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Validation of Endogenous Biomarkers for Renal Tubular Transporter PEPT2

Yile Li^{1,2†}, Fenglin Ran^{1,3†}, Xueyan Gou¹, Xinan Wu^{1*}

¹ Department of Pharmacy, The First Hospital of Lanzhou University, Lanzhou 730000, China.

² School of Pharmacy, Lanzhou University, Lanzhou 730000, China.

³ Chongqing Hospital, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology 400010, China.

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ABSTRACT

Purpose: The aim of this study was to screen and validate endogenous biomarkers of renal tubular PEPT2.

Methods: Through metabolomics and literature searches, we initially identified two endogenous substrates of PEPT2: carnosine and 5-ALA. By evaluating the uptake of a PEPT2-overexpressing cell line, protein binding rates, and substrate specificity, carnosine was screened as a potential endogenous biomarker for PEPT2.

Results: Carnosine is primarily filtered by the glomeruli (with an albumin binding rate of 33%), and subsequently reabsorbed by the PEPT2 transporter, exhibiting appropriate affinity (Km of 59.04μM) and a half-life of 5.5±2.2 hours.

Conclusion: Carnosine is a suitable endogenous biomarker that can predict renal injury through the detection of its levels.

*Corresponding Author

Xinan Wu, Email: xinanwu6511@163.com, ORCID: 0000-0002-0371-0361

†These authors contributed equally to this work

Introduction

The kidney is a crucial excretory organ in the human body, mediating the elimination of various endogenous and exogenous substances.¹ The renal excretion process of drugs primarily consists of three parts: glomerular filtration, tubular secretion, and reabsorptions.² The kidney plays an irreplaceable role in the body, responsible for reabsorbing 99% of the glomerular filtrate every day, with about 60% of this process occurring mainly in the proximal tubule. This filtration includes water, inorganic salts, glucose, proteins, and drugs.³ However, renal reabsorption can be influenced by several factors, including age, disease, and drug interactions. Renal reabsorption is vital to the body, yet currently, no specific indicators exist to evaluate reabsorption function.⁴ Clinically, serum creatinine is often used to assess renal function, but its excretion involves glomerular filtration, tubular secretion, and reabsorption, making it unable to specifically reflect any one renal excretion pathway.^{5,6} Therefore, the commonly used clinical indicator of renal function, creatinine, cannot accurately assess renal reabsorption function.

The kidney mediates the elimination of many drugs and toxins from the body, and this elimination is the net result of glomerular filtration, tubular secretion, and tubular reabsorption.⁷ Approximately 32% of drugs are primarily cleared by the kidneys, with more than 90% secreted by transport proteins in the renal tubules.⁸ Among these, the peptide transporter 2 (PEPT2/SLC15A2) plays a critical role in reabsorption. PEPT2 is a high-affinity, low-capacity transporter expressed in various organs such as the kidney, brain, lung, eye, uterus, mammary gland, spleen, prostate, and skin.⁹ In the kidney, PEPT2 is expressed on the brush border of the S2 and S3 segments of the proximal tubules, mediating the reabsorption of nutrients, small peptides, and peptide-like drugs.¹⁰⁻¹³ PEPT2 mediates the reabsorption of several drugs, such as β -lactam antibiotics, angiotensin-converting enzyme inhibitors, anticancer drugs, and nucleotide drugs. PEPT2 is crucial in maintaining drug concentrations in the body, affecting drug pharmacokinetics, tissue distribution, and renal handling.^{14,15} PEPT2-mediated reabsorption helps maintain systemic drug exposure.

The relationship between endogenous biomarkers and transport proteins is closely intertwined, making them potential markers for studying human transport proteins.^{16,17} This is currently the safest and simplest method. It avoids the secondary harm that exogenous substances may cause to the body and provides an easier and more reliable clinical analysis method. Ideally, endogenous biomarkers used to evaluate PEPT2 function should be filtered primarily by the glomerulus and not secreted by the renal tubules. Compounds with a molecular weight of less than 50kDa can freely pass through the glomerulus. However, many compounds in the body can bind to albumin (molecular weight 66kDa), forming larger complexes that cannot be filtered by the glomerulus and are instead secreted through tubular secretion. Therefore, low-protein-binding compounds should be selected as candidates for tubular reabsorption markers.¹⁸⁻²⁰

This study aims to screen for potential endogenous biomarkers of PEPT2 through non-targeted metabolomics combined with literature review.²¹ Existing literature reports endogenous substrates of PEPT2, such as carnosine, Gly-Gln, and 5-ALA, but it remains unclear whether these compounds can serve as markers to evaluate PEPT2 function. Through in vitro and in vivo validation, we aim to identify biomarkers that can evaluate PEPT2-mediated drug excretion and establish a foundation for determining or adjusting clinical drug dosages.

2 MATERIALS AND METHODS

2.1 Chemicals and Reagents

Gentamicin sulfate injection (H20045446) was supplied by Shanghai Modern Hassan Pharmaceutical Co.,

Ltd (Shangqiu, China). Furosemide (F129560), *p*-aminohippurate (A112651), and inulin (I111088) were purchased from Aladdin Industrial Corporation (Shanghai, China). 5-fluorouracil (F8301) and metformin (D9351) were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China). Creatinine was provided by Sigma-Aldrich (St Louis, MO, USA). Indole-3-acetic acid was obtained from ZZBIO Co., Ltd. (Shanghai, China). All other reagents were of analytical grade and commercially available.

2.2 Animals

Male Sprague-Dawley (SD) rats (weighing 240-280 g) were purchased from the Gansu Animal Science Institute in Lanzhou, China, and housed in the SPF animal facility at Lanzhou University at a temperature of 25°C, with humidity maintained at 50% ± 5%, and free access to food and water under a 12-hour light/dark cycle. All procedures followed the NIH guidelines for the care and use of laboratory animals and were approved by the Animal Ethics Committee of the First Hospital of Lanzhou University (Approval code: LDYYLL2016-0036). The compound was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.3 Apparatus

The following equipment was used in the experiments: a time-of-flight mass spectrometry system (Pegasus HT, Leco Corp.), a carbon dioxide incubator (MCO-18AC, Panasonic), an automatic cell counter (IC1000, Shanghai Ruiyu Biotechnology Co., Ltd.), and an LC-MS/MS system (Agilent LC 1260-Agilent MS 6460, Agilent Technologies).

2.4 Construction of PEPT2 Overexpressing Cell Line and Uptake by PEPT2 Overexpressing Cells

Human renal tubular PEPT2 overexpressing cell lines were constructed using lentiviral vectors provided by GeneChem, Shanghai. Serum samples from 187 patients with chronic kidney disease (serum creatinine levels 443-706μM) treated at the Department of Nephrology of the First Hospital of Lanzhou University between July and October 2019 were collected. The samples were pooled, pre-treated with ethanol, vortexed, and centrifuged at 1500 rpm for 10 minutes. The supernatant was dried using a rotary evaporator at 45°C and 1600 rpm. The resulting serum powder was stored for long-term use. All procedures followed the ethical standards set by the Ethics Committee of the First Hospital of Lanzhou University (Approval code: NO.LDYYLL2018-32).

The HEK293T-MOCK and HEK293T-PEPT2 cell lines were seeded at a concentration of 5×10^5 cells/mL in poly-L-lysine-coated 12-well plates. After 24 hours, the complete culture medium was removed. Each tube of serum powder was dissolved in 1 mL of buffer, filtered through a 0.22μm membrane, and incubated in the cells for 30 minutes. After washing the cells with blank buffer and air drying, 200μL of purified water was added, and the cells were lysed using an ultrasonic cell disruptor. Protein concentrations were quantified using BCA, and proteins were precipitated using methanol. Gas chromatography-time-of-flight mass spectrometry (GC-TOF/MS) was then used for non-targeted metabolomics analysis to identify metabolites significantly increased in PEPT2-overexpressing cell lines.

