuren. ÖSTE *et al.* (1987) schlossen in ihrer Tierversuchsstudie mit Ratten ebenfalls auf eine Interaktion von Pyrralin mit den Transportsystemen für Lysin, da die intestinale Aufnahme von Lysin in Anwesenheit von Pyrralin vermindert war.

Die Inhibierung des L-[^{3}H]Lysin-Transportes auf bis zu 62% der Kontrolle in Anwesenheit der Dipeptid-Derivate wurde nicht erwartet (*Studie 1*: HELLWIG *et al.* 2011, Tabelle 1). Während der Inkubationszeit könnte es apikal zur enzymatischen Spaltung der Dipeptide in die jeweiligen freien MRPs gekommen sein, welche wiederum mit den spezifischen L-Lysin-Transportsystemen interagiert und zu einer Hemmung der L-[^{3}H]Lysin-Aufnahme geführt haben könnten.

Aus diesen Ergebnissen kann geschlussfolgert werden, dass wenige der freien, aber keine Dipeptid-gebundenen MRPs mit den Transportsystemen für kationische Aminosäuren interagieren. Im Gegensatz dazu interagierten die freien, aber vor allem die Dipeptid-gebundenen MRPs zum Teil sehr stark mit hPEPT1: In Anwesenheit eines nach innen

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[4] K_i-Werte sind die inhibitorischen Konstanten, welche aus den ermittelten IC_{50}-Werten (Konzentration eines Inhibitors die nötig ist, um die spezifische Aufnahme eines Standardsubstrates um 50% zu hemmen), unter Annahme einer kompetitiven Inhibierung, konvertiert werden (CHENG und PRUSOFF 1973).

gerichteten Protonengradienten hemmten die freien und Dipeptid-gebundenen MRPs die [^{14}C]Gly-Sar-Aufnahme in Caco-2-Zellen um 7-67% bzw. 40-91%. In einer konzentrationsabhängigen Inhibierung des [^{14}C]Gly-Sar-Transportes wurden die Affinitätskonstanten der Substanzen zum hPEPT1-Protein ermittelt (*Studie 1*: HELLWIG *et al.* 2011, Tabelle 1). Entsprechend der Klassifizierung von BRANDSCH *et al.* (2008) können die Substanzen als niedrig-, mittel- bzw. hoch-affine Liganden des PEPT1 eingeordnet werden (Tabelle 5.1).

Tabelle 5.1. Klassifizierung der freien und Dipeptid-gebundenen MRPs nach ihrer Affinität zum hPEPT1-Protein.

Affinität	hoch (K_i < 0,5 mM)	mittel (K_i 0,5-5 mM)	niedrig (K_i 5-15 mM)	keine
Testsubstanzen	Ala-Pyrr Pyrr-Ala Ala-Fom Fom-Ala Mal-Ala Ala-CML Ala-CEL Ala-Apy Apy-Ala	Ala-Mal Ala-(MG-H1) (MG-H1)-Ala CML-Ala CEL-Ala FL-Ala Tagatoselysin	Argpyrimidin Laktuloselysin Fruktoselysin Ala-FL	CML CEL Formylin Pyrralin Maltosin MG-H1
Kontrollsubstanzen	Ala-Lys Lys-Ala Ala-Arg Arg-Ala	Gly-Sar		Lys Arg

Ala, Alanin; Apy, Argpyrimidin; Arg, Arginin; CEL, Carboxyethyllysin; CML, Carboxymethyllysin; Fom, Formylin; FL, Fruktoselysin; Gly-Sar, Glycylsarkosin; Lys, Lysin; Mal, Maltosin; MG-H1, *methylglyoxal-derived hydroimidazolone 1;* Pyrr, Pyrralin

Die Modifizierung der Seitenketten (Lysin bzw. Arginin zu MRPs) hat nur geringen Einfluss auf die Affinitäten zu hPEPT1, da diese Affinitäten mit denen nicht-modifizierter Dipeptide vergleichbar ist (KNÜTTER *et al.* 2004, BIEGEL *et al.* 2006). Die Dipeptide mit einer Pyrrol-Seitenkette (Ala-Fom, Fom-Ala bzw. Ala-Pyrr und Pyrr-Ala) wiesen hierbei die höchsten Affinitäten auf. Diese Affinitätssteigerung kann, wie auch in anderen Arbeiten gezeigt (DANIEL *et al.* 1992, TATEOKA *et al.* 2001, KNÜTTER *et al.* 2004), auf die Erhöhung der Hydrophobizität[5] der Dipeptide, bedingt durch die Modifizierung, zurückgeführt werden. Die hydrophoberen Seitenketten können mit den hydrophoben Bindungstaschen des PEPT1-Proteins in Wechselwirkung treten (DANIEL *et al.* 1992). Durch die Glykierung und damit

[5] Log$P_{o/w}$-Werte für Ala-Lys/Lys-Ala -1,81, Ala-Pyrr/Pyrr-Ala -1,11, Ala-Fom/Fom-Ala -0,6 (aus GEISSLER *et al.* 2010, persönliche Mitteilung von MICHAEL HELLWIG: Werte berechnet mittels ChemDraw Pro 5.0 (Cambridgesoft, Cambridge, MA)).

Blockierung der ε-Aminogruppe in Ala-Lys und Lys-Ala gehen ebenso die sonst von der Bindungsdomäne im Transportprotein weniger gut tolerierten elektrostatischen Wechselwirkungen verloren (BRANDSCH *et al.* 1999, TATEOKA *et al.* 2001, KNÜTTER *et al.* 2004, VIG *et al.* 2006).

Des Weiteren interagierten, bis auf die Derivate von CEL und CML, alle Ala-MRP-Dipeptide mit einer um das 2-6fach geringeren Affinität mit dem hPEPT1-Protein, als die entsprechenden MRP-Ala-Dipeptide. KNÜTTER *et al.* (2004) berichteten ebenfalls, dass eine Modifizierung der Lysylreste in der *N*-terminalen Seitenkette von Dipeptiden zu höheren K_i-Werten führte, als eine Modifizierung der Seitenkette im *C*-Terminus. Bei den Dipeptid-Derivaten von CEL und CML zeigte sich, dass die Ala-MRP-Dipeptide höher affin gegenüber PEPT1 waren, als die MRP-Ala-Dipeptide. Möglicherweise resultiert dies aus der durch die Carboxyalkylierung bedingten negativen Ladung in den Lysyl-Seitenketten, wodurch eine ungleiche Ladungsverteilung in den asymmetrischen Bindungsregionen von PEPT1 entstehen könnte (KOTTRA *et al.* 2002).

Die höher affinen MRP-Ala-Dipeptide zeigen im Vergleich zu den entsprechenden Ala-MRP-Dipeptiden eine bis zu vierfach niedrigere intestinale Aufnahmerate, wobei die Derivate von CEL und CML wiederum den umgekehrten Fall aufweisen. Eine höhere Affinität zum PEPT1 könnte daher zusätzlich mit einer stärkeren Bindung am Protein einhergehen, was sich in einem langsameren Entlassen aus der Bindung ergeben und sich folglich in einem langsameren Flux wiederspiegeln würde.

Diesen Ergebnissen nach werden MRPs nicht in freier Form, aber Dipeptid-gebunden intestinal transepithelial transportiert. Die freien Glykierungsprodukte interagieren nur gering mit intestinalen Transportsystemen für L-Lysin und L-Arginin, werden aber in Dipeptid-gebundener Form zu mittel- bis hoch-affinen PEPT1-Liganden (Substrate oder Inhibitoren). Die Daten lassen daher den Schluss zu, dass PEPT1 als wesentliches Transportsystem an der intestinalen Absorption der Dipeptid-Derivate beteiligt zu sein scheint. Die zellulär aufgenommenen Dipeptid-Derivate werden in die jeweiligen freien glykierten Aminosäuren hydrolysiert und intrazellulär akkumuliert. Die für CEL, CML, Maltosin und MG-H1 gezeigte intrazelluläre Akkumulation von über 50% der Ausgangskonzentration (*Studie 1*: HELLWIG *et al.* 2011, Abbildung 1) spricht ebenfalls für eine Beteiligung von PEPT1 an der Translokation der modifizierten Dipeptid-Derivate, da PEPT1 seine Substrate erwiesenermaßen entgegen eines Konzentrationsgradienten transportiert (GANAPATHY und LEIBACH 1985). Die Hydrolyseprodukte werden an der abluminalen Zellseite durch entsprechende Aminosäure-

Transporter oder entlang des Konzentrationsgradienten aus den Zellen transportiert. Sowohl die Position der modifizierten Aminosäure-Seitenkette im Dipeptid, als auch die Art der Seitenketten-Modifizierung bzw. -Blockierung haben Einfluss auf die Affinität und den intestinalen transepithelialen Transport (KNÜTTER *et al.* 2004).

