ROLE, RELEVANCE AND REGULATION OF PEPT1 IN PEPTIDE INTESTINAL ABSORPTION

by

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To my parents

Mrs. Ronghua Yang (1940-2001)

Mr. Jisheng Ma

For their endless love

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TABLE OF CONTENTS

DEDICATION ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

LIST OF FIGURES ..... **viii**

LIST OF TABLES ..... **xi**

LIST OF APPENDICES ..... **xii**

CHAPTER I-RESEARCH OBJECTIVES **1**

References **6**

CHAPTER II- BACKGROUND AND LITERATURE REVIEW **9**

2.1 Proton-Coupled Oligopeptide Transporter 1 (Pept1) **9**

2.1.1 Descrption and Importance 9

2.1.2 Molecular Structure 11

2.1.3 Genetic Polymorphism 14

2.1.4 Tissue and Cellular Localization 15

2.1.5 Regulation.....17

2.1.6 Substrate Specificity.. 30

2.1.7 Transport Mechanism.. 35

2.1.8 Clinical Relevance.. 36

2.2 Transporters In Small Intestine And Colon.....37

2.2.1 Introduction.....37

2.2.2 Influx Transporters.....38

2.2.3 Efflux Transporters. 45

2.2.4 Intestinal Transporters and Fasting.....46

2.2.5 Mice with Targeted Disruption of Intestinal Transporters ...47

2.3 Small Peptide Renal Reabsorption And Transporters.....47

References.....52

CHAPTER III-PEPTIDE TRANSPORTER 1 IS RESPONSIBLE FOR THE *IN VITRO* INTESTINAL UPTAKE OF GLYCYLSARCOSINE IN WILD-TYPE VERSUS PEPT1 NULL MICE.....74

Abstract.....74

Introduction.....76

Materials and methods.....79

Results.....82

Discussion.....85

References.....97

CHAPTER IV-INFLUENCE OF FED-FASTED STATE ON INTESTINAL PEPT1 EXPRESSION AND *IN VIVO* PHARMACOKINETICS OF GLYCYLSARCOSINE IN WILD-TYPE AND PEPT1 KNOCKOUT MICE 104

Abstract 104

Introduction 106

Materials and methods 109

Results 114

Discussion 118

References 139

APPENDICES 144

LIST OF FIGURES

Figure 2.1 Structural model of PEPT1..... 50

Figure 3.1 Time-dependent uptake of ^{14}C-GlySar in the jejunum of wild-type mice (4 μM GlySar in external medium)…….. 90

Figure 3.2 Temperature-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). 91

Figure 3.3 Sodium-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). 92

Figure 3.4 pH-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).. 93

Figure 3.5 Concentration-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (0.1-40 mM total GlySar in external medium).. 94

Figure 3.5 Effect of potential inhibitors (10 mM) on the uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).. 95

Figure 4.1 PEPT1 relative mRNA expression in 5 different intestinal segments during fed and fasted conditions of wild-type mice.. ..... .124

Figure 4.2 PEPT1 protein expression in 5 different intestinal segments of wild-type mice during fed-fasted conditions 125

Figure 4.3A Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after intravenous bolus administration of dipeptide at 5 nmol/g body weight..... 126

Figure 4.3B Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after intravenous bolus administration of drug at 5 nmol/g body weight. 127

Figure 4.4A Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight. 128

Figure 4.4B Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight. 129

Figure 4.5A Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after oral administration of dipeptide at 5 nmol/g body weight 130

Figure 4.5B Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after oral administration of dipeptide at 5 nmol/g body weight 131

Figure 4.6A Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight..... 132

Figure 4.6B Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight 133

Figure 4.7 Upper gastrointestinal transit in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, in which a charcoal meal was administered by gavage to each mouse..... 134

Figure 4.8 Histology of jejunum in fed PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice. 135

Figure 4.9 Histology of jejunum in fasted PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice..... 136

LIST OF TABLES

Table 2.1 Amino Acid Residues Involved in PEPT1 Transport Activity and the Expected Effect of Mutation ……..... 51

Table 3.1 Uptake kinetics of GlySar in wild-type and PEPT1 null mice ……..... 96

Table 4.1 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g intravenous bolus dose of dipeptide in $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice during fed-fasted conditions ……..... 137

Table 4.2 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g oral dose of dipeptide in $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice during fed-fasted conditions... 138

LIST OF APPENDICES

APPENDIX A INDIVIDUAL DATA FROM CHAPTER III…...……...…… 144

APPENDIX B INDIVIDUAL DATA FROM CHAPTER IV…...……...…..…… 151

CHAPTER I

RESEARCH OBJECTIVES

As a major organ of nutrition, the small intestine was first identified to express various amino acid transport systems like L, y+, y+L, A, ASC (Acevedo and Armstrong, 1987). With the understanding of protein digestion, a large quantity and variety of short- and medium-sized peptides were observed to absorb intact through epithelium although they were not substrates of amino acid transporters. Typically, peptides with three to six amino acid residues were corresponded to a concentration of 120 mM to 145 mM and free amino acids were only 30 mM to 60 mM in the digest after administration of model proteins such as albumin (Daniel, 2004). Approximately, 400 dipeptides and 8,000 tripeptides could be generated from protein digestion. Those di-, tripeptides and a wide range of drugs such as *β*–lactam antibiotics and angiotensin-converting enzymes (ACE) inhibitors could be transported in the gut lumen (Radhakrishnan, 1977).

The discovery of the intestinal oligopeptide transporter, designated as PEPT1, has provided molecular evidence for the functional activity of intestinal peptide transport. As the exclusive oligopeptide transporter on the brush-border membrane of the intestinal mucosa so far, PEPT1 was identified as the first H^+-coupled transporter in vertebrates, independent of extracellular Na^+, K^+ and Cl^- (Fei et al., 1994). It is predicted to contain 12 transmembrane domains with both the C- and N-termini localized inside the cell, in which the first 4 transmembrane regions from amino-terminal and domains 7 to 9 are

responsible for the substrate affinity and PEPT1 unique characteristic features (Doring et al., 2002). As a member of proton coupled oligopeptide transporters (POT) family, PEPT1 is classified as a low-affinity, high-capacity transporter and demonstrates broad substrate specificities ranging from different molecular size, polarity, to charge and conformation. PEPT1 is distinguished from PEPT2, which is delieanated as a high-affinity, low-capacity transporter primarily located in the kidney (Shen et al., 2001); even though both isoforms share 50% overall sequence identity and 70% similarity (Saito et al., 1996). In addition, PEPT1 is a conserved gene among species: human H^+/peptide cotransporter is highly homologous (81% identity and 92% similarity) to rabbit PEP1 (Liang et al., 1995) and it exhibits a high degree of homology (83% identity, 86% similarity) to mouse PEPT1 on amino acid level (Fei et al., 2000).

Shortly after its discovery, PEPT1, either in Caco-2 cells or in *Xenopus* oocytes expressing PEPT1, had been utilized to analyze hundreds of substrates in competition assays with tracer dipeptides. Several high-affinity type inhibitors, but not substrates, of PEPT1 had been revealed such as active sulfonylurea antidiabetics and nonsulfonylurea insulin secretagogue (Sawada et al., 1999; Terada et al., 2000) to competitively block dipeptide transport via PEPT1. Based on the structure, the substrates of PEPT1 can be classified into two categories: substrates with peptide bonds and those without peptide bonds. In substrates carrying peptide bonds, they can be sub grouped into α–amino group presenters such as glycylsarcosine (GlySar) and substrates with other groups like *β*–lactams and ACE-inhibitors (Boll et al., 1994). To be efficiently bound and eletrogenic transported by PEPT1, its substrates with α–amino peptide bond require an essential structure with two oppositely charged head groups (i.e., amino and carboxyl groups)

separated by a carbon backbone with a distance of 5.5 to 6.3Å between the centers of the head groups (Doring et al., 1998). More interestingly, simple omega-amino fatty acids such as Delta-aminolevulinic acid (ALA) can be transported via PEPT1 and show similar affinities as dipeptides, which contribute to the broad range of substrates for PEPT1 (Doring et al., 1998). In addition, PEPT1 is stereoseletive to its substrates. Peptides with solely D-enantiomers of amino acids are not transported via PEPT1 (Wenzel et al., 1995).

Better understanding of PEPT1 will facilitate drugs to improve their bioavailability. One successful example was valacyclovir. As the prodrug of acyclovir, valacyclovir showed a three to five-fold increase in bioavailability comparing to its parent drug, acyclovir, and utilization of PEPT1 has been demonstrated to contribute to this improvement (Weller et al., 1993).

Normally, PEPT1 has very little expression in the colon tissues and is undetectable in the stomach. Besides its expression in the small intestine, PEPT1 can be detected in kidney, mainly in early regions of the proximal tubules (Shen et al., 1999), brain, mammary gland and lung (Fei et al., 1994; Lu and Klaassen, 2006). In the kidney, PEPT1 and PEPT2 are cooperating in the reabsorption of peptides, first by the low-affinity, high-capacity PEPT1 in S1 segment and then by the high-affinity, low-capacity PEPT2 in S2 and S3 segments of the proximal tubule (Daniel and Rubio-Aliaga, 2003). In addition, PEPT1 has been shown to express in the tumor cell lines from extrahepatic biliary duct for the endogenous photosensitizer δ–aminolevulinic acid transport (Neumann et al., 2003).

Besides its broad substrate specificity, another interesting characteristic of PEPT1 is its regulation and adaptation to numerous physiological and pharmacological

conditions. Hormones such as insulin (Thamotharan et al., 1999) and leptin (Buyse et al., 2001) could stimulate dipeptides uptake into Caco-2 cells with an increase in the Vmax and no change of Km. 3,5,3'-Triiodothyronine (T3), on the other hand, would reduce the Vmax for GlySar influx to Caco-2 cells (Ashida et al., 2002). PEPT1 will be induced in the stomach and colon in disease conditions (i.e. short-bowel syndrome), suggesting its compensatory effect in the absorption of dietary amino acids as well as some drugs (Ford et al., 2003). Other factors like bacterial infection alter PEPT1 expression level and thus its function in the colon tissue (Marquet et al., 2007). In addition, the expression PEPT1 will be induced after a brief fast or sustained starvation (Thamotharan, et al., 1999) and after malnourishment (Ihara, et al., 2000),

Knockout technology, a valuable research tool, has been developed for twenty years. Targeted disruption of PEPT1 gene will conclusively determine the relative significance of this peptide transporter in small intestines as well as kidney, the two major expression organs of PEPT1. We hypothesized that: 1) PEPT1 deletion will vitiate the intestinal absorption of dipeptides as reflective of the apical localization of the transporter in the enterocytes, 2) disruption of PEPT1 gene will result in significant changes in the pharmacokinetic profiles, especially absorption of dipeptides and 3) the absence of PEPT1 will lead to different responses towards physiological and pathological conditions such as fasting in the absorption of dipeptides.

Based on these hypotheses, the specific objectives of this study were:

1) To determine the role of PEPT1 in the *in vitro* intestinal uptake of GlySar from transgenic mice.

2) To determine the influence of PEPT1 on *in vivo* pharmacokinetics, tissue distribution and systemic exposure of GlySar under fed and fasted conditions in transgenic mice.

3) To explore possible physiological and/or pathological changes in transgenic mice towards fasting treatments.

To reach these goals, studies were performed using a combination of complementary techniques such as molecular and pharmacokinetic models like *in vitro* vs *in vivo*. The results from this project provide exclusive understanding about the relative significance of PEPT1-mediated transport in the body. These studies imply that PEPT1 might be used as a target to improve bioavailability of poor absorbed drugs by pharmaceutical companies. This project could have important clinical applications in the instruction of oral drug administration. Finally, these studies provide insight into the importance of PEPT1 in animal survival.

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CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Proton-Coupled Oligopeptide Transporter 1 (PEPT1)

2.1.1 Description and Importance

The proton-coupled peptide cotransporters PEPT1 (SLC15A1) and PEPT2 (SLC15A2) are the best characterized and possibly the pharmaceutically most relevant peptide transporter systems in the Proton-Coupled Oligopeptide Transporters (POT) superfamily. In addition to PEPT1 and PEPT2, the histidine/peptide transporters, PHT1 (SLC15A4) and PHT2 (SLC15A3), are the other members in this superfamily. The human PEPT1 consists of 708 amino acid residues and shares a 50% overall sequence identity and 70% similarity to hPEPT2. PHT1, on the other hand, has weak similarity to PEPT1 and PEPT2 (32% and 27%, respectively) on amino acid levels. It was cloned from rat brain (Yamashita et al., 1997) and expresses in retina (Ocheltree et al., 2003), skeletal muscle and spleen (Botka et al., 2000) as well. A recent paper (Romano et al., 2009) showed its expression in rat thyroid tissue, while its function was less important than PEPT2 in peptide transport, largely due to its intracellular localization. rPHT2 encodes a protein of 582 amino acids with 49% identity to PHT1 and it mainly expresses in spleen, thymus and lung (Sakata et al., 2001). PHT1 and PHT2 were shown to transport free histidine and certain di-, tripeptides, but it is little known about their roles

in the systemic exposure of substrates. None was done about their specific pharmaceutical or pharmacological relevance (Daniel and Kottra, 2004).

PEPT1 was the first mammalian oligopeptide transporter cloned from rabbit intestine in 1994 using the *X. laevis* oocyte expression cloning method (Fei et al., 1994). The gene encoding hPEPT1 is located on chromosome 13q33-34 with 23 exons (Liang et al., 1995). Shortly after identification of PEPT1, PEPT2 was isolated by expression cloning and homology screening from a kidney cDNA library (Liu et a., 1995; Boll et al., 1996). PEPT2 mainly expresses in the kidney for the reabsorption of di-, tripeptides (Ocheltree et al., 2005) and choroid plexus for neuropeptide homeostasis and the efflux of peptides/mimetics from cerebrospinal fluid (Shen et al., 2007).

After a protein meal, di-, tripeptides, instead of free amino acids, are the major components in the gut lumen. PEPT1, predominantly expressing on the apical membrane of enterocytes, has been suggested for the translocation of dipeptides from gut lumen to cytoplasm in the enterocytes, where they will be degraded and/or transported into the systemic circulation. Besides its normal functionality in nutrients and drugs absorption, PEPT1also plays an important role in pathological states as listed examples below.

Cystinuria, an autosomal recessive disorder, is characterized with impaired transport of essential amino acids like cystine, lysine, arginine and ornithine in the GI tract and the proximal renal tubule due to the dysfunction of $b^{0,+}AT$ (Dello Strologo et al., 2006). Hartnup disease is another autosomal recessive abnormality in renal and GI systems marked with poor absorption of histidine, tryptophan, and phenylalanine (Broer et al., 2005). Surprisingly, patients suffering from these heritage diseases did not show much malnutrition such as essential amino acid deficiency. The main reason is that they

received the essential amino acids like lysine in di-, tripeptide forms via peptide transporters, which have been confirmed in *in situ* jejunal perfusion from patients providing lysine and leucine in their free forms or in dipeptide leucyl-lysine (Hellier et al., 1971). It evidently establishes that PEPT1 is important or even essential to human beings, especially when they suffer from inherited gene defects.

Because of their nature, single amino acids like glutamine and tyrosine are either unstable or poorly absorbed. Steinhardt, et al. (1986) compared the absorption of 12 amino acids either in free amino acid mixtures with glycine or in glycyl-dipeptide forms by human jejunum. They found that all amino acids except for arginine were absorbed significantly higher in dipeptide forms than in free amino acid mixtures, especially for amino acids with poor absorptive characteristics like histidine and tryptophan.

Numerous examples showed that PEPT1 played important roles in peptidomimetic drug absorption clinically used for the treatment of infection (e.g., β-lactam antibiotics), hypertension (e.g., ACE inhibitor and rennin inhibitors) and cancer (e.g., bestatin). One successful application of PEPT1 was to improve bioavailability of drugs in fighting viral infection (e.g., valacyclovir). Overall, PEPT1 is pharmaceutically and pharmacologically relevant.

2.1.2 Molecular Structure

PEPT1 cDNA from human, rabbit, rat and mouse encode highly homologous proteins containing 708 (human) (Liang et al., 1995), 707 (rabbit) (Fei et al., 1994), 710 (rat) (Erickson et al., 1995) or 709 (mouse) (Fei et al., 2000), respectively. Due to the lack of PEPT1 crystal structure, the transmembrane (TM) model of PEPT1 was generated by a two-approach computer modeling method, based on minimizing the interactions of

the faces of TMs in a pairwise fashion (Bolger et al., 1998; Yeung et al., 1998). The protein contains 12 putative transmembrane spanning domains (TMD) with both N-, C-termini facing in the cytosolic side. A large extracellular loop is predicted between TMD 9 and 10 (Figure 2.1). From the amino-terminal region, the first 4 transmembrane regions and the domains 7 to 9 are critical for its substrate affinity and other characteristic features (Doring et al., 1996). PEPT1 is a heavily glycosylated protein with multiple N-glycosylation sites. Human PEPT1 contains two potential sites for protein kinase C phosphorylation, while rat and rabbit PEPT1 have singly potential site for protein C-dependent phosphorylation (Liang et al., 1995). In rabbit and rat PEPT1, there is a single potential site for protein kinase A phosphorylation. Interestingly, human PEPT1 is lack of any site for protein kinase A. The molecular weight of hPEPT1 is 78.81 kilodaltons and an isoelectric point of 8.6. Chromosomal assignment studies with somatic cell hybrid analysis and *in situ* hybridization identified that human PEPT1 gene is on chromosome 13 q33-34 (Liang et al., 1995).

Both mouse and human PEPT1 genes are ~32 kb long and contain 23 exons and 22 introns. In mouse PEPT1, the putative regulatory region contains three GC boxes locating at nucleotide positions -88, -322, and -352, which are believed to be the binding sites for the transcription activator SP1. Instead, a TATA box-like sequence, CAATAAATA, is present at nucleotide -813, suggesting its less importance on PEPT1 transcriptional expression (Fei et al., 2000). Human PEPT1 promoter regions are like mouse counterpart with GC boxes being at -29 bp and a couple of others within 300 bp. TATA boxes are locating at 511 bp and 517 bp upstream from the transcription start site. Shortly after the cloning of human PEPT1 gene, a closely related transcript, hPEPT1-RF

(for hPEPT1-regulatory factor) was isolated from human duodenum cDNA library (Saito et al., 1997). The 18-195 amino acid residues of this 208-amino acid protein are identical to the 8-185 residues of hPEPT1, whereas the 1-17 and 196-208 residues are unique. hPEPT1-RF alone does not induce peptide transport activity; rather, it shifts pH sensitivity profile of hPEPT1-mediated dipeptide transport when coexpressing with hPEPT1 in *Xenopus* oocytes, suggesting its regulatory function on hPEPT1 activity.

Site-directed mutagenesis has been extensively used to determine the substrate affinity of PEPT1. H57, H121 and H260 were the first spot mutations tested on hPEPT1. H57 is locating near the extracellular surface of the second putative transmembrane domain and is suggested to participate in the binding and translocation of proton. H57Q almost completely abolish PEPT1 transport function, while the other mutations had little effects on PEPT1 functionality (Fei et al., 1997). hPEPT1 with Y167A in transmembrane domain 5 lost its ability to transport reference compound GlySar even though Y167A-hPEPT1 expression level by western blot analysis and cell surface expression by immunofluorescence microscopy were similar to those of the wild type (Yeung et al., 1998). Mutation of W294A in TM 7 showed much larger Km and decreased Km when hPEPT1 transported GlySar. Mutants G595A (locating in TM 10), Y12A (locating in TM1), Y167A (locating in TM5), and R28A (locating in TM 7) affected Vmax, but not Km, of hPEPT1 in transporting GlySar (Bolger et al., 1998). Computer modeling of PEPT1 predicted that TMD 7 was essential in forming the putative central aqueous channel for substrate to traverse through. Based on this prediction, a couple of single amino acids in TMD 7 were individually mutated to test PEPT1 function changes. Three mutants F293C, L296C, and F297C of hPEPT1 showed negligible GlySar uptake

activity, suggesting their importance in defining the overall hPEPT1 structure. In K278C mutant, GlySar transport decreased about 40% (Kulkarni et al., 2003). R282E-rPEPT1 had similar binding affinity to its substrate, but lost its responsiveness to extracellular pH changes in transporting D-Phe-L-Gln, suggesting the relevance of arginine 282 in cotransport of protons and peptides by wild type PEPT1 (Meredith, 2004). Mutations such as Y587F (locating in TM10) and D341R (locating in TM8) had little apparent effect on PEPT1-mediated peptide transport (Meredith and Price, 2006). Mutation of Y91F-rPEPT1 reduced 80% of its activity when transporting model dipeptide compounds, with mechanistic basis on transporter-proton interactions being interrupted (Meredith and Price, 2006). A recent paper tested a couple of conserved tyrosine residues in rabbit PEPT1 and summarized that Y12F (locating in TM1), Y56F (locating in TM5), Y91F (locating in TM3), Y167F (locating in TM5), and Y345F (locating in TM8) altered rPEPT1 transport functions (Pieri et al., 2009). A complete list of site mutations is shown in Table 2.1.

2.1.3 Genetic Polymorphism

Classified as a conserved gene, PEPT1 (SLC 15A1) shows little genetic polymorphisms among various ethnic groups. A genetic screening of 44 ethnically diverse individuals by a DNA polymorphism discovery panel identified 9 nonsynonumous and four synonymous coding-region single-nucleotide polymorphisms (SNPs) in hPEPT1 (Zhang et al., 2004). Among nine nonsynonymous SNPs, only a single SNP (P586L) demonstrated significantly reduced transport activity. A subsequent study tested genetic polymorphisms on 247 individuals of various ethnic origins and only a new, low-frequency hPEPT1-F28Y variant was identified. Functional studies in Cos7

cells transiently transfected with this variant showed a great reduction in transporting cephalexin uptake (Anderle et al., 2006).

Largely due to the low frequency of genetic polymorphisms, little information is known about the clinical relevance of PEPT1 SNPs to oral drug bioavailability. One study was conducted to correlate a pharmacokinetics of valacyclovir, a believed PEPT1 substrate, to genetic polymorphisms from 16 healthy volunteers (Phan et al., 2003). Though interindividual differences were observed in valacyclovir bioavailability, PEPT1 genetic variation alone could not explain this clinical divergence. Conclusively, SLC15A1 genetic polymorphisms are unlikely to have clinical impact on the drug absorption.

2.1.4 Tissue and Cellular Localization

PEPT1 expresses in a variety of tissues with different subcellular localization. Among all tissues it expresses, small intestines have the highest amount of PEPT1 than any other tissues from mRNA to protein expression levels. In the small intestines, it is locating on apical membranes of epithelial cells. In rats, PEPT1 mRNA expression levels are comparable in all duodenal, jejunal and ileal segments (Howard et al., 2004). In human beings, mRNA levels are the highest in duodenum, followed by jejunum and least being found in ileum (Terada et al., 2005). PEPT1 is locating on the apical sides of epithelial cells in S1 segments of the proximal tubule in the kidney (Shen et al., 1999). Its renal expression is species-dependent: moderate levels of PEPT1 mRNA were detected in rat kidney, while its expression was very low in mice. In addition, protein and function of PEPT1 were also confirmed in renal lysosomal membrane to transport di-, tripeptides from cytosol to lysosome for their hydrolysis (Zhou et al., 2000).

In pancreas, PEPT1 has been demonstrated to express in nuclei of smooth muscle cells in the wall of arterioles; in nuclei of Schwanna cells in unmyelinated pancreatic nerves, and lysosomes in acinar cells by immunohistological analysis (Bockman et al., 1997). Its function in lysosome was to mediate small peptides from lysosome to cytoplasm following intralysosomal protein degradation. But the nature of the transporter function in the nucleus remained unknown. In the liver, PEPT1 also expressed on the lysosomal membrane to translocate small peptides from lysosome to cytoplasm after protein degradation (Thamotharan et al., 1997). PEPT1 mRNA transcripts, however, were low to undetectable in spleen, thymus, brain, pituitary, prostate, and uterus (Lu and Klaassen, 2006).

For PEPT1 expressions in extrahepatic biliary duct, all data were from cancer cell lines derived from this organ (Knutter et al., 2002; Neumann and Brandsch 2003). It expressed on apical membranes of epithelial cells to transport the endogenous photosensitizer delta-aminolevulinic acid into tumor cells. In addition, mRNA and function of PEPT1 were confirmed in pheochromocytoma neuroendocrine cell lines, suggesting its possible location in adrenal gland (Hussain et al., 2001). PEPT1 mediated dipeptide transport into human full-term placental brush-border membrane vesicles (BBMV) (Meredith and Laynes, 1996).

Recently, mRNA, protein and function of PEPT1 were confirmed in nonpolarized monocytic cell line and macrophages isolated from human peripheral blood (Charrier et al., 2006). Unlike PEPT1 having maximal activity at acidic environments like pH 6.0 in other tissues, hPEPT1 in nonpolarized immune cells functioned best at physiological pH

7.2. In addition, mRNA of rPEPT1 was confirmed in lactating mammary gland, possibly transporting both nutrients and drugs into breast milk (Gilchrist and Alcorn, 2009).

2.1.5 Regulation

One of the most interesting characteristics of PEPT1 is that it responds to a variety of factors ranging from its substrates to pharmacological agents. The regulation of PEPT1 expression has summarized into below categories:

Signal transduction components:

Caco-2 cells, the human colon carcinoma cell line, has been widely used in the research of PEPT1 since this cell line will differentiate into polarized cell monolayers with PEPT1, other transporters and enzymes expressing on the apical membrane. When treated with phorbol esters, an activator of protein kinase C, Caco-2 cells minimized its uptake of GlySar with a decrease in the maximal velocity and Km remaining unaltered. This inhibition could be blocked if Caco-2 cells were co-treated with staurosporine, an inhibitor of protein kinase C, suggesting the PEPT1 transport system was under the regulatory control of protein kinase C (Brandsch et al., 1994). Cholera toxin could increase cAMP levels in the Caco-2 cells, which inhibited the activity of PEPT1 in a dose-dependent manner. A decrease of Vmax was observed in the treatment as a reason for inhibition. Furthermore, the inhibitors of protein kinase A and protein kinase C would block the inhibitory effect of cholera toxin. This study indicated that cAMP, once activated, would work on its downstream receptors, PKA or PKC, to inhibit the activity of PEPT1 (Muller et al., 1996).

PEPT1 substrates:

When exposed to PEPT1 substrates (i. e., 10 mM GlySar for 24 hours), mRNA and protein expressions of PEPT1 in Caco-2 cells would increase three and two folds, respectively. Moreover, glycylglutamine (GlyGln) uptake by Caco-2 cells was significantly increased with two-fold increase in Vmax and no changes in Km. In the presence of trans-Golgi network inhibitor (brefeldin), the stimulation was not achieved, indicating that dipeptides would boost the membrane population of PEPT1 (Thamotharan et al., 1998). Similar stimulation of dipeptide uptake by PEPT1 was observed when feeding Caco-2 cells with 4 mM GlyGln for 3 days (Walker et al., 1998). Further study demonstrated that dipeptides such as GlyPhe and certain free amino acids (i.e., Phe) could directly stimulate the rat PEPT1 promoter and increase expression of PEPT1 in Caco-2 cells. In addition, brush border membrane vesicles (BBMV) from rats fed with high protein had higher GlySar uptake compared to those from rats without protein in diet (Shiraga et al., 1999).

Hormones and growth factors:

As a key hormone in metabolism, physiological conditions of insulin (5 nM) were shown to stimulate GlyGln uptake through Caco-2 cells by the increase of velocity (Vmax), while no alteration in capability (Km) in the Michaelis-Menten constant of its transport was observed. Genistein, a blocker of the insulin signal pathway, could counteract its stimulatory effect. In addition, PEPT1 protein, but not mRNA in Caco-2 cells was increased after insulin treatment. It was suggested that the increase of

membrane PEPT1 by insulin stimulation was because of accelerating PEPT1 translocation from a preformed cytoplasmic pool (Thamotharan et al., 1999). Comparing to non-treatment control, streptozotocin-induced diabetic rats had significantly lower GlySar influx rate when performing an *in situ* perfusion in the jejunum. Insulin treatment in diabetic rats could increase the influx rate of GlySar to the similar level observed in normal rats (Bikhazi et al., 2004).

Leptin, the ob gene product, is secreted by adipose tissue and works on hypothalamus for energy homeostasis by altering energy intake and expenditure. Subsequent studies have demonstrated that leptin can be produced by stomach and it remains active when reaching the small intestine. Buyse et al. (2001) showed that leptin receptor was present in Caco-2 cells as well as brush border membranes of small intestine. Caco-2 cells were demonstrated to increase GlySar and cephalexin (CFX) transport after treated with leptin. The Vmax was increased and Km remained unaffected in GlySar transport comparing to Caco-2 controls. Moreover, CFX blood concentration was increased when intrajejunally perfusing with leptin in rats. Excess GlyGly could diminish leptin's stimulatory effect on CFX transport, presumably by competing for PEPT1 transporters.

Another important hormone, thyroid hormone T3, regulates growth, development and normal energy levels such as through increasing metabolism glucose. T3 treatment demonstrated a completely opposite effect on PEPT1 as compared to the effects of insulin or leptin on GlySar uptake in Caco-2 cells. The reduction in GlySar uptake was due to the decrease of Vmax but not Km in the GlySar transport system (Ashida et al., 2002). Both mRNA and protein of PEPT1 had been shown to decrease in hyperthyroid

than euthyroid rats. GlySar uptake by everted small intestinal rings decreased in hyperthyroid rats with reduction of Vmax and unaffected Km. The reduced GlySar uptake was also confirmed in rats by *in situ* intestinal perfusion (Ashida et al., 2004).