2.5 Non-Targeted Metabolomics Analysis

Non-targeted metabolomics analysis was performed on the XploreMET platform provided by Shanghai ApBio. The samples were thawed on ice, and 800μL of pre-cooled methanol/chloroform (v/v = 3/1) was added, vortexed for 15 minutes, and centrifuged at $14,000 \times g$ at 4°C for 20 minutes. The 700μL supernatant was briefly evaporated using a Centrивap vacuum concentrator (Labconco, Kansas City, MO, USA) to remove chloroform, followed by further freeze-drying using a FreeZone freeze dryer (Labconco, Kansas City, MO, USA). The dried samples were derivatized with 50μL of methoxyamine pyridine (20 mg/mL) at 30°C for 2 hours, followed by further derivatization with 50μL of MSTFA (1% TMCS) containing fatty acid methyl esters (FAMES) at 37.5°C for 1 hour before analysis.

Gas chromatography-time-of-flight mass spectrometry (GC-TOF/MS) was performed using a Restek Rxi-5ms capillary column (30 m×250µm, 0.25µm). The inlet and interface temperatures were set to 270°C, and the ion source temperature was set to 220°C. Helium was used as the carrier gas at a flow rate of 1.0 mL/min, and a 1 µL injection volume was used. Measurements were taken in full-scan mode (m/z 50-500) using electron impact ionization (70 eV).

2.6 Quantitative Analysis by LC-MS/MS

Quantitative analysis was performed using an Agilent 6460 triple quadrupole mass spectrometer. The chromatographic column used was an Agilent ZORBAX RR StableBond C18 analytical guard column (4.6 mm×100 mm, 3.5 µm). The mobile phase consisted of 0.1% formic acid in water and methanol (40:60, v/v) at a flow rate of 0.5 mL/min. The column temperature was maintained at 40°C, and the injection volume was 2µL. The ion source was an ESI source, with a drying gas temperature of 300°C, drying gas flow rate of 7 L/min, nebulizer pressure of 45 psi, sheath gas temperature of 350°C, and sheath gas flow rate of 11 L/min. The analysis was performed in positive ion mode using multiple reaction monitoring (MRM) mode with a capillary voltage of 4000 V and a nozzle voltage of 1500 V.

Sample Preparation: Blood samples: Collected arterial blood from rats, left to stand for 2 hours, and centrifuged at 8000×g at 4°C for 10 minutes to collect the supernatant. Urine samples: Measured the weight of the urine and tube, then divided the urine into aliquots for storage at -80°C. Kidney samples: Weighed 200 mg of kidney tissue, added 400µL of purified water and magnetic beads, and used a tissue homogenizer to break down the tissue. The homogenate was centrifuged at 14,000×g at 4°C for 10 minutes, and the supernatant was collected for further analysis.

Preparation of blank matrices: Prepared blank blood, urine, and kidney homogenate samples by incubating with activated charcoal at 4°C for 12 hours, followed by centrifugation at 14,000×g at 4°C for 10 minutes. This process was repeated three times to obtain blank matrices with absorbed endogenous substances.

LC-MS Sample Processing: Added 100µL of methanol to 50µL of the cell sample, vortexed for 1 minute, and centrifuged at 14,000×g at 4°C for 10 minutes. The supernatant was filtered through a 0.22µm membrane before injection.

Serum, urine, and kidney homogenate samples: Added 150µL of methanol to 50µL of the sample, vortexed for 1 minute, and centrifuged at 14,000×g at 4°C for 10 minutes. The supernatant was filtered through a 0.22µm membrane before injection.

Calibration Curve Preparation: Weighed approximately 1.0 mg of standard, dissolved it in methanol or purified water, and stored the stock solution (1µg/mL) at -80°C. The stock solution was diluted in a 1:2 gradient to obtain ten concentrations of working solutions. Calibration curve solutions were prepared by mixing 50µL of the working solution with 50µL of the corresponding blank matrix and 100µL of methanol. The solutions were vortexed for 1 minute, centrifuged at 14,000×g at 4°C for 10 minutes, and the supernatants were filtered through a 0.22µm membrane before injection.

2.7 In Vitro Protein Binding Rate Measurement

A 4% human albumin (HSA) solution was prepared using PBS, along with a 1nM solution of the standard compound. A mixture of 360µL of 4% HSA and 40µL of the standard solution was vortexed at a low speed for 2 minutes and incubated in a shaking incubator at 37°C for 2 hours. A 300µL aliquot of the incubation solution was transferred to a 30kDa ultrafiltration tube and centrifuged at 3500×g at 37°C for 10 minutes. The filtrate was collected to measure the concentration of free drug. Additionally, 100µL of the incubation solution was used to determine the total concentration. The protein binding rate was calculated using the following formula:

Protein binding rate = (Total concentration - Free concentration) / Total concentration × 100%

2.8 Cell Uptake

Validation of differential metabolites: A 0.01% poly-L-lysine solution was prepared using sterile water, filtered through a 0.22µm membrane, and stored at 4°C. The solution can be reused after each experiment. For each well, 500µL was added to a 12-well plate, which was then incubated at 37°C for 10 minutes. The poly-L-lysine was recovered, and the plate was sterilized under UV light and air-dried. HEK293T-MOCK and HEK293T-PEPT2 cells were seeded at a concentration of 5×10^5 cells/mL, with 1 mL per well in the poly-L-lysine-coated 12-well plates. After 24 hours, the complete medium was removed, and the cells were pre-incubated with blank Krebs-Henseleit buffer (118mM NaCl, 23.8mM NaHCO₃, 4.8mM KCl, 1.0mM KH₂PO₄, 1.2mM MgSO₄, 12.5mM HEPES, 5mM glucose, and 1.5mM CaCl₂, pH7.4) for 20 minutes. The buffer was removed, and buffer containing 100µM of the differential metabolites was added. The cells were incubated for 30 minutes, then washed with blank buffer and air-dried in a sterile workbench. The cells were lysed with 200µL of purified water for 10 minutes, followed by cell disruption using an ultrasonic cell disruptor. Protein concentrations were determined by BCA assay, and proteins were precipitated with methanol. Differential metabolite concentrations were analyzed by LC-MS/MS.

Uptake experiments at 4°C and 37°C: HEK293T-MOCK and HEK293T-PEPT2 cells were seeded at 5×10^5 cells/mL in poly-L-lysine-coated 12-well plates, with 1 mL per well. After 24 hours, the complete medium was removed, and the cells were incubated with blank buffer at either 4°C or 37°C for 20 minutes. The buffer was discarded, and buffer containing 100 µM of the endogenous substrate was added. After a 30-minute incubation at 4°C and 37°C, the cells were washed with blank buffer, air-dried, and lysed with 200µL of purified water. The cells were then disrupted by an ultrasonic cell disruptor. Protein concentrations were determined by BCA assay, and the drug content was analyzed by LC-MS/MS.

Uptake inhibition experiments: HEK293T-MOCK and HEK293T-PEPT2 cells were seeded at 5×10^5 cells/mL in poly-L-lysine-coated 12-well plates, with 1 mL per well. After 24 hours, the complete medium was removed, and buffer containing 100µM of the endogenous substrate and different doses of the PEPT2 inhibitor cefadroxil (0, 100, 500µM) was added. The cells were incubated for 30 minutes at 37°C, washed with blank buffer, air-dried, and lysed with 200µL of purified water. After protein quantification by BCA assay, the drug content was analyzed by LC-MS/MS.

Transport kinetics experiments: HEK293T-MOCK and HEK293T-PEPT2 cells were seeded at 5×10^5 cells/mL in poly-L-lysine-coated 12-well plates, with 1 mL per well. After 24 hours, the complete medium was removed, and buffer containing 100µM of the endogenous substrate was added. The cells were incubated at 37°C for 2, 5, 10, 20, or 30 minutes. After incubation, the cells were washed with blank buffer, air-dried, and lysed with 200µL of purified water. After protein quantification by BCA assay, the drug content was analyzed by LC-MS/MS to determine the linear uptake time. The same procedure was followed to obtain affinity kinetics constants using 1, 5, 10, 50, 100, 500, and 1000µM of the endogenous substrate.