Da die bisherigen Daten aber weder die direkte Interaktion mit dem PEPT1-Protein noch den tatsächlichen PEPT1-vermittelten aktiven Transport der modifizierten Dipeptid-Derivate beweisen, sollte dies in nachfolgenden Untersuchungen gezeigt werden. Hierbei standen die in der fortgeschrittenen Phase der Maillard-Reaktion entstehenden AGEs Pyrralin (*Studie 3*; HENLE *et al.* 1996) und Maltosin (*Studie 4*; LEDL *et al.* 1989) im Fokus.

Pyrralin, als ein quantitativ in Lebensmitteln vorkommendes AGE, wird nach oraler Aufnahme nahezu vollständig renal wieder ausgeschieden (FÖRSTER und HENLE 2003, FÖRSTER *et al.* 2005). Pyrralin beeinflusst nicht nur die Aktivität proteolytischer Enzyme, sondern gilt auch als potentiell mutagen (OMURA *et al.* 1983, ÖSTE *et al.* 1987). Maltosin hingegen besitzt komplexbildende Eigenschaften und ist hoch-affin gegenüber Eisenionen (REHNER und WALTER 1991, SEIFERT 2008). Eine hohe Maltosin-Konzentration könnte nach intestinaler Aufnahme zu einer vermehrten Eisenausscheidung über den Harn führen.

Für die Reproduktion der an Caco-2-Zellen ermittelten Affinitätskonstanten und um zeigen zu können, dass die Interaktion der Dipeptid-Derivate von Pyrralin und Maltosin tatsächlich am PEPT1-Protein von statten geht, wurden Kompetitionsstudien an hPEPT1-transfizierten HeLa-Zellen durchgeführt. Die Dipeptid-Derivate, jedoch nicht die freien glykierten Aminosäuren Pyrralin und Maltosin, inhibierten konzentrationsabhängig die [^{14}C]Gly-Sar-Aufnahme in die transfizierten Zellen (*Studie 3*: GEISSLER *et al.* 2010, Abbildung 2; *Studie 4*: GEISSLER *et al.* 2011, Abbildung 4B). Die Derivate sind entsprechend der Klassifizierung von BRANDSCH *et al.* (2008) hoch-affine Liganden des hPEPT1-Proteins. Die ermittelten K_i-Werte sind ähnlich denen an Caco-2-Zellen ermittelten (*Studie 3*: GEISSLER *et al.* 2010, Tabelle 1; *Studie 4*: GEISSLER *et al.* 2011, Tabelle 1). Die inhibitorischen Effekte der Dipeptid-Derivate resultieren daher ausschließlich aus der direkten Interaktion der modifizierten Dipeptide mit dem Transportprotein.

Ob die Pyrralin- und Maltosin-Dipeptide tatsächlich auch hPEPT1-vermittelt transportiert werden oder nur an PEPT1 binden, sollten die elektrophysiologischen Untersuchungen an hPEPT1-exprimierenden *Xenopus laevis*-Oozyten zeigen. Die Derivate erzeugten an den hPEPT1-exprimierenden Oozyten positive einwärts gerichtete Ströme. Diese Dipeptid-Derivate interagieren demnach nicht nur mit dem hPEPT1-Protein, sondern werden auch tatsächlich hPEPT1-vermittelt im Kotransport mit Protonen transportiert. Obwohl die Ströme

signifikant[6] waren, entsprachen sie nur etwa 17-38% des Stromes der nicht-modifizierten Kontrolldipeptide, unabhängig von der Seitenketten-Modifizierung (*Studie 3*: GEISSLER *et al.* 2010, Abbildung 3; *Studie 4*: GEISSLER *et al.* 2011, Abbildung 6). Allerdings zeigte sich auch bei KNÜTTER *et al.* (2004), dass Seitenketten-modifizierte Dipeptide trotz mittlerer bis hoher Affinität zum PEPT1-Protein einwärts gerichtete Ströme von 16-84% des nicht-modifizierten Kontroll-Dipeptides erzeugten. Die Erhöhung der Hydrophobizität durch eine Seitenketten-Modifizierung könnte für den verminderten Transport der Derivate verantwortlich sein (KNÜTTER *et al.* 2004, TATEOKA *et al.* 2001). Wahrscheinlicher ist jedoch eine sterische Behinderung des Transportes aufgrund der voluminösen Seitenketten (Modifizierung von Lysin zu Pyrralin bzw. Maltosin). Denkbar wäre ebenso ein temporär stabilisierter *Carrier*-Substrat-Komplex, welcher die Konformationsänderung des „Substrat-beladenen" hPEPT1 beeinflussen könnte.

Zusätzlich wurden in einem weiteren, unabhängigen Versuch zur eindeutigen Bestimmung der hPEPT1-vermittelten Translokation der Dipeptid-Derivate von Pyrralin und Maltosin hPEPT1-transfizierte HeLa-Zellen herangezogen und mittels HPLC untersucht. Intrazellulär konnte keines der Dipeptid-gebundenen Derivate, jedoch die jeweiligen freien Aminosäure detektiert werden. Die Konzentrationen an freiem Pyrralin bzw. Maltosin lagen in hPEPT1-exprimierenden HeLa-Zellen 4-7fach bzw. 10-16fach höher als in den Leervektor-transfizierten Kontrollzellen (*Studie 3*: GEISSLER *et al.* 2010, Abbildung 4; *Studie 4*: GEISSLER *et al.* 2011, Abbildung 7). Aus diesen Untersuchungen kann daher geschlossen werden, dass die Dipeptid-Derivate von Pyrralin und Maltosin in intakter Form aktiv, im Kotransport mit Protonen, hPEPT1-vermittelt in die Zellen aufgenommen und durch intrazelluläre Dipeptidasen in freies Pyrralin bzw. Maltosin und Alanin hydrolysiert werden. Diese Ergebnisse sind der direkte Beweis, dass ausschließlich PEPT1 an der Translokation dieser Dipeptid-gebundener MRPs verantwortlich ist.

Die dargelegten Ergebnisse könnten als Modell einer *in vivo* Absorption betrachtet und wie folgt zusammengefasst werden (schematische Darstellung in Abbildung 5.1):

1. Die freien glykierten Aminosäuren werden möglicherweise nur durch einfache Diffusion entlang des Konzentrationsgefälles in intestinale Zellen aufgenommen.
2. In Anwesenheit eines nach innen gerichteten Protonen-Gradienten interagieren die Dipeptid-Derivate der MRPs, in Abhängigkeit von der Seitenketten-Modifizierung

[6] Ströme werden als signifikant angesehen, wenn diese (*i*) Transporter-spezifisch, d.h. kein Strominduktion durch eine Substanz an wasserinjizierten Oozyten, (*ii*) mindestens 5% der Ströme der Referenzdipeptide ausmachen und (*iii*) statistisch signifikant verschieden von Null sind (KNÜTTER *et al.* 2008).

und der Position der Modifizierung im Dipeptid, mit mittlerer bis hoher Affinität mit PEPT1,

3. werden aktiv im Kotransport mit Protonen PEPT1-vermittelt in die intestinalen Epithelzellen aufgenommen und
4. intrazellulär in das jeweilige freie MRP und Alanin hydrolysiert.
5. Der basolaterale Efflux der freien MRPs erfolgt entweder durch Aminosäure-Transporter oder durch einfache Diffusion entlang ihres Konzentrationsgefälles.