Inflammation:

In short-bowel syndrome patients, mucosal biopsy showed PEPT1 mRNA abundance was more than five-fold higher than that from healthy human controls, indicating the remaining colon tissue might compensate to accommodate di- and tripeptide absorption (Ziegler et al., 2002). In addition, PEPT1 expression was induced in colon tissues in patients with chronic ulcerative colitis and Crohn's disease. Bacteria, such as *Escherichia coli*, secrete n-formyl peptides like formyl-Met-Leu-Phe (fMLP) as chemotactic substances to attract neutrophils to the inflammatory sites. hPEPT1 can transport fMLP so that its upregulation in chronic colon disease might induce intestinal inflammation (Merlin et al., 2001). A recent work revealed possible mechanisms for the upregulation of hPEPT1 in colon diseases. In this regard, tumor necrosis factor - α (TNF - α) and interferon - γ (IFN - γ), inflammatory mediators, could enhance hPEPT1 protein density and activity as indicated by the increase of GlySar influx rate in a concentration-dependent manner in Caco-2 cells. After intraperitoneal injection of TNF - α and/or IFN - γ, mouse colon, instead of small intestine, showed higher GlySar uptake compared to untreatment controls. Furthermore, mRNA and protein of hPEPT1 in colon tissues, but not small intestine, were upregulated after treatment (Vavricka et al., 2006). A recent report demonstrated that butyrate, a short-chain fatty acid from commensal bacteria, upregulated PEPT1 mRNA, protein and functions in Caco2-BBE cells (Dalmasso et al.,

2008). In addition, when mice were treated with 5mM butyrate for 24 h, colonic PEPT1 was observed increases in its mRNA and protein expression levels as well as its transport activity in colonic apical membrane vesicles.

Cryptosporidium parvum, a parasitic protozoa infection is thought to be the reason for intestinal malabsorptive syndrome, which leads to malnutrition and/or growth failure. When *C. parvum* was administered to neonatal Sprague-Dawley rats, PEPT1 protein and its ability to transport GlySar from BBMV were reduced at the peak time of infection and they returned to normal level after parasites were spontaneously cleared. IFN- γ was believed to mediate this PEPT1 change (Marquet et al., 2007).

Physiological factors:

Most physiological, biochemical, and behavior processes in mammals observe diurnal rhythms. Whether peptide transport obeyed this rule was also investigated (Pan et al., 2002). Wister rats were raised in a room with 12-hr photo-period. GlySar plasma concentrations in portal vein were higher in the dark phase than in the light phase. PEPT1 protein and mRNA from small intestines were the highest at 8:00 pm and decreased gradually to the lowest point at 8:00 am. GlySar transport by *in situ* intestinal loop and everted intestinal rings gradually decreased from 12:00 am and dropped to the lowest at 12:00 pm. This pattern change of PEPT1 activity was believed as an adaptation to the feeding habits at night in the nocturnal animals. Renal PEPT1, on the other hand, had not shown such a pattern. A recent study isolated mRNA transcripts of PEPT1 from duodenal, jejunal and ileal segments in rats and tested their changes in 24 hours (Qandeel et al., 2009). Surprisingly, PEPT1 mRNA expression and its corresponding transport

functions varied diurnally in duodenum and jejunum, but not in ileum. After abdominal vagotomy was performed, diurnal variations in PEPT1 protein expression and function (e.g., GlySar uptake), but not mRNA, were absent in duodenum and jejunum, indicating diurnal rhythm of PEPT1 was partially mediated by vagal innervations (Qandeel et al., 2009). On the molecular mechanistic basis, clock-controlled gene, albumin D site-binding protein (DBP) has been demonstrated to play a critical role in the circadian oscillation of PEPT1 by regulating PEPT1 promotor activity (Saito et al., 2008).

Interestingly, long-term exposure to different glucose levels in culture medium could alter Caco-2 ability to transport dipeptides such as GlySar (D'souza et al., 2003). Caco-2 cells cultured in 5.5 mM (physiological concentration) glucose concentrations had higher uptake rate of GlySar comparing to Caco-2 cells with 25 mM glucose in the culture medium. Furthermore, high glucose treatment diminished Vmax, increased passive diffusion (Kd) but not Km in GlySar transport system. Changes of PEPT1 activity with high glucose concentration were unclear since PEPT1 mRNA in confluent Caco-2 cells remained the same and protein levels were not reported. When co-treated with thiol antioxidant DTT, Caco-2 cells in high glucose medium restored PEPT1 ability by increasing Vmax to the similar levels as that from low glucose concentration. This suggested that long term exposure to high glucose may induce oxidative damage in carriers and/or proteins involved in regulating PEPT1 functional activity.

Because of food deprivation and medical requirements, mammalians may experience fed and fasted conditions. After rats were briefly fasted (1 d), mRNA and protein levels of PEPT1 in brush-border membranes from jejunum had been shown to increase three-fold as compared to fed rats. Vmax, but not Km, in GlyGln transport

system was upregulated by two-fold in BBMV (Thamotharan et al., 1999). Subsequent studies further investigated the relationships among PEPT1, feeding conditions and diurnal rhythm. Consistent with the increase of mRNA and protein after a brief fast, PEPT1 mRNA and protein from rat small intestines were significantly induced after a 4-d deprivation of food. In normal feeding condition, intestinal PEPT1 mRNA and protein levels were highest at 8:00 pm and lowest at 8: 00 am. However, there was no difference in PEPT1 protein levels between 8:00 pm and 8:00 am in rats deprived of food for 2-4 d. More interestingly, food deprivation did not alter PEPT1 mRNA diurnal variation and it still expressed the highest at 20pm and lowest at 8am. Mechanisms for inconsistent changes between PEPT1 mRNA and protein levels had not been addressed yet. After rats were refed for 2 d, PEPT1 protein restored its diurnal oscillation. When intraintestinally administered with an oral antibiotic ceftibuten, a pharmacological substrate of PEPT1, fed rats, but not 4-d fasted rats, had higher Tmax, Cmax and $AUC_{0\text{-}3h}$ at 8:00 pm than at 8:00 am. Moreover, 4-d fasted rats showed significantly higher Tmax, Cmax and $AUC_{0\text{-}3h}$ both at 8:00 pm and at 8:00 am than fed rats (Pan et al., 2004; Pan et al., 2003).

PEPT1 mRNA and protein levels changed consistently in rats in different development stages. PEPT1 expression reached maximum 3 to 5 days after birth in the duodenum, jejunum, and ileum and declined rapidly to adult levels, which were approximately 70% of those observed on d 3-5. PEPT1 expressed similar level in colons with that in small intestines in the first week after birth, but it was undetectable thereafter through adulthood (Shen et al., 1999). PEPT1 expression was less regulated in rat kidney. Its protein level gradually increased from embryo to pups and reached plateau around d 14 after birth.

Pharmacological agents:

Antitumor drugs, such as 5-fluorouracil (5-FU), have long been known to cause deleterious effects on the intestinal mucosa so that absorption via small intestines will be vitiated. Intestinal peptide transport system, on the other hand, is resistant to tissue damage. To address mechanism of this resistance, rats treated with 5-FU were used to explore the possibilities (Tanaka et al., 1998). After 5-FU treatment, BBMV from small intestine kept its ability to transport GlySar, while absorption of free amino acid like glycine was dramatically reduced. Western blot and immunoblot analysis showed that PEPT1 protein levels and localization in the 5-FU treated rat were similar with untreatment control while other transporters like Na+-dependent glucose transporter almost vanished. In addition, PEPT1 mRNA was slightly increased after 5-FU treatment, indicating that accelerated PEPT1 synthesis may explain its resistance to tissue injury.

α_2-agonist clonidine was observed to induce a two-fold increase in intestinal absorption of CFX when performing a single-pass jejunal perfusion in rats (Berlioz et al., 1999). Since α_2–adrenergic receptors were also present in intestinal epithelial cells, Caco-2 cells stably expressed with α_{2A}–adrenergic receptors at a density similar to that found in normal mucosa, (i. e., Caco-2 3B cells), were used to clarify the enhancement of CFX absorption after clonidine treatment (Berlioz et al., 2000). When treated with clonidine, Caco-2 3B, but not Caco-2 cells, showed a two-fold increase in CFX transport comparing to the untreated cells and the stimulatory effect would be abolished by excess GlySar and α_2–antagonists like yohimbine and RX821002. Clonidine-treated Caco-2 3B cells demonstrated increased Vmax and unaffected Km in CFX transport system. In

addition, disturbing microtubule integrity by colchicine could block the stimulatory effect on CFX transport by clonidine.

Since σ receptors are expressed in non-neuronal tissues (i.e., gastrointestinal tract) as well as the central nervous system, its physiological role in the small intestine was also investigated. When treated with (+) pentazocine, a selective σ 1 receptor ligand, Caco-2 cells displayed an increase of GlySar uptake mediated by PEPT1 in a concentration-dependent manner. Further analysis of GlySar kinetics showed that Vmax, but not Km was enhanced twice after (+) pentazocine treatment. In addition, mRNA of PEPT1 was increased correspondingly as (+) pentazocine concentration increased in Caco-2 cells (Fujita et al., 1999).

When coadministered with Ca^{2+} channel blockers (i.e., nifedipine), healthy volunteers showed a 30% increase in the absorption rate of cefiximem, an oral antibiotic for the treatment of respiratory tract infection (Duverne et al., 1992). After treated with either verapamil, nifedipine, diltiazem, or bepridil, Caco-2 cells had higher uptake rate of cefixime while the rate reduced when Ca^{2+} ionophores like ionomycin and A23187 were added to the cells. Analysis of cefixime transport kinetics in Caco-2 cells showed that Ca^{2+} channel blockers increased Vmax with Km unaltered. Further measurement of intracellular pH indicated that Ca^{2+} channel blockers may affect pH regulatory systems, such as Na^{+}/H^{+} exchanger on the apical membrane so that the driving force was boosted for uptake of cefixime (Wenzel et al., 2002).

In contrast to Ca^{2+} channel blockers, dietary phosphodiesterase inhibitors caffeine, theophylline, and pentoxifylline would decrease GlySar uptake in Caco-2 cells by showing a reduction in Vmax and unaffected Km. The mechanism of this reduction

was not due to its direct inhibition on PEPT1, but rather through inhibition of Na^+/H^+ exchanger. Since driving force was minimizing for H^+–coupled solute transport, GlySar influx rate was vitiated in Caco-2 cells (Anderson et al., 2007).

Because of its dipeptide-like structure, nateglinide, a nonsulfonylurea insulin secretagogue, was investigated for its interaction with PEPT1 in Caco-2 and LLC-rPEPT1 cells (rat PEPT1-transfectants). Nateglinide could potently inhibit GlySar uptake by Caco-2 and LLC-rPEPT1 cells with a reduction in Vmax and unaltered Km. Analysis of the kinetics on GlySar transport revealed that nateglinide was noncompetitively inhibited GlySar uptake. Moreover, the PEPT1-mediated influx rate of nateglinide was negligible, suggesting that although not a substrate, nateglinide could inhibit PEPT1. Its inhibitory mechanism remains unclear (Terada et al., 2000). Various sulfonylureas, such as glibenclamide, tolbutamide demonstrated a similar inhibition to that of nateglinide on dipeptide transport via PEPT1 (Sawada et al., 1999).

Transcription factors:

Since PEPT1 is a highly responsive gene, its molecular mechanisms are under investigation. After PEPT1 promoters from various species had been cloned, they were used to reveal the direct regulation on PEPT1 by different factors. Dipeptides and certain amino acids like phenylalanine could stimulate rPEPT1 promoter activities in Caco-2 cells after transfection with PEPT1 promoter-luciferase constructs. The working region spanned from -351 to -171 on the promoter (Shiraga et al., 1999). Serial deletion analysis of hPEPT1 promoter showed that the region of -172 to -35 bp was critical for the basal transcriptional activity. Though lacking a TATA-box, this region contained GC-

rich sites and the transcription factor Sp1 was found to work on hPEPT1 promoter through direct binding to some GC-rich sites (Shimakura et al., 2005).

Since Sp1 is expressed ubiquitously, it could not be the transcription factor responsible for intestine-specific PEPT1 expression. To its clarify tissue-specific factors, several transcription factors were under investigation and only caudal-related homeobox protein 2 (Cdx 2) was revealed to trans-activate hPEPT1 promoter. Further deletion analysis showed that Cdx 2 responsive element was located between -172 to -35 bp. Chromatin immunoprecipitation confirmed that Cdx 2 associated with the hPEPT1 promoter. Protein-protein interactions suggested that Cdx 2 and Sp1 were associated in a protein complex in Caco-2 cells. In addition, analysis of human gastric samples with intestinal metaplasia indicated that mRNA levels of PEPT1 were highly correlated with that of Cdx 2 (r^2=0.8665 in linear regression) (Shimakura et al., 2006).

As one of the prominent characteristics, PEPT1 is highly inducible in fasting conditions. To elucidate the mechanism underlying PEPT1 induction, pexisome proliferator-activated receptor α (PPARα) were intensely explored, which played a pivotal role in the adaptive response to fasting in the liver and other tissues. mRNA levels of PPARα were markedly increased in the rat small intestine after fasted for 48-hr, accompanied by an increase of PEPT1. When treated with PPARα ligand, WY-14643, Caco-2 cells showed enhanced PEPT1 mRNA levels and a higher transport rate of GlySar. Rats dosed with WY-14643 for 5 d demonstrated an increased mRNA level of PEPT1. Moreover, PPARα null mice had no mRNA changes in fasting condition, indicating that PPARα was a regulator for the inducible PEPT1 in the small intestines (Shimakura et al., 2006).

As shown before, PEPT1 would be induced after leptin treatment. After the possible signal-transduction pathways were analyzed, Nduati et al (2007) revealed that cAMP-response element-binding protein (CREB) and CDX2 transcription factors were interacted and docked on hPEPT1 promoter to upregulate its expression. The following study about leptin and PEPT1 demonstrated that MAPK pathway and ribosomal protein S6 activation were involved in the regulation of PEPT1 in the transcriptional and translational levels, respectively (Hindlet et al., 2009).

Since food effect on PEPT1 is one of our research targets, its regulations by diets and fasting will be listed separately.

Diet:

After rats were fed with protein-free, 5% casein, 20% casein, or 50% casein diets for 3 days, PEPT1 mRNA and protein expression levels were upregulated in 20% casein and 50% casein diet treatments comparing those to from protein-free fed rats (Shiraga et al., 1999). GlySar uptakes into BBMVs were increased correspondingly. Similar increases were observed in rats fed with diets containing 10% Phe or 20% GlyPhe.

When mice were fed with hypercaloric diet (i. e., 36% fat in the diet) for 4 weeks, a 30% decrease in PEPT1 protein and a 50% decrease in PEPT1 mRNA were observed in the jejunal segments. PEPT1 - mediated GlySar transport in the jejunal loop showed a 46% reduction than that from standard laboratory chow-fed mice (i.e., 3% fat in the diet) (Hindlet et al., 2009). These changes in PEPT1 were suggested to be associated with leptin receptor down-regulation in the small intestines.

Fasting:

In the first paper about fasting on PEPT1, Thamotharan et al. (1999) observed three-fold and two-fold increase of PEPT1 mRNA and protein expressions, respectively, in intestinal mucosa and brush-border membrane after rats were fasted for 1 day. In addition, GlyGln uptake rate by BBMVs from 1-d fasted rats were increased by two-fold in Vmax with Km unchanged. A parallel research used western blot analysis and immunogold electron microscopic techniques to demonstrate that PEPT1 protein expression levels were elevated in the apical microvillous plasma membrane of the absorptive epithelial cells of the rat jejunum after 4-d fasting. But dietary administration of amino acids slightly reduced the amount of peptide transporter present (Ogihara et al., 1999).

In 2002, Naruhashi et al. measured PEPT1 mRNA abundance and its transport of cefadroxil in upper, middle and lower intestinal regions from 2-d fasted rats by Real-time PCR and Ussing Chamber Method, respectively. Whole small intestine was arbitrarily divided into 8 segments with the first two segments being upper region, the middle 4 segments being middle region, and the rest two being lower region. As a result, PEPT1 mRNA amount and cefadroxil transports were correlatively increased in the upper and middle regions, but not in the lower segments. After rats were fasted for 2 to 4 days, diurnal rhythms of PEPT1 protein expression and transport activity, but not mRNA, were disrupted (Pan et al., 2003).

PEPT1 expressions in different intestinal regions responded differently to total parenteral nutrition (TPN) in rats. Ihara et al. (2000) revealed that mRNA and protein abundance of PEPT1 were both increased in the jejunal mucosa after rats were given

TPN for 10 days. The following study demonstrated that PEPT1 mRNA expression was unaffected in the duodenum and increased in the ileum in rats administered with TPN diet for 7 days (Howard et al., 2004).

Molecular mechanisms underlying the augmentation of PEPT1 by fasting are unclear yet. Shimakura et al. (2006) claimed that peroxisome proliferator-activated receptor alpha was mediated the induction of PEPT1 expression in fasting condition through the increase of PEPT1 mRNA abundance by WY-14643, a synthetic PPARalpha ligand, in rats and the abolishment of PEPT1 mRNA augmentation in fasted PPARalpha-null mice. While in an independent study, PEPT1 mRNA was unaffected in wild-type or PPARalpha-null mice orally administered with PPARalpha-specific agonists (Hirai et al., 2007).

Finally, a recent report demonstrated higher plasma and brain concentrations of oseltamivir, an ester-type prodrug of the neuraminidase inhibitor, were observed in fasted overnight rat pups (1-week-old with body weight 16.5 to 20.6 grams) than their fed littermates after oral administration (Ogihara et al., 2009). Oseltamivir was suggested as a substrate of PEPT1. In Caco-2 cells, its uptake was greatly inhibited by GlySar. In adult rats, its absorption was markedly reduced when simultaneously administered with milk, casein or GlySar.

2.1.6 Substrate Specificity

As the best known reference compound, synthesized [14C] or [3H]-glycylsarcosine (GlySar) has been extensively to test whether unknown compounds are the substrates of PEPT1. GlySar is relatively stable against intra- and extracellular

enzymatic hydrolysis and its transport activity has been characterized in almost all tissues known to express PEPT1. Since an inhibitor on GlySar uptake can be competitive, noncompetitive, or even by breaking down the H+ gradients to reduce its uptake, a test compound will be performed by other techniques such as the two-microelectrode voltage-clamp technique in X. oocytes expressing PEPT1 to confirm its substrate specificity.

Di- and tripeptides

PEPT1 has a broad substrate spectrum (Daniel and Kottra, 2004) and accept all possible di- and tripeptides in a stereospecific manner with a few exceptions. It cannot transport amino acids or tetraepeptides. For di- and tripeptides, the N-terminal and C-terminal charged head groups have to separate between 500 to 635 pm (Rubio-Aliaga and Daniel, 2008). PEPT1 has preferences to transport peptides containing L-amino acid residues to those composing of D-amino acids (Daniel et al., 1992). For those peptides solely consisting of D-stereoisomers, they will not be transported by PEPT1. When proline is locating in N-terminal position in peptides, they are typically not good substrates of PEPT1. Studies have demonstrated that PEPT1 in Caco-2 cells had little, if any, affinity towards Pro-Ala, Pro-Asp, Pro-Ser, Pro-Glu and Pro-Gly as its substrates (Brandsch et al., 1999; Vig et al., 2006). Largely because of the cyclic structure of the proline, it provides an exceptional conformational rigidity so that PEPT1 can not bind well to those peptides.

For peptides containing only basic amino acids such as Arg-Arg or Lys-Lys, they will not bind to PEPT1 when performing at a medium pH of 6 (Biegel et al., 2006; Vig et al., 2006). The possible explanation is that both protonated charged side-chains will repel

each other. But in a previous study, Lys-Lys was transported in oocyte-expressing PEPT1 when the medium pH was above 7.4 (Amasheh et al., 1997). It reiterates that the protonation state of a substrate at a given pH conclusively determines the substrate binding specificity to PEPT1.

When modifying the N-terminal amino group or C-terminal carboxyl group by methylation, acetylation or other substitutions, the modified peptides shows reduced affinity for PEPT1 (Hartrodt et al., 1998; Meredith et al., 2000). In addition, the N-terminus modification decreases the affinity to a higher extent than that of C-terminus (Biegel et al., 2006). When either or both of amino and carboxyl termini is removed, peptides will totally lose their affinity to PEPT1.

A peptide bond is not an essential structural requirement for PEPT1 substrates. δ-AA possesses a ketomethylene group instead of a peptide bond, but it is a substrate of PEPT1 (Irie et al., 2001). Ala-ψ[CS-N]-pro, which has an isosteric thioxo peptide bond, can be recognized and transported by PEPT1 (Brandsch et al., 1998). In addition, ω-amino fatty acids are substrates of PEPT1 though different affinities were reported among laboratories (Doring et al., 1998; Terada et al., 2000; Biegle et al., 2006).

Rubio-Aliaga and Daniel (2008) summarized essential structural features for di-, tripeptides to be substrates of PEPT1, which contain: 1) L-amino acid, 2) an acidic or hydrophobic function at the C-terminus, 3) a weakly basic group in the α-position at the N-terminus, 4) a ketomethylene or acid amide bond, and 5) a trans conformation of peptide bonds.

Xenobiotics

Largely due to structural resemblance to physiologically occurring peptides, foreign compounds are potential substrates of PEPT1, which include amino β-lactam antibiotics of the cephalosporin and penicillin classes, certain angio-tensin-converting enzyme (ACE) inhibitors, selected renin inhibitors, antitumor agents such as bestatin, dopamine receptor antagonists such as sulpiride and various amino acid ester prodrugs.

Back to 1970s, Addison et al. (1975) showed that high dose of cephalexin significantly inhibited GlySarSar *in vitro* uptake by hamster jejunum. Not all cephalosporins are substrates of PEPT1. Cephalosporins with an α-amino group increase recognition of PEPT1, but this structure is not an absolute requirement for substrate interaction (Raeissi et al., 1999). Ceftibuten (Ganapathy et al., 1997) and cyclacillin (Fei et al., 1994) interact with PEPT1 in high affinity (Ki < 1mM). Cefadroxil (Boll et al., 1994), cefalor, cephalexin, ampicillin and others are moderately binding to PEPT1 (Ki = 7-14 mM), while cefapirin, cefuroxime, benzylpenicillin, ceftriaxone, cefesoludin have no affinity to PEPT1 (Ki > 20mM) (Bretschneider et al., 1999). Uptake assays on Caco-2 cells (Bretschneider et al., 1999) and molecular modeling studies (Biegel et al., 2005) suggested structural features of β-lactam antibiotics to be recognized by PEPT1: 1) sterical resemblance to the tripeptide backbone, 2) N-terminal peptide bond with α-amino group, 3) carboxyl group at dihydrothiazine ring of cephalosporins or thiazolidine of penicillin, and 4) substituents on and saturation of the N-terminal ring systems.

Just as for β-lactam antibiotics, recognitions of ACE inhibitors by PEPT1 have been modified with more evidences published and the transport of some ACE inhibitor compounds is still a matter of controversy. For instance, enalapril showed high affinity to PEPT1 in an Ussing Chamber test (Swaan et al., 1995), while Moore et al. (2000) and

many others found no affinity of enalapril for PEPT1. Similar discrepancies were observed in captopril (Thwaites et al., 1995; Brandsch et al., 2004).

In contrast, fosinopril is undoubtedly a substrate of PEPT1 (Moore et al., 2000). It is transported intact by PEPT1 in a saturable process and intracellular accumulation of fosinopril is 3 to 4 times higher from the apical side than from the basolateral side. Quinapril, on the other hand, is suggested as a noncompetitive, non-transported inhibitor of PEPT1 (Chen et al., 1999).

Because of its high capacity and broad substrate specificities, PEPT1 has been used as a route or mediator to enhance oral bioavailability. Acyclovir, an antiviral compound, is poorly absorbed. Its prodrug, valacyclovir, showed 3 to 5-fold increase in its bioavailability (Weller et al., 1993). hPEPT1-mediated uptake of valacyclovir into intestinal cells was first demonstrated as the underlying mechanism for its elevated absorption (Smiley et al., 1996), which was then confirmed by various following studies (Balimane et al., 1998; Ganapathy et al., 1998). Similarly, valganciclovir, the valine ester of ganciclovir, has been demonstrated to be a substrate of PEPT1 (Sugawara et al., 2000). The prodrug strategy has also been applied to L-α-methyl-dopa. PEPT1 has been revealed to be involved in the translocation of L-dopa-L-Phe and the prodrug will be hydrolysed to the active drug within the intestinal cells (Tamai et al., 1998).

As an endogenous photosensitizer for fluorescence diagnosis and photodynamic tumor therapy, has gained interest and been shown to be well absorbed in the gastrointestinal track. It contains a ketomethylene group instead of a peptide bond. Doring et al (1998) and Irie et al (2001) reported that PEPT1 served as a mediator for δ-ALA good absorption rate. Since photodynamic tumor therapy is used to treat tumors of

extrahepatic biliary duct, δ-ALA has been suggested to accumulate in bile duct tumor cells via PEPT1 before photodynamic therapy (Neumann and Brandsch, 2003). In addition, bestatin, an inhibitor of aminopeptidases, has been confirmed as a substrate of PEPT1 (Daniel and Adibi 1994; Faria et al., 2004).

2.1.7 Transport Mechanism

Intestinal dipeptide uptake was first indicated to be driven by an inwardly directed H^+ gradient by Ganapathy and Leibach (1983) using brush-border membrane vesicles and radiolabelled dipeptide. The advanced techniques like two-electrode voltage-pump in oocytes expressing hPEPT1 or rPEPT1 were able to record electric currents through membrane. It is universally believed that PEPT1 mediates electrogenic uphill transport of its substrates into cells. A transmembrane electrochemical H^+ gradient is the driving force for PEPT1 transport activity with intervillous pH at jejunum being 6.1 to 6.6 (Daniel et al., 1989) and intercellular pH of enterocytes being 7.3. The acidic microclimate on the luminal surface of intestinal epithelium is generated and maintained by the activity of the apical Na^+/H^+ antiporter (Aronson et al., 1982). Coupling of Na^+/H^+ antiporter and PEPT1 in peptide transport has been demonstrated unequivocally (Thwaites et al., 1994; Thwaites and Anderson, 2007). Possible involvement of other H^+ translocators such as V-type H^+ pumps in the acidic microclimate is under investigation (Beyenbach and Wieczorek, 2006). As the driving force for apical Na^+/H^+ antiporter, inwardly directed Na^+ gradient is established by Na^+ - K^+ - ATPase on the basolateral membrane of polarized epithelial cells. It is not clear yet about driving forces for the basolateral efflux of peptides except for the concentration gradient.

As already discussed, PEPT1 has a broad substrate spectrum regardless of the substrate's net charge. But PEPT1 has a preference to transport substrates that carries no net charge and they are translocated with a 1:1 stoichiometry in proton to substrate flux (Steel et al., 1997). Generally, PEPT1 will have higher activity when transporting anionic substrates at more acidic microclimate, and when transporting cationic substrates at more neutral or slightly alkaline extracellular pH. For dipeptides with glutamate or aspartate residues, two protons are required to enter cells with each substrate molecule and the second proton is believed to protonate the side chain carboxyl group prior to transport (Kottra et al., 2002). When transporting cationic peptides containing lysine or arginine groups, PEPT1 will prefer their neutral form to the charged form with a 1:1 stoichiometry in both cases.

Being a proton-coupled transporter, PEPT1 demonstrates a bell-shaped pH dependence with the maximal activity in pH values of 6.5 to 6.0 of the extracellular microclimate (Amesheh et al., 1997). Its transport activity decreases when extracellular pH becomes neutral or decreases from 6.0. However, this pH-dependence is solely observed when substrates are zwitterionic and at low concentration. In addition, PEPT1 is proposed as a bidirectional transporter with its single proton binding site being symmetrically accessible from both sides of the membrane. Membrane potential will determine PEPT1 transporting direction and rate (Nussberger et al., 1997).

2.1.8 Clinical Relevance

PEPT1 has pharmaceutical importance for oral drug delivery. In addition, it is clinically relevant for enteral nutrition support. Small peptide mixtures are intestinally

absorbed faster than comparable free amino acid composition, largely due to high capacity of PEPT1 (Ganapathy et al., 1994; Daniel 2004). Consequently, higher concentrations of amino acids are detected in blood after absorption from peptide mixture than from amino acid mixture. For insoluble or unstable essential amino acids like tyrosine, glutamine and cysteine, those amino acids will be obtained in the gastrointestinal track in the form of dipeptides (Adibi, 1997). In addition, enteral nutrients in the peptide form will reduce the tonicity to avoid diarrheal complications of enteral nutrition, which is mainly caused by the hyperosmolar free amino acids in the solution.

Genetic disorders such as Hartnup disease and cystinuria, amino acid transporters are dysfunctional for the disrupted essential amino acid intestinal absorption (Seow et al., 2004; Palacin et al., 2005). Surprisingly, patients do not show evidences of malnutrition because the amino acids are absorbed adequately in the form of small peptides (Daniel, 2004).

PEPT1 has not been reported for genetic disorders. But numerous evidences demonstrate its role in diseases such as short-bowel syndrome, chronic ulcerative colitis and Crohn's disease since PEPT1 is pathologically upregulated in colon tissues.

2.2 Transporters in Small Intestine and Colon

2.2.1 Introduction

In the gastrointestinal track, nutrients and water from food and drinks will be digested and absorbed. Simultaneously, this organ serves as a host defense/detoxification barrier to reduce harmful exogenous substances. Long-term exposures to those nutritious

and harmful stimuli enable small intestine and colon to equip with influx transporters, metabolic enzymes and efflux transport systems. In addition, those transporters and/or enzymes have flexibility to accommodate to different dietary uptakes. For instance, in a high protein meal study, human volunteers having 320 to 480 g of protein, which was equivalent to 1.5 to 2.8 kg of lean meat, within 8 hours did not yield evidence of a limitation of protein assimilation (Daniel, 2004).

In this chapter, various intestinal and colonic transporter categories will be discussed.

2. 2. 2 Influx Transporters

Amino acid transporters on brush-border membrane

After human volunteers were administered with albumin, a model protein, the jejunal contents recovered were constituted by 120 to 145 mM of three to six amino acid residues and 0.6 to 16 mM of individual free amino acids. The total concentration of all amino acids was between 30 to 60 mM (Adibi and Mercer, 1973). A number of amino acid transporters have been identified by molecular cloning and assigned to different amino acid transport system based on their genetic family, driving force, substrate specificity and cellular localization. Unlike peptide transporters, amino acid transporters on both apical and basolateral sides of intestinal epithelial cells have been well characterized (Halestrap and Meredith, 2004). For amino acid transporters on the brush-border membrane, they primarily mediate free amino acid transport from the lumen into the enterocytes. Amino acid transport systems on the basolateral side, on the other hand, perform a dual function: they transport free amino acids from the enterocytes into the

blood in the absorptive process; in addition, in harsh conditions such as starvation, they translocate amino acid from blood back into enterocytes.