Specificity validation: HEK293T-MOCK and HEK293T-OAT1, OAT3, OCT2, MATE1, MRP4, URAT1, OAT4, and PEPT1 cell lines were seeded at 5×10^5 cells/mL in poly-L-lysine-coated 12-well plates, with 1 mL per well. After 24 hours, the complete medium was removed, and buffer containing 100µM of the endogenous substrate was added. The cells were incubated for 30 minutes at 37°C, washed with blank buffer, air-dried, and lysed with 200µL of purified water. Protein concentrations were determined by BCA assay, and the drug content was analyzed by LC-MS/MS.

2.9 Animal Experiments

In vivo inhibition experiment: Male SD rats (weighing 200-240 g) were randomly divided into control, cefadroxil (30 mg/kg), cefadroxil (60 mg/kg), and cefadroxil (120 mg/kg) groups, with six rats per group. The rats were orally administered cefadroxil suspensions (prepared with CMC-Na) at 30 mg/kg, 60 mg/kg, and 120 mg/kg, respectively, twice a day for seven days. The control group received an equivalent amount of 0.5% CMC-Na daily. On the sixth day, the rats were fasted for 12 hours. On the seventh day, after the last dose, urine was collected at 0-2, 2-4, 4-6, 6-8, 8-10, and 10-12 hours. Another group of rats was anesthetized 1 hour after the last dose using pentobarbital sodium (5 mg/100 g) for blood and kidney collection.

d4-carnosine administration experiment: Male SD rats (weighing 200-240 g) were randomly divided into control and cefadroxil (120 mg/kg) groups, with six rats per group. The rats were orally administered 0.5% CMC-Na or cefadroxil suspension (120 mg/kg), twice a day for seven days. On the seventh day, at the time of the last dose, 2 mg/kg of d4-carnosine was injected via the tail vein, and urine was collected at 0-2, 2-4, 4-6, 6-8, 8-10, and 10-12 hours. The pharmacokinetic parameters of d4-carnosine were calculated using DAS2.0.

Carnosine excretion in kidney injury model: Male SD rats (weighing 200-240 g) were randomly divided into control, Genta (50 mg/kg), Genta (75 mg/kg), and Genta (100 mg/kg) groups, with six rats per group. The rats were intraperitoneally injected with saline or gentamicin at 50 mg/kg, 75 mg/kg, and 100 mg/kg, respectively, once a day for seven days. After a 12-hour fast on the seventh day, urine was collected at 0-2, 2-4, 4-6, 6-8, 8-10, and 10-12 hours. Another group of rats was anesthetized using pentobarbital sodium (5 mg/100 g) for blood and kidney collection.

2.10 q-PCR

Kidney tissues (10-20 mg) were extracted, and total RNA was isolated using the Eastep® Super Total RNA Extraction Kit. RNA concentration was measured with a nucleic acid quantification instrument, and the RNA concentration should be between 1.5-3.0 µg/mg. The A260/A280 absorbance ratio should be between 1.9-2.1, and the A260/A230 absorbance ratio should be between 2.0-2.5. RNA samples were diluted to the same concentration (50-150 ng/µL) with nuclease-free water. cDNA was synthesized from RNA using the RevertAid First Strand cDNA Synthesis Kit by reacting at 42°C for 60 minutes, heating at 70°C for 5 minutes, and then cooling to room temperature. The samples and corresponding primers were prepared according to the UltraSYBR Mixture kit instructions, and q-PCR was conducted using the BIORAD CFX96 real-time PCR instrument. The reaction conditions were as follows: pre-denaturation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute for 40 cycles. A melting curve analysis was performed (95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, and 60°C for 15 seconds), followed by cooling at 25°C for 5 minutes. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.11 Statistical Analysis

Pharmacokinetic parameters were calculated using DAS 2.0, and GraphPad Prism 7 software was used for graph plotting. Statistical analysis was performed using Student's t-test (two-tailed). Data were expressed as mean $\pm$ standard deviation (mean $\pm$ S.D.), and differences were considered statistically significant if $p < 0.05$ or $p < 0.01$.

3 RESULT

3.1 PEPT2-Mediated Uptake and Metabolomics Results

A PEPT2-overexpressing cell line was constructed by transfecting HEK293T cells with lentiviral vectors carrying the PEPT2 gene. HEK293T-MOCK and HEK293T-PEPT2 cells were imaged using an inverted

fluorescence microscope. Under normal light, HEK293T-MOCK and HEK293T-PEPT2 cells were observed. Under blue light (excitation wavelength 460-495 nm), the green fluorescent protein (GFP) carried by HEK293T-MOCK and HEK293T-PEPT2 cells emitted green fluorescence. Nearly all cells in the same field of view displayed fluorescence, indicating successful transfection. The transfected cells expressed the flag protein, which was verified by Western blot using an anti-flag antibody. The experiment confirmed the successful transfection of the flag gene carried by the lentivirus, with consistent results across three repetitions.

In a cell uptake experiment using cefadroxil, a classic PEPT2 substrate, it was observed that the uptake of cefadroxil in HEK293T-PEPT2 cells was significantly higher compared to the HEK293T-MOCK group. Specifically, cefadroxil uptake in HEK293T-PEPT2 cells increased 14-fold compared to the HEK293T-MOCK group, demonstrating that the PEPT2-overexpressing cell line was successfully constructed and functionally active(S1).

After incubating the PEPT2-overexpressing cell line with lyophilized serum powder from chronic kidney disease patients, a non-targeted metabolomics study was conducted, followed by data analysis using OPLS-DA and volcano plots. The results showed that 12 differential metabolites were increased in the PEPT2-overexpressing cell line, namely: serine, threonine, pyroglutamic acid, leucine, isoleucine, trans-4-hydroxyproline, glutamic acid, aspartic acid, valine, alanine, asparagine, and inositol (Table.1).

Table.1 Differential Metabolites Identified through Non-Targeted Metabolomics in PEPT2-Overexpressing Cells Incubated with Serum from Chronic Kidney Disease Patients (n=6)

Compounds	MEAN Control	MEAN PEPT2	p value
Serine	72.85242166	77.78333572	0.002164502
Threonine	52.33440495	58.11325914	0.002164502
Pyroglutamic acid	399.6293722	464.2265304	0.002439169
Leucine	67.14061643	75.12023646	0.005507556
Isoleucine	25.26752634	27.77461335	0.009027617
trans-4-Hydroxyproline	2.588127589	3.674794775	0.010159096
Glutamic acid	51.94834449	59.71560799	0.010562766
Aspartic acid	13.75212253	16.92663122	0.013213651
Valine	61.8222692	66.91831415	0.013620759
Alanine	687.7785316	744.0111224	0.026569568
Asparagine	9.110262669	10.21034908	0.035155405
Inositol	449.9021993	508.2839187	0.041125541

3.2 Literature Search Results

Using the specified keywords, searches were conducted in both Chinese and international databases. The Chinese databases yielded carnosine and 5-ALA, while the international databases identified carnosine,^{22,23} Gly-Sar, Gly-Gln, and 5-ALA. In total, four endogenous substrates of PEPT2 were identified: carnosine, Gly-Gln,²⁴ Gly-Sar,²⁵ and 5-ALA.²⁶

3.3 Validation of Potential Biomarkers

The uptake results of differential metabolites in the PEPT2-overexpressing cell line. Compared to HEK293T-MOCK, there was no significant difference ($p > 0.05$) in the uptake of serine, threonine, pyroglutamic acid, leucine,

isoleucine, trans-4-hydroxyproline, glutamic acid, aspartic acid, valine, alanine, asparagine, and inositol in the HEK293T-PEPT2 cell line. For the PEPT2 endogenous substrates identified through the literature search, the uptake of carnosine and 5-ALA was significantly increased in the PEPT2-overexpressing cell line compared to the control group ($p < 0.01$). Therefore, carnosine and 5-ALA can be considered potential endogenous biomarkers for PEPT2(Fig.1).