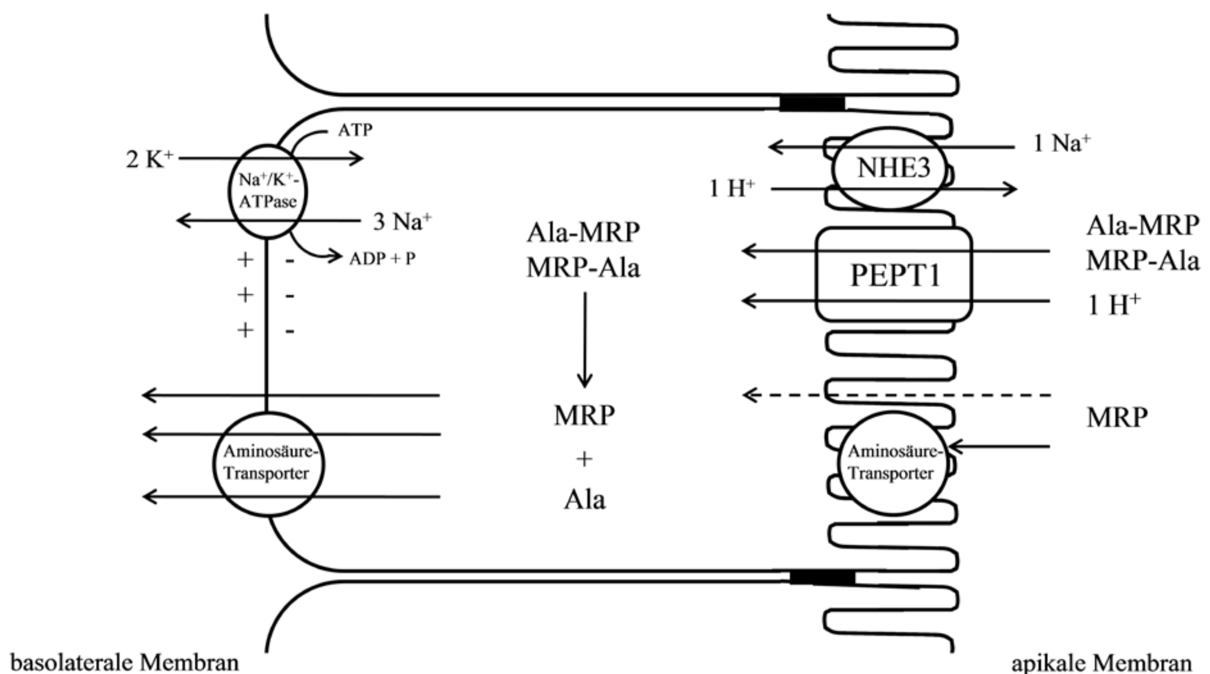

Abbildung 5.1. Schematische Darstellung des Transportes freier und Dipeptid-gebundener Maillard-Reaktionsprodukte (MRP) an intestinalen Epithelzellen. NHE3, Na^+/H^+-Austauscher; Ala, Alanin; PEPT1, Protonen-abhängiger Peptidtransporter 1.

Diese Untersuchungen zeigen erstmals den aktiven PEPT1-vermittelten Transport Dipeptid-gebundener MRPs und liefern somit neue Erkenntnisse über den Mechanismus der intestinalen Absorption von MRPs. Anhand der Daten könnte die orale Bioverfügbarkeit von MRPs, v.a. des Pyrralins (FÖRSTER und HENLE 2003), in Protein-gebundener Form erklärt werden. Voraussetzung wäre hierbei die Hydrolyse Protein-gebundener MRPs in absorbierbare Einheiten durch luminale oder Membran-gebundene Proteasen. Nach ADIBI *et al.* (1973) liegt nach der Aufnahme Protein-reicher Nahrung die Mehrheit von Aminosäuren als kleine Peptide und nicht in freier Form vor. Daher ist es wahrscheinlich, dass auch alimentär

aufgenommene Protein-gebundene MRPs hauptsächlich Dipeptid-gebunden[7] intestinal vorliegen würden und als solches auch tatsächlich absorbiert werden könnten.

Ähnlich wie Pyrralin und Maltosin interagierten auch die AGEs Formylin und Argpyrimidin mit hoher Affinität mit hPEPT1 und werden ebenso Dipeptid-gebunden intestinal transepithelial transportiert. Daher kann vermutet werden, dass auch die Dipeptid-Derivate von Formylin und Argpyrimidin nach der Verdauung aus glykierten Nahrungsproteinen nicht nur an PEPT1 binden, sondern auch aktiv PEPT1-vermittelt aufgenommen werden könnten.

5.3 Die Interaktion mit renalen Transportsystemen und der renale transepitheliale Transport

Die Ergebnisse der intestinalen Transportexperimente verdeutlichen, dass glykierte Aminosäuren intestinal ausschließlich Dipeptid-gebunden aufgenommen werden, aber nur als freie Aminosäure auf die abluminale Zellseite gelangen. Demzufolge wäre das Erscheinen der glykierten Aminosäuren im Primärharn ausschließlich in freier Form zu erwarten.

Alimentär aufgenommenes Peptid-gebundenes Pyrralin wird in der Tat nahezu vollständig in freier Form über den Harn wieder ausgeschieden (FÖRSTER und HENLE 2003, FÖRSTER *et al.* 2005). Die renale Eliminierung von Maltosin konnte bisher aus den Ergebnissen der Studie von REHNER und WALTER (1991) aufgrund der erhöhten Eisenausscheidung nach oraler Aufnahme von Maltosin nur vermutet werden. Pyrralin und möglicherweise auch Maltosin unterliegen *in vivo* keiner renalen Rückresorption. Dennoch sollte eine Interaktion mit renalen Transportsystemen und der renale transepitheliale Transport an der renalen Zelllinie OK untersucht werden, um mögliche Zusammenhänge *in vivo* verdeutlichen zu können.

In Kompetitionsstudien interagierten Pyrralin und Maltosin im Vergleich zur Referenzaminosäure L-Lysin nur schwach mit den Transportsystemen für kationische Aminosäuren (*Studie 2*: HELLWIG *et al.* 2009, Abbildung 2D, Tabelle 2; *Studie 4*: GEISSLER *et al.* 2011, Abbildung 5, Tabelle 2). Der transepitheliale Transport war im Vergleich zu [^{14}C]Mannitol nicht wesentlich höher (Pyrralin) bzw. sogar niedriger (Maltosin). Die Reabsorption von Pyrralin und Maltosin an renalen Zellen kann daher als gering eingeschätzt werden. Beide Aminosäuren würden folglich unter physiologischen Bedingungen hauptsächlich mit dem Harn ausgeschieden werden. Diese Ergebnisse könnten demzufolge die hohe renale Ausscheidung von Pyrralin *in vivo* erklären und die Vermutung von Maltosin

[7] Die Ergebnisse einer durchgeführten simulierten gastrointestinalen Verdauung des mit 3-Deoxyglucosulose glykierten β-Caseins zeigen, dass der Großteil des entstandenen Pyrralins Dipeptid-gebunden, hauptsächlich als Serylpyrralin und Valylpyrralin, vorlag (GEISSLER *et al.* 2010).

bezüglich der Ausscheidung unterstützen (REHNER und WALTER 1991, FÖRSTER und HENLE 2003).

In *Studie 2* wurde zusätzlich die Interaktion der Dipeptid-gebundenen Pyrralin-Derivate mit dem renalen Peptidtransporter PEPT2 an SKPT-Zellen untersucht. Anhand der durchgeführten Kompetitionsstudien können Ala-Pyrr und Pyrr-Ala entsprechend der Klassifizierung von BRANDSCH *et al.* (2008) als hoch-affine PEPT2-Liganden eingeordnet werden. Neben dem PEPT2 wird auch PEPT1 an der apikalen Membran des Nierenepithels exprimiert. Beide Transportsysteme könnten demnach die Dipeptid-gebundenen MRPs aus dem Primärharn wieder rückresorbieren. Anhand der dargelegten Ergebnisse ist eine Rückresorption der MRPs in Dipeptid-gebundener Form durch die Peptidtransporter unter physiologischen Bedingungen irrelevant, da die MRPs nach oraler Aufnahme nur in freier Form die intestinalen Zellen verlassen und somit auch nur in freier Form in den Harn gelangen können. Eine renale Rückresorption Dipeptid-gebundener MRPs wäre demzufolge endogen gebildeten Dipeptid-Derivaten vorbehalten, wobei die enzymatische Aktivität Membran-gebundener Peptidasen der renalen Epithelzellen mit berücksichtigt werden müsste.