B^0AT1 (SLC6A19) is a major apical neutral amino acid transporter in the intestines. It is a Na^+ - dependent amino acid co-transporter (cotransporting one Na^+ per amino acid) and comprised of 634 amino acids (Broer et al., 2004). In functional studies, B^0AT1 transports all neutral amino acids, although its affinities are different (Preston et al., 1974). Functionally related to B^0AT1, B^0AT2 (SLC6A15) expresses on the apical membrane of epithelial cells to transport neutral amino acid (Broer et al., 2006; Takanaga et al., 2005). It mainly transports branched- chain amino acids plus proline with affinities from 40 to 200 μM. In addition, it shows low affinity to phenylalanine and analine.

$ATB^{0,+}$ (SLC6A14) is a Na^+ and Cl^- dependent transporter to transport neutral and cationic amino acids with a steoichiometry of 2 Na^+ : 1 Cl^- : 1 amino acid (Sloan and Mager, 1999). Its expression in the small intestine has not been confirmed yet, but it is abundant in the colon for amino acids colonic absorption (Nakanishi et al., 2001).

$b^{0,+}AT$ mRNA and rBAT protein have been identified in the microvillus of small intestine in 1999 (Pickel et al., 1999). $b^{0,+}AT$ (SLC7A9) encodes a polytopic transmembrane protein comprised of 487 amino acid with 12 putative transmembrane helices (Chairoungdua et al., 1999; Pfeiffer et al., 1999). rBAT (SLC 3A1) encodes a highly glycosylated membrane protein with 685 amino acids and a large extracellular domain (Bertran et al., 1993; Tate et al., 1992). These two units are linked by a disulfide bond to form a complete transport system $b^{0,+}$ (Palacin and Kanai, 2004). Cationic amino acids and cysteine show high affinity (Km being ~100 μM) to this Na^+ - independent transport system. It can also transport neutral amino acids in low affinity fashion. In

addition, gabapentin, an anti-epileptic agent, is a substrate of $b^{0,+}$ AT-rBAT to show its pharmaceutical relevance (Stewart et al., 1993).

EAAT3 (SLC1A1) is an anionic amino acid transporter, encoded by 524 amino acids (Kanai et al., 1992). It cotransports 3 Na^+ and 1 H^+ together with each glutamate molecule (Zerangue et al., 1996). As a high-affinity transporter, it shows a preference for D-aspartate to L-glutamate.

The proton amino acid transporter PAT1 (SLC36A1) is an imide acid carrier with proline, glycine, alanine, β-alanine, betaine, sarcosine, MeAIB and GABA being its substrates (Anderson et al., 2004; Boll et al., 2003). It cotransports 1 H^+ together with each amino acid. PAT has been classified as a high-capacity, low-affinity transporter, with Km in the mM range. PAT1 shows similar affinity to D- and L- isoforms of its substrates (Boll et al., 2003). Besides free amino acids, PAT1 also accepts short-chain fatty acids (Foltz et al., 2004).

IMINO (SLC6A20) is another imide acid transporter and functions differently to PAT1 among species. In rat intestines, PAT1 is the major transporter for proline absorption, whereas IMINO is dominant in rabbit intestines (Anderson et al., 2004; Miyauchi et al., 2005). Comparing to broad substrate spectrum of PAT1, IMINO has a limited transport activity: it accepts only amino acids with secondary, tertiary, or quanternary amines, such as proline, sarcosine, betaine, MeAIB (Kowalczuk et al., 2005; Takanaga et al., 2005); it cannot carry glycine (Crugeiras et al., 2008); and it is stereoselective for L-amino acids (Zvilichovsky et al., 2004). In addition, IMINO is Na^+ and Cl^-- coupled transporter (Kowalczuk et al., 2005).

TauT (SLC6A6), a Na^+ and Cl^-- dependent transporter, is the major carrier for β-amino acids and taurine (Uchida et al., 1992). It is comprised of 620 amino acids and shows high affinity to its substrates (with Km in μM range) (Shimizu and Satsu, 2000).

Amino acid transporters on basolateral membrane transporters

4F2hc/LAT2 (SLC3A2/SLC7A8) forms a heteromeric amino acid transporter on the basolateral membrane in the intestine, especially in the jejunum and ileum (Pineda et al., 1999). In this transport system, the 4F2hc heavy chain protein is highly glycosylated with 520- 530 amino acids depending on the species (Sagawa et al., 1999). LAT2 is the light chain protein, comprising of 500- 530 amino acid residues. These proteins are connected by a disulfide bond extracellularly (Pfeiffer et al., 1998). Though 4F2hc/LAT2 accepts all neutral amino acids except proline (Sagawa et al., 1999), it is not the major efflux pathway for neutral amino acids in the epithelial cells. Functional analysis showed that LAT2 binds to the outside substrates with Km in μM range, whereas, cytosolic affinity was in mM range (Sagawa et al., 1999; Meier et al., 2002). Further study verified its role in cysteine efflux through exchange of other extracellular neutral amino acids (Bauch et al., 2003).

TAT1 (SLC16A10) is the major aromatic amino acid transporter on the basolateral side of epithelial cells. It is comprised of 534 amino acid residues and a Na^+-independent, facilitative transporter (Kim et al., 2001). Besides aromatic amino acids, TAT1 accepts N-methylated derivatives of these amino acids and L-dopa with low affinity, Km in mM range (Ramadan et al., 2006).

Like $b^{0,+}$ AT-rBAT, 4F2hc/y^+LAT1 (SLC3A2/SLC7A7) forms a heteromeric transporter and is the carrier of cationic amino acid efflux in epithelial cells (Pfeiffer et al., 1999). It is a high affinity, Na^+- independent transporter for cationic amino acids (Km in μM range) (Torrents et al., 1998). When transporting neutral amino acid, 4F2hc/y^+LAT1 demonstrates much higher affinity in the presence of Na^+. In the absence of Na^+, proton gradient is the driving force (Kanai et al., 2000). Interestingly, this transport system exports cationic amino acids in exchange for extracellular neutral amino acids (Bauch et al., 2003). The mediator for the subsequent efflux of neutral amino acids is not identified yet. 4F2hc/y^+LAT2 (SLC3A2/SLC7A6), similarly to 4F2hc/y^+LAT1, is identified in the intestines, but it functions minimally due to its relatively low expression level (Shoji et al., 2002).

Organic anion transporters and organic cation transporters

Anionic drug intestinal absorption is mainly through passive diffusion because intestinal lumen is more acidic than the intracellular pH. Most of organic anion transporters (OAT) have been characterized extensively in the tissues other than intestine (Vanwert et al., 2009). For instance, there are OAT1- 10 and Urat1 in OAT family and only mRNA expression of OAT10 (SLC22A13) was detected in intestine and colon tissues (Sweet et al., 1997; Youngblood and Sweet, 2004; Yokoyama et al., 2008; Bahn et al., 2008). Besides OAT family, organic anion transporting polypeptide (OATP/oatp) family can accept organic anions as well as conjugated and unconjugated bile salts in a Na^+- independent manner (Meier and Stieger, 2002). OAPTB (SLC21A9) is located at the apical membrane of intestinal epithelial cells in human (Kobayashi et al., 2003).

OATs and OAPTs have wide range of substrates from endogenous bile salts to xenobiotics and drugs including dexofenadine, methotrexate, pravastatin and ouabain (Bossuyt et al., 1996; Cvetkovic et al., 1999; Dresser et al., 2002).

Polyspecific organic cation transporters belong to the SLC22 family and the MATE (the multidrug and toxin extrusion) H^+/drug antiporters family. In SLC22 family, OCT1 (SLC22A1) (Chen et al., 2001), OCT2 (SLC22A2) (Gorboulev et al., 1997) and OCT3 (SLC22A3) (Kummer et al., 2006) are passive diffusion organic cation transporters, whereas the cation and carnitine transporter OCTN1 (SLC22A4) is a proton-cation exchanger (Peltekova et al., 2004). OCTN2 (SLC22A5) is a Na+-dependent, high affinity transporter for L-carnitine, acetyl-L-carnitine and cephaloridine (Tamai et al., 1998). When transporting cationic substrates such as TEA, choline, verapamil and pyrilamine, it is Na+-independent (Ohashi et al., 2001; Wagner et al., 2000). OCTN3 (SLC22A9) was only cloned from mouse and is expressed on the basolateral membrane of the enterocytes as a Na+-independent transporter (Tamai et al., 2000; Duran et al., 2005). MATE1 and 2 strongly express in organs such as liver, kidney, skeletal muscle, instead of small intestines (Otsuka et al., 2005; Hiasa et al., 2006).

OCT1, a 556- amino- acid membrane protein, expresses on intestinal serosal membranes (Grundemann et al., 1994; Sekine et al., 1998). OCT2 is comprised of 593 amino acid residues (Okuda et al., 1996). It shares 67% identity with OCT1 and predominantly expresses in the basolateral membrane of renal proximal tububules (Karbach et al., 2000). OCT2 mRNA transcript is not detected in the small intestine (Okuda et al., 1996). The cDNA of OCT3 encodes a 551-amino- acid protein (Kekuda et al., 1998). It is a potential-sensitive organic cation transporter and mediates cation uptake

across the luminal membrane (Muller et al., 2005). OCTN1, a protein of 553 amino acids, shares 75% similarity to OCTN2, which is comprised of 557 amino acids (Wu et al., 2000; Tamai et al., 1998). In human intestines, all OCT3, OCTN1 and OCTN2 are expressing on the brush-border membrane to transport organic cations and carnitines from the intestinal lumen, whereas their efflux across the basolateral side is mediated by OCT1 (Koepsell et al., 2007).

Other influx transporters

Nutritional intestinal glucose absorption is mediated by sodium-dependent Na+/glucose co-transporters (SGLT) 1 (SLC5A1) on the brush-border membrane (Hediger and Rhoads, 1994). Facilitated transporter GLUT5 (SLC2A5) is responsible for intestinal D-fructose absorption (Hediger et al., 1987). Efflux of these hexoses from serosal membrane is via facilitated transporter GLUT2 (SLC2A2) (Gould and Holman, 1993). Long chain fatty acids provide energy especially for heart and muscle tissues in fasting conditions. FATP4 (SLC27A4) is the primary transporting protein in the apical membrane for fatty acid intestinal absorption (Hui et al., 1998). Bile acids are synthesized in the liver, secreted into the bile, and passed to the intestinal lumen. Above 95% of bile acids are re-absorbed passively in the proximal small intestine, actively in the distal ileum, and passively in the colon (Dawson et al., 2009). ASBT/ISBT (SLC10A2) is well characterized as a Na^+- and potential- driven transporter on the apical membrane to translocate bile acids from intestinal lumen into epithelial cells in the ileum (Hagenbuch and Dawson, 2004; Shneider 2001). Organic solute transporter alpha-beta (OSTα-OSTβ) mediates bile acids exit from serosal membrane into the portal circulation

(Dawson et al., 2005; Ballatori et al., 2005). Monocarboxylate transporter MCT1 (SLC16A1) is a H^+- coupled transporter to accept a wide variety of anions, including the vitamin B3, nicotinate, short-chain fatty acis butyrate, and acetate (Halestrap and Meredith, 2004). mRNA expression of MCT1 has been detected from stomach to the distal colon with the greatest intensity in the caecum (Iwanaga et al., 2006). Its cellular localization is still controversial. Apical and/or basolateral membrane of enterocytes in different species have been immunoblotted with MCT1 (Iwanaga et al., 2006; Tamai et al., 1999; Gill et al., 2005).

2. 2. 3 Efflux Transporters

Three major ATP-binding cassette efflux transporters express on the apical membrane of intestinal epithelial cells to determine oral bioavailability, intestinal efflux clearance and drug-drug interaction, especially in cancer treatment, since most of anticancer agents are recognized by those efflux transporters (Borst et al., 2000; Chan et al., 2004). The most extensively studied efflux transporter is P-glycoprotein (P-gp), which is encoded by human multidrug resistance gene (MDR) 1 and named as ABCB1 (Ling and Thompson, 1974; Riordan et al., 1985). It is comprised of 1280 amino acids with two equal homologous parts, two ATP-binding domains and 12 putative transmembrane domains (Chen et al., 1986). P-gp expresses in various tissues such as adrenal, kidney, liver, and brain (Fojo et al., 1987). In human intestine, P-gp is highly expressed in the ileum and colon, and its expression gradually decreases proximally into jejunum, duodenum, and stomach (Ho et al., 2003). Multidrug resistance-associated protein (MRP) 2 (ABCC2) is the second member of human MRP family. In this family,

9 members from named MRP1- 9 have been identified (Takano et al., 2006). MRP2 is a 1545-amino-acid protein, comprising of two ATP- binding domains and 17 transmembrane regions (Buchler et al., 1996). Unlike P-gp, the expression of MRP2 is highly expressed in the proximal regions, and decreases gradually from jejunum to ileum (Rost et al., 2002). In colon tissue, its expression is minimal (Rost et al., 2002). Breast cancer resistance protein (BCRP, ABCG2) contains 655 amino acids (Miyake et al., 1999). Comparing to P-gp or MRP2, it only has a single ATP-binding domain at the amino terminus and 6 transmembrane domains at carboxyl terminus (Doyle and Ross, 2003). In addition, BCRP almost evenly expresses in the whole intestine (Maliepaard et al., 2001). Efflux transporters have diverse and broad substrate spectrum. A summary of their substrates is listed by Takano et al (2006). MRP3 (ABCC3) is expressed in the basolateral membrane of enterocytes and its expression level increases gradually from proximal duodenum to the distal ileum and colon for the efflux of its substrates from enterocytes to the portal blood (Rost et al., 2002).

2. 2. 4 Intestinal Transporters and Fasting

A recent study used the affymetrix GeneChip Mouse Genome 430A array to test intestinal transporters and phase I/II metabolism gene changes after mice were fasted for 24 hours (van den Bosch et al., 2007). Among them, 243 SLC transporters and 40 ABC transporters were analyzed. No SLC or ABC transporters described in this part have been detected mRNA changes after 24-hr fasting. mRNA of Oatp2a1 (SLCo2A1) on the apical side had increased 1.4 folds and MCT4 (SLC16A3) on the basolateral membrane

had decreased 1.6 folds in 24-hr fasted mice. But both transporters do not function predominantly in their family.

2. 2. 5 Mice with Targeted Disruption of Intestinal Transporters

Knockout mice for OCT1 and OCT2 were generated in 2001 (Jonker et al., 2001; Zwart et al., 2001) and double knockout mouse for OCT1 and OCT2 was cloned in 2003 (Jonker et al., 2003). All three strains were fertile and had no phenotypic abnormality. Because GLUT2 expresses in the plasma membrane of pancreatic β-cells, hepatocytes and kidney besides small intestine, GLUT2 knockout mice usually died between two and three weeks of age (Guillam et al., 1997). OSTα$^{-/-}$ mice were viable and fertile, but they showed small intestinal hypertrophy and growth retardation (Ballatori et al., 2008). OAT1 knockout mice were generated in 2006 (Eraly et al., 2006). They are physiologically similar to wild-type mice. OAT3 knockout mice, on the other hand, showed a 10 to 15% lower blood pressure than wild-type mice (Vallon et al., 2008). MDR 1a$^{-/-}$ (Schinkel et al., 1994), MDR 1b$^{-/-}$ (Schinkel et al., 1997), MDR 1a/1b$^{-/-}$ (Schinkel et al., 1997) and BCRP1$^{-/-}$ (Jonker et al., 2002) have been generated, but none of them had physiological abnormalities. MRP3$^{-/-}$ mice were healthy and showed indistinguishable from wild-type mice (Belinsky et al., 2005).

2.3 Small Peptide Renal Reabsorption and Transporters

Renal elimination of drugs involves glomerular filtration, tubular secretion, and tubular reabsorption. For renal tubular drug secretion, organic anion transport system,

organic cation transport system and ATP-dependent primary active transporters are the major transport systems. OAT1 (SLC22A6), OAT2 (SLC22A7), and OAT3 (SLC22A8) are locating on the basolateral membranes of renal proximal tubules (Bahn et al., 2005; Sweet, 2005; Rizwan and Burckhardt, 2007). In addition, OAT3 is also found in the thick ascending limb of Henle's loop, distal convoluted tubule, and collecting duct in rats (Rizwan and Burckhardt, 2007). OAT5 (SLC22A19), OAT10 (SLC2213), and human URAT1 (SLC22A12) are expressing on the brush-border membranes in the urinary lumen of renal proximal tubule (Sweet, 2005). Substrates of OATs are including methotresate, cidofovir, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Cundy et al., 1996).

For organic cation transporters, OCT1 (SLC22A1), OCT2 (SLC22A2) and OCT3 (SLC22A3) are localized on the basolateral membrane of epithelial cells in renal proximal tubules (Koepsell et al., 2007). The carnitine and cation transporter OCTN2 (SLC22A5) is expressing on the apical membrane of proximal tubular epithelial cells.

Small peptides are mainly reabsorbed from the urinary lumen via peptide transporters, PEPT1 and PEPT2. In rats, PEPT1 is localized to the brush-border membranes in S1 segments of proximal tubules. In contrast, PEPT2 is primarily expressed in brush-border membranes of the S3 segments of proximal tubules. When small peptides are reabsorbed, they will be transported by the high-capacity, low-affinity PEPT1 in early regions of the proximal tubules (pars convolute), and then by the low-capacity, high-affinity PEPT2 in the later regions of proximal tubules (pars recta) (Inui et al., 2000). In PEPT2 null mice study, Ocheltree et al (2005) demonstrated that PEPT2 accounted for 86% and PEPT1 accounted for 14% of reabsorbed GlySar. For drugs such

as cefadroxil, PEPT2 was responsible for 95% and PEPT1 contributed to 5% of reabsorbed cefadroxil (Shen et al., 2007). Recently, when GlySar was tested in PEPT1 null mice, the clearance of GlySar only decreased about 7% after PEPT1 was abolished, suggesting that PEPT2 is the major transporter for GlySar reabsorption in the kidney (Hu et al., 2008).

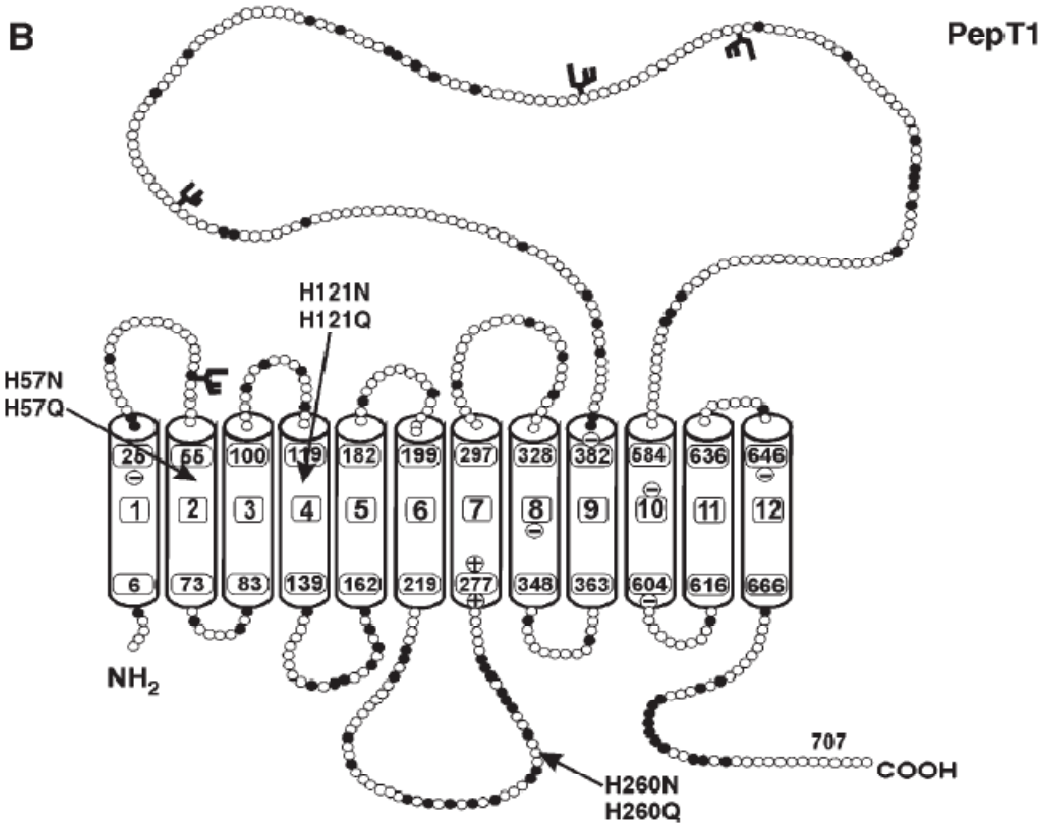

Figure 2.1 Structural model of PEPT1 with mutations indicated. Histidine residues are shown to be subjected to mutagenesis. Amino acid residues are defined using the single-letter code and residue number, and branched tree represents *N*-glycosylation sites. Figure obtained from Steel A and Hediger MA (1998) *News Physiol Sci* 13: 123-131.

Table 2.1 Amino Acid Residues Involved in PEPT1 Transport Activity and the Expected Effect of Mutation.

Amino Acid	TM Location	Expected Function in Dipeptide Uptake	Major Expected Result of Mutation	Actual Result of Mutation
W294	7	Participates in initial binding	Affects Km	W294A-hPEPT1 Km↑ Vmax↓
Y12	1	Regulates Passage	Affects Vmax	Y12A-hPEPT1 Vmax↓ by 25%
E595	10	Regulates passage	Affects Vmax	E595A-hPEPT1 no transport
Y91	3	Interacts with proton	Affects pH dependence	Y91F-rPEPT1 transport↓by 80%
Y167	5	Regulates passage	Affects Vmax	Y167A-hPEPT1 no transport
H57	2	Regulates passage	Affects Vmax	H57Q-hPEPT1 no transport
F293	7	Regulates passage	Affects Vmax	F293C-hPEPT1 no transport
L296	7	Regulates passage	Affects Vmax	L296C-hPEPT1 no transport
F297	7	Regulates passage	Affects Vmax	F297C-hPEPT1 no transport
K278	7	Regulates passage	Affects Vmax	K278C-hPEPT1 transport↓by 40%
Y12	1	Regulates passage	Affects Vmax	Y12F-rPEPT1 transport↓by 70%
Y91	3	Participates in initial binding	Affects Km	Y91F-rPEPT1 Km↑ Vmax↓
Y167	5	Regulates passage	Affects Vmax	Y167F-rPEPT1 transport↓by 90%
Y345	8	Regulates passage	Affects Vmax	Y345F-rPEPT1 transport↓by 15%
Y56	2	Participates in initial binding	Affects Km	Y56F-rPEPT1 Km↑ Vmax↓

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CHAPTER III

PEPTIDE TRANSPORTER 1 IS RESPONSIBLE FOR THE *IN VITRO* INTESTINAL UPTAKE OF GLYCYLSARCOSINE IN WILD-TYPE VERSUS PEPT1 NULL MICE

Abstract

The H^+-coupled peptide cotransporter 1, PEPT1, is primarily expressed at brush-border membranes of intestinal absorptive epithelial cells, suggesting that it may play a role in the transport of small peptides (di- and tri-peptides). To determine the role and relevance of PEPT1 in the uptake of peptides/peptidomimetics in mouse small intestinal tissue, we used glycylsarcosine (GlySar) as a model compound to compare its uptake in everted jejunual rings from wild-type and PEPT1 null mice. Our findings showed that 4 μM GlySar uptake was linear through 30 sec in wild-type mice and reached a plateau value of 1.2 pmol/mg at 7.5 min. Compared to wild-type mice, PEPT1 null mice exhibited a 78% reduction in GlySar uptake ($p < 0.001$) under physiologic conditions (pH 6.0, 37 ^oC). The 4 μM GlySar uptakes showed pH dependence with peak values between pH 6.0 to 6.5 in wild type-mice, while no such tendency was observed in PEPT1 null mice. GlySar exhibited Michaelis-Menten uptake kinetics with a Vmax of 233 pmol/mg/20 s, a Km of 10 mM and a minor nonsaturable component (i.e., Kd) in wild-type mice. In contrast, PEPT1 null mice demonstrated that GlySar jejunual uptake was governed by a nonsaturable component alone (i.e., Kd). A wide variety of substrates like

dipeptides (i.e., carnosine, GlyGly and GlySar), amniocephalosporins (i.e., cephradine and cefadroxil), angiotensin-converting enzyme inhibitors (i.e., lisinopril, captopril and enalapril) and the peptidomimetic prodrug valacyclovir significantly inhibited GlySar uptake in wild-type mice ($p<0.01$), while no such inhibition was observed in the uptake of GlySar in PEPT1 null mice. These findings demonstrate that PEPT1 plays a critical role in the uptake of GlySar in the small intestine. This study indicates that PEPT1 might be the major transporter responsible for the small peptides absorption in the small intestines. Moreover, the ability of PEPT1 to transport small peptides/peptidomimetics in the small intestine may also be useful as a targeting protein for drug delivery.

Introduction

As mammalian small intestine has acid microclimate, usually pH 5-6.8, at its luminal surface (Daniel et al., 1985; Shimada and Hoshi 1988; McKie et al., 1988; McConnell et al., 2008), transporters carrying various solutes via uphill transport across intestinal brush-border membranes have been found to utilize proton/sodium gradients as driving forces. Transporters include the excitatory amino acid transporter EAAC1 (SLC1A1 in the SoLute Carrier (SLC) gene nomenclature system by the Human Genome Organization) for L-glutamate, L- and D-aspartate, and L-cysteine absorption (Kanai and Hediger, 2004); sodium-coupled glucose cotransporter SGLT1 (SLC5A1) to transport D-glucose and D-galactose (Wright and Turk, 2004); monocarboxylate transporter MCT1 (SLC16A1) to translocate short-chain fatty acids (Tamai et al., 1995) and di/tripeptide transporter PEPT1 (SLC15A1) to transport small oligopeptides (Fei et al., 1994).

PEPT1 belongs to mammalian proton-coupled oligopeptide transporter (POT) superfamily. Besides PEPT1, this superfamily consists of PEPT2 (SLC15A2), PHT1 (SLC15A3) and PHT2 (SLC15A4). PEPT1 was the first proton-coupled organic solute transporter identified in vertebrate (Fei et al., 1994). A complementary DNA screening from rabbit intestinal library showed that this gene encoded a predicted 707-amino-acid transmembrane protein on the brush-border membrane. A subsequent topology study indicated that PEPT1 has 12-transmembrane domains (TMD) with its N- and C-termini facing the cytosol (Covitz et al., 1998). Site-directed mutations in PEPT1 suggested that His-57 in the second putative TMD (Fei et al., 1997), Arg-282 in the seventh TMD (Meredith 2004; Meredith 2008), Tyr-167 in the fifth TMD, Trp 294 in the seventh TMD

and Glu-595 in the tenth TMD (Bolger et al., 1998) were critical for its binding with protons and substrates. PEPT1 will utilize one proton when cotransporting one neutral or cationic dipeptide molecule (Kottra et al., 2002; Steel et al., 1997). If anionic dipeptides are in their neutral forms, PEPT1 will require one proton for their transport to enterocytes. Two proton molecules are necessary for PEPT1 when translocating charged anionic dipeptides (Machenzie et al., 1996). PEPT1 has a wide spectrum of substrates variable in molecular size, net charge and solubility from di- and tripeptides (Liang et al., 1995), peptide-like drugs such as beta-lactam antibiotics (Wenzel et al., 1995) and selected angiotensin converting enzyme (ACE) (Knutter et al., 2008). It has been classified as a low affinity (i.e., mM Km values), high capacity transporter (Mackenzie et al., 1996). In addition, PEPT1 has been successfully used as a tool to improve poor bioavailability of antiviral drugs (Ganapathy et al., 1998; Han et al., 1998; Sugawara et al., 2000); low-permeability in rebamipide (Kikuchi et al., 2008), and polarity of anticancer drug gemcitabine (Song et al., 2005) via their prodrug forms. Though mainly expressed in the small intestine (Freeman et al., 1995), PEPT1 has been shown to be responsible for uptake of delta-amniolevulinic acid (δ-ALA) in cancer cells of human extrahepatic biliary duct. ALA is a precursor of cellular porphyrin synthesis and used in photodynamic tumor therapy. Rather than a peptide bond, δ-ALA contains a ketomethylene group (Peng et al., 1997).

PEPT2 was cloned from human kidney cDNA library through probing from rabbit PEPT1 (Liu et al., 1995) and has been characterized as a high affinity, low capacity transporter (Boll et al., 1996). Human PEPT1 and PEPT2 proteins share 50% identity and 70% similarity in their primary structures and have comparably wide spectrum of

substrates (Biegel et al., 2006). PEPT2 primarily functions in proximal tubule of kidney (Shen et al., 1999) and brain, especially apical membrane of choroid plexus epithelial cells (Shen et al., 2003), for renal reabsorption of small peptides (Shen et al., 2007) and removal of di- and tripeptides from cerebrospinal fluid (Ocheltree et al., 2005), respectively. In contrast to the extensively studied PEPT1 and PEPT2, PHT1 and PHT2 are far less explored. Their substrate specificity has not been fully described, yet PHT1 and PHT2 do accept free histidine as a substrate (Sakata et al., 2001).

The recent generation of PEPT1 knockout mice by our laboratory (Hu et al., 2008) has provided a research tool to thoroughly examine the functional role of PEPT1 in the transport of peptide/mimetics in its expressed organ systems under physiological conditions. The functional properties of PEPT1-mediated transport were evaluated through comparison of jejunual uptake of glycylsarcosine (GlySar) in wild-type and PEPT1 null mice. Our findings are novel in demonstrating that a lack of PEPT1 abolished pH-dependency in the uptake of dipeptides; vitiation of PEPT1 resulted in a nonsaturable transport process; and absence of PEPT1 was affected little if any by a variety of inhibitors when transporting dipeptides.