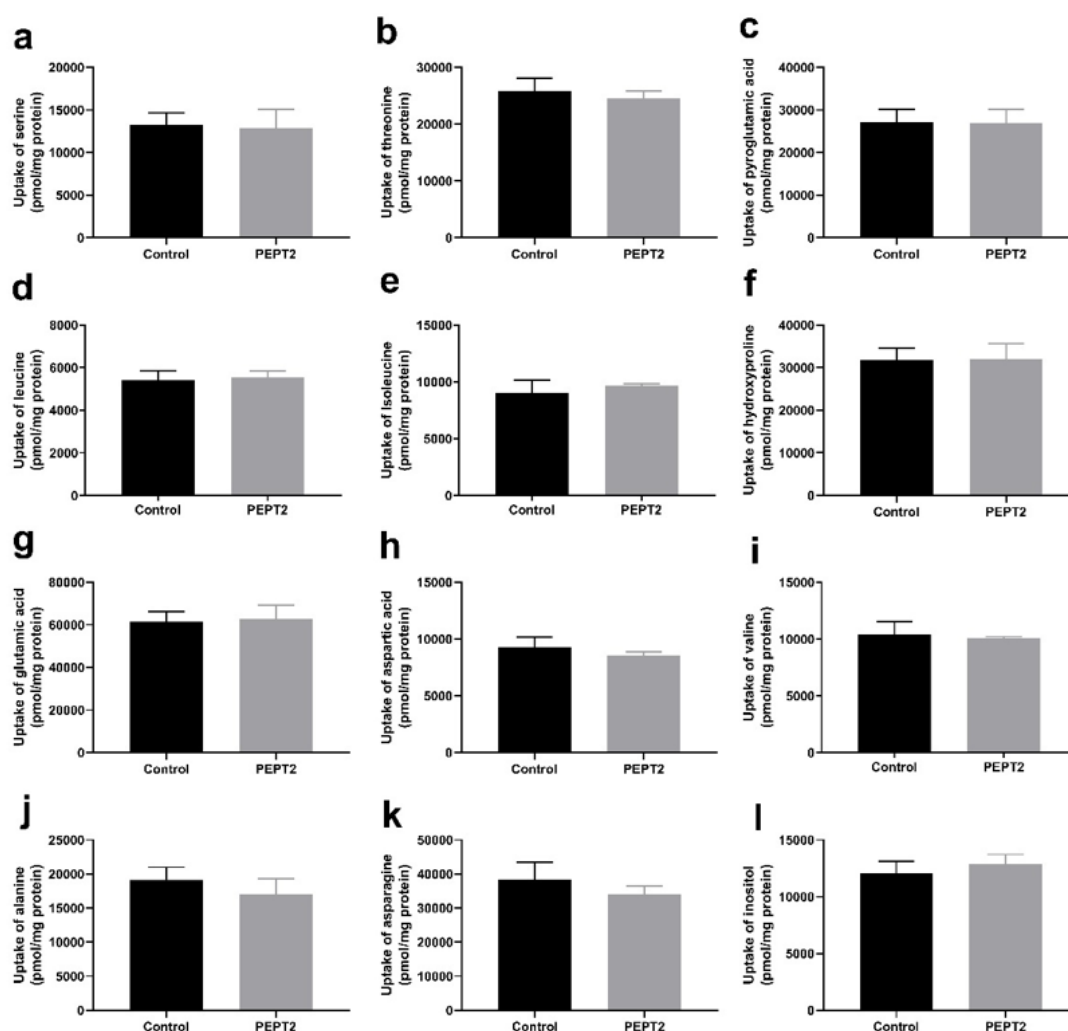


Figure.1 Uptake Results of Differential Metabolites in HEK293T-MOCK and HEK293T-PEPT2 Cells (n=6; mean $\pm$ S.D., compared to the control group)

3.4 In Vitro Validation of Potential Biomarkers

In vitro albumin binding rate experiment(S2): The in vitro incubation of endogenous substrates with albumin at 37°C showed the results in Table 2. The binding rates of carnosine and 5-ALA to albumin were $33 \pm 9\%$ and $9 \pm 2\%$, respectively. Both showed relatively low binding rates to HSA in vitro, indicating that they can be filtered through the glomerulus.

Table 2. Protein Binding Rates of Potential Biomarkers in Vitro

Endogenous substance	HAS binding <i>in vitro</i> (%) (n=5)
Carnosine	33 ± 9
Gly-Sar	0
5-ALA	9 ± 2

Uptake Experiment at 4°C and 37°C: At both 4°C and 37°C, the uptake of carnosine and 5-ALA in HEK293T-PEPT2 cells was significantly increased compared to the control group ($p < 0.01$). Furthermore, compared to the uptake at 4°C, the uptake at 37°C in the PEPT2-overexpressing cell line was even more pronounced ($p < 0.01$).

Inhibition Experiment with Different Concentrations (S3): The PEPT2 inhibitor cefadroxil significantly inhibited the uptake of carnosine and 5-ALA mediated by PEPT2 ($p < 0.01$), and the uptake of these substrates showed a dose-dependent relationship with the concentration of cefadroxil.

Transport Kinetics (Fig.2): The uptake of Gly-Sar in the PEPT2-overexpressing cell line showed a Km of 299.6μM and a Vmax of 595.8pmol/mg protein/min. The linear uptake time for 5-ALA was also 0-5 minutes, with a Km of 24,886μM and a Vmax of 17,515pmol/mg protein/min. The linear uptake time for carnosine was 0-5 minutes, and the concentration-dependent uptake results showed a Km of 59.4μM and a Vmax of 2003pmol/mg protein/min. The uptake of carnosine in the PEPT1-overexpressing cell line showed a Km of 127.2μM and a Vmax of 0.06932pmol/mg protein/min.