5.4 Die ernährungsphysiologische und pharmazeutische Relevanz der Ergebnisse

Während der in Lebensmitteln stattfindenden Maillard-Reaktion wird die Seitenkette von L-Lysinen und L-Argininen durch die Reaktion mit Kohlenhydraten bzw. deren Abbauprodukten irreversibel modifiziert. Diese Modifizierung mindert die biologische Wertigkeit von Proteinen, da diese essentiellen und semi-essentielle Aminosäuren L-Lysin bzw. L-Arginin für den Organismus nicht mehr verfügbar sind. Andererseits können freie glykierte Aminosäuren die orale Bioverfügbarkeit von nicht-modifizierten L-Lysinen und L-Argininen durch Interaktion mit demselben Transportsystem vermindern. Das gilt vor allem für Pyrralin, welches einen effektiven, nicht-transportierten Inhibitor intestinaler Transportsysteme für kationische Aminosäuren darstellt. Ein Mangel bzw. ein Verlust essentieller bzw. semi-essentieller Aminosäure ist in westlichen Industrieländern, aufgrund der ohnehin schon die Empfehlungen übersteigenden täglichen Proteinaufnahme[8], wahrscheinlich weniger relevant.

Das während der Maillard-Reaktion gebildete Maltosin stellt nicht nur eine Prozess-induzierte Nahrungsmittelkomponente dar, sondern besitzt auch die Eigenschaft Komplexe mit Metallionen zu bilden. Das mit der Nahrung aufgenommene Maltosin könnte in freier

[8] Laut Nationaler Verzehrsstudie II (2008) liegt die tägliche Proteinzufuhr bei Kindern und Erwachsenen im Alter zwischen 14 und 80 Jahren um ca. 30% (Frauen) bzw. 40% (Männer) über der von der D-A-C-H empfohlen Menge (Nationale Verzehrsstudie II, Ergebnisbericht Teil 2, Hrsg. Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, 2008.

Form bei gleichzeitiger Aufnahme eisenhaltiger oder -reicher Lebensmittel präresorptiv mit Metallionen komplexieren. Postresorptiv wäre dies nach intestinaler Aufnahme von Maltosin in Dipeptid-gebundener Form und anschließender Hydrolyse in die freie Form möglich. Ernährungsphysiologisch bedeutsam wäre hierbei, dass das für den Organismus essentielle Eisenion als Maltosin-Metallionen-Komplex wahrscheinlich entweder über den Fäzes oder den Harn wieder ausgeschieden werden würde und folglich dem Organismus nicht mehr zur Verfügung stünde.

In neuesten Studien zeigte sich, dass die Komplexbildungskonstante von Maltosin um acht bis neun Größenordnungen höher liegt (SEIFERT 2008), als die von dem zur Behandlung von Eisenspeichererkrankungen therapeutisch eingesetzten 1,2-Dimethyl-3-Hydroxy-4-Pyridinon (Deferipron; KONTOGHIORGHES *et al.* 1987). Maltosin könnte daher einen möglichen neuen Wirkstoff darstellen, welcher als effektiver Metall-Chelator pharmazeutische Relevanz in der Behandlung von Eisenspeichererkrankungen erlangen könnte. Dies setzt die orale Bioverfügbarkeit von Maltosin voraus, welche in Dipeptid-gebundener Form, als sogenanntes *Prodrug*[9], gegeben wäre. Die in den vorliegenden Untersuchungen dargelegten Daten der hPEPT1-vermittelten intestinalen Absorption des Dipeptid-gebundenen Maltosins und dessen möglicher Einsatz als *Prodrug* in der Behandlung von Eisenspeichererkrankungen, führten zusätzlich zur Beantragung eines Patents[10].

Die gleichzeitige Aufnahme von Dipeptid-gebundenen AGEs und Arzneistoffen könnte nachteilig für die Wirkung der Arzneistoffe sein. PEPT1 transportiert nicht nur Dipeptid-gebundene AGEs, sondern ist auch für die orale Bioverfügbarkeit von β-Lactam-Antibiotika oder anderen Peptidmimetika (BRETSCHNEIDER *et al.* 1999) verantwortlich. Da oftmals empfohlen wird Medikamente zur Mahlzeit einzunehmen, könnten die Dipeptid-gebundenen AGEs und die Wirkstoffe um die Bindungsplätze am Transport-Protein konkurrieren. Dies könnte wiederum die orale Verfügbarkeit der Wirkstoffe beeinflussen.

Die Ergebnisse der Studien sind ernährungswissenschaftlich und physiologisch relevant und sollten als Teil der „Risikobewertung“ von MRPs, speziell AGEs, angesehen werden. In weiterführenden Studien sollten der hepatische Metabolismus der intestinal absorbierbaren MRPs sowie weitere wichtige MRPs auf ihre intestinale Absorption, renale Rückresorption oder Eliminierung untersucht werden. Hierbei wäre interessant, wie sich die inhibitorischen

[9] *Prodrug* = wenig oder inaktiver pharmakologischer Stoff, welcher im Organismus zu einem aktiven Wirkstoff metabolisiert wird. Sie stellen eine Alternative zu oral nicht verfügbaren Wirkstoffen dar.

[10] Patent: Hellwig M., Geissler S., Brandsch M., Henle T. (2010) Peptidgebundene α-Hydroxy-4(1H)-pyridinon-Derivate, Verfahren zu deren Herstellung und deren Verwendung. Patentanmeldung beim DPMA, Anmeldenummer DE 10 2010 036 261.1, Anmeldetag 24.08.2010.

und Transport-Eigenschaften der MRPs an intestinalen Epithelzellen im Hinblick auf den Austausch des Alanins im Dipeptid verändern würden.

Verdauungsstudien von Proteinen, welche durch die Maillard-Reaktion modifiziert werden, könnten zeigen, ob diese Proteine intestinal durch Membran-gebundene Peptidasen in absorbierbare Einheiten hydrolysiert werden. Eine anschließende intestinale Absorption der MRPs wird in Dipeptid-gebundener Form begünstigt und je hydrophober die MRPs sind, desto schneller würden diese bedingt durch den basolateralen Efflux in die Blutzirkulation gelangen.

6 Zusammenfassung

Die während der Lagerung und des Erhitzens von Lebensmitteln ablaufende nicht-enzymatische Bräunung bzw. Glykierung, die sogenannte Maillard-Reaktion, führt zur Bildung erwünschter Farb- und Aromaverbindungen. Reduzierende Zucker und deren Abbauprodukte reagieren hierbei mit der ε-Aminogruppe von freien, Peptid- oder Protein-gebundenen L-Lysinen oder L-Argininen unter der Bildung stabiler Endprodukte, den *advanced glycation end products* (AGE).

Metabolische Studien implizieren durch die beobachtete renale Ausscheidung von Maillard-Reaktionsprodukten (MRPs) nach alimentärer Zufuhr eine stattgefundene intestinale Absorption. Die MRPs könnten demnach parazellulär durch einfache Diffusion oder transzellulär durch Diffusion, endozytotisch oder vermittelt durch Transportproteine intestinal aufgenommen worden sein. Aufgrund der Struktur der MRPs – freie oder Peptid-gebundene glykierte Aminosäuren – können diese sowohl Liganden für intestinale Aminosäure-Transportsysteme, als auch für den intestinalen Peptidtransporter (PEPT) 1 darstellen. Letztgenanntes Transportsystem transportiert neben natürlichen Di- und Tripeptiden auch modifizierte Aminosäuren und Dipeptide. Durch die Strukturvielfalt der MRPs wird ein unterschiedliches Interaktionsverhalten mit den Transportsystemen erwartet.