Materials and Methods

Materials. [^{14}C]GlySar (106 mCi/mmol) was purchased from Amersham Biosciences (Chicago, IL) and [^{3}H] mannitol (20 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). PEPT1 null mice were generated on a C57BL/6 mouse background as described by Hu et al. (2008). Glycylsarcosine, glycylglycine, histidine, sarcosine, glycine, carnosine, cephapirin, cephalothin, cephradine, cefadroxil, lisinopril, captopril, enalapril, tetraethylammonium (TEA), 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid (SITS) were obtained from Sigma-Aldrich (St. Louis, MO). Acyclovir and valacyclovir were kind gifts from GlaxoSmithKline Company (Durham, NC). Hyamine hydroxide was purchased from ICN Pharmaceuticals (Costa Mesa, CA). All other chemicals were obtained from standard sources and were of the highest quality available.

Preparation of Everted Small Intestinal Ring. Gender and weight-matched transgenic mice of each genotype (n≥3, PEPT1$^{+/+}$; n≥3, PEPT1$^{-/-}$) were anesthetized with sodium pentobarbital (65 ug/g ip). Two 2-cm segments from the proximal end of jejunum were isolated from each mouse for uptake study. After isolation, jejunual segments were transferred to ice-cold incubation medium. Composition of the incubation medium was as follows (in mM): 129 NaCl, 5.1 KCl, 1.4 $CaCl_2$, 1.3 NaH_2PO_4, and 1.3 Na_2HPO_4 (pH 6.0) (Pan et al., 2002). For pH-dependent analysis, different combination of 10 mM Tris and/or MES were obtained to achieve pH values among 5.5 to 8.0, with osmolarity and concentration of Na^+ being held constant. After a rapid wash, the jejunual segments were everted and fixed over glass rods 3 mm in diameter by surgical threads. Everted jejunual segments were equilibrated in incubation medium gassed with 5% CO_2-

95% O_2 at 37°C (water bath) for 5 min. After the recovery period, each jejunual segment was placed in 1 ml of pre-warmed incubation medium containing 4 μM ^{14}C-GlySar and 2 μM ^{3}H-mannitol (an extracellular marker), or different concentrations of ^{14}C-GlySar shown in the concentration- dependent study at 37°C. The uptake was terminated by transferring each segment to ice-cold wash buffer (the composition was the same as the incubation medium). The jejunual segments were washed for 20 seconds, blotted on filter paper, weighed and soaked overnight in 0.33 ml of 1 M hyamine hydroxide (as a tissue solubilizer). Radioactivity was determined in 6 ml of CytoScint (+) scintillation cocktail by a dual-channel liquid scintillation counter.

The uptake of radiolabeled GlySar into small intestine, in microliters per milligram of tissue weight, was calculated according to the following equation:

Substrate Uptake =

$$\left[S(GlySar) - S(Mannitol) * (GlySar / Mannitol)\right] / S(Media) / TissueWeight / Time$$

Where *S(GlySar)* is the total substrate (GlySar) concentration in the everted jejunal ring and *S(Media)* is the concentration of substrate in the external media. The term *S(Mannitol)*(GlySar/Mannitol)* is to correct extracellular space, where *S(Mannitol)* is the total mannitol concentration in the everted jejunal ring. Multiplying *S(Mannitol)* by the ratio of ^{14}C-GlySar to ^{3}H-mannitol in the external medium provides an estimate of the extracellular content of GlySar.

Data analysis. For kinetic studies, the concentration-dependent uptake of GlySar by wild-type and PEPT1 null mice was simultaneously fit to Michaelis-Menten relationship with a same nonsaturable component:

$$V = \frac{V\max \cdot C}{Km + C} + Kd \cdot C$$

Where Vmax was the maximal rate of saturable uptake, Km was the Michaelis constant, Kd was the rate constant for nonsaturable processes and C was the substrate concentration.

GlySar uptake in two jejunual segments was combined for an average number to indicate uptake in one mouse. All data were expressed as the mean ± S.E. of at least 3 independent experiments ($n \geq 3$). Statistical comparisons between null and wild-type groups were performed using an analysis of variance (ANOVA), and pairwise comparisons with the control values were processed using Dunnett's test. A probability of $p \leq 0.05$ was considered statistically significant. In the analyses for linear and nonlinear regression, the quality of fit was determined by evaluating the coefficient of determination (r^2) and the coefficient of variation of the parameter estimates, and by visual inspection of the residuals. All statistical analyses were performed using Prism version 5 (GraphPad Software, Inc., San Diego, CA).

Results

Time-Dependent Uptake of GlySar. The uptake of GlySar (4 μM) was first studied in whole everted jejunual tissue isolated from wild-type mice. As shown in Figure 3.1, GlySar showed a linear uptake for approximately 30 sec ($r^2 = 0.999$). The y-intercept value was not significantly different from zero ($p \geq 0.05$), suggesting that nonspecific binding was negligible. Based on these results and to maximize radiotracer uptake in the linear region, an uptake time of 20 sec would be used to perform the subsequent experiments in wild type and PEPT1 null mice. GlySar reached a plateau value of approximately 1.2 pmol/mg at 7.5 min to 15 min into the experiment.

Temperature-Dependent, Sodium-Independent Uptake of GlySar. Compared to $PEPT1^{+/+}$ mice, the $PEPT1^{-/-}$ mice demonstrated a 78% reduction ($p < 0.0001$) in 4 μM GlySar uptake by everted jejunual rings at 37°C. In addition, GlySar uptake in both genotypes, at 4°C, was less than 10% of the value observed in wild type mice at physiological temperature. In contrast, no statistical difference was observed between genotypes at the lower temperature. These results suggest that PEPT1 is responsible for the majority (about 80%) of GlySar uptake into the jejunum of wild type mice, with only a small percentage being governed by nonspecific processes (about 10- 20%). GlySar uptakes in sodium-containing and low sodium incubation buffers were comparable in both $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice, indicating that PEPT1 is a Na^+-independent transporter in our *in vitro* system (Figure 3.3).

pH-Dependent Uptake of GlySar. Since PEPT1 is a proton-coupled transporter, GlySar uptake was explored as a function of pH (pH from 5.5 to 8.0) in the buffer system in both genotypes. As shown in Figure 3.4, the GlySar uptake profile in $PEPT1^{+/+}$ mice

resembled a bell-shape with peak numbers at pH 6.0 to pH 7.0 and a minimal value at pH 8.0. In contrast, GlySar uptake did not alter as a function of pH in PEPT1$^{-/-}$ mice.

Concentration-Dependent Uptake of GlySar. A series of concentrations from 0.1- 40 mM of GlySar was investigated in both genotypes under physiological conditions. As shown in Figure 3.5, PEPT1$^{+/+}$ mice demonstrated saturable transport with a maximum velocity (Vmax) of 233 pmol mg^{-1} 20 sec^{-1}, a Michaelis constant (Km) of 9.96 mM, and a nonsaturable rate constant (Kd) of 0.0081 µl mg^{-1} 20 sec^{-1}. In contrast, PEPT1$^{-/-}$ mice exhibited a linear uptake with Kd of 0.0081 µl mg^{-1} 20 sec^{-1}. The carrier-mediated component accounted for 74.3% of transport, while the nonspecific component accounted for 25.7% of the total GlySar uptake in PEPT1$^{+/+}$ mice. The complete kinetic data are summarized in Table 3.1.

Inhibitor Analysis of GlySar Uptake. Potential inhibitors were classified into six groups: A) amino acids, B) di- and tripeptides, C) cephalosporins, D) ACE inhibitors, E) organic anions and cations, and F) antiviral prodrug. As shown in Figure 3.6, L-histidine, sarcosine and glycine had no inhibition of GlySar uptakes in either wild type or PEPT1 null mice relative to control values. The dipeptides, carnosine, GlyGly and GlySar, showed significant reduction on GlySar uptake in PEPT1$^{+/+}$ mice (GlySar uptake dropped to 62.4%, 47.4%, and 39.2%, respectively), while inhibition was absent in PEPT1$^{-/-}$ mice. Based on the presence of α-amino carbons, cephalosporins were divided into two groups, cephradine and cefadroxil containing α-amino carbons whereas cephapirin and cephalothin do not. Both cephardine and cefadroxil remarkably reduced GlySar uptake in PEPT1$^{+/+}$ mice (GlySar uptakes were reduced to 60.8%, and 53.6%, respectively), where GlySar uptake remained unchanged in the presence of cephalothin

and cephapirin in PEPT1$^{+/+}$ mice compared with the control. All cephalosporins showed no inhibitory effects on GlySar uptake in PEPT1$^{-/-}$ mice. ACE inhibitors like lisinopril, captopril, and enalapril significantly inhibited the uptake of GlySar in PEPT1$^{+/+}$ mice (GlySar uptakes were inhibited to 66.0%, 34.0%, and 30.9%, respectively). No effect of ACE inhibitors was observed in PEPT1$^{-/-}$ mice. SITS and TEA showed no effect in the GlySar uptake in both PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice. Finally, valacyclovir, but not acyclovir, inhibited GlySar uptake in PEPT1$^{+/+}$ mice to 60.8% of control values.

Discussion

Protein assimilation in the gut stems from its degradation by a spectra of proteases and peptidases, which are in their free forms in intestinal lumen and in the bound forms on brush border membrane of enterocytes (Daniel 2004). The subsequent oligopeptides and individual amino acids will be absorbed through passive diffusion (Mizuma et al., 1997) and transporter mediated processes (Buyse et al., 2001). Various transporters such as di-, tripeptide transporter PEPT1 and amino acid transporter B(0)AT1 have been shown to express along human digestive tract in different expression patterns (Terada et al., 2005). Though PEPT1 could be induced for more peptide/mimetics absorption (Hindlet et al., 2007), there is no solid evidence to show how dipeptides are absorbed in the small intestines if PEPT1 is absent. Gene knockout is an elegant approach to exclusively characterize physiological and pharmacologic significance of a transporter in its gene families. By using our PEPT1 knockout mice, we could get better insight into the role and relevance of PEPT1 in the absorption of small peptide/mimetics in the small intestines.

As a neutral dipeptide, GlySar has been widely confirmed to be a substrate of PEPT1 and been used as a model compound to indicate dipeptide absorption via PEPT1 (Boll et al., 1994; Hindlet et al., 2007). In our preliminary study to describe PEPT1 null animals (Hu et al., 2008), we found that the *in vitro* jejunual uptake GlySar uptake (4 μM) was vitiated by about 80% after PEPT1 has been knocked out. To more fully describe how jejunum tackled small peptides in the absence of PEPT1, we performed this study and observed different behaviors of jejunum in transport of GlySar in PEPT1 null mice. In our concentration-dependent study, we demonstrated that PEPT1 contributed

about 75% to GlySar intestinal transport, while passive diffusion only counted for about 25%. The role of passive diffusion in β-lactam antibiotics and di-/tripeptides absorption in the small intestines has long been debatable (Yamashita et al., 1986; Sugawara et al., 1990). Cephalexin has been widely used to study PEPT1 since it is believed to be absorbed mainly via PEPT1 (Covitz et al., 1996; Okamura et al., 2003). A recent paper (Hironaka et al., 2008) compared cephalexin absorption in different intestinal segments by *in situ* closed loop and *in vivo* study in the absence or presence of PEPT1 competitor. They claimed that although total absorption of cephalexin was 50% due to PEPT1, the passive diffusion could compensate for PEPT1-mediated absorption of cephalexin after PEPT1 ablation or saturation. Similar results were reported in the oral absorption of ampicillin in everted gut sac model in rats (Lafforgue et al., 2008). These studies highlighted that passive diffusion might be underappreciated, at least for β-lactam antibiotic absorption like cephalexin and ampicillin in the small intestine.

Paracellular permeability is largely influenced by the dynamic structures of tight junctions. Tight junctions are composed of multiple transmembrane, scaffolding, and signaling proteins. A variety of stimuli has been demonstrated to alter tight junction properties. Cytokines like IFN-γ (Youakim et al., 1999), TNF-α (Ozaki et al., 1999), IL-1 (Marcus et al., 1996), IL-4 (Ahdieh et al., 2001), IL-6 (Yang et al., 2001) and IL-13 (Prasad et al., 2005) would induce actin remodeling to increase permeability in epithelial barriers. Growth factors (Grant-Tschudy et al., 2005; Singh et al., 2007) would either enhance or reduce paracellular permeability based on different cellular environments. Other factors include drugs (Oshima et al., 2008, Amesheh et al., 2008) and hormones (Savidge et al., 2007).

Similarly, PEPT1 is a regulatory protein responding to environmental changes in the GI track (Thamotharan et al., 1999), drug treatments (Hirai et al., 2007), and disease conditions (Bikhazi et al., 2004). Although the absorption route of cephalexin is debatable using *in vitro* research and *in situ* perfusion, this *β*-lactam antibiotic has been used extensively as a model compound when testing PEPT1 regulation. Its permeability was correlated to PEPT1 expression levels in Caco-2 cells after they were infected with different titers of adenoviral hPEPT1 (Chu et al., 2001). When PEPT1 was stimulated by leptin in Caco-2 cells, cephalexin diffusion was enhanced correspondingly (Hindlet et al., 2007). The pdzk1 gene encodes postsynaptic density95/disk-large/ZO-1(PDZ) domain-containing protein PDZK1 and is an upstream gene to govern PEPT1 expression. PDZK1 knockout mice showed decreased expression levels of PEPT1 along with delayed absorption of cephalexin in the GI track after oral administration (Sugiura et al., 2008). These studies indicate that cephalexin absorption is via PEPT1, even though a change of passive diffusion may not be ruled out.

In Caco-2 cells, the affinity of PEPT1 for cephalexin was 10- to 15-fold less than GlySar (Brandsch et al., 1997). Cephalexin only modestly inhibited PEPT1-mediated GlySar uptake in LLC-PK1 cells stably transfected with PEPT1 cDNA (Terada et al., 1997). Sodium ampicillin was calculated to have 95% zwitterionic form at pH 6.0 and this percentage was reduced as solution pH increased due to its pK values of 2.5 and 7.3 (Lafforgue et al., 2008). Since pH of gastric fluid rises along GI tract, PEPT1 is predicted to have less important role in transporting ampicillin, while paracellular route might dominate over PEPT1 for the absorption of ampicillin in the distal small intestine.

In our concentration-dependent analysis, we revealed an uptake curve of GlySar (from 4 μM to 40 mM) in wild-type mice, which could be clearly divided into saturable (i.e., PEPT1) and nonsaturable (i.e., passive diffusion) components. After PEPT1 ablation, the GlySar absorption profile resembled a linear curve, presumably only the paracellular route remaining. From what we observed, PEPT1 plays a predominant role in small peptide absorption from jejunum, which could suggest a minor passive diffusion of small peptide absorption.

In our system, the everted jejunual rings were intact with mucosa being exposed to the drug solution, and submucosa and serosa adhering to the glass rod. The everted jejunual rings maintained good viability and functionality over time according to their GlySar uptake analysis at different time points, and supported by other research (Molina et al., 2007). The maximal pH effects on GlySar uptake in the small intestine of wild-type mice were found at pH 6.0 to pH 6.5, similar to the pH range of GlySar uptake in Caco-2 monolayers and dropping dramatically at pH 7.0 (Thwaites et al., 1993). Due to our using the entire tissue and unstirred water layers in the *in vitro* jejunal ring model, GlySar uptake did not change as much to pH as observed in Caco-2 monolayers. A less dramatic change was also observed in amino acid absorption in rat everted intestinal rings (Inigo et al., 2006). After PEPT1 was abolished, GlySar uptake was insensitive to pH changes in buffer, indicating no pH responsive uptake of GlySar in PEPT1 null mice.

After cDNA cloning for PHT1 and PHT2 (Yamashita et al., 1997; Sakata et al., 2001), little has been discovered about their functionality in the small intestines. We used L-histidine to probe the possible involvement of PHT1/2 in the jejunal uptake of GlySar. We found no inhibition in both wild-type and PEPT1 null mice, indicating that

PHTs were not involved in the jejunual transport of GlySar. Cephalosporins with α-amino carbons (cephradine and cefadroxil) demonstrated inhibitory effects on GlySar uptake in wild-type mice, while little, if any, change was observed in PEPT1 null mice. The increasing permeability of valacyclovir, but not acyclovir, in small intestine was attributed to the involvement of several carrier-mediated pathways, one of which was believed to be PEPT1 (Ganapathy et al., 1998; Han et al., 1998; Thomsen et al., 2004). In our system, valacyclovir inhibited GlySar transport in wild-type mice, confirming the previous reports. However, valacyclovir also showed inhibitory effect on GlySar uptake in PEPT1 null mice. We are unsure of the mechanism for this interaction, but it may be an artifact of measuring few uptakes.

In conclusion, our novel findings have confirmed that PEPT1 is the primary transporter responsible for small peptide uptake in the jejunum. Without PEPT1, transport of di-, tripeptides was provided by nonspecific, pH-independent processes. Future studies will be directed at the absorption of cefadroxil in wild-type and PEPT1 knockout mice to clarify the relative roles of PEPT1 and passive diffusion in peptide-like drug absorption.

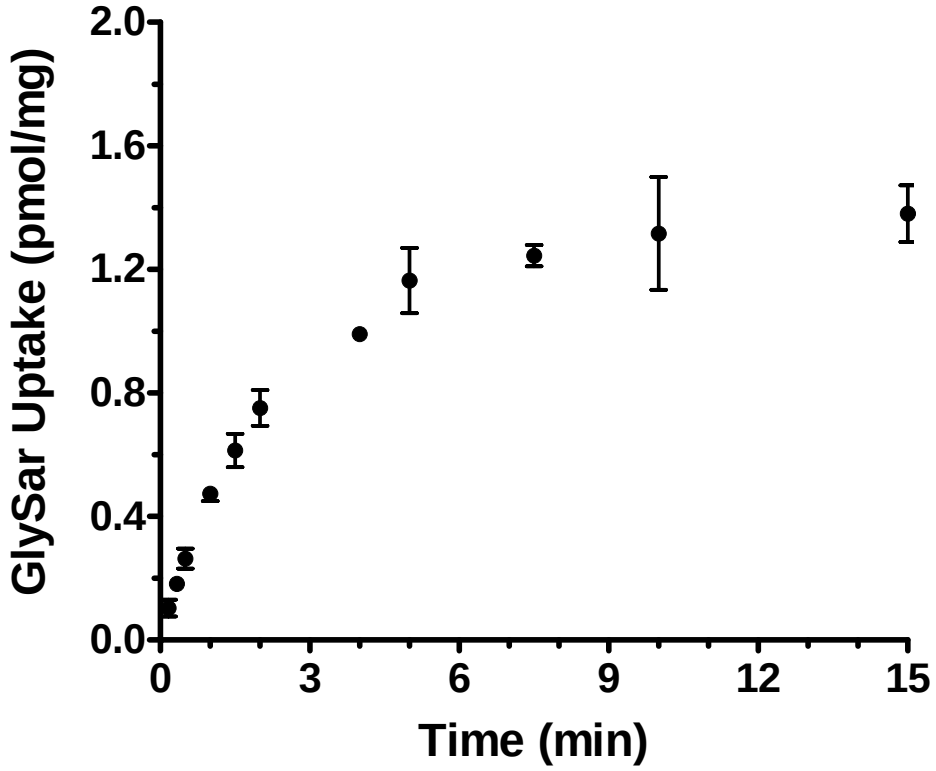

Figure 3.1 Time-dependent uptake of ^{14}C-GlySar in the jejunum of wild-type mice (4 μM GlySar in external medium). Studies were performed at 37^{o}C in small intestine incubation buffer (pH 6.0). Data are expressed as mean ± S.E. (n=3-4).

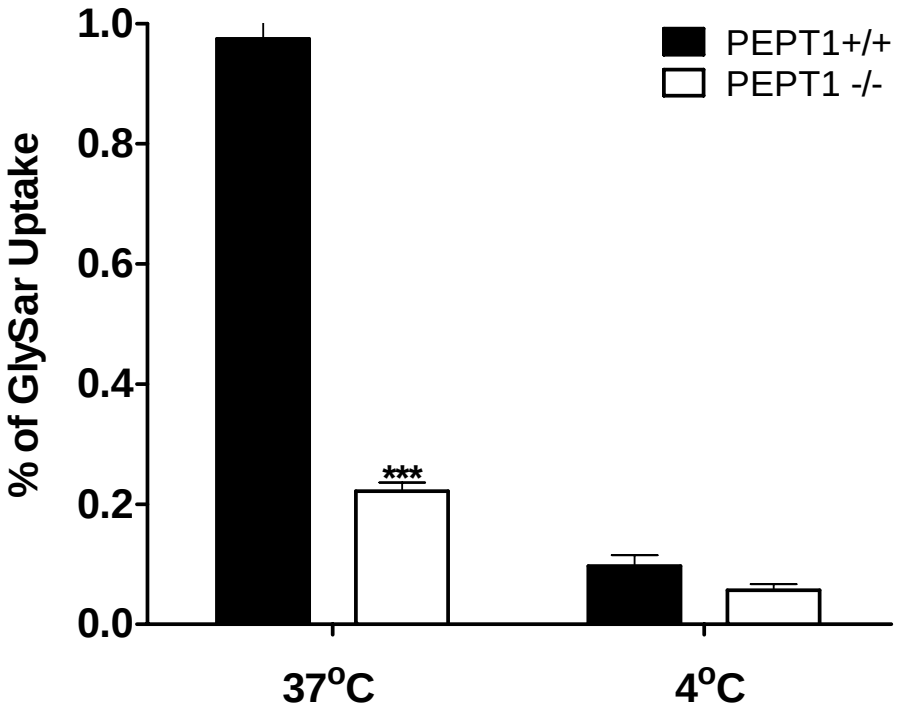

Figure 3.2 Temperature-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). Studies were performed at 37°C and 4°C in small intestine incubation buffer (pH 6.0). Data are expressed as mean ± S.E. (n=3-4). ***p< 0.001 for null versus wild-type animals at 37°C.

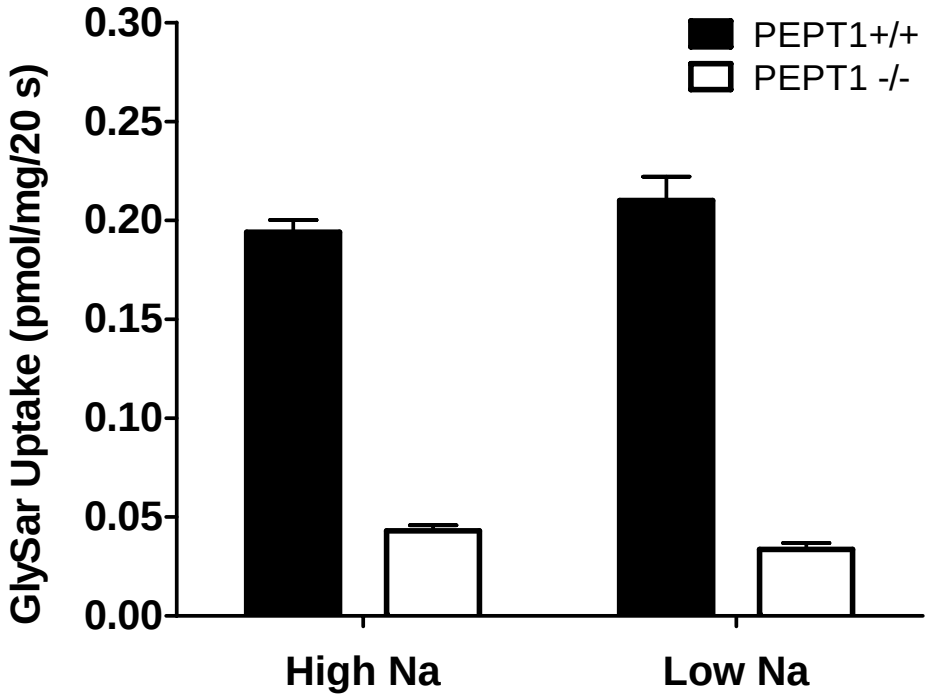

Figure 3.3 Sodium-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). Studies were performed at 37^{o}C under normal sodium and low sodium conditions (pH 6.0). Data are expressed as mean ± S.E. (n=3-4).

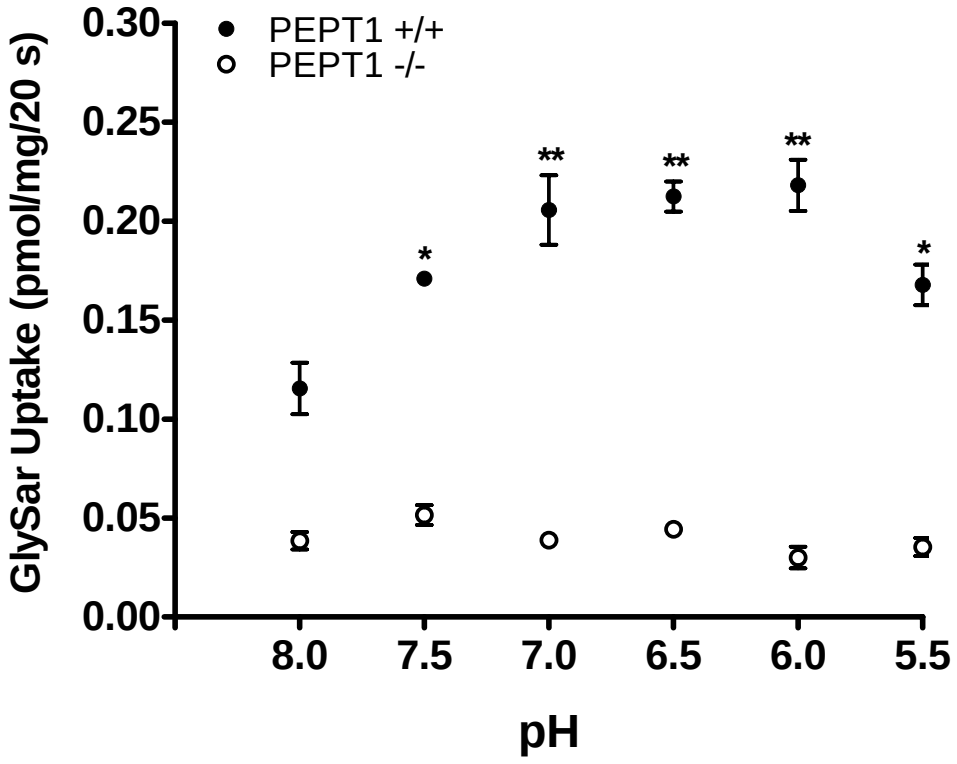

Figure 3.4 pH-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). Studies were performed at 37^{o}C in small intestine Tris-MES incubation buffer. Data are expressed as mean ± S.E. (n=3). * $p < 0.05$, ** $p < 0.01$ as compared to pH 8.0 for each genotype.

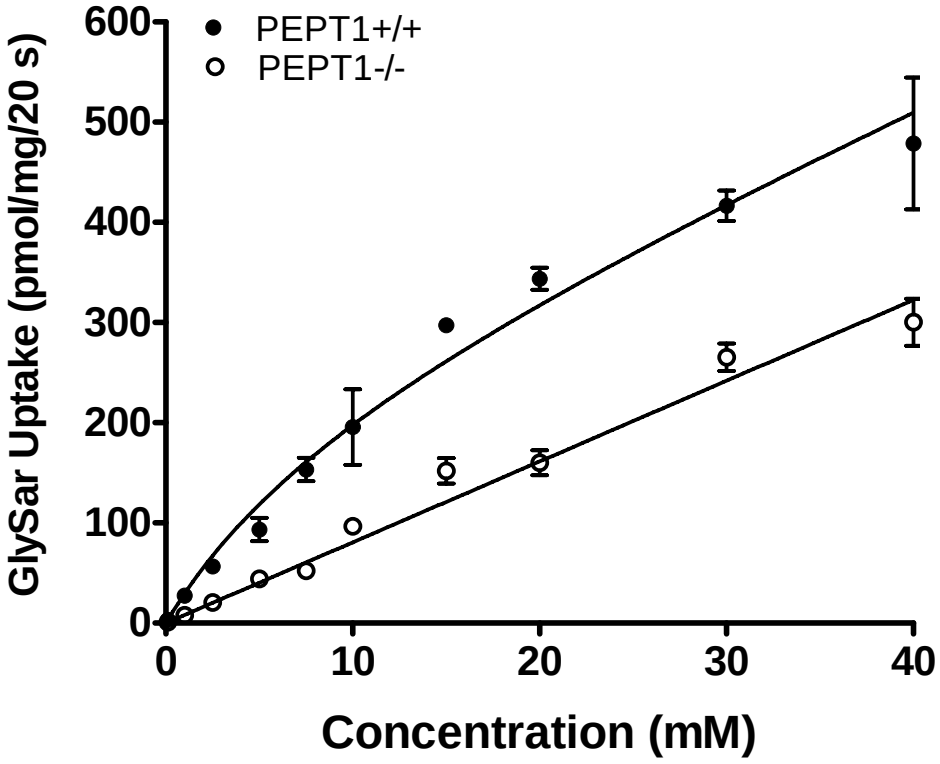

Figure 3.5 Concentration-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (0.1-40 mM total GlySar in external medium). Studies were performed at 37^{o}C in small intestine incubation buffer (pH 6.0). Data are expressed as mean ± S.E. (n=3).