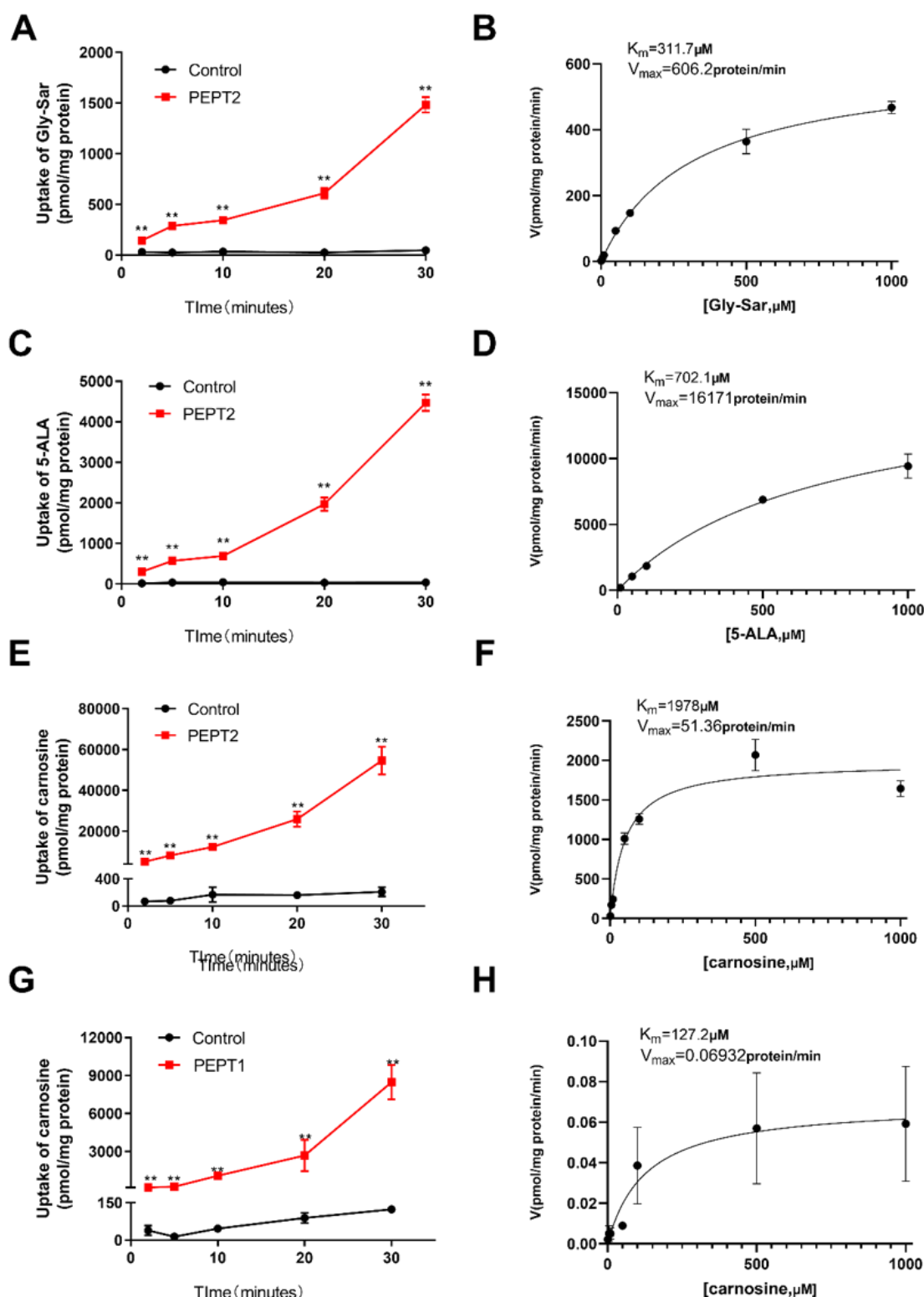


Figure.2 Time-Dependent (a) and Concentration-Dependent (b) Uptake of Gly-Sar in HEK293T-PEPT2 Cells (n=6, mean $\pm$ S.D., **p < 0.01 compared to the control group); Time-Dependent (c) and Concentration-Dependent (d) Uptake of 5ALA in HEK293T-PEPT2 Cells (n=6, mean $\pm$ S.D., **p < 0.01 compared to the control group); Time-Dependent (e) and Concentration-Dependent (f) Uptake of Carnosine in HEK293T-PEPT2 Cells (n=6, mean $\pm$ S.D., p < 0.01 compared to the control group); Time-Dependent (g) and Concentration-Dependent (h) Uptake of Carnosine in HEK293T-PEPT1 Cells (n=6, mean $\pm$ S.D., p < 0.01 compared to the control group)

Specificity Validation (Fig.3): The uptake of carnosine and 5-ALA was evaluated using the overexpressing cell lines HEK293T-OAT1, OAT3, OCT2, MATE1, MRP4, URAT1, OAT4, and PEPT1. The results indicate that

carnosine is neither taken up nor excreted via OAT1, OAT3, OCT2, MATE1, MRP4, URAT1, or OAT4, but can be taken up by PEPT1. The uptake of 5-ALA is mediated by both OAT1 and PEPT1 transporters. Therefore, carnosine can serve as a potential endogenous biomarker for PEPT2.

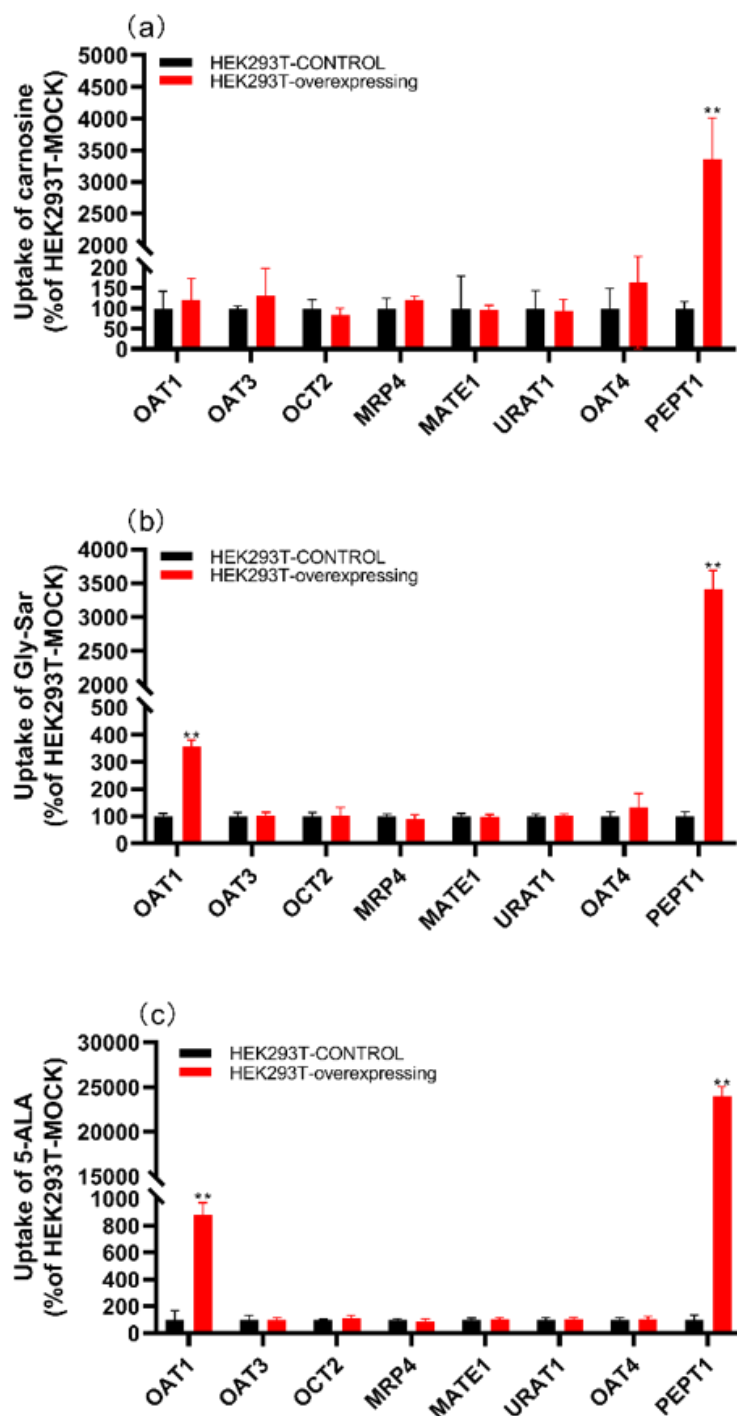


Figure.3 Uptake of carnosine(a) Gly-Sar(b) and 5-ALA(c) in overexpressing cell lines HEK293T-OAT1, OAT3, OCT2, MRP4, MATE1, URAT1, OAT4, and PEPT1 (n=6; mean $\pm$ S.D., **indicates p<0.01 compared to the control group). Uptake of Gly-Sar in overexpressing cell lines HEK293T-OAT1, OAT3, OCT2, MRP4, MATE1, URAT1, OAT4, and PEPT1 (n=6; mean $\pm$ S.D.)

3.5 In Vivo Validation of Potential PEPT2 Biomarkers

Based on the screening and validation using the PEPT2 overexpressing cell line, carnosine can be considered an endogenous biomarker for PEPT2. Therefore, further validation was conducted in rats using a PEPT2 inhibitor. After administering different doses of cefadroxil to the rats (Fig. 4-a), the serum levels of carnosine at different concentrations of cefadroxil indicated no significant changes compared to the control group ($p>0.05$). The cumulative urinary excretion of carnosine (Fig. 4-b) significantly increased in all dosage groups compared to the control group ($p<0.01$) and was dose-dependent concerning cefadroxil. Figure (Fig. 4-c) shows the renal concentration of carnosine, which also exhibited no significant changes compared to the control group ($p>0.05$). Biochemical assay results (Fig. 4-d-f) indicated that the serum levels of creatinine and urea nitrogen showed no significant changes ($p>0.05$), while cystatin C levels increased in the group receiving 60 mg/kg of cefadroxil ($p<0.05$).