Ziel der vorliegenden Untersuchungen war es neue Informationen bezüglich des intestinalen und renalen Transportes von MRPs zu gewinnen. Die sowohl in freier, als auch Dipeptid-gebundener Form (Ala-MRP, MRP-Ala) synthetisierten MRPs wurden auf deren intestinale und renale Interaktion mit spezifischen Transportsystemen, sowie deren transepithelialer Transport untersucht.

Die in den Untersuchungen eingeschlossenen MRPs werden in freier Form nicht intestinal transepithelial über Caco-2-Zellen transportiert. Dipeptid-gebunden werden diese MRPs allerdings in die intestinalen Zellen aufgenommen, schnell in die jeweilige freie Aminosäure hydrolysiert und akkumuliert. Die freien MRPs verlassen die Zellen auf der basolateralen Zellseite entweder über Transportsysteme für Aminosäuren oder durch einfache Diffusion entlang des Konzentrationsgefälles. Des Weiteren interagieren die freien MRPs nicht mit den apikalen Transportsystemen für kationische Aminosäuren, ausgenommen das AGE Pyrralin, welches als hoch-affiner Ligand des Transportsystems eingeordnet werden kann. Dipeptid-gebundene MRPs interagieren ebenso nicht mit diesen Aminosäure-Transportsystemen, dafür aber mit PEPT1 mit ähnlichen Affinitäten nicht-modifizierter Dipeptide und stellen mögliche Substrate für dieses *Carrier*-Protein dar. Dipeptide mit einem Pyrrol in der Seitenkette

(Pyrralin, Formylin) wiesen hierbei die höchsten Affinitäten auf. Die MRP-Ala-Dipeptide sind höher affin zum PEPT1 als die entsprechenden Ala-MRP-Dipeptide. PEPT1 kann als beteiligtes Transportsystem bei der intestinalen Translokation Dipeptid-gebundener MRPs diskutiert werden.

Weiterhin wurden Pyrralin, als das mengenmäßig in Lebensmitteln dominierende AGE, und Maltosin, welches Komplexe mit Metallionen eingehen kann, für weitere Untersuchungen hinsichtlich ihres tatsächlichen PEPT1-vermittelten Transportes einbezogen. Beide AGEs binden spezifisch an das PEPT1-Protein. Elektrophysiologische Messungen an hPEPT1-exprimierenden *Xenopus laevis*-Oozyten ergaben, dass die Dipeptid-Derivate beider Aminosäuren außerdem aktiv im H^+-Kotransport PEPT1-vermittelt transportiert werden. Auch die zelluläre Aufnahme und die Akkumulation der freien glykierten Aminosäuren an hPEPT1-transfizierten HeLa-Zellen resultiert aus deren Transport in Dipeptid-gebundener Form und anschließender intrazellulärer Hydrolyse in die jeweilige freie Aminosäure.

Pyrralin und Maltosin werden ausschließlich als freie glykierte Aminosäure aus den intestinalen Epithelzellen transportiert. Das Erscheinen der glykierten Aminosäuren wäre daher im Primärharn ausschließlich in freier Form zu erwarten. Untersuchungen zur Interaktion von Pyrralin und Maltosin mit renalen Transportsystemen und der transepitheliale Transport an OK-Zellen zeigten, dass beide glykierte Aminosäuren schwach mit den Transportsystemen für kationische Aminosäuren interagierten. Der transepitheliale Transport war ebenso gering. Die Reabsorption kann daher als gering eingeschätzt werden. Pyrralin und Maltosin werden vermutlich renal wieder ausgeschieden.

Unter physiologischen Bedingungen würden alimentär aufgenommene MRPs in Dipeptid-gebundener Form PEPT1-vermittelt in die Enterozyten aufgenommen, schnell hydrolysiert und akkumuliert werden, sowie als freie MRPs über basolaterale Aminosäure-Transporter oder entlang ihres Konzentrationsgradienten in die Blutzirkulation gelangen. Renal könnten diese möglicherweise mit dem Harn wieder ausgeschieden werden. Diese Ergebnisse könnten die hohe Bioverfügbarkeit bzw. die renale Exkretion von Pyrralin *in vivo* erklären.

Diese Ergebnisse liefern neue Erkenntnisse über den intestinalen und renalen Transport von MRPs, welche ausschließlich Dipeptid-gebunden oral bioverfügbar sind. Des Weiteren konnte zum ersten Mal der tatsächliche hPEPT1-vermittelte Transport Dipeptid-gebundener MRPs gezeigt werden, welcher die hohe orale Bioverfügbarkeit von MRPs *in vivo* erklären könnte. Maltosin, ein effektiver Eisen-Chelator, könnte zudem in Dipeptid-gebundener Form als *Prodrug* oral bioverfügbar gemacht werden und somit pharmazeutische Relevanz in der Therapie von Eisenspeichererkrankungen erlangen.

7 Summary

During storage or thermal processing of food the non-enzymatic browning or glycation, the so-called Maillard reaction occurs which results in coloured and aromatic compounds. Reducing carbohydrates and their degradations products react with the ε-amino groups of free, peptides or protein-bound L-lysins or L-arginins to form stable end products, called “advanced glycation end products” (AGE).

Metabolic studies have shown that after oral intake of food rich in Maillard reaction products (MRP), the MRPs are excreted with the urine and implicated an intestinal absorption. The MRPs can cross the intestinal epithelial barrier paracellularly (simple diffusion) or transcellularly by diffusion, endocytosis or mediated by transport proteins. Because of the structure of the MRPs – free or peptid-bound glycated amino acids – they could represent ligands (inhibitors or substrates) for intestinal amino acid transporters and also for the intestinal peptide transporter PEPT1. The latter accepts natural occurring di- and tripeptides as well as modified amino acids and dipeptides. MRPs due to their structural diversity must interact quite differently with the intestinal and renal transport systems for amino acids and peptides.

The purpose of the studies was to characterize the interaction of MRPs with membrane transport proteins and to provide new information about the intestinal and renal transport of MRPs. MRPs were synthesized as free and dipeptide-bound modified amino acids (Ala-MRP, MRP-Ala). The intestinal and renal interaction with specific transport systems as well as the transepithelial transport was examined.

The investigated MRPs were taken up into intestinal cells across the apical membrane bound in dipeptides but not in its free form. Intracellularly, the dipeptide derivatives were rapidly hydrolysed to free MRP and alanine as well as accumulated. Free MRPs permeates the basolateral cell membrane by simple diffusion down its concentration gradient or by the action of basolateral amino acid transporters. With the exception of pyrraline, none of the free MRPs interacted with the amino acid transporters for cationic amino acids. Pyrraline was considered as a high affinity ligand for this transport system. Similarly the dipeptide derivatives did not interacted with the transporters but interacted with high affinities with PEPT1 comparable to those obtained from natural dipeptides. The dipeptide-bound MRPs are putative substrates for the PEPT1 protein. Dipeptides with pyrrole side-chains (pyrraline, formyline) showed the highest affinities towards PEPT1. The MRP-Ala dipeptides exhibit 2-6 fold higher affinities towards PEPT1 than the corresponding Ala-MRP dipeptides. PEPT1

could be discussed as the transport system involved in the intestinal translocation of MRPs bound in dipeptides.

Furthermore, pyrraline the quantitative dominating AGE in foods, and maltosine which forms complexes with metal ions were investigated for the actual PEPT1 mediated transport. The dipeptide derivatives of both compounds interacted specific with the PEPT1 protein. Electrophysiological measurements at *Xenopus laevis* oocytes expressing hPEPT1 showed that the dipeptide-derivatives of pyrraline and maltosine also generated inward directed currents. Thus, the modified dipeptides were actively transported by the human PEPT1 in an electrogenic manner, i.e., a proton symport. The cellular uptake and the accumulation of the free modified amino acids at hPEPT1-transfected HeLa cells could be explained by the transport of the dipeptide derivatives by PEPT1 following by the intracellular hydrolyses to the corresponding free amino acids.