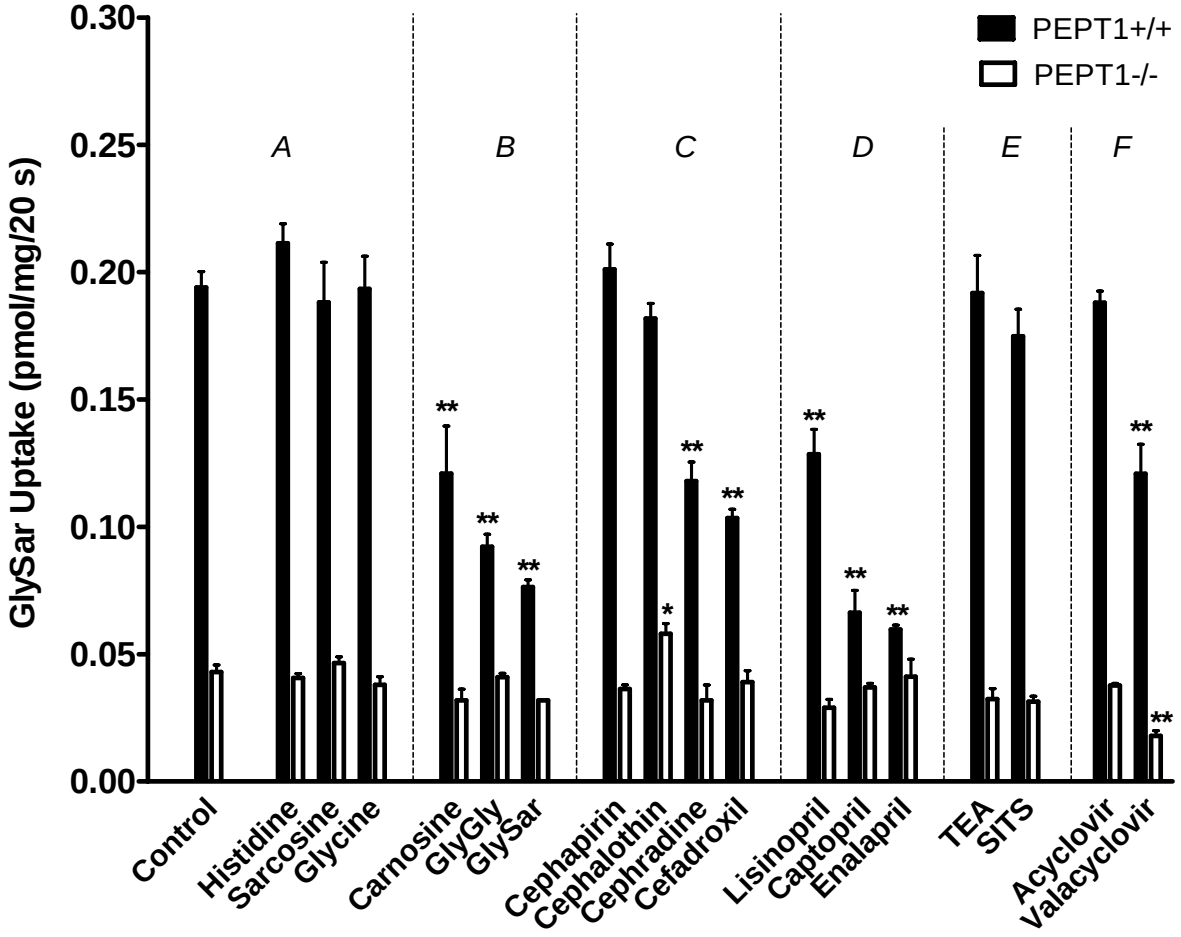

Figure 3.6 Effect of potential inhibitors (10 mM) on the uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium). Studies were performed at 37^{o}C in small intestine incubation buffer (pH 6.0). GlyGly represents glycylglycine, GlySar represents glycylsarcosine, TEA represents tetraethylammonium and SITS represents 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid. Data are expressed as mean ± S.E. (n=3-6).

Table 3.1 Uptake kinetics of GlySar in wild-type and PEPT1 null mice.

Parameter*	PEPT1$^{+/+}$	PEPT1$^{-/-}$
Vmax (pmol mg^{-1} 20sec^{-1})	**233 (36)**	-
Km (mM)	**9.96 (3.59)**	-
Kd (µl mg^{-1} 20sec^{-1})	**0.0081 (0.0003)**	**0.0081 (0.0003)**
Vmax/Km (µl mg^{-1} 20sec^{-1})	**0.0234**	-
% Kd in (Kd+ Vmax/Km)	**25.7%**	-
% (Vmax/Km) in (Kd+ max/Km)	**74.3%**	-
r^2	**0.9382**	**0.9564**

*Parameter estimates (± SE) were calculated by nonlinear (or linear) least square regression models. r^2 is the coefficient of determination.

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CHAPTER IV

INFLUENCE OF FED-FASTED STATE ON INTESTINAL PEPT1 EXPRESSION AND *IN VIVO* PHARMACOKINETICS OF GLYCYLSARCOSINE IN WILD-TYPE AND *PEPT1* KNOCKOUT MICE

Abstract

The proton-oligopeptide cotransporter 1 PEPT1 is the major protein for di-,tri-peptide uptake in small intestines, and its expression has been shown to be responsive to dietary intake. The aim of this study was to investigate whether food and fasting conditions can alter the regional expression of PEPT1 in intestine, and if these changes can influence the *in vivo* systemic exposure of a model dipeptide, glycylsarcosine (GlySar). The *in vivo* pharmacokinetics of GlySar was determined in wild-type (WT) and *PEPT1* knockout (KO) mice after fed and fasted conditions: 1) mice fed a liquid diet *ad libitum* (control), and 2) mice fasted for 16 hours (16-hr fasted). [^{14}C]GlySar was administered (5 nmol/g body weight) by gavage and intravenous injection to both genotypes following the two conditions. Serial blood samples were obtained over 120 min after intravenous dosing and over 360 min after oral dosing. PEPT1 transcript and protein levels were measured longitudinally in mouse small intestine and colon by real time-PCR and immunoblot analyses, respectively. Upper small intestinal transit was detected by the distance traveled after a charcoal meal, 30 min post administration in fed-

fasted states. Histopathological analysis was reported by a pathologist after small intestines from wild-type and PEPT1 knockout mice were prepared in Swiss rolls. Although minimal changes were observed between WT control and 16-hr fasted mice for intestinal PEPT1 mRNA, protein levels of PEPT1 were increased 2.3-fold in the duodenum ($p<0.001$), 2.4-fold in the jejunum ($p<0.01$), and 1.6-fold in the ileum ($p<0.001$) of fasted animals. After oral dosing, the Cmax of GlySar was significantly increased in 16-hr fasted mice (from 1.7 to 2.8 μM; $p<0.0001$) as was area under the plasma concentration-time curve, AUC (from 415 to 581 μM · min; $p<0.01$). In contrast, no significant differences in Cmax or AUC were observed between KO control and 16-hr fasted mice. As expected, the Cmax and AUC values of WT mice > KO animals under both fed and 16-hr fasted conditions ($p<0.001$). With respect to intravenous dosing, there were no differences in Cmax or AUC as a function of genotype and fed-fasted states. Upper small intestine quotients were about 75% in wild-type and PEPT1 knockout mice in both fed and fasted conditions. No phenotypical abnormality was observed in PEPT1 knockout or in fasted condition. These findings reveal that during brief fasting conditions, the intestinal uptake of GlySar is significantly increased because of greater PEPT1 protein expression in all regions of small intestine. In the absence of PEPT1, food has little if any effect on GlySar absorption.

Introduction

As a regulatory gene, PEPT1 responds to a variety of factors ranging from food intake like high-protein diet (Shiraga et al., 1999); hormones such as leptin (Hindlet et al., 2009) and hyperthyroidism condition (Ashida et al., 2004); pathological states, for instance, Crohn's disease and short-bowel syndrome (Adibi, 2003) for the elevated or reduced absorption of its substrates. Because of proton being its driving force, any alteration of the pH gradients or membrane voltage will affect PEPT1 transport activity (Wenzel U et al., 2002; Rexhepaj et al., 2009).

PEPT1 predominantly expresses on the apical membrane of enterocytes in the small intestines with comparable expression levels from duodenal to ileal segments in rats (Qandeel et al., 2009). In kidney, it is located on the apical membranes of epithelial cells in S1 segments of proximal tubule (Shen et al., 1999). In addition, the expression and/or function of PEPT1 have been detected in nuclei of vascular smooth muscle cells in pancreas (Bockman et al., 1997); apical membrane of epithelial cells in bile duct (Knutter et al., 2002); plasma membrane of nonpolarized immune cells (Charrier et al., 2006). PEPT1 transports a broad spectrum of substrates from all possible di- and tripeptides in a stereospecific manner except for a few exceptions; amino β-lactam antibiotics of the cephalosporin and penicillin classes; certain angiotensin-converting enzyme inhibitors to various amino acid ester pro-drugs (Brandsch et al., 2008).

Back to 1999, mRNA and protein levels of PEPT1 in the intestinal mucosa from rats were induced 3- to 4-fold, respectively, after 1 day of fasting (Thamotharan et al., 1999), along with a 2-fold increase in the uptake of a model compound Gly-Gln by brush

border membrane vesicles. Subsequent studies demonstrated that intestinal PEPT1 expression and function were upregulated after 2-d fasting (Naruhashi et al., 2002) and 4-d fasting (Pan et al., 2003) in rats. A recent paper described higher plasma and brain concentrations of oseltamivir, an ester-type prodrug of the neuaminidase inhibitor, in overnight fasted rat pups than their milk-fed littermates after its oral administration. This prodrug was suggested as a substrate of PEPT1 and its absorption was greatly reduced when simultaneously administered with milk, casein or GlySar in adult rats (Ogihara et al., 2009). Although the data suggested an upregulation of PEPT1 in intestine under fasting condition, PEPT1 protein expression was not determined nor was it correlated to its *in vivo* function.

Meal intake may change gastric emptying and/or gastric pH so that the absorption rate of drugs will be altered (Chen et al., 2007). In addition, food may affect the expression of intestinal transporters and/or enzymes to modify the absorption rate and/or extent of oral drugs (Grenier et al., 2006). P-gp, an efflux transporter in the small intestine, has been extensively studied with respect to its regulation by dietary components (Zhang et al., 2009). Little research, however, has been performed to explore the possible relationship between food, drug and PEPT1 expression/activity.

Keeping its function and regulation in mind, we tried to explore whether fed and fasted conditions would alter regional expression of PEPT1 in the small intestine. If so, to what extent would it affect the *in vivo* systemic exposure of the model compound, glycylsarcosine (GlySar). Our unique PEPT1 knockout mice enabled us to specifically investigate the food-PEPT1 interaction on drug absorption. In addition, we examined any

physiological changes that might occur in our wild-type and knockout mice during fed and fasted conditions.

Materials and Methods

Materials. ^{14}C-GlySar (106 mCi/mmol) was purchased from Amersham Biosciences (Chicago, IL) and ^{3}H-dextran 70,000 (265 mCi/gm) from American Radiolabeled Chemicals (St. Louis, MO). PEPT1 knockout mice were generated on a C57BL/6 mouse background as described by Hu et al. (2008). Glycylsarcosine was obtained from Sigma-Aldrich (St. Louis, MO). Diet LD 101 was obtained from TestDiet (Richmond, IN). It arrived as a dry powder and a liquid suspension was prepared afterwards for rodents. In this liquid diet, crude protein, crude fat, crude fiber and ash were no less than 16.0%, 15.0%, 10.0% and 5.0%, respectively. The growth rate of rodents maintained on this diet was similar to that maintained on a good quality, standard rodent solid diet. Hyamine hydroxide was obtained from ICN Pharmaceuticals (Costa Mesa, CA). All other chemicals were obtained from standard sources.

Diet Treatment. Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health. Gender- and weight-matched PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, 6 to 8 weeks of age, were used for all phenotypic analyses unless otherwise noted. The mice were kept in a temperature-controlled environment with a 12-h light and 12-h dark cycle, and received a standard diet and water *ad libitum* (Unit for Laboratory Animal medicine, University of Michigan, Ann Arbor, MI). Before conducting fed-fasted experiments, mice were fed with Diet LD101 instead of standard chow for 4 days (Anji and Kumari, 2008). On average, a male and a female mouse consumed about 15 ml/ 24 hr and 13.5 ml/ 24 hr, respectively, of Diet LD101. For the fed control group, mice were maintained on a liquid diet. In the fasted group, liquid diet was removed from cages at 5

pm and water was accessible to the mice *ad libitum*. Experiments were performed at 9 am the next day, which represented 16-hr of fasting to mice. The experimental design for fed-fasted treatments is shown below:

	Liquid Diet				
	Day 1	Day 2	Day 3	Day 4	Day 5
Fed group	LD101	LD101	LD101	LD101	Experiments
Fasted group	LD101	LD101	LD101	LD101, fasted overnight	Experiments

Real-Time PCR Analysis. After mice during fed-fasted conditions were anesthetized with sodium pentobarbital (65 ug/g ip), the intestine and colon were opened longitudinally and the mucus layer was gently scraped off from duodenal, jejunal, ileal, proximal and distal colonic segments by glass slides. Total RNA was isolated separately according to the manufacturer's protocol using TriZol reagent (Invitrogen, Carlsbad, CA). After genomic DNA was removed from total RNA by DNAse I treatment (Ambion, Austin, TX), first strand cDNA was synthesized by reverse-transcriptase III (Invitrogen, Carlsbad, CA). PEPT1 and house-keeping gene 18s rRNA primers were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). Forward and reverse primers for PEPT1 were CTTGGAGCCACCACAATGG and ACAGAATTCATTGACCACGATGA; forward and reverse primers for 18s rRNA were GGCGTCCCCCAACTTCTTA and GGGCATCACAGACCTGTTATTG. Thermal profile for real-time PCR was 1 cycle at 50 ^{o}C for 2 min, 1 cycle at 95 ^{o}C for 10 min, 40 cycles at 95 ^{o}C for 15s and 60 ^{o}C for 1 min. Relative abundance of PEPT1 transcripts were calculated based on the Ct cycles and normalized for 18s rRNA.

Immunoblot Analysis. Total protein of the mucus layer was resolved on 7.5% SDS-PAGE gel. After it was transferred to PVDF membrane, polyclonal rabbit anti - mouse PEPT1 antisera (raised against the COOH- terminal region, KGIGKENPYSSLEPVSQTNM; Lampire Biological Laboratories, Pipersville, PA) (1:1000 dilution) was used to detect specific PEPT1 expression, followed by goat anti - rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) (1:3000 dilution). For loading control β-actin, the same membrane was blotted with a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1: 1000 dilution), and then incubated with goat anti - mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) (1:3000 dilution). The membrane bound with specific antibodies was detected with Immobilon Western Chemiluminescent Substrate (Millipore, Billerica, MA).

***In Vivo* Phamacokinetic Studies.** Gender-matched PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice in fed-fasted conditions were anesthetized with sodium pentobarbital (65 ug/g ip) prior to administration of ^{14}C-GlySar (5 nmol/g body weight) by tail vein injection. For the oral study, conscious mice were administered ^{14}C-GlySar (5 nmol/g body weight) by gavage. After intravenous dosing, serial blood samples were collected at 0.25, 1, 5, 15, 30, 60, 90 and 120 min; after oral dosing, serial blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 240 and 360 min. Blood samples (15 to 20 µl) were obtained via tail transections and the plasma was harvested. Animals after oral gavage were returned to their cages in between blood sampling with free access to water. Radioactivity in plasma was measured by a dual-channel liquid scintillation counter.

Tissue Distribution Studies. At the end of blood sampling, ^{3}H-dextran 70,000 (2 μCi/mouse) was administered i.v. 2 min prior to tissue harvest to correct extracellular space for GlySar tissue concentration. The mouse was immediately decapitated and multiple tissues/organs were obtained (i. e., cerebral cortex, eye, lung, heart, liver, stomach, duodenum, jejunum, ileum, colon, spleen, kidney, skeletal muscle and testis/ovary). Tissue samples were blotted dry, weighted, and digested in 0.33 ml of 1 M hyamine hydroxide (a tissue solubilizer) at 37^{o}C. A 20-μL aliquot of 33% H_2O_2 was added to each solubilized tissue followed by Ecolite (+) liquid scintillation cocktail (MP Biomedicals). Radioactivity in each sample was detected by a dual-channel liquid scintillation counter (Beckman LS 3801; Beckman Coulter, Fullerton, CA). Corrected tissue concentrations of GlySar (nmol/g of wet weight) were calculated as C_{tiss} - DS·C_b, where C_{tiss} is the uncorrected GlySar tissue concentration (nmol/g), DS is the dextran space, and C_b is the GlySar blood concentration (nmol/ml).

Pharmacokinetics of GlySar Plasma Concentrations. The plasma concentration-time curves were fitted using a noncompartmental approach after oral and intravenous administrations in WinNonlin (version 5.2; Pharsight, Mountain View, CA).

Gastrointestinal Transit. Gender-matched PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice in their fed-fasted states were orally administered a meal of 10% activated charcoal (Sigma, St. Louis, MO) in 5% gum Arabic solution at a volume of 10 μl/g body weight. At 30 min, animals were sacrificed by cervical dislocation after brief anesthesia. The stomach and small intestine were separated from the omentum and dissected. The distance traveled by the leading edge of the charcoal suspension and the total length of small intestine from pyloric sphincter to ileocecal junction were measured. Gastrointestinal transit for each

animal was determined as the percentage of the distance traveled by charcoal relative to the total length of the small intestine: percent transit = (charcoal distance)/ (small intestine length) × 100 (Matsuda et al., 2006).

Morphometric Analysis. Duodenum, jejunum, ileum and colon were obtained from mice in fed-fasted conditions in both genotypes. Samples were fixed overnight in 10% formaldehyde at 4^{o}C, transferred to 70% (vol/vol) ethanol, and then sent to the Cancer Center at the University of Michigan to prepare ematoxylin and eosin stained sections. Prepared sections were analyzed by the Core Pathology Laboratory at the University of Michigan and histopathological reports were provided.

Statistical Analysis. Data are reported as mean ± S.E. One way analysis of variance (ANOVA) was performed to test the statistic difference among multiple treatments for a given parameter. If ANOVA showed significant difference among treatments, the Tukey method of multiple comparisons was used to determine differences among groups. All statistical analyses were operated using Prism version 5 (GraphPad Software, Inc., San Diego, CA).

Results

PEPT1 mRNA Expression in Wild-Type Intestinal Mucosa after 16-Hour Fasting. PEPT1 mRNA expression in the 3 segments of small intestine and 2 segments of colon were determined by real-time PCR in wild-type fed control and 16-hr fasted animals. PEPT1 mRNA level of duodenal mucosa in fed group was arbitrarily assigned a value of unity. All other mucosal PEPT1 mRNA expressions were compared to this arbitrary unit 1. As shown in Figure 4.1, mRNA expression of PEPT1 in jejunal mucosa in fed mice was the highest and about 3-fold greater than that in duodenal site. Ileum, proximal colon and distal colon had expression values that were 1.6-fold, 0.07-fold and 0.50-fold of duodenal PEPT1 in fed mice, respectively. When mice were fasted for 16 hr, PEPT1 mRNA expression values decreased in the duodenal and distal colonic segments ($p < 0.05$) and increased (not significantly) in jejunal, ileal and proximal colonic segments compared to their corresponding levels in fed animals.

PEPT1 Protein Expression in Wild-Type Intestinal Mucosa after 16-Hour Fasting. PEPT1 proteins from small intestinal and colonic mucosa were detected by immunostaining with our specific rabbit anti-mouse PEPT1 antibody. The level in fed group in each segment was arbitrarily assigned a value of unity. Compared to the fed control, PEPT1 protein levels increased about 2.3-fold ($p < 0.001$), 2.4-fold ($p < 0.01$) and 1.6-fold ($p < 0.001$), respectively, in duodenal, jejunal and ileal mucosa after mice were fasted for 16 hr. In fed or fasted mice, no PEPT1 signals were detected in either proximal or distal colon (Figure 4.2).

Pharmacokinetics of Intravenously Administered GlySar after 16-Hour Fasting. As shown in Figure 4.3 (A and B), GlySar plasma concentration-time curves

were almost superimposable in fed and fasted wild-type mice. A similar pattern was observed in PEPT1 null mice. In addition, the plasma concentration-time profiles were similar across genotypes. Pharmacokinetic parameters derived from i.v. administration for each genotype in their fed-fasted conditions are summarized in Table 4.1. PEPT1 ablation or diet in the fed state did not affect the iv pharmacokinetics of GlySar. However, differences were observed in the terminal half-life (t1/2), volume of distribution steady-state (Vdss), and mean residence time (MRT) between wild-type and PEPT1 null mice in their fasted condition.

Tissue Distribution of Intravenously Administered GlySar at 120 Min after 16-Hour Fasting. After i.v. bolus injection of GlySar, different tissues were harvested at 2 hr and GlySar tissue concentrations were corrected for vascular space by dextran 70,000. Figure 4.4 A displays the concentrations of GlySar in the tissues and blood. Although GlySar blood concentrations were similar at 120 min in all four groups, GlySar concentrations in ileum were higher in wild-type fasted over fed mice; lung, stomach, duodenum, colon, spleen and teste/ovary had higher GlySar concentrations in PEPT1 null fed over fasted mice; liver, stomach, duodenum, jejunum, and ileum showed higher GlySar concentrations in fasted wild-type over PEPT1 null mice. Tissue concentrations of GlySar:blood ratios are shown in Figure 4.4 B.

Pharmacokinetics of Orally Administered GlySar after 16-Hour Fasting. Figure 4.5 (A and B) displays GlySar plasma concentration-time profiles in wild-type and PEPT1 null mice during fed-fasted conditions. A summary of the pharmacokinetic parameters for all treatment groups is presented in Table 2. GlySar peak concentrations (C_{max}) and area under the plasma concentration-time curve ($AUC_{0\text{-}360min}$) in fasted wild-

type mice were about 1.5-fold of that in fed animals. The C_{max} and $AUC_{0\text{-}360min}$ from fed wild-type mice were significantly higher than values in PEPT1 null. In addition, the plasma concentration-time curves were superimposable in PEPT1 null mice in fed-fasted conditions, as shown by the comparable C_{max} and $AUC_{0\text{-}360min}$ values in these two groups.

Tissue Distribution of Orally Administered GlySar at 360 Min after 16-Hour Fasting. As shown in Figure 4.6 A, after oral administration, GlySar distributed evenly in all tissues except for testis/ovary in wild-type and PEPT1 null mice as a function of diet. Tissue concentration of GlySar:blood ratios are shown in Figure 4.6 B.

Gastrointestinal Transit (upper GI motility). A charcoal meal was administered orally as a method to compare intestinal transit times in wild-type and PEPT1 knockout mice during fed-fasted conditions. Transit was reported as percent of the leading edge of charcoal travelled relative to the total length of the small intestine, 30 min after the meal. As shown in Figure 4.7, the small intestinal transit was about 75% in all four groups. No significant differences were observed between genotypes or as a function of diet.

Morphometric Study. Duodenum, jejunum, ileum and colon were isolated from wild-type and PEPT1 knockout mice during fed-fasted conditions. Small intestinal and colonic segments were prepared as Swiss rolls (Freeman et al., 1995) and interpreted by the Core Pathology Laboratory at the University of Michigan. Mucosal height and composition, proprial lymphoplasmacytic cells, degree of mitotic activity, submucosal and muscular thickness, appearance, and overall morphology were analyzed in each group (n=6). No significant histological differences were observed between wild-type and PEPT1 knockout mice or as a function of diet. Morphology of both wild-type and

PEPT1 null mice were normal. Representative histologies of jejunum in wild-type and PEPT1 null mice during fed-fasted conditions are shown in Figure 4.8 and Figure 4.9, respectively.

Discussion

We have previously shown that PEPT1 deletion reduced the oral absorption of GlySar by about 50% following gastric gavage and that plasma concentration-time profiles of GlySar after intravenous dosing were superimposable in wild-type and PEPT1 null mice (Hu et al., 2008). Since PEPT1 is inducible after a brief fast (Naruhashi et al., 2002), we tried to explore how food affects the expression of PEPT1, the correlation of PEPT1 expression to its functionality, and the food-PEPT1 interaction on drug absorption.

In PEPT1 studies performed in rats, the duration of fasting has been explored from 1- 4 days (Ogihara et al., 1999; Thamotharan et al., 1999). Because mice have relatively smaller body weights than rats, and because drug absorption studies in mouse and human are usually performed after overnight fast (Sheikh Hassan et al., 2009; Scallion and Moore, 2009), we chose a 16-hr fast for our research design. A model compound, GlySar, was dissolved in isotonic saline solution for intravenous and oral dosing. To minimize any potential solid food effects, we changed the regular chow to a liquid diet (crude protein content was no less than 16%) in our fed-fasted studies, which has been shown to provide satisfactory nutrition to rodents and been tested previously (Anji and Kumari, 2008). In addition, a previous study showed that expression of PEPT1 was comparable in rats fed with an amino acid liquid mixture as those fed with regular chow (Ogihara et al., 1999).

We first examined the expression of PEPT1 transcripts in small intestine and colon of wild-type mice. In both fed and fasted animals, mRNA levels were highest in the jejunum, followed by ileum and then duodenum. Compared to its abundant

expression in the small intestine, colonic segments had very low expression levels of PEPT1. The PEPT1 mRNA expression pattern observed in our mouse study is consistent with that of a previous study in humans (Englund et al., 2006). In rats, it expressed similarly in duodenum, jejunum and ileum (Howard et al., 2004). With respect to human PEPT1 mRNA expression in the intestines, it was reported variably. Terada et al (2005) used normal human mucosal specimens to claim that PEPT1 mRNA levels were highest in the duodenum, followed by jejunum and then ileum (duodenum> jejunum> ileum), while another study revealed that the mRNA expression levels were comparable in the duodenum and ileum from human histologically normal biopsies (Meier et al., 2007). It is widely believed that normal human colonic tissues express low levels of PEPT1. In disease states such as colon cancer, the colonic mRNA expression of PEPT1 is significantly induced (Anderson et al., 2009).

After 16-hr fasting, PEPT1 mRNA increased slightly in jejunal and ileal segments (p> 0.05) but not in duodenal tissue (Figure 4.1), which was inconsistent with changes observed in rat PEPT1. In 2-d fasted rats, the PEPT1 mRNA increased from 2- to 5-fold in different intestinal segments (Naruhashi et al., 2002). However, when we measured PEPT1 protein levels in the same tissue segments, we found that PEPT1 increased in duodenum, jejunum and ileum on the order of 1.6- to 2.4-fold (Figure 4.2).

A 5 nmol/g intravenous bolus dose of GlySar was administered to wild-type and PEPT1 null mice during fed and fasted conditions to explore if diet would affect the GlySar plasma concentration-time profiles of dipeptide. We found all four plasma profiles to be almost superimposable (Figure 4.3) with AUC and CL showing no differences (Table 1). Since renal PEPT1 is not regulated during fasted conditions (Pan

et al., 2003), our observation confirmed this characteristic by showing no changes of CL in fed vs fasted wild-type mice. Previously, we reported that PEPT2 accounted for 84% of the total reabsorption of GlySar in kidney when studied in wild-type and PEPT2 null mice after a 50 nmol/g iv bolus dose of dipeptide (Ocheltree et al., 2005). In the present study, we revealed that the CL of GlySar was comparable between wild-type and PEPT1 null mice in both the fed and fasted states. This finding emphasizes that PEPT1 is a minor transporter for GlySar renal reabsorption and indicates that the functionality of PEPT2 in kidney was not altered after PEPT1 was abolished.

Due to the expression of PEPT1 on apical membranes of enterocytes, a 5 nmol/g oral dose of GlySar was administered to wild-type and PEPT1 null mice during fed and fasted conditions. As shown in the plasma concentration-time profiles (Figure 4.5), we observed different degrees of GlySar absorption between genotypes and feeding conditions, with the greatest systemic exposure of GlySar occurring in fasted wild-type mice (i.e., AUC of 580 μM · min and Cmax of 2.80 μM) followed by fed wild-type animals (i.e., AUC of 415 μM · min and Cmax of 1.67 μM). After PEPT1 ablation, GlySar absorption was comparable in fed and fasted states, both of which were the lowest in all four test groups (i.e., AUCs of 268 μM · min and 302 μM · min, and Cmaxs of 1.15 μM and 1.25 μM, respectively, for fed and fasted PEPT1 null mice). We also observed that PEPT1 protein levels were upregulated after the 16-hr fast (Figure 4.2). By comparing the absorption of GlySar between genotypes and feeding conditions, we concluded that the increase of GlySar systemic exposure was due to an upregulation of PEPT1 protein in wild-type mice. We also observed that changes in protein expression of PEPT1 corresponded to similar changes in *in vivo* functionality. Therefore, food

deprivation served to upregulate intestinal PEPT1 expression, which then served to increase GlySar absorption.

Food-drug interactions have been extensively studied (Welling 1996; Custodio et al., 2008). Since the small intestines express a variety of influx and efflux transporters (Takano et al., 2006), food effects on drugs through regulated transporters have been addressed as well. For instance, grapefruit juice (GFJ) reduced the oral bioavailability of fexofenadine probably through its inhibition of influx-directed organic anion transporter polypeptides (OATPs) (Greenblatt 2009). Because of its efflux activity and broad substrate spectrum, P-gp is of great interest in transporter-based drug interaction profiles. In this case, the bioavailability (F) of dextromethorphan, a substrate of P-gp, increased significantly when taken orally with GFJ in human healthy volunteers, largely due to GFJ's inhibitory effects on P-gp (Di Marco, et al., 2002). A recent review (Zhang et al., 2009) listed potential effects of food components on P-gp.

In our tissue distribution study after oral administration, we observed little change in the tissue concentrations of GlySar (at 360 min) between genotypes or feeding conditions. With the advancement of technology, gastric emptying and gastrointestinal transit times were shown to be longer than previously thought. Schwarz et al (2002) used nuclear magnetic resonance to demonstrate that the exponential decay constant for stomach emptying in mice was 74 min, ant that there was a small amount of reagent remaining in the stomach for as long as 3 days. In addition, they showed that 20% of the contrast agent remained in the GI track even after 10 hours post administration. In light of these findings, it should not be surprising that GlySar exhibited a slow rate of absorption in our study.

When we examined the plasma concentration-time profiles of GlySar, we found that GlySar peak time (Tmax) did not change because of genotype or feeding conditions, averaging about 50 min. We, thereby, examined the upper small intestinal transit in our four groups by oral administration of a charcoal meal. Upper intestinal transit, represented by the percent of charcoal meal travelled relative to the length of the small intestine (30 min after the meal), have been extensively used as a parameter for intestinal physiology (Charoenthongtrakul et al., 2009). The upper small intestine transit times measured by a charcoal meal were laboratory-dependent in fasted 16-hr mice, and ranged from 50% to 85% (Matwyshyn et al., 2006; Matsuda et al., 2006). In the current study, we observed an upper small intestine transit time of 75% in all four groups.