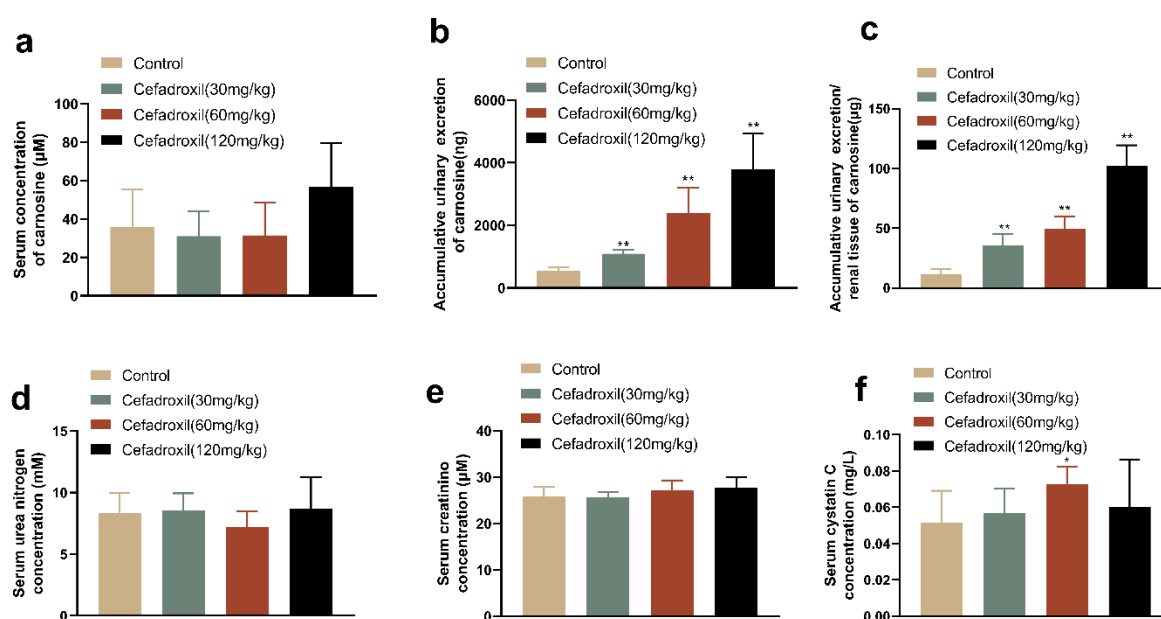


Figure 4 The effects of the PEPT2 inhibitor cefadroxil on serum levels of carnosine and cumulative urinary excretion in rats (a: Carnosine serum concentration; b: Cumulative urinary excretion of carnosine; c: Renal concentration of carnosine; $n=6$; mean $\pm$ S.D., **indicates $p<0.01$ compared to the control group). The effects of the PEPT2 inhibitor cefadroxil on serum biochemical levels in rats (d: Serum urea nitrogen levels; e: Serum creatinine levels; f: Serum cystatin C levels; $n=6$; mean $\pm$ S.D., *indicates $p<0.05$ compared to the control group).

The cumulative urinary excretion curve of d4-carnosine after administration of the inhibitor (S4) shows that the elimination half-life ($t_{1/2}$) of d4-carnosine in rats is 5.5 ± 2.2 hours. After administering 120 mg/kg of cefadroxil as an inhibitor, the cumulative urinary excretion of d4-carnosine significantly increased ($p<0.01$), with an elimination half-life of 1.2 ± 0.1 hours.

In the acute renal injury model induced by different doses of gentamicin, the kidney HE staining results are shown. The histopathological results indicate that the degree of kidney injury increases with the rising doses of gentamicin, showing cell swelling, renal tubular dilation, desquamation of renal tubular epithelial cells, and widespread inflammatory infiltration. Regarding the biochemical indicators in serum (Fig.5), there were no significant changes in the biochemical indicators in the 50 mg/kg gentamicin group, while creatinine and urea

nitrogen levels significantly increased in the 75 mg/kg and 100 mg/kg dose groups ($p < 0.01$). Cystatin C levels also significantly increased in the 100 mg/kg dose group ($p < 0.01$), indicating successful establishment of the acute renal injury model induced by gentamicin.

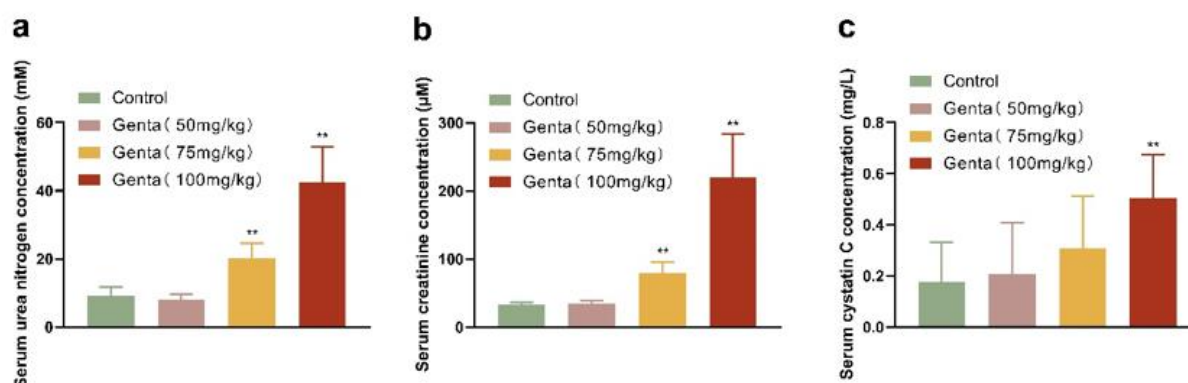


Figure 5 HE staining of kidneys in acute renal injury models induced by different doses of gentamicin. The effects of different doses of gentamicin on serum biochemical levels in rats (a: Serum urea nitrogen levels; b: Serum creatinine levels; c: Serum cystatin C levels; $n=6$; mean $\pm$ S.D., *indicates $p < 0.05$, **indicates $p < 0.01$ compared to the control group).

The serum levels of carnosine are shown (Fig. 6-a), with a significant increase in the 100 mg/kg gentamicin dose group ($p < 0.01$), while no significant changes were observed in the other dose groups. (Fig. 6-b) shows the cumulative urinary excretion of carnosine, where there was no significant difference in the 50 mg/kg gentamicin group ($p > 0.05$). Compared to the control group, the 75 mg/kg dose group showed an increase in cumulative urinary excretion of carnosine, with increases at 2, 4, 10, and 12 hours ($p < 0.05$), and significant increases at 6 and 8 hours ($p < 0.01$). In contrast, the 100 mg/kg dose group exhibited a decrease in cumulative urinary excretion of carnosine compared to the control group, with a reduction at 6 hours ($p < 0.05$) and significant decreases at 8, 10, and 12 hours ($p < 0.01$). (Fig. 6-c) illustrates the renal concentration of carnosine, which decreased in the 50 mg/kg and 75 mg/kg gentamicin dose groups ($p < 0.05$), while the 100 mg/kg dose group showed a significant decline. The cumulative urine volume over 12 hours in the 100 mg/kg gentamicin dose group was significantly reduced ($p < 0.01$), while no significant changes were observed in the other groups (Fig. 6-d).