Pyrralin and Maltosin permeate the basolateral cell side only as free amino acids and could appear in the primary filtrate only in the free form. Therefore, the interaction of free pyrraline and maltosine with renal transport systems and the transepithelial transport at the renal cell line OK was investigated. Both compounds exhibit only weak interactions with renal amino acid transport systems for cationic amino acids. The transepitheliale transport was also only slightly. Pyrraline and maltosine were probably excreted with the urine.

It could be speculated that under physiological conditions the dipeptide-bound MRPs would transported intestinal by PEPT1. Intracellulary, the dipeptides would hydrolysed to free MRPs and alanine as well as accumulated. The free MRPs would appear in the blood and possibly excreted *via* the urine. These results could explain the high oral availability and the excretion of pyrraline *in vivo*.

The results provide new information about the intestinal absorption and the renal handling of MRPs which were exclusively orally available bound in dipeptides. Furthermore, this work demonstrated for the first time the actual proton-coupled transport of dipeptide-bound MRPs by hPEPT1. These results might explain the high oral bioavailability of MRPs *in vivo*. Moreover, maltosine, an effective metal chelating agent, is orally available bound in dipeptides and could obtain pharmaceutical importance as powerful prodrug for the treatment of iron storage diseases.

8 Literatur

Addison J.M., Burston D., Matthews D.M., Evidence for active transport of the dipeptide glycylsarcosine by hamster jejunum in vitro. *Clin. Sci.* **1972**, 43, 907-911.

Addison J.M., Burston D., Matthews D.M., Carnosine transport by hamster jejunum in vitro and its inhibition by other di- and tripeptides. *Clin. Sci.* **1973**, 45, 3P-4P.

Adibi S.A., Mercer D.W., Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. *J. Clin. Invest.* **1973**, 52, 1586-1594.

Agu R., Cowley E., Shao D., Macdonald C., Kirkpatrick D., Renton K., Massoud E., Proton-coupled oligopeptide transporter (POT) family expression in human nasal epithelium and their drug transport potential. *Mol. Pharm.* **2011**, 8, 664-672.

Ahmed M.U., Thorpe S.R., Baynes J.W., Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.* **1986**, 261, 4889-4894.

Ahmed M.U., Brinkmann Frye E., Degenhardt T.P., Thorpe S.R., Baynes J.W., N-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.* **1997**, 324, 565-570.

Ahmed N., Thornalley P.J., Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes Obes. Metab.* **2007**, 9, 233-245.

Ames J.M., Evidence against dietary advanced glycation endproducts being a risk to human health. *Mol. Nutr. Food Res.* **2007**, 51, 1085-1090.

Asatoor A.M., Cheng B., Edwards K.D., Lant A.F., Matthews D.M., Milne M.D., Navab F., Richards A.J., Intestinal absorption of two dipeptides in Hartnup disease. *Gut* **1970**, 11, 380-387.

Brandsch M., Brandsch C., Prasad P.D., Ganapathy V., Hopfer U., Leibach F.H., Identification of a renal cell line that constitutively expresses the kidney-specific high-affinity H^+/peptide cotransporter. *FASEB J.* **1995**, 9, 1489-1496.

Brandsch M., Knütter I., Thunecke F., Hartrodt B., Born I., Börner V., Hirche F., Fischer G., Neubert K., Decisive structural determinants for the interaction of proline derivatives with the intestinal H^+/peptide symporter. *Eur. J. Biochem.* **1999**, 266, 502-508.

Brandsch M., Knütter I., Bosse-Doenecke E., Pharmaceutical and pharmacological importance of peptide transporters. *J. Pharm. Pharmacol.* **2008**, 60, 543-585.

Brant S.R., Yun C.H., Donowitz M., Tse C.M., Cloning, tissue distribution, and functional analysis of the human Na^+/H^+ exchanger isoform, NHE3. *Am. J. Physiol.* **1995**, 269, C198-C206.

Bretschneider B., Brandsch M., Neubert R., Intestinal transport of β-lactam antibiotics: analysis of the affinity at the H^+/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. *Pharm. Res.* **1999**, 16, 55-61.

Bröer A., Wagner C.A., Lang F., Bröer S., The heterodimeric amino acid transporter 4F2hc/y^+LAT2 mediates arginine efflux in exchange with glutamine. *Biochem. J.* **2000**, 349, 787-795.

Chen X.Z., Zhu T., Smith D.E., Hediger M.A., Stoichiometry and kinetics of the high-affinity H^+-coupled peptide transporter PepT2. *J. Biol. Chem.* **1999**, 274, 2773-2779.

Cheng Y., Prusoff W.H., Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction, *Biochem. Pharmacol.* **1973**, 22, 3099-3108.

Christensen H.N., Handlogten M.E., Thomas E.L., Na^+-facilitated reactions of neutral amino acids with a cationic amino acid transport system. *Proc. Natl. Acad. Sci. USA* **1969**, 63, 948-955.

Closs E.I., Boissel J.P., Habermeier A., Rotmann A., Structure and function of cationic amino acid transporters (CATs). *J. Membr. Biol.* **2006**, 213, 67-77.

Cohen M.P., Clements R.S., Measuring glycated proteins: clinical and methodological aspects. *Diabetes Technol. Ther.* **1999**, 1, 57-70.

Covitz K.M., Amidon G.L., Sadée W., Membrane topology of the human dipeptide transporter, hPEPT1, determined by epitope insertions. *Biochemistry* **1998***,* 37, 15214-15221.

Daniel H., Neugebauer B., Kratz A., Rehner G., Localization of acid microclimate along intestinal villi of rat jejunum. *Am. J. Physiol.* **1985**, 248, G293-G298.

Daniel H., Morse E.L., Adibi S.A., The high and low affinity transport systems for dipeptides in kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. *J. Biol. Chem.* **1991**, 266, 19917-19924.

Daniel H., Morse E.L., Adibi S.A., Determinants of substrate affinity for the oligopeptide/H^+ symporter in the renal brush border membrane. *J. Biol. Chem.* **1992**, 267, 9565-9573.

Daniel H., Molecular and integrative physiology of intestinal peptide transport. *Annu. Rev. Physiol.* **2004**, 66, 361-384.

Daniel H., Kottra G., The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. *Pflugers Arch.* **2004**, 447, 610-618.

Devés R., Chavez P., Boyd C.A.R., Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. *J. Physiol.* **1992**, 454, 491-501.

Devés R., Boyd C.A.R., Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol. Rev.* **1998**, 78, 487-545.

Döring F., Dorn D., Bachfischer U., Amasheh S., Herget M., Daniel H., Functional analysis of a chimeric mammalian peptide transporter derived from the intestinal and renal isoforms. *J. Physiol.* **1996**, 497, 773-779.

Döring F., Martini C., Walter J., Daniel H., Importance of a small N-terminal region in mammalian peptide transporters for substrate affinity and function. *J. Membr. Biol.* **2002**, 186, 55-62.

Dyer J., Beechey R.B., Gorvel J.P., Smith R.T., Wootton R., Shirazibeechey S.P., Glycyl-L-proline transport in rabbit enterocyte basolateral-membrane vesicles. *Biochem. J.* **1990**, 269, 565-571.

Dyer D.G., Blackledge J.A., Thorpe S.R., Baynes J.W., Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine *in vivo*. *J. Biol. Chem.* **1991**, 25, 266, 11654-11660.

Erbersdobler H.F., Brandt A., Scharrer E., Von Wangenheim B., Transport and metabolism studies with fructose amino acids. *Prog. Food Nutr. Sci.* **1981**, 5, 257-263.

Erbersdobler H.F., Lohmann M., Buhl, K., Utilization of early Maillard reaction products by humans. *Adv. Exp. Med. Biol.* **1991**, 289, 363-370.

Erbersdobler H.F., FaistV., Metabolic transit of Amadori products. *Nahrung* **2001**, 45, 177-181.

Fei Y.J., Liu W., Prasad P.D., Kekuda R., Oblak T.G., Ganapathy V., Leibach F.H., Identification of the histidyl residue obligatory for the catalytic activity of the human H^+/peptide cotransporters PEPT1 and PEPT2. *Biochemistry* **1997**, 36, 452 - 460.

Fei Y.J., Ganapathy V., Leibach F.H., Molecular and structural features of the proton-coupled oligopeptide transporter superfamily. *Prog. Nucleic Acid Res. Mol. Biol.* **1998,** 58, 239-261.