In contrast to the present study, Mittelstadt and Spruell observed that the gastrointestinal transit time was significantly shorter in fasted rats (50% versus 40% in fed rats) (2005). One of the primary differences between the two studies is the fasting period. We fasted mice for 16-hr, whereas, in the previous study, food was removed from rats for only 6-hr by claiming this time period was adequate enough for the stomach to be emptied. In another study, a gastric half-emptying time of 86.9 ± 8.4 min was observed in overnight fasted mice (Moechars et al., 2006). It is possible that fasting period could affect the physiology of the gastrointestinal tract, that is small intestine transit was rapid after a short fast (i.e., 6-hr), while it slowed down to its original level as in fed condition after a prolonged fasting (i.e., 16-hr).

Another difference between the present study and Mittelstadt and Spruell (2005) is the animal diet. Here, we used a liquid diet to replace the regular rodent chow. Gastric half-emptying time was shorter for liquid meals than for solid meals in mice (Symonds et

al., 2003). It is also possible that charcoal molecules release faster from a liquid-filled stomach than from a stomach containing solid food. In addition, species differences may contribute to this inconsistency. Regardless, the upper small intestine transit time was not different in fed versus fasted conditions for our mice.

The colony of PEPT1 knockout mice had no obvious phenotypical abnormality. The intestines from wild-type and PEPT1 knockout mice had no significant histological differences, in fed or fasted states, thereby questioning the importance of PEPT1 in nutrition and/or survival. A similar finding was observed in mice carrying targeted disruption(s) of other intestinal transporter genes such as mdr1a (Schinkel et al., 1994), mdr1b (Schinkel et al., 1997), mdr1a/1b (Schinkel et al., 1997), and bcrp1 (Jonker et al., 2002). However, like our PEPT1 knockout mice, those other null mice demonstrated significant differences in drug exposure, emphasizing their importance in pharmacological and pharmaceutical fields.

In concluding, our findings conclusively demonstrate that PEPT1 protein expression is induced in duodenum, jejunum and ileum of wild-type mice after 16-hr fast; no changes are observed in the pharmacokinetics of GlySar after iv dosing as a function of genotype or diet; the AUC and Cmax of GlySar are significantly increased in wild-type after oral dosing during a 16-hr fast, while no such pattern is observed after PEPT1 ablation; the small intestinal transit (at 30 min) is about 75%, regardless of diet and genotypes. These findings support PEPT1 as a pharmaceutically relevant transporter, in which its upregulation correlates to changes in functionality (i.e., increased intestinal absorption of GlySar).

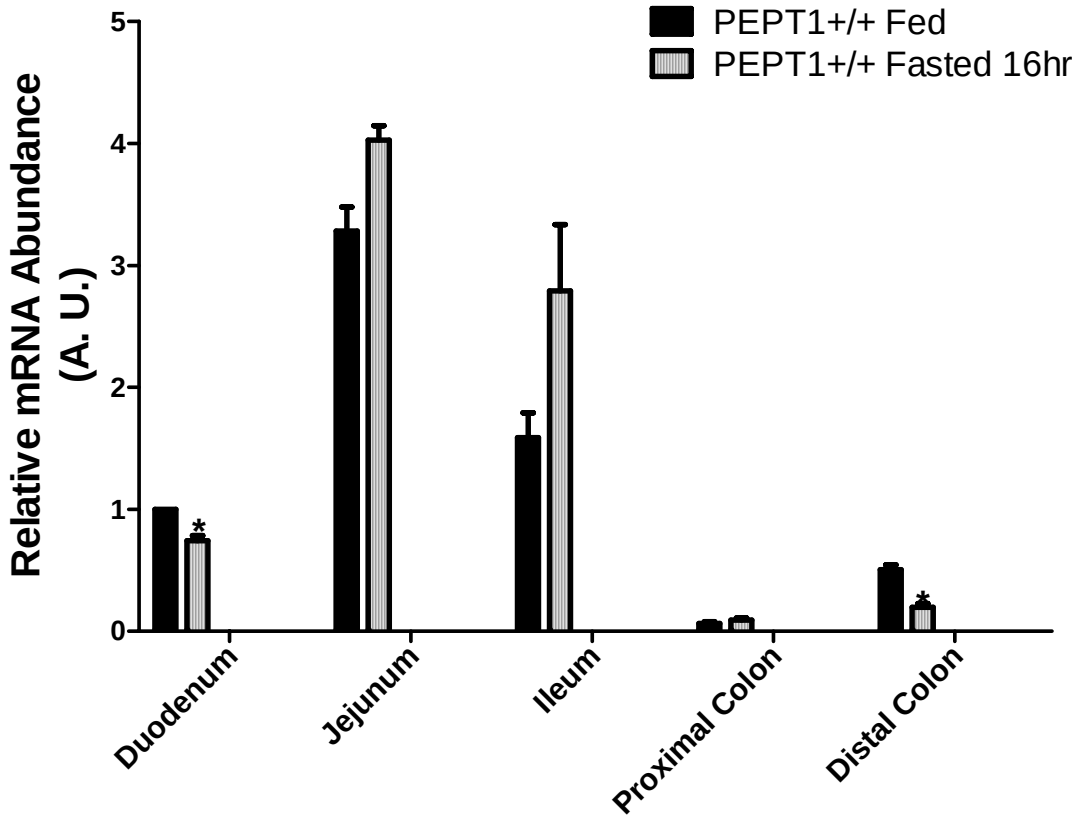
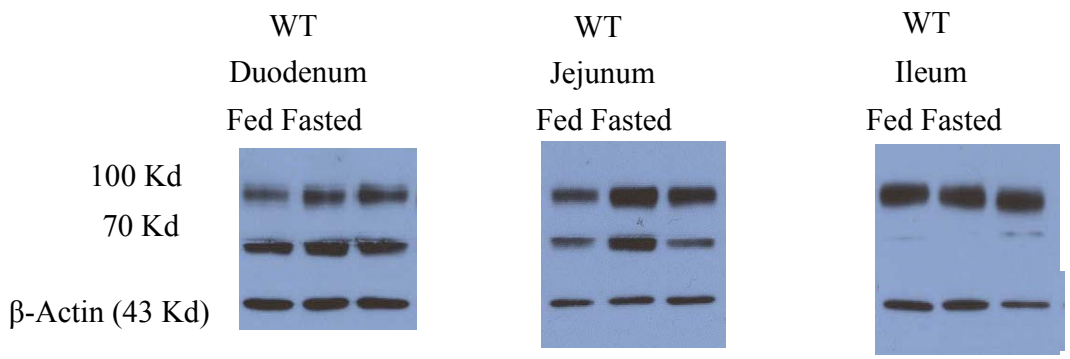


Figure 4.1 PEPT1 mRNA expression in 5 different intestinal segments during fed and fasted conditions of wild-type mice. Data are reported as mean ± SE (n = 6). The mRNA expression in duodenum from fed mice was considered as a control value and arbitrarily assigned a value of unity. All other expression values were compared to the control. Statistical analyses were performed between treatments for a specific intestinal segment by student t-test with $p \leq 0.05$ being significant.



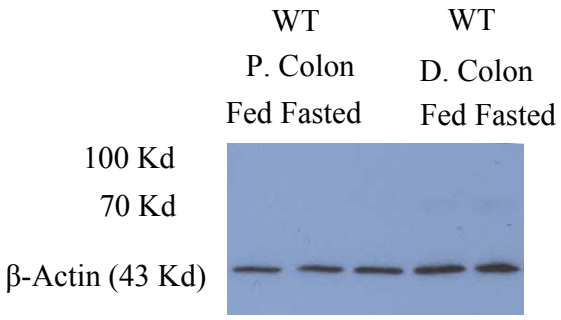


Figure 4.2 PEPT1 protein expression in 5 different intestinal segments of wild-type mice during fed-fasted conditions. Data are reported as mean ± SE (n = 6). The protein expressions in fed mice were the controls and arbitrarily assigned a value of unity. Expression values of fasted mice were compared to their corresponding controls. Statistical analyses were performed by student t-test with $p \leq 0.05$ being significant.

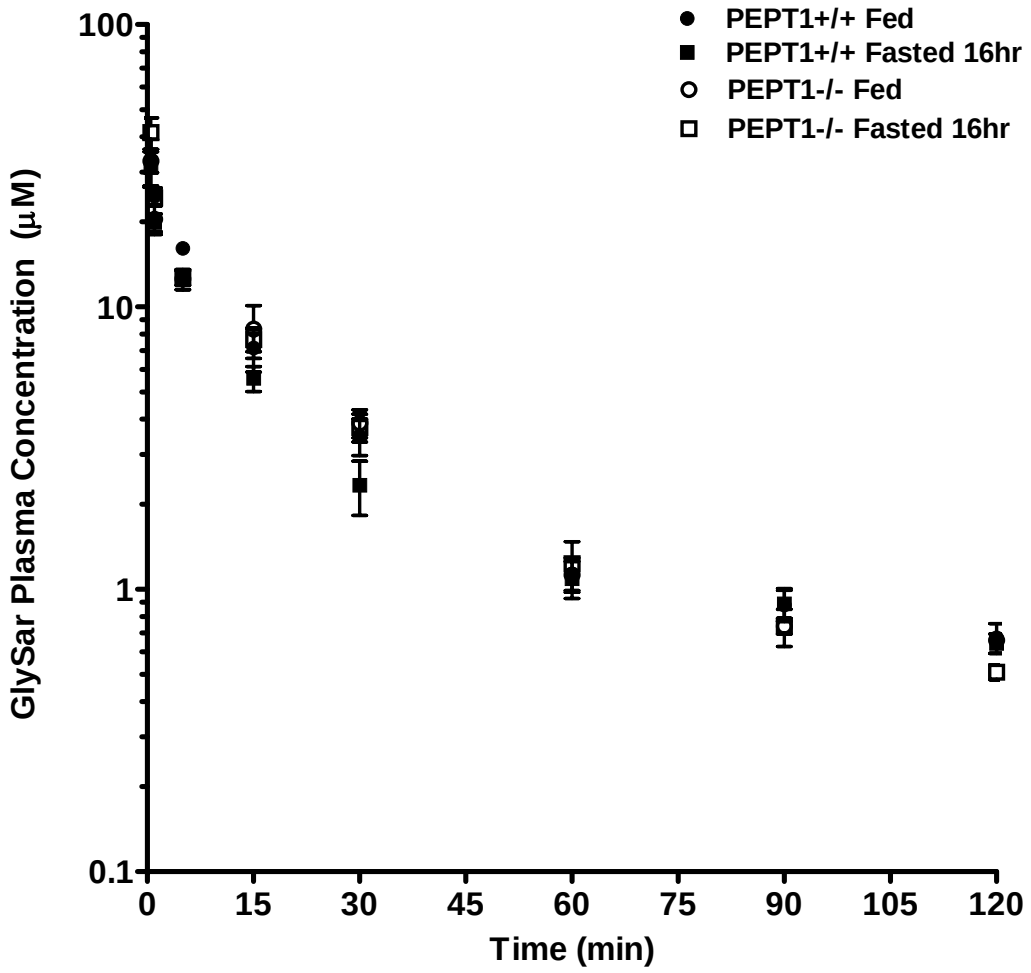


Figure 4.3A Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after intravenous bolus administration of dipeptide at 5 nmol/g body weight (y-axis shown as log scale). Data are reported as mean ± SE (n = 6).

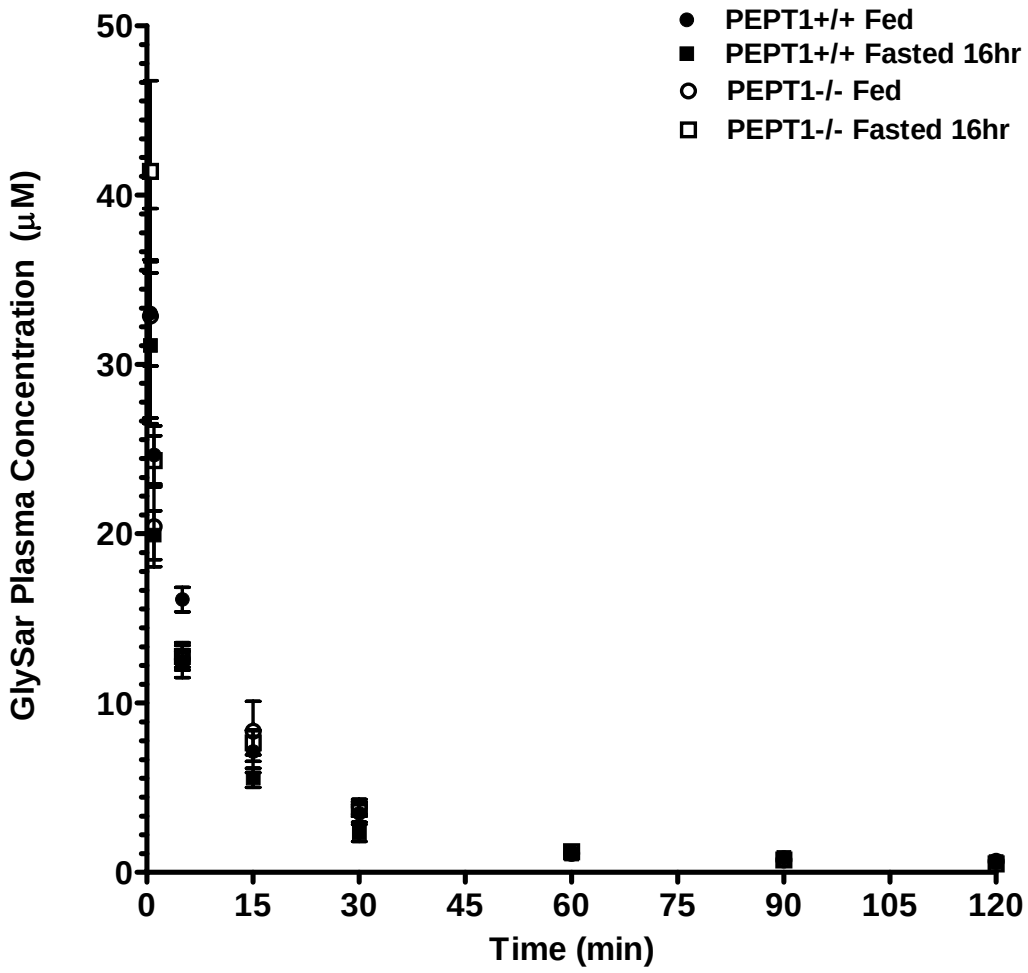


Figure 4.3B Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after intravenous bolus administration of drug at 5 nmol/g body weight (y-axis shown as linear scale). Data are reported as mean ± SE (n = 6).

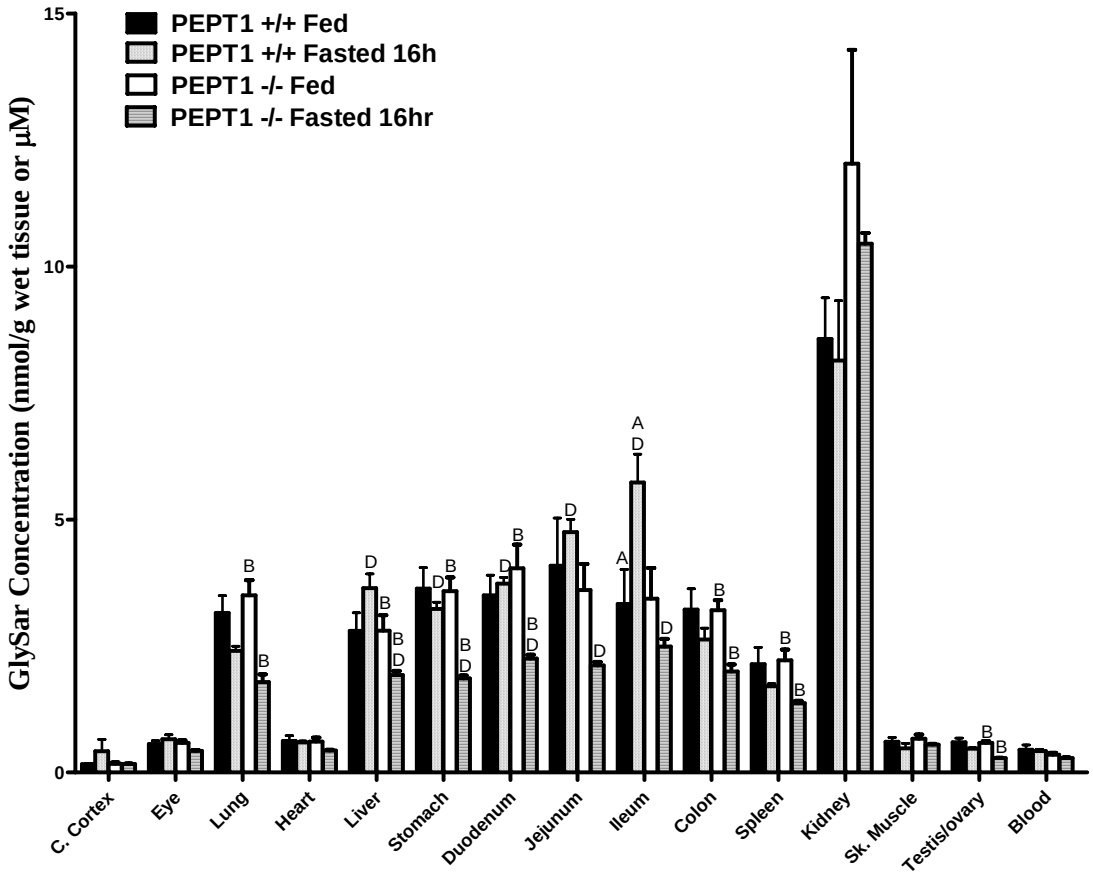


Figure 4.4A Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight. Data are reported as mean ± SE (n = 6). Statistical analyses were performed by one-way analysis of variance (ANOVA)-Tukey test. ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted conditions.

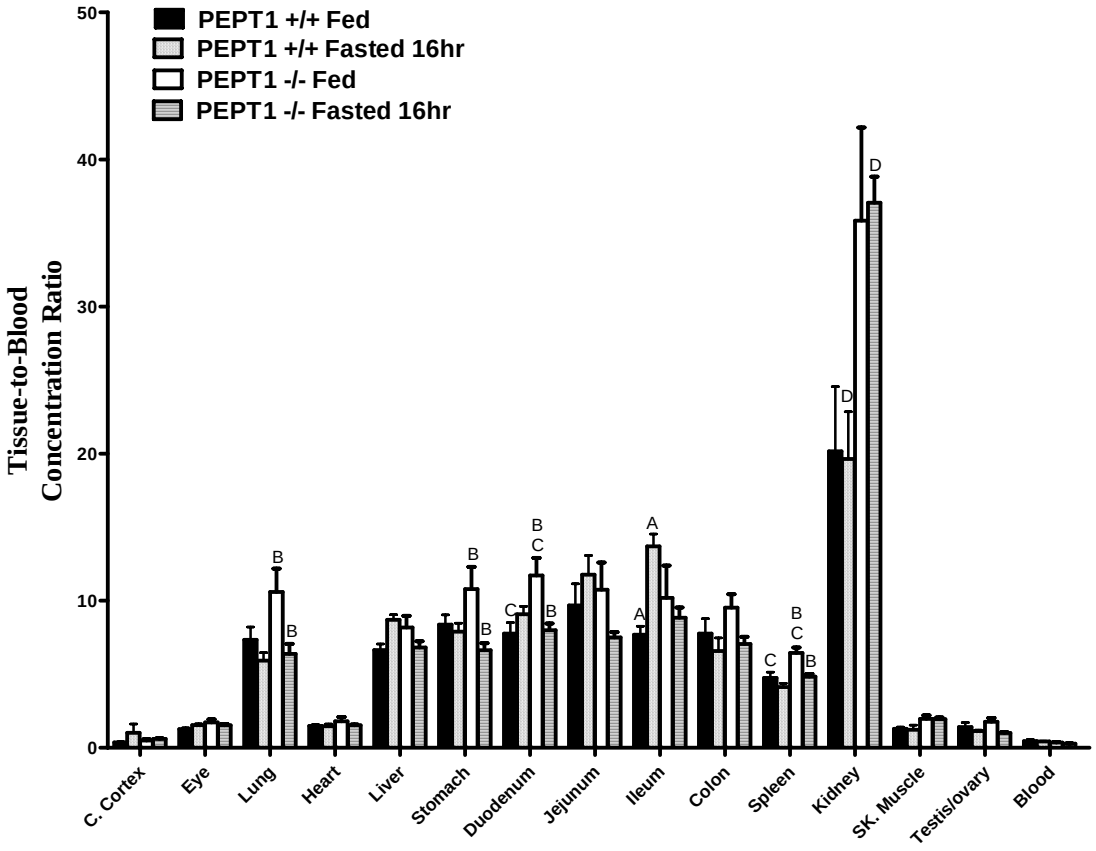


Figure 4.4B Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight. Data are reported as mean ± SE (n = 6). Statistical analyses were performed by one-way analysis of variance (ANOVA)-Tukey test. ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted conditions.

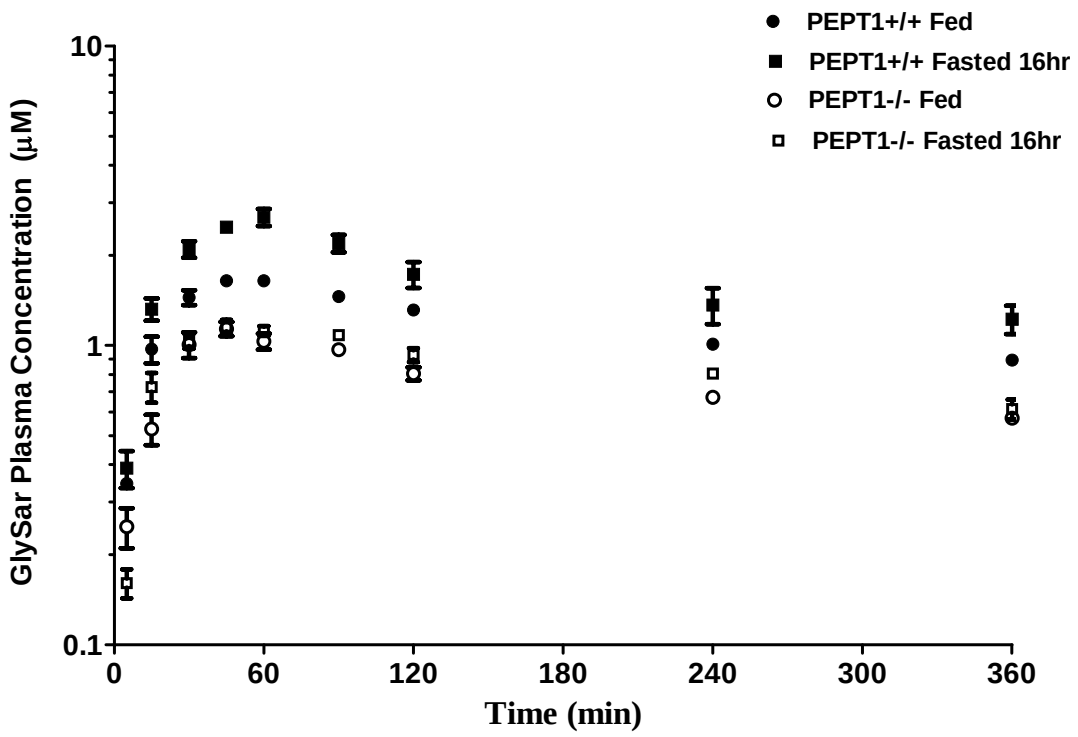


Figure 4.5A Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after oral administration of dipeptide at 5 nmol/g body weight (y-axis shown as log scale). Data are reported as mean ± SE (n = 6).

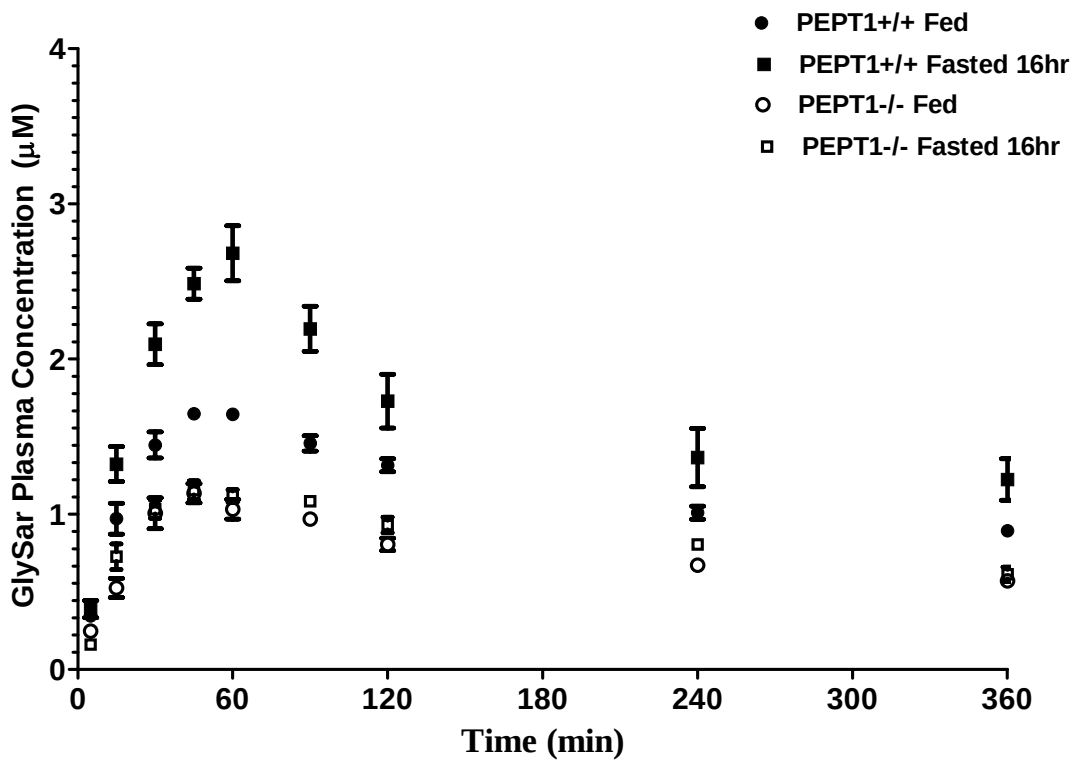


Figure 4.5B Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after oral administration of dipeptide at 5 nmol/g body weight (y-axis shown as linear scale). Data are reported as mean ± SE (n = 6).

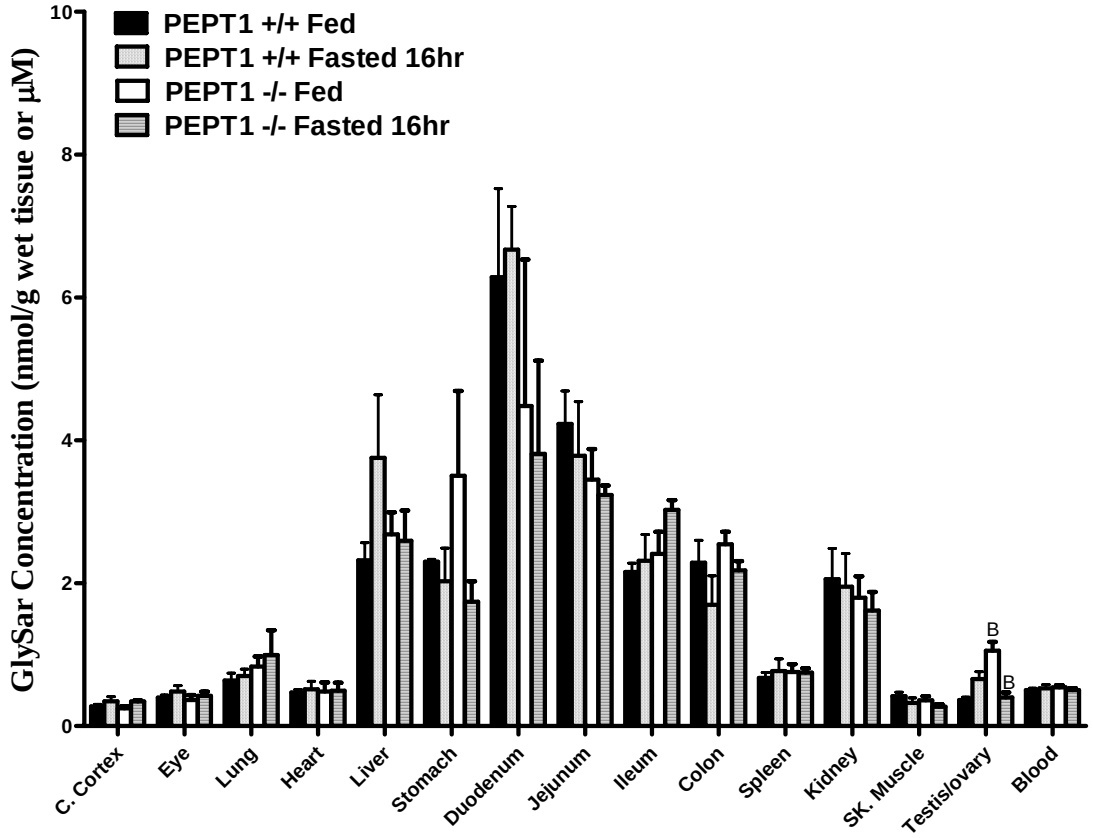


Figure 4.6A Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight. Data are reported as mean ± SE (n = 6). Statistical analyses were performed by one-way analysis of variance (ANOVA)-Tukey test. ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted conditions.