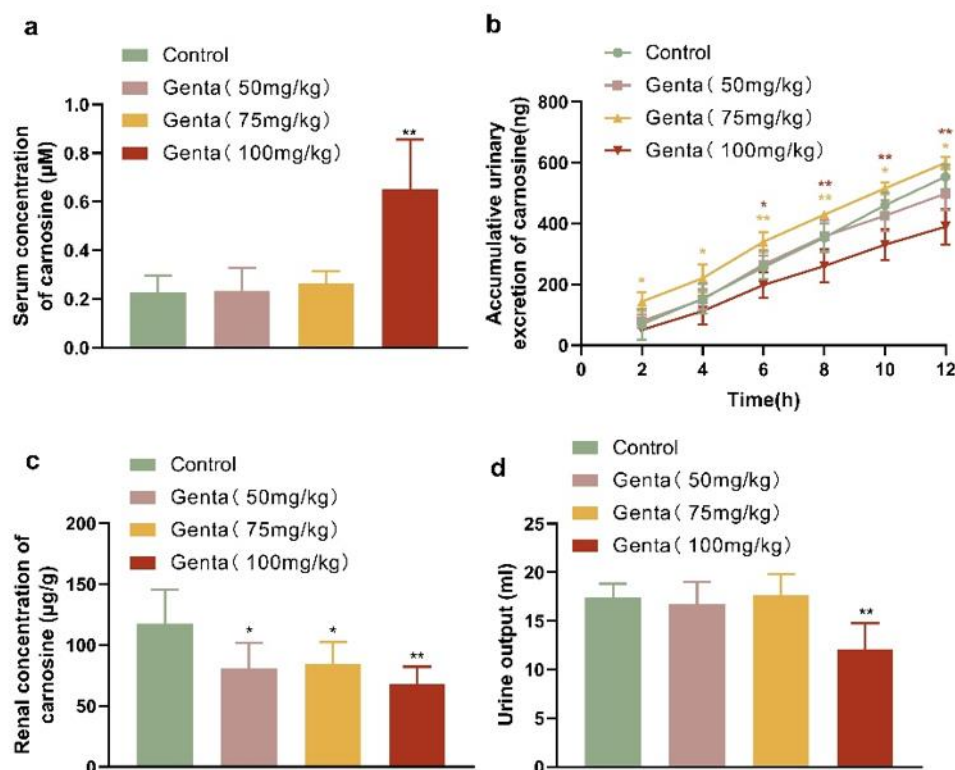


Figure 6 The effects of different doses of gentamicin-induced acute renal injury models on serum concentration of carnosine in rats (a), cumulative urinary excretion curve of carnosine (b), renal concentration of carnosine (c), and urine volume (d) (n=6; mean $\pm$ S.D., *indicates $p < 0.05$, **indicates $p < 0.01$ compared to the control group).

In the acute renal injury model induced by different doses of gentamicin, the expression of renal PEPT2 protein mRNA is shown. The results indicate that the expression of PEPT2 protein mRNA is decreased in the 100 mg/kg gentamicin dose group ($p < 0.05$). The specificity of the GAPDH and PEPT2 primers is presented, along with the melt curves for GAPDH and PEPT2 primers.

4 Discussion

Renal tubular secretion and reabsorption are mediated by numerous transporters. Renal tubular secretion is primarily facilitated by uptake transporters located on the basolateral membrane of renal tubular epithelial cells and efflux transporters at the brush border of the renal tubular epithelial cells. These transporters are responsible for the uptake of drugs from the blood into the cells and the excretion of intracellular drugs into the tubular lumen. Key transporters include organic anion transporters (OATs), organic cation transporters (OCTs), organic anion transporting polypeptide 4C1 (OATP4C1), multidrug and toxin extrusion proteins (MATEs), multidrug resistance-associated proteins (MRPs), and P-glycoprotein (P-gp). Additionally, the reabsorption process in the renal tubules is mediated by uptake transporters located at the brush border, such as peptide transporters (PEPTs), urate transporter 1 (URAT1), organic anion transporter 4 (OAT4), and glucose transporter 9 (GLUT9).²⁷

Currently, the commonly used biomarker in clinical practice, creatinine, primarily assesses glomerular filtration function.²⁸ Carnosine is a key biomarker for diabetic nephropathy.²⁹ Supplementation with carnosine (1000 mg/day for 12 weeks) can significantly reduce the urine albumin-to-creatinine ratio (UACR). Carnosine also significantly decreases the levels of serum advanced glycation end products (AGEs) and oxidative stress markers (MDA).³⁰ In the subgroup of diabetic patients not taking ACEIs or ARBs (standard renal protective drugs), carnosine supplementation significantly reduced urinary albumin excretion. It cannot accurately assess the overall status of kidney function. Creatinine is a stable metabolic product of muscle, primarily produced from food

metabolism and synthesized in muscle tissue. Creatinine is mainly formed through the dehydration and cyclization of creatine and phosphocreatine, with approximately 1.7% of creatine being catalyzed to creatinine daily. Additionally, muscle is the primary site for creatine synthesis. As a terminal metabolic product, creatinine is primarily excreted in its original form via the kidneys. The view that glomerular filtration is the main route of creatinine excretion is widely recognized; however, some studies suggest that creatinine may also undergo renal tubular secretion.^{31,32} This may involve the secretion by transporters such as OCT,^{33,34} OAT1, OAT3,³⁵ OAT2,³³ MATE1 and MATE2-K.³⁶ However, other studies have reached opposite conclusions, suggesting that the transporters OCT2,³¹ OAT1³⁷ and OAT2³² are not involved in the transport of creatinine. Our previous research has demonstrated that creatinine is secreted via OCT2.³⁸ In addition, research by Imamura et al. found that creatinine is also a substrate of OAT4, suggesting that there may be a small amount of reabsorption of creatinine.³² Apart from this, OAT1 and OAT3 are located on the blood side and serve as the primary gateways for drugs and toxins to enter the kidneys for clearance.³⁹ OAT4, on the other hand, is located on the urine side and is more involved in reabsorption.⁴⁰ Therefore, when discussing the systemic clearance of most exogenous drugs/toxins by the kidneys, OAT1 and OAT3 are the transporters of primary concern.

Thus, it is evident that there is still controversy regarding the mechanisms of creatinine excretion in the kidneys. The renal excretion of creatinine may involve three pathways: glomerular filtration, renal tubular secretion, and reabsorption. As such, creatinine may reflect a composite value that includes all three excretory routes, but it cannot specifically indicate any one of these pathways. Therefore, the current clinical marker for assessing kidney function, creatinine, cannot be used to evaluate renal reabsorption function. Reabsorption plays a crucial role in the kidneys; however, there are currently no indicators available in clinical practice to assess tubular reabsorption function. The PEPT2 transporter plays a significant role in renal reabsorption and mediates the reabsorption of various drugs.⁴¹ Therefore, accurately assessing the function of PEPT2 is crucial for precise dosing and predicting drug-drug interactions.

In adult human kidneys, the PEPT2 protein is specifically expressed in the S3 segment of the proximal tubules, while it is almost not expressed in the S1 and S2 segments.⁴² In rat kidneys, PEPT2 mRNA and protein are expressed in both the S2 and S3 segments of the proximal tubules.⁴³ Despite the differences in expression locations, the S3 segment is a common region with high PEPT2 expression in both species. Therefore, using deuterated creatine to probe the functional integrity of the S3 segment remains reasonable and effective in cross-species studies. This study shows, the functionality of the PEPT2-overexpressing cells, confirms successful transfection and good functionality. To improve the screening hit rate, it is essential to start with a sample rich in various endogenous compounds. Therefore, this study selected serum from patients with chronic kidney disease, as these patients have renal dysfunction and exhibit a significant accumulation of endogenous substances in their serum.⁴⁴ To identify new endogenous substrates, we employed non-targeted metabolomics. A total of 12 differential metabolites were screened, along with 3 endogenous compounds obtained through literature searches, and the PEPT2-overexpressing cell line was used for reuptake verification. The results indicated that the metabolomics analysis did not yield suitable endogenous biomarkers; however, significant differences were observed for carnosine and 5-ALA in the PEPT2-overexpressing cell line. The functionality of the PEPT2-overexpressing cell line was consistently good during each cell uptake experiment. The potential reasons for the failure of the metabolomics screening could be attributed to the non-targeted metabolomics approach, which focuses on overall analysis. Despite its capability to detect signals from a large number of metabolites and covering a broad range, it suffers from poor sensitivity and quantitative accuracy, leading to false positive results

that rendered the screening ineffective. Furthermore, it is important to acknowledge a methodological limitation of our screening strategy. While non-targeted metabolomics provided a broad, hypothesis-generating approach for identifying potential PEPT2 substrates, the initial screen itself did not yield viable candidates. The subsequent identification of carnosine and 5-ALA relied primarily on a literature search rather than a confirmatory, targeted re-analysis of our metabolomics data. Therefore, our study lacks orthogonal validation of the non-targeted platform for these specific biomarkers. Additionally, the low content of nucleoside compounds and inadequate sample preprocessing may have contributed to the inability to detect effective PEPT2 endogenous substrates. Ultimately, the results demonstrated significant differences for both carnosine and 5-ALA, indicating that they are substrates of PEPT2.