Finot P.A., Magnenat E., Metabolic transit of early and advanced Maillard products. *Prog. Food Nutr. Sci.* **1981**, 5, 193-207.

Förster A., Henle T., Glycation in food and metabolic transit of dietary AGEs: studies on the urinary excretion of pyrraline. *Biochem. Soc. Transact.* **2003**, 31, 1383-1385.

Förster A., Kühne Y., Henle T., Studies on absorption and elimination of dietary Maillard reaction products. *Ann. N. Y. Acad. Sci.* **2005**, 1043, 474-481.

Friedmann M., Food browning and its prevention: an overview. *J. Agric. Food Chem.* **1996**, 44, 631-653.

Ganapathy V., Leibach F.H., Is intestinal peptide transport energized by a proton gradient? *Am. J. Physiol.* **1985**, 249, G153-G160.

Ganapathy M.E., Brandsch M., Prasad P.D., Ganapathy V., Leibach F.H., Differential recognition of β-lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. *J. Biol. Chem.* **1995**, 270, 25672-25677.

Geissler S., Hellwig M., Zwarg M., Markwardt F., Henle T., Brandsch M., Transport of the advanced glycation end products alanylpyrraline and pyrralylalanine by the human proton-coupled peptide transporter hPEPT1. *J. Agric. Food Chem.* **2010**, 58, 2543-2547.

Geissler S., Hellwig M., Markwardt F., Henle T., Brandsch M., Synthesis and intestinal transport of the iron chelator maltosine in free and dipeptide form. *Eur. J. Pharm. Biopharm.* **2011**, 78, 75-82.

Grunwald S., Krause R., Bruch M., Henle T., Brandsch M., Transepithelial flux of early and advanced glycation compounds across Caco-2 cell monolayers and their interaction with intestinal amino acid and peptide transport systems. *Br. J. Nutr.* **2006**, 95, 1221-1228.

Hellier M.D., Holdsworth C.D., Perrett D., Thirumalai C., Intestinal dipeptide transport in normal and cystinuric subjects. *Clin. Sci.* **1972**, 43, 659-668.

Hellwig M., Geissler S., Peto A., Knütter I., Brandsch M., Henle T., Transport of free and peptide-bound pyrraline at intestinal and renal epithelial cells. *J. Agric. Food Chem.* **2009**, 57, 6474-6480.

Hellwig M., Henle T., Formyline, a new glycation compound from the reaction of lysine and 3-deoxypentosone. *Eur. Food Res. Technol.* **2010**, 230, 903-914.

Hellwig M., Geissler S., Matthes R., Peto A., Silow C., Brandsch M., Henle T., Transport of free and peptide-bound glycated amino acids: synthesis, transepithelial flux at Caco-2 cell monolayers, and interaction with apical membrane transport proteins. *ChemBioChem.* **2011**, 12, 1270-1279.

Hellwig M., Henle T., Formylin, eine neue Maillard-Verbindung in Lebensmitteln. 39. Deutscher Lebensmittelchemikertag, Stuttgart 2010, *Lebensmittelchemie* **2011,** 65, 53.

Henle T., Walter A.W., Haeßner R., Klostermeyer H., Detection and identification of a protein-bound imidazolone resulting from the reaction of arginine residues and methylglyoxal. *Z. Lebensm. Unters. Forsch.* **1994a**, 199, 55-58.

Henle T., Walter A.W., Klostermeyer H., Simultaneous determination of protein-bound Maillard products by ion-exchange chromatography and photodiode array detection. In *Maillard Reactions in Chemistry, Food, and Health*; Labuza, T.P., Ed.; The Royal Society of Chemistry: Cambridge, UK, **1994b**; pp 195-200.

Henle T., Bachmann A., Synthesis of pyrraline reference material. *Z. Lebensm. Unters. Forsch.* **1996**, 202, 72-74.

Henle T., Schwarzenbolz U., Klostermeyer H., Detection and quantification of pentosidine in foods. *Lebensm. Unters. Forsch. A* **1997**, 204, 95-98.

Henle T., AGEs in foods: do they play a role in uremia? *Kidney Int. Suppl.* **2003**, 63, S145-S147.

Henle T., Miyata T., Advanced glycation end products in uremia. *Adv. Ren. Replace Ther.* **2003**, 10, 321-331.

Henle T., Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. *Amino Acids* **2005**, 29, 313-322.

Henle T., Dietary advanced glycation end products - A risk to human health? A call for an interdisciplinary debate. *Mol. Nutr. Food Res.* **2007**, 51, 1075-1078.

Hidalgo I.J., Raub T.J., Borchardt R.T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **1989**, 96, 736-749.

Hodge J.E., Chemistry of Browning Reactions in Model Systems. *Agric. Food Chem.* **1953**, 1, 928-943.

Kennedy D.J., Leibach F.H., Ganapathy V., Thwaites D.T., Optimal absorptive transport of the dipeptide glycylsarcosine is dependent on functional Na^+/H^+ exchange activity. *Pflugers Arch.* **2002**, 445, 139-146.

Kim J.W., Closs E.I., Albritton L.M., Cunningham J.M., Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* **1991**, 352, 725-728.

Knütter I., Hartrodt B., Theis S., Foltz M., Rastetter M., Daniel H., Neubert K., Brandsch M., Analysis of the transport properties of side chain modified dipeptides at the mammalian peptide transporter PEPT1. *Eur. J. Pharm. Sci.* **2004**, 21, 61-67.

Kontoghiorghes G.J., Aldouri M.A., Sheppard L., Hoffbrand A.V., 1,2-Dimethyl-3-hydroxypyrid-4-one, an orally active chelator for treatment of iron overload, *Lancet* **1987**, 1, 1294-1295.

Kottra G., Stamfort A., Daniel H., PEPT1 as a paradigm for membrane carriers that mediate electrogenic bidirectional transport of anionic, cationic, and neutral substrates. *J. Biol. Chem.* **2002**, 277, 32683-32691.

Koschinsky T., He C.-J., Mitsuhashi T., Bucala R., Liu C., Bünting C., Heitmann K., Vlassara H., Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. *Proc. Natl. Acad. Sci.* **1997,** 94, 6474-6479.

Ledl F., Osiander H., Pachmayr O., Severin T., Formation of maltosine, a product of the Maillard reaction with a pyridone structure. *Z. Lebensm. Unters. Forsch. A* **1989**, 188, 207-211.

Ledl F., Schleicher E., Die Maillard-Reaktion in Lebensmitteln und im menschlichen Körper - neue Ergebnisse zu Chemie, Biochemie und Medizin. *Angew. Chemie* **1990**, 102, 597-734.

Lee K., Erbersdobler H.F., Balance experiments on human volunteers with ε-fructoselysine (FL) and lysinoalanine (LAL). In: *Maillard reactions in chemistry, food and health* (Hrsg.: Labuza T.P., Reineccius G.A., Monnier V.M., O´Brien J., Baynes J.W.), *Royal Society of Chemistry*, Cambridge, **1994**, 358-363.

Lindenmeier M., Faist V., Hofmann T., Structural and functional characterization of pronyl-lysine, a novel protein modification in bread crust melanoidins showing in vitro antioxidative and phase I/II enzyme modulating activity. *J. Agric. Food Chem.* **2002**, 50, 6997-7006.

Lindsey J.B., Cipollone F., Abdullah S.M., McGuire D.K., Receptor for advanced glycation end-products (RAGE) and soluble RAGE (sRAGE): cardiovascular implications. *Diab. Vasc. Dis. Res.* **2009**, 6, 7-14.

Mackenzie B., Loo D.D., Fei Y., Liu W.J., Ganapathy V., Leibach F.H., Wright E.M., Mechanisms of the human intestinal H^+-coupled oligopeptide transporter hPEPT1. *J. Biol. Chem.* **1996**, 271, 5430-5437.

Maillard L.C., Action des acides aminés sur les sucres: formation des melanoidines par voie methodique. *C.R. Acad. Sci. Ser.* **1912**, 154, 66-68.