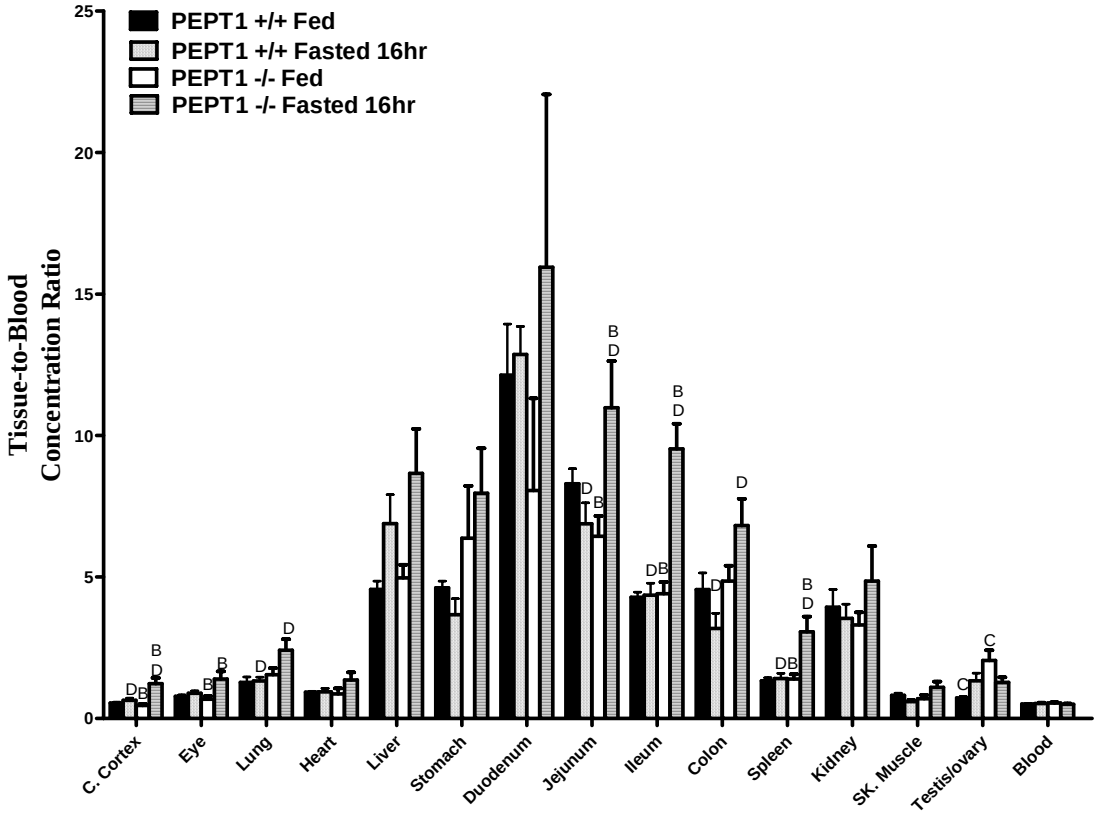


Figure 4.6B Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight. Data are reported as mean ± SE (n = 6). Statistical analyses were performed by one-way analysis of variance (ANOVA)-Tukey test. ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted conditions.

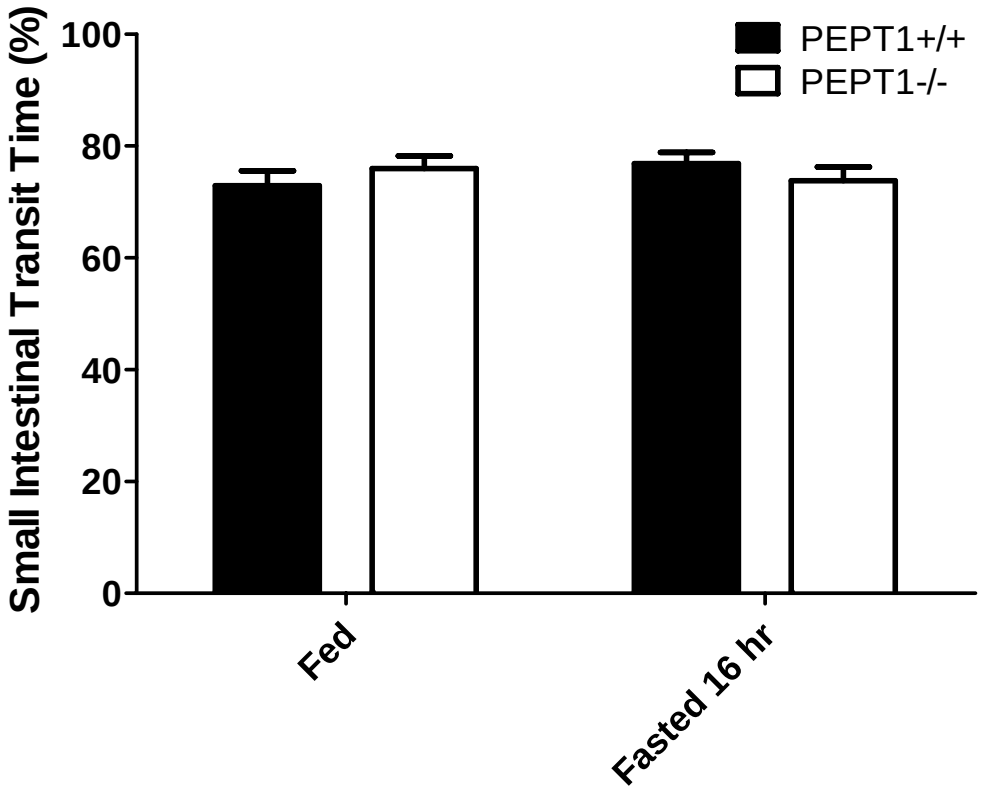


Figure 4.7 Upper gastrointestinal transit in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, in which a charcoal meal was administered by gavage to each mouse. Thirty minutes later, mice were sacrificed and GI transit was expressed as percent of the distance travelled by the charcoal relative to that of total small intestinal length. Data are reported as mean ± S.E. (n=6). Statistical analyses were performed by one-way analysis of variance (ANOVA)-Tukey test.

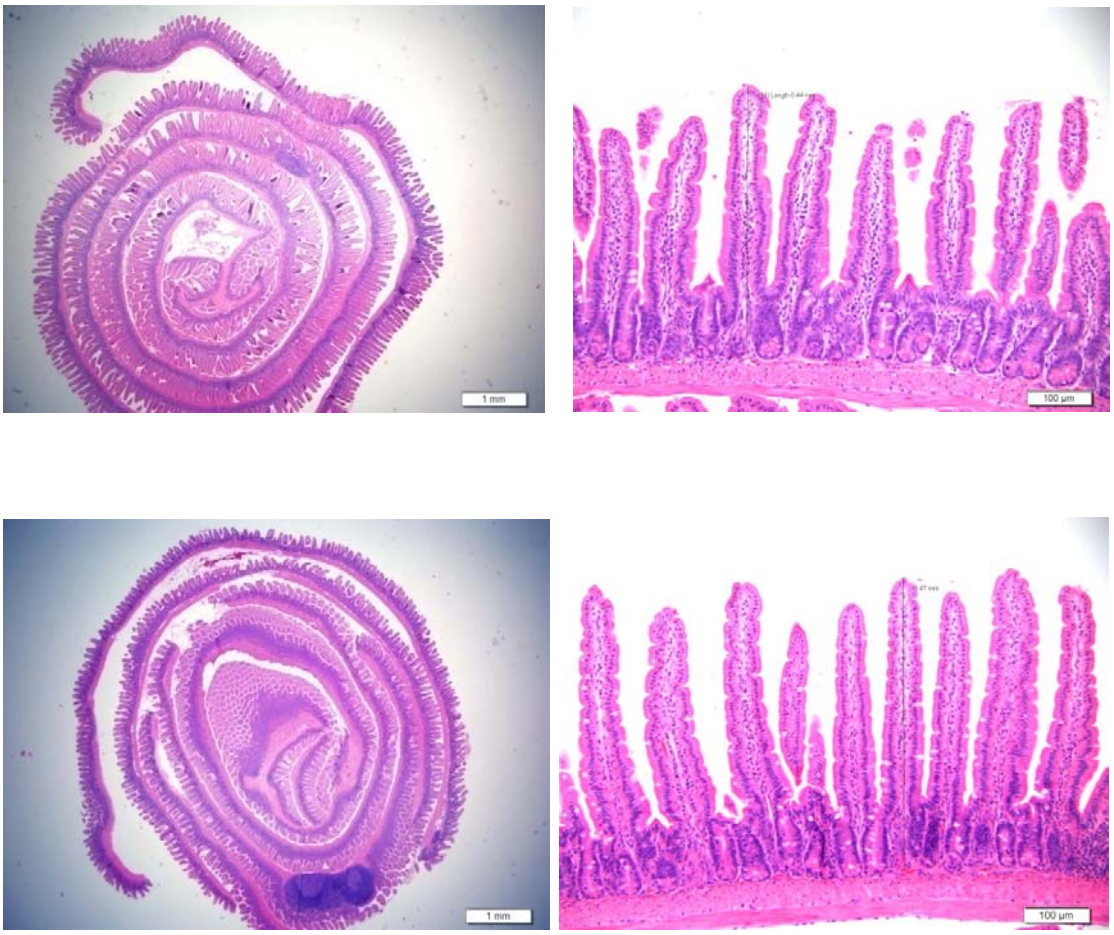


Figure 4.8 Histology of jejunum in fed PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice. Sections were stained with H &E and examined by light microscopy. For PEPT1$^{+/+}$ mice, the bar = 1 mm (A) and bar = 100 μm (B). For PEPT1$^{-/-}$ mice, the bar = 1 mm (C) and bar = 100 μm (D).

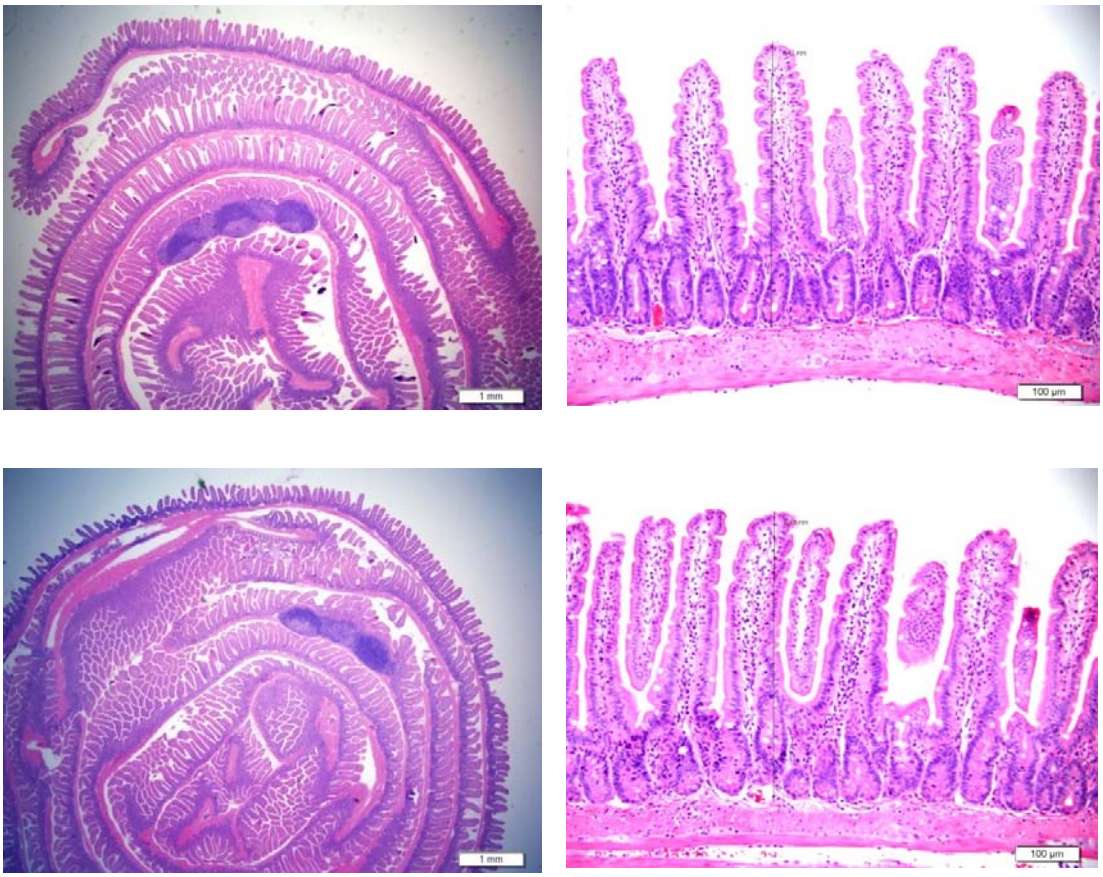


Figure 4.9 Histology of jejunum in fasted PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice. Sections were stained with H &E and examined by light microscopy. For PEPT1$^{+/+}$ mice, the bar = 1 mm (A) and bar = 100 µm (B). For PEPT1$^{-/-}$ mice, the bar = 1 mm (C) and bar = 100 µm (D).

Table 4.1 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g intravenous bolus dose of dipeptide in $PEPT1^{+/+}$ and $PEPT1^{-/-}$ mice during fed-fasted conditions.

Parameters	$PEPT1^{+/+}$		$PEPT1^{-/-}$	
	Fed	Fasted 16 hr	Fed	Fasted 16 hr
CL (µl/min)	216 (19)	253 (21)	223 (13)	221 (11)
Vdss (ml)	8.6 (1.2)	13.2 (2.0)^{D}	8.1 (0.8)	6.9 (0.6)^{D}
MRT (min)	33.5 (1.8)	39.1 (2.0)^{D}	32.9 (1.6)	29.0 (1.6)^{D}
$t_{1/2}$ (min)	44.6 (5.5)	59.1 (6.3)^{D}	37.9 (2.6)	34.1 (2.4)^{D}

Data are represented as mean (± SE) (n = 6)

CL, total plasma clearance; Vdss, volume of distribution steady-state; MRT, mean residence time; $t_{1/2}$, terminal half-life

One-way ANOVA-Tukey test was performed to analyze differences among groups; ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted condition.

Table 4.2 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g oral dose of dipeptide in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice during fed-fasted conditions.

Parameters	PEPT1$^{+/+}$		PEPT1$^{-/-}$	
	Fed	Fasted 16 hr	Fed	Fasted 16 hr
$AUC_{0\text{-}360min}$ (μM · min)	415 (11)^{A,C}	581 (46)^{A,D}	268 (8)^{C}	302 (13)^{D}
C_{max} (μM)	1.67 (0.03)^{A,C}	2.80 (0.10)^{A,D}	1.15 (0.07)^{C}	1.21 (0.03)^{D}
T_{max} (min)	47.5 (4.6)	52.5 (3.4)	45.0 (3.9)	55.0 (7.4)
$t_{1/2}$ (min)	350 (16)	410 (47)	374 (31)	401 (47)

Data are represented as mean (± SE) (n = 6)

AUC, area under the plasma concentration-time curve; Cmax, peak concentration; T_{max}, time to peak concentration; $t_{1/2}$, terminal half-life

One-way ANOVA-Tukey test was performed to analyze differences among groups; ^{A}Represents significant differences between fed and fasted conditions in wild-type mice, ^{B}represents significant differences between fed and fasted conditions in PEPT1 null mice, ^{C}represents significant differences between wild-type and PEPT1 null mice in fed condition, and ^{D}represents significant differences between wild-type and PEPT1 null mice in fasted condition.

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APPENDIX A

INDIVIDUAL DATA FROM CHAPTER III

Table A.1 Time-dependent uptake of ^{14}C-GlySar in the jejunum of wild-type mice (4 μM GlySar in external medium).

Time (min)	0.167	0.333	0.5	1.0	1.5	2.0	4.0	5.0	7.5	10.0	15.0
Trial 1	0.084	0.227	0.183	0.460	0.550	0.725	0.960	1.132	1.312	1.113	1.304
Trial 2	0.054	0.158	0.305	0.440	0.570	0.890	0.980	1.354	1.198	1.207	1.562
Trial 3	0.180	0.160	0.240	0.520	0.720	0.780	1.030	0.880	1.224	1.085	1.273
Trial 4	0.095	0.180	0.325	-	-	0.610	-	1.290	-	1.860	-
Mean	0.103	0.181	0.263	0.473	0.613	0.751	0.990	1.164	1.244	1.316	1.380
SE	0.027	0.016	0.032	0.024	0.054	0.058	0.021	0.106	0.034	0.183	0.092

Table A.2 Temperature-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).

PEPT1$^{+/+}$		
Temperature (^{o}C)	**37^{o}C**	**4^{o}C**
Trial 1	0.936	0.088
Trial 2	1.034	0.072
Trial 3	1.072	0.131
Trial 4	0.859	-
Mean	0.975	0.097
SE	0.048	0.018

PEPT1$^{-/-}$		
Temperature (^{o}C)	**37^{o}C**	**4^{o}C**
Trial 1	0.232	0.057
Trial 2	0.240	0.075
Trial 3	0.193	0.039
Mean	0.222	0.057
SE	0.014	0.010

Table A.3 pH-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).

PEPT1$^{+/+}$						
pH	**8.0**	**7.5**	**7.0**	**6.5**	**6.0**	**5.5**
Trial 1	0.090	0.167	0.204	0.227	0.194	0.169
Trial 2	0.127	0.177	0.176	0.209	0.238	0.185
Trial 3	0.130	0.170	0.237	0.202	0.224	0.150
Mean	0.116	0.171	0.206	0.213	0.218	0.168
SE	0.013	0.003	0.018	0.008	0.013	0.010

PEPT1$^{-/-}$						
pH	**8.0**	**7.5**	**7.0**	**6.5**	**6.0**	**5.5**
Trial 1	0.046	0.056	0.036	0.045	0.025	0.027
Trial 2	0.031	0.057	0.036	0.038	0.024	0.038
Trial 3	0.039	0.042	0.045	0.050	0.041	0.042
Mean	0.039	0.052	0.039	0.044	0.030	0.035
SE	0.004	0.005	0.003	0.003	0.006	0.005

Table A.4 Sodium-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).

PEPT1$^{+/+}$		
Sodium concentration	**High**	**Low**
Trial 1	0.182	0.193
Trial 2	0.201	0.234
Trial 3	0.208	0.204
Trial 4	0.186	-
Mean	0.194	0.210
SE	0.006	0.012

PEPT1$^{-/-}$		
Sodium concentration	**High**	**Low**
Trial 1	0.045	0.034
Trial 2	0.047	0.028
Trial 3	0.038	0.039
Mean	0.043	0.034
SE	0.003	0.003

Table A.5 Concentration-dependent uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (0.1-40 mM total GlySar in external medium).

PEPT1$^{+/+}$										
Concentration (mM)	**0.1**	**1.0**	**2.5**	**5.0**	**7.5**	**10.0**	**15.0**	**20.0**	**30.0**	**40.0**
Trial 1	2.919	27.91	63.01	78.64	152.5	180.6	283.5	365.2	427.4	536.5
Trial 2	3.471	30.53	60.99	85.52	174.0	139.1	303.0	328.6	386.4	552.1
Trial 3	2.953	24.14	45.94	116.2	133.7	267.3	305.5	337.3	436.2	347.5
Mean	3.114	27.53	56.65	93.44	153.4	195.7	297.3	343.7	416.6	478.7
SE	0.179	1.854	5.384	11.53	11.66	37.78	6.96	11.03	15.35	65.77

PEPT1$^{-/-}$										
Concentration (mM)	**0.1**	**1.0**	**2.5**	**5.0**	**7.5**	**10.0**	**15.0**	**20.0**	**30.0**	**40.0**
Trial 1	0.843	7.751	26.00	42.87	58.98	87.54	154.1	137.1	291.4	253.6
Trial 2	1.403	9.205	13.90	46.41	43.70	104.6	129.2	179.8	245.0	322.8
Trial 3	1.040	7.213	21.64	43.49	54.49	97.65	173.0	163.8	260.2	324.8
Mean	1.095	8.056	20.51	44.26	52.39	96.61	152.1	160.2	265.5	300.4
SE	0.164	0.595	3.538	1.092	4.534	4.966	12.71	12.44	13.66	23.43

Table A.6 Effect of potential inhibitors (10 mM) on the uptake of ^{14}C-GlySar in the jejunum of PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice (4 μM GlySar in external medium).

	PEPT1$^{+/+}$							
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Mean**	**SE**
Control	0.182	0.201	0.208	0.186	-	-	0.194	0.006
Histidine	0.200	0.226	0.208	-	-	-	0.211	0.008
Sarcosine	0.158	0.196	0.211	-	-	-	0.188	0.016
Glycine	0.177	0.219	0.186	-	-	-	0.194	0.013
Carnosine	0.141	0.084	0.138	-	-	-	0.121	0.019
GlyGly	0.102	0.086	0.089	-	-	-	0.092	0.005
GlySar	0.072	0.082	0.076	-	-	-	0.076	0.003
Cephapirin	0.191	0.192	0.221	-	-	-	0.201	0.010
Cephalothin	0.178	0.175	0.194	-	-	-	0.182	0.006
Cephradine	0.108	0.133	0.114	-	-	-	0.118	0.007
Cefadroxil	0.109	0.097	0.105	-	-	-	0.104	0.003
Lisinopril	0.116	0.148	0.123	-	-	-	0.129	0.010
Captopril	0.060	0.056	0.084	-	-	-	0.066	0.009
Enalapril	0.063	0.057	0.060	-	-	-	0.060	0.002
TEA	0.221	0.172	0.184	-	-	-	0.192	0.015
SITS	0.172	0.195	0.158	-	-	-	0.175	0.011
Acyclovir	0.192	0.179	0.193	-	-	-	0.188	0.005
Valacyclovir	0.102	0.120	0.142	-	-	-	0.121	0.012

	PEPT1$^{-/-}$							
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Mean**	**SE**
Control	0.045	0.047	0.038	-	-	-	0.043	0.003
Histidine	0.040	0.038	0.044	-	-	-	0.041	0.002
Sarcosine	0.047	0.051	0.042	-	-	-	0.047	0.002
Glycine	0.043	0.039	0.032	-	-	-	0.038	0.003
Carnosine	0.023	0.036	0.037	-	-	-	0.032	0.004
GlyGly	0.043	0.038	0.043	-	-	-	0.041	0.002
GlySar	0.032	0.032	0.032	-	-	-	0.032	0.000
Cephapirin	0.033	0.038	0.039	-	-	-	0.036	0.002
Cephalothin	0.052	0.066	0.057	-	-	-	0.058	0.004
Cephradine	0.027	0.025	0.044	-	-	-	0.032	0.006
Cefadroxil	0.036	0.048	0.034	-	-	-	0.039	0.005
Lisinopril	0.026	0.036	0.026	-	-	-	0.029	0.003
Captopril	0.035	0.036	0.040	-	-	-	0.037	0.002
Enalapril	0.032	0.037	0.055	-	-	-	0.041	0.007
TEA	0.029	0.041	0.028	-	-	-	0.032	0.004
SITS	0.036	0.030	0.029	-	-	-	0.031	0.002
Acyclovir	0.036	0.038	0.039	-	-	-	0.038	0.001
Valacyclovir	0.017	0.022	0.025	0.017	0.017	0.011	0.018	0.002

APPENDIX B

INDIVIDUAL DATA FROM CHAPTER IV

Table B.1 PEPT1 relative mRNA expression in 5 different intestinal segments during fed and fasted conditions of wild-type mice.

PEPT1$^{+/+}$ Fed					
Intestinal Segment	**Duodenum**	**Jejunum**	**Ileum**	**P. Colon**	**D. Colon**
Trial 1	1.050	3.720	2.100	0.110	0.360
Trial 2	0.950	2.350	1.560	0.040	0.660
Trial 3	1.050	3.540	0.810	0.020	0.560
Trial 4	0.950	3.370	1.860	0.100	0.500
Trial 5	1.050	3.460	1.970	0.040	0.490
Trial 6	0.950	3.250	1.230	0.080	0.450
Mean	1.000	3.282	1.588	0.065	0.503
SE	0.022	0.197	0.201	0.015	0.041

PEPT1$^{+/+}$ Fasted					
Intestinal Segment	**Duodenum**	**Jejunum**	**Ileum**	**P. Colon**	**D. Colon**
Trial 1	0.580	4.240	4.750	0.160	0.310
Trial 2	0.660	4.320	1.850	0.060	0.099
Trial 3	0.880	3.500	1.870	0.042	0.170
Trial 4	0.780	4.020	4.230	0.089	0.220
Trial 5	0.810	4.110	1.990	0.100	0.140
Trial 6	0.740	3.980	2.060	0.096	0.240
Mean	0.742	4.028	2.792	0.091	0.197
SE	0.044	0.118	0.542	0.017	0.031

Table B.2 PEPT1 protein expression in 5 different intestinal segments of wild-type mice during fed-fasted conditions.

PEPT1$^{+/+}$ Fed					
Intestinal Segment	**Duodenum**	**Jejunum**	**Ileum**	**P. Colon**	**D. Colon**
Trial 1	1.050	1.050	1.050	-	-
Trial 2	0.950	0.950	0.950	-	-
Trial 3	1.050	1.050	1.050	-	-
Trial 4	0.950	0.950	0.950	-	-
Trial 5	1.050	1.050	1.050	-	-
Trial 6	0.950	0.950	0.950	-	-
Mean	1.000	1.000	1.000	-	-
SE	0.022	0.022	0.022	-	-

PEPT1$^{+/+}$ Fasted					
Intestinal Segment	**Duodenum**	**Jejunum**	**Ileum**	**P. Colon**	**D. Colon**
Trial 1	2.004	2.046	1.561	-	-
Trial 2	2.782	2.598	1.782	-	-
Trial 3	2.120	2.678	1.307	-	-
Trial 4	2.256	3.786	1.450	-	-
Trial 5	2.253	1.589	1.869	-	-
Trial 6	2.647	1.738	1.580	-	-
Mean	2.344	2.406	1.592	-	-
SE	0.125	0.330	0.085	-	-

Table B.3 Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after intravenous bolus administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed								
Time (min)	**0.5**	**1**	**5**	**15**	**30**	**60**	**90**	**120**
Trial 1	42.15	26.60	15.60	8.44	4.50	1.39	-	0.80
Trial 2	21.25	18.92	14.41	6.49	2.33	0.99	0.81	0.75
Trial 3	37.86	30.64	16.32	8.72	3.71	1.36	1.03	0.96
Trial 4	29.10	22.23	16.55	3.55	1.78	0.60	0.59	0.58
Trial 5	38.04	27.08	19.28	11.66	5.16	1.63	1.23	0.54
Trial 6	29.85	22.48	14.59	4.01	3.48	0.85	0.74	0.41
Mean	33.04	24.66	16.12	7.15	3.50	1.13	0.88	0.67
SE	3.14	1.72	0.72	1.26	0.52	0.16	0.11	0.08

PEPT1$^{+/+}$ Fasted								
Time (min)	**0.5**	**1**	**5**	**15**	**30**	**60**	**90**	**120**
Trial 1	22.91	20.82	15.23	5.10	1.91	0.89	0.68	0.66
Trial 2	17.64	13.73	8.52	4.17	1.54	0.68	0.62	0.59
Trial 3	47.82	22.06	13.00	6.10	2.01	1.44	1.05	0.86
Trial 4	32.64	24.01	12.42	5.43	2.15	1.06	0.93	0.67
Trial 5	35.48	18.48	14.08	8.10	4.83	1.70	1.36	0.52
Trial 6	30.25	20.48	11.48	4.72	1.58	0.78	0.69	0.55
Mean	31.12	19.92	12.45	5.59	2.34	1.09	0.89	0.64
SE	4.28	1.44	0.95	0.56	0.51	0.16	0.12	0.05

PEPT1$^{-/-}$ Fed								
Time (min)	**0.5**	**1**	**5**	**15**	**30**	**60**	**90**	**120**
Trial 1	28.11	18.12	12.62	7.50	3.49	1.08	-	0.61
Trial 2	23.62	18.80	11.16	6.62	3.49	1.16	0.65	0.64
Trial 3	40.95	18.67	14.38	17.15	5.33	0.96	0.66	0.66
Trial 4	22.96	19.55	13.30	6.46	5.01	1.35	0.77	0.76
Trial 5	60.95	31.88	11.58	5.83	2.48	1.14	0.91	0.65
Trial 6	20.56	15.49	12.48	6.47	3.49	1.05	0.72	0.64
Mean	32.86	20.42	12.59	8.34	3.88	1.12	0.74	0.66
SE	6.36	2.36	0.48	1.78	0.44	0.05	0.05	0.02

PEPT1$^{-/-}$ Fasted								
Time (min)	**0.5**	**1**	**5**	**15**	**30**	**60**	**90**	**120**
Trial 1	54.48	25.99	15.49	10.38	5.03	0.73	0.53	0.43
Trial 2	48.33	24.84	12.19	6.91	3.30	1.06	1.02	0.53
Trial 3	29.66	25.70	12.32	7.64	4.54	2.39	-	0.47
Trial 4	28.11	18.12	12.62	7.50	3.49	1.08	-	0.61
Trial 5	56.41	28.62	9.63	5.10	2.11	0.87	0.60	0.44
Trial 6	31.48	22.69	14.30	8.41	4.02	1.26	0.81	0.57
Mean	41.41	24.33	12.76	7.66	3.75	1.23	0.74	0.51
SE	5.35	1.47	0.82	0.71	0.42	0.24	0.11	0.03

Table B.4 Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.103	0.204	0.201	0.122	0.140	0.198	0.162	0.018
Eye	0.368	0.613	0.782	0.447	0.459	0.700	0.561	0.066
Lung	2.333	3.261	4.383	2.193	3.002	3.747	3.153	0.341
Heart	0.384	0.694	1.070	0.472	0.412	0.700	0.622	0.106
Liver	1.776	3.214	4.278	2.242	2.410	2.874	2.799	0.359
Stomach	2.435	3.877	5.367	2.887	3.504	3.741	3.635	0.412
Duodenum	2.223	3.789	4.804	2.507	3.589	4.102	3.502	0.399
Jejunum	2.000	5.258	8.026	3.136	2.008	4.103	4.089	0.939
Ileum	1.492	3.673	6.140	2.685	2.015	3.987	3.332	0.682
Colon	2.326	3.335	4.932	2.154	3.026	3.547	3.220	0.409
Spleen	1.589	1.747	3.649	1.464	2.001	2.401	2.142	0.331
Kidney	9.156	9.625	11.788	7.113	6.871	6.874	8.571	0.810
Sk. Muscle	0.391	0.576	0.868	0.450	0.500	0.853	0.606	0.084
Testis/Ovary	0.714	0.521	0.910	0.335	0.600	0.503	0.597	0.081
Blood	0.242	0.424	0.893	0.343	0.401	0.399	0.450	0.093

PEPT1$^{+/+}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.135	0.148	0.275	0.192	0.174	1.590	0.181	0.021
Eye	0.508	0.549	1.073	0.572	0.574	0.677	0.659	0.086
Lung	2.448	2.311	2.198	2.545	2.756	2.143	2.400	0.094
Heart	0.555	0.723	0.556	0.593	0.504	0.631	0.594	0.031
Liver	2.879	3.200	4.617	4.101	3.077	4.000	3.646	0.282
Stomach	3.524	2.760	3.147	3.619	3.000	3.345	3.232	0.133
Duodenum	3.224	3.811	4.083	3.891	3.542	3.842	3.732	0.124
Jejunum	4.606	5.958	4.249	4.527	4.424	4.736	4.750	0.251
Ileum	4.714	5.649	8.386	4.850	5.777	5.003	5.730	0.560
Colon	3.382	2.792	2.159	1.853	2.569	3.006	2.627	0.228
Spleen	1.587	1.750	1.881	1.824	1.572	1.600	1.702	0.055
Kidney	7.723	11.007	9.081	9.638	2.660	8.742	8.142	1.182
Sk. Muscle	0.534	0.947	0.278	0.346	0.340	0.400	0.474	0.101
Testis/Ovary	0.460	0.476	0.541	0.446	0.403	0.490	0.470	0.019
Blood	0.367	0.355	0.595	0.451	0.360	0.402	0.422	0.038

PEPT1$^{-/-}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.103	0.117	0.212	0.260	0.140	0.198	0.172	0.025
Eye	0.368	0.675	0.638	0.652	0.459	0.700	0.582	0.055
Lung	2.333	3.938	4.421	3.562	3.002	3.747	3.500	0.301
Heart	0.384	0.541	0.749	0.860	0.412	0.700	0.608	0.078
Liver	1.776	2.803	2.857	4.087	2.410	2.874	2.801	0.309
Stomach	2.435	3.418	4.466	3.923	3.504	3.741	3.581	0.275
Duodenum	2.223	4.482	4.078	5.730	3.589	4.102	4.034	0.468
Jejunum	2.000	4.120	4.566	4.828	2.008	4.103	3.604	0.518
Ileum	1.492	3.101	4.920	5.090	2.015	3.987	3.434	0.610
Colon	2.326	3.261	3.379	3.688	3.026	3.547	3.204	0.199
Spleen	1.589	2.289	1.913	3.104	2.001	2.401	2.216	0.213
Kidney	9.156	14.856	13.451	20.982	6.871	6.874	12.030	2.248
Sk. Muscle	0.391	0.605	0.743	0.907	0.500	0.853	0.666	0.083
Testis/Ovary	0.714	0.527	0.492	0.662	0.600	0.503	0.583	0.037
Blood	0.242	0.350	0.245	0.462	0.401	0.399	0.350	0.037

PEPT1$^{-/-}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.153	0.205	0.166	0.165	0.140	0.175	0.168	0.009
Eye	0.483	0.396	0.441	0.423	0.400	0.430	0.429	0.013
Lung	1.367	2.440	1.527	1.857	1.649	1.874	1.786	0.153
Heart	0.435	0.444	0.496	0.388	0.401	0.430	0.433	0.015
Liver	2.270	1.789	1.936	1.847	2.001	1.699	1.923	0.082
Stomach	1.869	2.090	1.785	1.905	1.678	1.842	1.862	0.056
Duodenum	2.308	2.097	2.545	2.206	1.998	2.348	2.250	0.079
Jejunum	2.270	2.028	2.393	2.014	1.987	2.004	2.116	0.070
Ileum	2.018	2.457	3.122	2.553	2.248	2.547	2.491	0.152
Colon	1.912	1.688	2.587	1.876	1.705	2.187	1.993	0.140
Spleen	1.344	1.354	1.577	1.246	1.285	1.402	1.368	0.047
Kidney	10.969	10.519	11.176	10.019	9.985	10.025	10.450	0.215
Sk. Muscle	0.537	0.508	0.630	0.552	0.540	0.524	0.549	0.017
Testis/Ovary	0.285	0.277	0.306	0.284	0.284	0.269	0.284	0.005
Blood	0.294	0.292	0.317	0.220	0.300	0.287	0.285	0.014

Table B.5 Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 120 min after intravenous bolus administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.426	0.480	0.225	0.357	0.451	0.287	0.371	0.041
Eye	1.522	1.444	0.875	1.303	1.184	1.241	1.262	0.093
Lung	9.639	7.689	4.906	6.399	5.502	9.940	7.346	0.863
Heart	1.587	1.637	1.198	1.379	1.222	1.844	1.478	0.104
Liver	7.337	7.579	4.789	6.544	6.777	6.884	6.652	0.403
Stomach	10.06	9.142	6.007	8.425	6.809	9.759	8.367	0.669
Duodenum	9.186	8.934	5.378	7.317	5.883	9.834	7.755	0.755
Jejunum	8.263	12.399	8.984	9.152	4.519	14.823	9.690	1.452
Ileum	6.167	8.660	6.873	7.837	6.653	9.943	7.689	0.580
Colon	9.610	7.864	5.521	6.286	5.556	11.712	7.758	1.018
Spleen	6.567	4.119	4.085	4.272	4.469	5.004	4.752	0.388
Kidney	37.84	22.70	13.20	20.76	15.58	20.91	21.83	3.524
Sk. Muscle	1.615	1.358	0.972	1.312	0.927	1.594	1.296	0.121
Testis/Ovary	2.951	1.228	1.018	0.979	1.137	1.142	1.409	0.311
Blood	0.242	0.424	0.893	0.343	0.443	0.402	0.458	0.092

PEPT1$^{+/+}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.369	0.416	0.463	0.426	0.484	3.958	1.019	0.588
Eye	1.383	1.547	1.803	1.270	1.597	1.686	1.548	0.080
Lung	6.671	6.509	3.695	5.648	7.665	5.337	5.921	0.557
Heart	1.511	2.036	0.934	1.316	1.402	1.572	1.462	0.147
Liver	7.844	9.012	7.761	9.100	8.557	9.962	8.706	0.341
Stomach	9.601	7.772	5.290	8.032	8.343	8.330	7.895	0.581
Duodenum	8.784	10.733	6.863	8.635	9.849	9.567	9.072	0.540
Jejunum	12.55	16.78	7.143	10.05	12.30	11.79	11.77	1.296
Ileum	12.84	15.91	14.10	10.76	16.07	12.46	13.69	0.847
Colon	9.216	7.863	3.629	4.111	7.144	7.486	6.575	0.904
Spleen	4.324	4.928	3.162	4.049	4.372	3.985	4.137	0.238
Kidney	21.04	31.00	15.27	21.39	7.397	21.77	19.64	3.202
Sk. Muscle	1.454	2.666	0.468	0.767	0.946	0.997	1.216	0.318
Testis/Ovary	1.253	1.342	0.910	0.991	1.122	1.221	1.140	0.067
Blood	0.367	0.355	0.595	0.451	0.360	0.402	0.422	0.038

PEPT1$^{-/-}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.426	0.333	0.868	0.563	0.349	0.497	0.506	0.081
Eye	1.522	1.929	2.609	1.413	1.144	1.756	1.729	0.208
Lung	9.639	11.25	18.07	7.714	7.482	9.398	10.59	1.599
Heart	1.587	1.546	3.062	1.863	1.028	1.756	1.807	0.277
Liver	7.337	8.009	11.68	8.852	6.007	7.209	8.182	0.798
Stomach	10.06	9.765	18.26	8.497	8.733	9.383	10.78	1.515
Duodenum	9.186	12.81	16.67	12.41	8.945	10.29	11.72	1.188
Jejunum	8.263	11.77	18.67	10.46	5.005	10.29	10.74	1.855
Ileum	6.167	8.86	20.12	11.03	5.022	10.00	10.20	2.190
Colon	9.610	9.317	13.81	7.989	7.540	8.897	9.528	0.915
Spleen	6.567	6.539	7.819	6.724	4.987	6.022	6.443	0.378
Kidney	37.84	42.44	54.99	45.45	17.12	17.24	35.85	6.332
Sk. Muscle	1.615	1.729	3.036	1.964	1.246	2.140	1.955	0.250
Testis/Ovary	2.951	1.506	2.013	1.434	1.496	1.262	1.777	0.256
Blood	0.242	0.350	0.245	0.462	0.401	0.399	0.350	0.037

PEPT1$^{-/-}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.522	0.702	0.523	0.751	0.467	0.610	0.596	0.046
Eye	1.645	1.357	1.390	1.922	1.334	1.496	1.524	0.092
Lung	4.655	8.357	4.817	8.441	5.495	6.520	6.381	0.692
Heart	1.480	1.522	1.565	1.764	1.338	1.498	1.528	0.057
Liver	7.731	6.126	6.106	8.397	6.669	5.909	6.823	0.415
Stomach	6.366	7.159	5.633	8.657	5.595	6.408	6.636	0.468
Duodenum	7.860	7.181	8.029	10.026	6.660	8.169	7.988	0.470
Jejunum	7.731	6.945	7.550	9.157	6.624	6.972	7.497	0.372
Ileum	6.874	8.415	9.850	11.605	7.494	8.861	8.850	0.696
Colon	6.512	5.783	8.162	8.527	5.682	7.609	7.046	0.500
Spleen	4.578	4.636	4.976	5.663	4.285	4.878	4.836	0.193
Kidney	37.36	36.03	35.26	45.54	33.28	34.88	37.06	1.783
Sk. Muscle	1.827	1.741	1.988	2.511	1.801	1.824	1.949	0.117
Testis/Ovary	0.969	0.948	0.967	1.292	0.947	0.937	1.010	0.057
Blood	0.294	0.292	0.317	0.220	0.300	0.287	0.285	0.014

Table B.6 Plasma concentration-time profiles of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, after oral administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed									
Time (min)	**5**	**15**	**30**	**45**	**60**	**90**	**120**	**240**	**360**
Trial 1	-	0.952	1.414	1.696	1.672	1.469	1.308	1.067	0.956
Trial 2	-	1.103	1.460	1.620	1.635	1.445	1.372	1.077	0.937
Trial 3	-	0.987	1.103	1.685	1.677	1.669	1.420	1.140	0.987
Trial 4	-	0.514	1.742	1.652	1.649	1.295	1.130	0.853	0.803
Trial 5	0.334	1.226	1.516	1.563	1.552	1.424	1.294	0.952	0.787
Trial 6	0.357	1.048	1.442	1.665	1.682	1.440	1.370	0.974	0.893
Mean	0.346	0.972	1.446	1.647	1.645	1.457	1.316	1.011	0.894
SE	0.011	0.100	0.084	0.020	0.020	0.049	0.042	0.042	0.034

PEPT1$^{+/+}$ Fasted									
Time (min)	**5**	**15**	**30**	**45**	**60**	**90**	**120**	**240**	**360**
Trial 1	0.327	1.334	2.127	2.137	2.706	2.317	2.201	1.833	1.538
Trial 2	0.279	0.976	1.813	2.297	3.175	2.059	1.933	1.665	1.460
Trial 3	0.212	1.022	2.023	2.830	2.763	2.446	1.719	0.982	0.813
Trial 4	0.508	1.382	2.564	2.624	2.544	2.254	1.068	0.929	0.993
Trial 5	0.473	1.604	1.703	2.481	1.916	1.551	1.416	0.938	0.998
Trial 6	0.537	1.618	2.342	2.548	2.987	2.540	2.037	1.847	1.543
Mean	0.389	1.323	2.095	2.486	2.682	2.195	1.729	1.366	1.224
SE	0.055	0.113	0.132	0.100	0.178	0.145	0.173	0.188	0.133

PEPT1$^{-/-}$ Fed									
Time (min)	**5**	**15**	**30**	**45**	**60**	**90**	**120**	**240**	**360**
Trial 1	0.199	0.378	0.877	1.108	1.113	1.089	0.772	0.691	0.575
Trial 2	0.203	0.579	1.059	1.112	0.999	0.936	0.677	0.637	0.636
Trial 3	0.285	0.445	0.902	1.012	0.851	0.841	0.758	0.633	0.522
Trial 4	0.187	0.478	0.852	1.005	0.903	0.900	0.891	0.712	0.541
Trial 5	0.423	0.804	1.479	1.421	1.282	1.050	0.952	0.690	0.579
Trial 6	0.192	0.471	0.874	1.159	1.048	1.000	0.784	0.669	0.574
Mean	0.248	0.526	1.007	1.136	1.033	0.970	0.806	0.672	0.571
SE	0.038	0.062	0.099	0.062	0.063	0.038	0.041	0.013	0.016

PEPT1$^{-/-}$ Fasted									
Time (min)	**5**	**15**	**30**	**45**	**60**	**90**	**120**	**240**	**360**
Trial 1	0.152	0.741	0.917	0.907	0.952	1.068	0.710	0.767	0.411
Trial 2	0.163	0.811	1.201	1.306	1.209	1.154	1.077	0.860	0.742
Trial 3	0.174	0.942	1.140	1.270	1.170	1.105	0.987	0.842	0.604
Trial 4	0.144	0.702	1.050	1.190	1.080	1.080	0.954	0.850	0.621
Trial 5	0.234	0.813	1.074	1.207	1.087	1.008	0.951	0.754	0.612
Trial 6	0.099	0.351	0.816	1.054	1.214	1.086	0.906	0.762	0.692
Mean	0.161	0.727	1.033	1.156	1.119	1.083	0.931	0.806	0.614
SE	0.018	0.082	0.058	0.061	0.041	0.019	0.050	0.020	0.046

Table B.7 Tissue distribution of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.276	0.253	0.256	0.230	0.416	0.222	0.276	0.029
Eye	0.429	0.411	0.356	0.332	0.559	0.299	0.398	0.038
Lung	0.919	0.961	0.478	0.487	0.345	0.648	0.640	0.103
Heart	0.589	0.494	0.375	0.378	0.584	0.402	0.470	0.041
Liver	2.848	2.453	1.875	1.948	3.167	1.648	2.323	0.245
Stomach	2.402	2.394	2.287	2.298	2.248	2.178	2.301	0.035
Duodenum	6.559	3.484	4.586	5.003	12.08	5.994	6.285	1.240
Jejunum	5.241	3.347	3.589	3.697	6.031	3.489	4.232	0.458
Ileum	2.155	2.301	1.987	1.985	2.683	1.845	2.159	0.123
Colon	1.492	3.385	1.979	2.000	3.116	1.748	2.287	0.316
Spleen	0.787	0.781	0.570	0.571	0.924	0.415	0.675	0.076
Kidney	2.263	2.028	1.024	1.120	3.935	1.987	2.060	0.429
Sk. Muscle	0.406	0.418	0.350	0.349	0.683	0.301	0.418	0.056
Testis/Ovary	0.363	0.332	0.332	0.322	0.560	0.287	0.366	0.040
Blood	0.567	0.494	0.426	0.452	0.593	0.500	0.505	0.026

PEPT1$^{+/+}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.468	0.145	0.382	0.359	0.544	0.200	0.350	0.063
Eye	0.572	0.238	0.486	0.427	0.839	0.345	0.485	0.085
Lung	0.927	0.429	0.864	0.763	0.838	0.398	0.703	0.094
Heart	0.432	0.325	0.510	0.442	1.059	0.324	0.516	0.113
Liver	2.751	2.727	3.379	3.681	8.002	1.998	3.756	0.882
Stomach	2.537	0.893	1.514	2.357	3.876	0.999	2.029	0.462
Duodenum	8.229	5.101	6.240	7.138	8.332	4.998	6.673	0.602
Jejunum	3.030	1.971	3.267	5.054	6.895	2.501	3.787	0.754
Ileum	1.657	1.877	2.484	2.292	3.996	1.589	2.316	0.366
Colon	1.177	1.454	1.237	1.400	3.717	1.204	1.698	0.406
Spleen	0.534	0.528	0.830	0.769	1.553	0.420	0.773	0.169
Kidney	1.761	1.142	1.780	1.870	4.141	1.026	1.953	0.461
Sk. Muscle	0.259	0.195	0.347	0.296	0.655	0.202	0.326	0.070
Testis/Ovary	1.084	0.686	0.413	0.376	0.778	0.604	0.657	0.106
Blood	0.500	0.333	0.594	0.593	0.706	0.452	0.530	0.053

PEPT1$^{-/-}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.281	0.112	0.278	0.256	0.322	0.231	0.247	0.030
Eye	0.256	0.361	0.300	0.298	0.274	0.703	0.365	0.069
Lung	0.557	0.948	0.569	0.574	0.930	1.430	0.835	0.141
Heart	0.370	0.385	0.253	0.260	0.559	1.080	0.485	0.128
Liver	2.198	2.712	2.003	1.998	3.467	3.732	2.685	0.310
Stomach	2.179	2.796	2.405	2.305	1.947	9.403	3.506	1.185
Duodenum	1.536	3.168	2.500	2.783	2.227	14.670	4.481	2.050
Jejunum	2.028	3.363	3.005	2.997	4.950	4.361	3.451	0.429
Ileum	2.647	1.442	2.005	1.998	3.624	2.751	2.411	0.312
Colon	2.473	2.834	2.648	2.658	1.723	2.938	2.546	0.177
Spleen	0.622	0.704	0.551	0.560	0.856	1.261	0.759	0.111
Kidney	1.722	1.790	1.136	1.201	1.784	3.166	1.800	0.298
Sk. Muscle	0.196	0.517	0.340	0.356	0.235	0.528	0.362	0.056
Testis/Ovary	0.682	1.596	1.005	1.127	0.989	0.936	1.056	0.124
Blood	0.599	0.459	0.558	0.413	0.606	0.612	0.541	0.035

PEPT1$^{-/-}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.395	0.341	0.350	0.349	0.371	0.270	0.346	0.017
Eye	0.389	0.347	0.332	0.340	0.422	0.712	0.424	0.059
Lung	0.756	0.742	0.500	0.500	0.759	2.707	0.994	0.346
Heart	0.425	0.378	0.289	0.300	0.545	1.034	0.495	0.114
Liver	2.196	2.238	1.913	1.900	2.705	4.623	2.596	0.423
Stomach	1.400	1.490	1.310	1.339	1.791	3.123	1.742	0.285
Duodenum	2.245	2.365	2.203	2.198	3.643	10.22	3.813	1.302
Jejunum	3.268	2.881	3.000	2.997	3.607	3.649	3.234	0.135
Ileum	2.478	2.966	3.001	2.998	3.480	3.247	3.028	0.137
Colon	2.335	1.938	2.100	2.113	2.738	1.863	2.181	0.130
Spleen	0.791	0.671	0.612	0.600	0.842	0.971	0.748	0.060
Kidney	1.678	1.550	0.977	0.980	1.804	2.711	1.617	0.262
Sk. Muscle	0.264	0.224	0.202	0.199	0.333	0.415	0.273	0.035
Testis/Ovary	0.354	0.313	0.320	0.315	0.361	0.752	0.402	0.070
Blood	0.543	0.499	0.413	0.448	0.555	0.568	0.504	0.026

Table B.8 Tissue/blood concentration ratios of GlySar in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, 360 min after oral administration of dipeptide at 5 nmol/g body weight.

PEPT1$^{+/+}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.487	0.512	0.602	0.510	0.701	0.444	0.543	0.038
Eye	0.756	0.832	0.835	0.735	0.943	0.598	0.783	0.048
Lung	1.621	1.946	1.123	1.078	0.582	1.295	1.274	0.193
Heart	1.039	1.000	0.882	0.837	0.985	0.803	0.924	0.039
Liver	5.026	4.966	4.404	4.308	5.344	3.294	4.557	0.299
Stomach	4.239	4.846	5.372	5.084	3.793	4.352	4.614	0.240
Duodenum	11.57	7.052	10.77	11.07	20.38	11.98	12.14	1.800
Jejunum	9.248	6.773	8.430	8.178	10.18	6.972	8.296	0.534
Ileum	3.802	4.656	4.668	4.391	4.526	3.687	4.288	0.177
Colon	2.633	6.852	4.647	4.425	5.257	3.494	4.551	0.595
Spleen	1.389	1.581	1.339	1.264	1.559	0.830	1.327	0.112
Kidney	3.993	4.105	2.404	2.478	6.640	3.971	3.932	0.628
Sk. Muscle	0.717	0.846	0.823	0.772	1.153	0.602	0.819	0.076
Testis/Ovary	0.641	0.671	0.781	0.713	0.945	0.574	0.721	0.053
Blood	0.567	0.494	0.426	0.452	0.593	0.500	0.505	0.026

PEPT1$^{+/+}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.938	0.435	0.643	0.605	0.771	0.442	0.639	0.079
Eye	1.144	0.713	0.819	0.721	1.188	0.763	0.891	0.088
Lung	1.856	1.288	1.455	1.286	1.186	0.880	1.325	0.132
Heart	0.864	0.977	0.860	0.746	1.500	0.717	0.944	0.118
Liver	5.505	8.185	5.691	6.207	11.33	4.417	6.889	1.022
Stomach	5.077	2.678	2.551	3.973	5.488	2.209	3.663	0.570
Duodenum	16.47	15.31	10.51	12.03	11.80	11.05	12.86	0.994
Jejunum	6.064	5.916	5.503	8.522	9.762	5.529	6.883	0.738
Ileum	3.316	5.633	4.184	3.864	5.658	3.513	4.361	0.424
Colon	2.355	4.364	2.083	2.361	5.262	2.663	3.181	0.534
Spleen	1.070	1.585	1.399	1.296	2.199	0.929	1.413	0.184
Kidney	3.524	3.428	2.998	3.153	5.862	2.269	3.539	0.499
Sk. Muscle	0.518	0.586	0.584	0.498	0.927	0.447	0.594	0.070
Testis/Ovary	2.170	2.058	0.696	0.634	1.101	1.336	1.332	0.269
Blood	0.500	0.333	0.594	0.593	0.706	0.452	0.530	0.053

PEPT1$^{-/-}$ Fed								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	0.470	0.244	0.499	0.621	0.532	0.377	0.457	0.054
Eye	0.428	0.785	0.539	0.722	0.452	1.149	0.679	0.111
Lung	0.929	2.064	1.020	1.392	1.534	2.338	1.546	0.229
Heart	0.617	0.839	0.454	0.631	0.923	1.766	0.872	0.192
Liver	3.667	5.904	3.591	4.844	5.719	6.100	4.971	0.459
Stomach	3.636	6.085	4.312	5.589	3.212	15.37	6.367	1.856
Duodenum	2.564	6.896	4.482	6.747	3.674	23.98	8.057	3.259
Jejunum	3.384	7.321	5.388	7.265	8.166	7.128	6.442	0.716
Ileum	4.417	3.138	3.594	4.845	5.979	4.496	4.411	0.406
Colon	4.126	6.169	4.748	6.444	2.842	4.803	4.855	0.543
Spleen	1.037	1.533	0.987	1.358	1.412	2.061	1.398	0.159
Kidney	2.874	3.895	2.037	2.911	2.942	5.174	3.305	0.444
Sk. Muscle	0.328	1.125	0.610	0.863	0.387	0.863	0.696	0.126
Testis/Ovary	1.138	3.475	1.802	2.732	1.631	1.531	2.051	0.358
Blood	0.599	0.459	0.558	0.413	0.606	0.612	0.541	0.035

PEPT1$^{-/-}$ Fasted								
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**SE**
C. Cortex	1.217	2.125	1.063	1.385	0.703	0.871	1.227	0.205
Eye	1.114	2.269	0.942	1.225	2.143	0.680	1.396	0.268
Lung	2.830	4.084	1.770	2.243	1.312	2.211	2.408	0.394
Heart	1.560	2.497	1.082	1.430	0.550	1.047	1.361	0.269
Liver	7.486	16.15	6.622	8.163	8.326	5.221	8.661	1.568
Stomach	5.822	15.33	7.175	8.666	4.276	6.500	7.962	1.588
Duodenum	8.275	19.60	8.044	10.20	44.70	4.878	15.95	6.102
Jejunum	9.749	16.37	9.442	12.50	13.29	4.545	10.98	1.654
Ileum	7.545	7.776	10.33	13.38	8.382	9.734	9.524	0.891
Colon	6.472	9.483	5.389	7.346	8.954	3.267	6.818	0.944
Spleen	2.542	5.209	2.414	2.995	3.843	1.372	3.063	0.541
Kidney	5.555	10.73	2.642	3.274	3.552	3.384	4.856	1.241
Sk. Muscle	0.853	1.792	0.806	1.019	1.609	0.529	1.101	0.202
Testis/Ovary	1.153	2.040	1.039	1.376	1.330	0.680	1.270	0.185
Blood	0.543	0.499	0.413	0.448	0.555	0.568	0.504	0.026

Table B.9 Upper gastrointestinal transit in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice, during fed-fasted conditions, in which a charcoal meal was administered by gavage to each mouse.

PEPT1$^{+/+}$		
Feeding condition	**Fed**	**Fasted**
Trial 1	83.21	84.40
Trial 2	71.43	77.78
Trial 3	71.11	79.02
Trail 4	76.06	69.84
Trial 5	72.22	75.63
Trial 6	63.57	74.49
Mean	72.93	76.86
SE	2.640	1.989

PEPT1$^{-/-}$		
Feeding condition	**Fed**	**Fasted**
Trial 1	81.89	64.90
Trial 2	65.73	73.28
Trial 3	77.94	79.07
Trail 4	78.62	71.14
Trial 5	75.17	82.05
Trial 6	76.56	72.27
Mean	75.99	73.79
SE	2.249	2.481

Table B.10 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g intravenous bolus dose of dipeptide in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice during fed-fasted conditions.

PEPT1$^{+/+}$ Fed				
PK parameter	**CL (µl/min)**	**Vdss (ml)**	**MRT (min)**	**$t_{1/2}$ (min)**
Trial 1	183.4	6.665	33.50	39.02
Trial 2	237.5	12.02	38.86	57.63
Trial 3	178.9	7.804	36.65	48.13
Trail 4	269.9	12.39	35.27	61.61
Trial 5	164.8	4.710	27.65	29.43
Trial 6	263.9	7.725	28.98	31.84
Mean	216.4	8.552	33.48	44.61
SE	18.9	1.243	1.793	5.464

PEPT1$^{+/+}$ Fasted				
PK parameter	**CL (µl/min)**	**Vdss (ml)**	**MRT (min)**	**$t_{1/2}$ (min)**
Trial 1	254.7	12.60	37.41	60.46
Trial 2	334.3	21.55	45.11	69.97
Trial 3	204.2	12.80	44.12	73.54
Trail 4	243.8	11.86	38.94	57.62
Trial 5	197.0	6.502	32.44	30.21
Trial 6	286.3	14.02	36.30	62.75
Mean	253.4	13.22	39.05	59.09
SE	21.07	1.980	1.969	6.267

PEPT1$^{-/-}$ Fed				
PK parameter	**CL (µl/min)**	**Vdss (ml)**	**MRT (min)**	**$t_{1/2}$ (min)**
Trial 1	234.1	8.399	32.76	38.57
Trial 2	250.8	9.366	34.60	36.69
Trial 3	164.5	4.583	25.54	31.22
Trail 4	217.3	8.075	36.05	33.36
Trial 5	218.0	8.456	33.14	49.20
Trial 6	252.2	9.669	35.25	38.12
Mean	222.8	8.091	32.89	37.86
SE	13.20	0.745	1.556	2.548

PEPT1$^{-/-}$ Fasted				
PK parameter	**CL (µl/min)**	**Vdss (ml)**	**MRT (min)**	**$t_{1/2}$ (min)**
Trial 1	189.8	4.387	22.14	27.11
Trial 2	222.9	7.279	30.35	37.64
Trial 3	198.0	6.290	31.64	27.14
Trail 4	233.7	8.431	32.91	38.75
Trial 5	266.6	8.057	27.08	40.76
Trial 6	212.1	6.708	29.89	33.04
Mean	220.5	6.858	29.00	34.07
SE	11.29	0.593	1.587	2.429

Table B.11 Pharmacokinetics of GlySar plasma concentrations after a 5 nmol/g oral dose of dipeptide in PEPT1$^{+/+}$ and PEPT1$^{-/-}$ mice during fed-fasted conditions.

PEPT1$^{+/+}$ Fed				
PK parameter	**$AUC_{0\text{-}360min}$ (µM · min)**	**C_{max} (µM)**	**T_{max} (min)**	**$t_{1/2}$ (min)**
Trial 1	426.1	1.696	45.00	391.9
Trial 2	431.3	1.635	60.00	385.6
Trial 3	446.9	1.685	45.00	374.2
Trail 4	369.9	1.742	30.00	314.1
Trial 5	400.1	1.563	45.00	303.1
Trial 6	416.7	1.682	60.00	331.0
Mean	415.2	1.667	47.50	350.0
SE	11.06	0.025	4.610	15.76

PEPT1$^{+/+}$ Fasted				
PK parameter	**$AUC_{0\text{-}360min}$ (µM · min)**	**C_{max} (µM)**	**T_{max} (min)**	**$t_{1/2}$ (min)**
Trial 1	690.9	2.706	60.00	402.7
Trial 2	641.5	3.175	60.00	346.5
Trial 3	518.4	2.831	45.00	165.1
Trail 4	469.5	2.624	45.00	212.3
Trial 5	454.6	2.481	45.00	319.4
Trial 6	708.0	2.987	60.00	356.6
Mean	580.5	2.801	52.50	300.4
SE	46.25	0.103	3.354	37.49

PEPT1$^{-/-}$ Fed

PK parameter	$AUC_{0\text{-}360min}$ (µM · min)	C_{max} (µM)	T_{max} (min)	$t_{1/2}$ (min)
Trial 1	269.0	1.113	60.00	323.0
Trial 2	257.3	1.112	45.00	492.1
Trial 3	244.9	1.012	45.00	415.9
Trail 4	267.3	1.005	45.00	390.3
Trial 5	306.0	1.479	30.00	275.3
Trial 6	265.0	1.159	45.00	351.8
Mean	268.3	1.147	45.00	374.7
SE	8.375	0.071	3.873	30.97

$PEPT1^{-/-}$ Fasted				
PK parameter	$AUC_{0\text{-}360min}$ (µM · min)	C_{max} (µM)	T_{max} (min)	$t_{1/2}$ (min)
Trial 1	249.5	1.068	90.00	247.4
Trial 2	339.3	1.306	45.00	416.0
Trial 3	320.0	1.270	45.00	328.4
Trail 4	311.0	1.190	45.00	383.1
Trial 5	299.4	1.207	45.00	365.1
Trial 6	293.9	1.214	60.00	390.1
Mean	302.2	1.209	55.00	355.0
SE	12.42	0.033	7.416	24.59