Mora C., Chillarón J., Calonge M.J., Forgo J., Testar X., Nunes V., Murer H., Zorzano A., Palacín M., The *rBAT* gene is responsible for L-cystine uptake via the $b^{0,+}$-like amino acid transport system in a "renal proximal tubular" cell line (OK cells). *J. Biol. Chem.* **1996**, 271, 10569-10576.

Murer H., Hopfer U., Kinne R., Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **1976**, 154, 597-604.

Nakayama T., Hayase F., Kato H., Formation of ε-(2-formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine in the Maillard reaction between D-glucose and L-lysine. *Agric. Biol. Chem.* **1980**, 44, 1201-1202.

Newey H., Smyth D.H., Cellular mechanisms in intestinal transfer of amino acids. *J. Physiol.* **1962**, 164, 527-551.

Öste R.E., Miller R., Sjöström H., Noren O., Effect of Maillard reaction products on protein digestion. Studies on pure compounds. *J. Agric. Food Chem.* **1987**, 35, 938-942.

Omura H., Jahan N., Shinohara K., Murakami H., Formation of mutagens by the Maillard reaction. *ACS Symposium Series* **1983**, 215, 537-563.

Palacín M., Kanai Y., The ancillary proteins of HATs: SLC3 family of amino acid transporters. *Pflugers Arch.* **2004**, 447, 490-494.

Pras E., Raben N., Golomb E., Arber N., Aksentijevich I., Schapiro J.M., Harel D., Katz G., Liberman U., Pras M., *et al.*, Mutations in the SLC3A1 transporter gene in cystinuria. *Am. J. Hum. Genet.* **1995**, 56, 1297-1303.

Paulsen I.T., Skurray R.A., The POT family of transport proteins. *Trends Biochem. Sci.* **1994**, 19, 404.

Rehner G., Walter T., Effect of Maillard products and lysinoalanine on the bioavailability of iron, copper and zinc. *Z. Ernährwiss.* **1991**, 30, 50-55.

Resmini P., Pellegrino L., Occurrence of protein-bound lysylpyrrolaldehyde in dried pasta. *Cereal Chem.* **1994**, 71, 254-262.

Saito H., Inui K., Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line Caco-2. *Am. J. Physiol.* **1993**, 265, G289-G294.

Schmidt A.M., Hori O., Cao R., Yan S.D., Brett J., Wautier J.L., Ogawa S., Kuwabara K., Matsumoto M., Stern D., RAGE: a novel cellular receptor for advanced glycation end products. *Diabetes* **1996**, 45, 3, S77-S80.

Schwenger V., Zeier M., Henle T., Ritz E., Advanced glycation endproducts (AGEs) as uremic toxins. *Nahrung* **2001**, 45, 172-176.

Seifert S., Synthese und Komplexbildungseigenschaften ausgewählter Maillard-Reaktionsprodukte. Dissertation, TU Dresden, **2008**.

Sell D.R., Monnier V.M., Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J. Biol. Chem.* **1989**, 264, 21597-21602.

Shipanova I.N., Glomb M.A., Nagaraj R.H., Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. *Arch. Biochem. Biophys.* **1997**, 344, 29-36.

Sloan J.L., Mager S., Cloning and functional expression of a human Na^{+} and Cl^{-}-dependent neutral and cationic amino acid transporter B^{0+}. *J. Biol. Chem.* **1999**, 274, 23740-23745.

Somoza V., Five years of research on health risks and benefits of Maillard reaction products: an update. *Mol. Nutr. Food Res.* **2005**, 49, 663-672.

Somoza V., Wenzel E., Weiß C., Clawin-Rädecker I., Grübel N., Erbersdobler H.F., Dose-dependent utilisation of casein-linked lysinoalanine, *N*(epsilon)-fructoselysine and *N*(epsilon)-carboxymethyllysine in rats. *Mol. Nutr. Food Res.* **2006**, 50, 833-841.

Steiner H.Y., Naider F., Becker J.M., The PTR family: a new group of peptide transporters. *Mol. Microbiol.* **1995**, 16, 825-834.

Sugano K., Kansy M., Artursson P., Avdeef A., Bendels S., Di L., Ecker G.F., Faller B., Fischer H., Gerebtzoff G., Lennernaes H., Senner F., Coexistence of passive and carrier-mediated processes in drug transport. *Nat. Rev. Drug Discov.* **2010**, 9, 597-614.

Tateoka R., Abe H., Miyauchi S., Shuto S., Matsuda A., Kobayashi M., Miyazaki K., Kamo N., Significance of substrate hydrophobicity for recognition by an oligopeptide transporter (PEPT1). *Bioconjug. Chem.* **2001**, 12, 485-492.

Teixeira S., Di Grandi S., Kühn L.C., Primary structure of the human 4F2 antigen heavy chain predicts a transmembrane protein with a cytoplasmic NH_2 terminus. *J. Biol. Chem.* **1987**, 262, 9574-9580.

Terada T., Saito H., Mukai M., Inui K.I., Identification of the histidine residues involved in substrate recognition by a rat H^{+}/peptide cotransporter, PEPT1. *FEBS Lett.* **1996**, 394, 196-200.

Terada T., Sawada K., Saito H., Hashimoto Y., Inui K., Functional characteristics of basolateral peptide transporter in the human intestinal cell line Caco-2. *Am. J. Physiol.* **1999**, 276, G1435-G1441.

Thwaites D.T., Markovich D., Murer H., Simmons NL., Na^+-independent lysine transport in human intestinal Caco-2 cells. *J. Membr. Biol.* **1996**, 151, 215-224.

Thwaites D.T., Kennedy D.J., Raldua D., Anderson C.M., Mendoza M.E., Bladen C.L., Simmons N.L., H^+/dipeptide absorption across the human intestinal epithelium is controlled indirectly via a functional Na^+/H^+ exchanger. *Gastroenterology* **2002**, 122, 1322-1333.

Torrents D., Estévez R., Pineda M., Fernández E., Lloberas J., Shi Y.B., Zorzano A., Palacín M., Identification and characterization of a membrane protein (y^+L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity y^+L. A candidate gene for lysinuric protein intolerance. *J. Biol. Chem.* **1998**, 273, 32437-32445.

Uribarri J., Cai W., Peppa M., Goodman S., Ferrucci L., Striker G., Vlassara H., Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *J. Gerontol. A Biol. Sci. Med. Sci.* **2007**, 62, 427-433.

Uribarri J., Woodruff S., Goodman S., Cai W., Chen X., Pyzik R., Yong A., Striker G.E., Vlassara H., Advanced glycation end products in foods and a practical guide to their reduction in the diet. *J. Am. Diet. Assoc.* **2010**, 110, 911-916.

Van Winkle L.J., Christensen H.N., Campione A.L., Na^+-dependent transport of basic, zwitterionic, and bicyclic amino acids by a broad-scope system in mouse blastocysts. *J. Biol. Chem.* **1985**, 260, 12118-12123.

Van Winkle L.J., Campione A.L., Gorman J.M., Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. *J. Biol. Chem.* **1988**, 263, 3150-3163.

Verrey F., Closs E.I., Wagner C.A., Palacin M., Endou H., Kanai Y., CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch.* **2004**, 447, 532-542.

Vig B.S., Stouch T.R., Timoszyk J.K., Quan Y., Wall D.A., Smith R.L., Faria T.N., Human PEPT1 pharmacophore distinguishes between dipeptide transport and binding. *J. Med. Chem.* **2006**, 49, 3636-3644.

Vlassara H., Cai W., Crandall J., Goldberg T., Oberstein R., Dardaine V., Peppa M., Rayfield E.J., Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc. Natl. Acad. Sci.* **2002**, 99, 15596-15601.

Wiame E., Delpierre G., Collard F., Van Schaftingen E., Identification of a pathway for the utilization of the Amadori product fructoselysine in *Escherichia coli*. *J. Biol. Chem.* **2002**, 277, 42523-42529.

White M.F., Gazzola G.C., Christensen H.N., Cationic amino acid transport into cultured animal cells. I. Influx into cultured human fibroblasts. *J. Biol. Chem.* **1982**, 257, 4443-4449.

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