Carnosine (β -alanyl-L-histidine) was discovered in 1900 and is a rich endogenous nitrogen-containing compound found in meat.⁴⁵ Carnosine is synthesized in the body from β -alanine and histidine through the action of carnosine synthase (CARNS), and it can be degraded by serum carnosinase-1 (dipeptide carnosinase 1, CN-1).⁴⁶ Carnosine is present in various meats, with the highest concentration found in red meat, making it a primary dietary source of carnosine.⁴⁷ β -Alanine is a precursor amino acid that is synthesized only in the liver through the degradation of uracil or supplementation.⁴⁸ Numerous studies have shown that supplementation with β -alanine alone can increase the concentration of carnosine in various tissues. Carnosine is primarily distributed in the skeletal muscle and nervous tissue of mammals.^{49,50} Additionally, it is widely found in the brain, cerebrospinal fluid, kidneys, liver, spleen, and heart. As an endogenous substance, carnosine has various biological functions in the body,^{51,52} such as buffering acidity and alkalinity, anti-inflammatory effects, antioxidant properties, anti-aging effects, and anti-tumor activities.⁵³ Research has shown that carnosine can promote the proliferation and differentiation of osteoblasts, inhibit osteoclasts, and protect chondrocytes. Additionally, carnosine can regulate the cell cycle of osteoprogenitor cells and the differentiation of bone marrow mesenchymal stem cells, accelerate fracture healing, and delay the progression of bone tumors.⁵⁴⁻⁵⁶ Carnosine can protect against renal injury in patients with diabetic nephropathy by scavenging reactive oxygen species.⁵⁷ Studies have shown that the pharmacokinetic parameters of carnosine in the choroid plexus are V_{max} of 73.9 pmol/mg/min and K_m of 39.3 mM, indicating a good affinity with the PEPT2 transporter.^{22,58}

5-ALA is a precursor of porphyrin and heme, and it is used as a photosensitizer in photodynamic therapy for various tumors. In PEPT2 knockout mice, the uptake of 5-ALA by the choroid plexus was reduced by 92%, indicating that the uptake of 5-ALA in the choroid plexus is primarily mediated by PEPT2.²⁶ Other studies have indicated that PEPT2 is also the primary transporter for the uptake of 5-ALA in astrocyte.³⁶

We conducted albumin assays, in vitro cell validations, transport kinetic parameter tests, and specificity tests for carnosine and 5-ALA. The in vitro results indicated that both are low-protein-binding compounds and endogenous substrates of PEPT2, with carnosine demonstrating appropriate affinity for the PEPT2 transporter. Additionally, biomarkers for PEPT2 should possess specific specificity exclusive to PEPT2 and not be mediated by other transporters. Due to the numerous transporters present in the human kidney, we could not construct them one by one; therefore, this study focused on transporters that are highly expressed in the kidneys to validate the specificity of these endogenous biomarkers. The specificity assessment included common renal uptake transporters OAT1, OAT3, and OCT2, efflux transporters MATE1 and MRP4, as well as reabsorption transporters URAT1, OAT4, and PEPT1. Carnosine was found to be exclusively reabsorbed by PEPT1, without mediation by any of the other seven transporters. Peptide transporter 1 (PEPT1/SLC15A1) shares a high degree of homology with PEPT2, and their substrates significantly overlap; the affinity of PEPT2 for substrates is 10-15 times greater than that of

PEPT1.⁵⁹ Furthermore, because PEPT1 is expressed less in the kidneys, studies have shown that the transporter primarily responsible for the reabsorption of peptide substances in the kidneys is PEPT2, rather than PEPT1.⁶⁰⁻⁶² Therefore, carnosine can be considered a potential biomarker for the PEPT2 transporter.

To observe whether the excretion of carnosine in vivo can be influenced by inhibitors, different doses of the PEPT2 inhibitor cefadroxil were administered to rats for in vivo experiments.⁶³ Pathological and biochemical indicators revealed that administering different doses of cefadroxil for 7 consecutive days did not cause renal damage in the rats, ruling out changes in carnosine levels due to renal injury. Continuous administration of various doses of cefadroxil for 7 days significantly increased the cumulative urinary excretion of carnosine, indicating that cefadroxil reduces the reabsorption of carnosine by competitively inhibiting the PEPT2 transporter, resulting in a significant increase in urinary excretion. However, there were no significant changes in the serum levels and kidney concentrations of carnosine, and the specific reasons for this remain to be further investigated.

As an endogenous substance, carnosine is in a dynamic equilibrium state in the body, making it difficult to measure its half-life. Therefore, we administered exogenous d4-carnosine to determine this parameter. Under the inhibitory effect of cefadroxil, the urinary excretion of d4-carnosine increased, and its clearance half-life was significantly shortened, further confirming that carnosine is a substrate of PEPT2. The half-life of d4-carnosine is 5.5 hours; if the half-life of an endogenous substrate is too long (greater than 28 hours), it may not reflect changes in transporter function in a timely manner. Conversely, if the half-life is too short, the fluctuations in its concentration within the body may be too great to reliably indicate changes in transporter function. Therefore, it can be concluded that carnosine possesses an appropriate half-life.

Finally, we examined the changes in the levels of potential endogenous biomarkers for PEPT2 in the context of renal injury. In a renal injury model induced by different doses of gentamicin, pathological and biochemical results indicated that there was no significant renal damage in the 50 mg/kg gentamicin group, while the 75 mg/kg and 100 mg/kg groups exhibited notable damage to the structure of renal tubular cells, suggesting successful construction of the renal injury model. Serum carnosine levels showed no significant changes in the 50 mg/kg and 75 mg/kg gentamicin groups; however, in the 100 mg/kg group, serum carnosine levels significantly increased, and urine volume significantly decreased, indicating severe renal damage and a decline in the glomerular filtration rate, which led to a marked reduction in the glomerular filtration of carnosine and a substantial increase in its serum levels. The cumulative urinary excretion of carnosine and its kidney concentration showed no significant changes in the 50 mg/kg gentamicin group, although kidney concentrations decreased. In the 75 mg/kg gentamicin group, damage to renal tubular cells reduced tubular reabsorption function, leading to an increase in the cumulative urinary excretion of carnosine, while kidney concentrations also decreased. In the 100 mg/kg gentamicin group, severe renal damage and reduced glomerular filtration function resulted in decreased blood volume filtered by the glomeruli, significantly lowering the cumulative urinary excretion of carnosine. This may be related to the marked decrease in urine volume at this dosage. The significant reduction in kidney concentrations indicates exacerbated damage to reabsorption function. Results from qPCR experiments on the rat kidneys revealed that as the degree of renal injury increased, the expression of PEPT2 protein mRNA gradually decreased.

In addition, AUC and systemic or renal clearance of d4-carnosine were not determined because the study was designed primarily to assess urinary excretion and elimination half-life, and the sampling schedule and available resources were insufficient for a comprehensive pharmacokinetic analysis. Therefore, although the increased urinary excretion and shortened elimination half-life support the involvement of PEPT2 in carnosine reabsorption, the absence of AUC and clearance data limits the quantitative interpretation and generalizability of these findings. Future studies should include intensive plasma sampling and noncompartmental analysis to determine AUC and

clearance.

5. Conclusion

This study identified carnosine and 5-ALA as potential biomarkers for PEPT2 through non-targeted metabolomics analysis and literature review. Subsequent in vivo and in vitro evaluations revealed that carnosine has a low protein binding rate, can be filtered through the glomeruli, and possesses suitable sensitivity and affinity. Although carnosine is also a substrate for PEPT1, its expression in the kidneys is relatively low, and PEPT2 plays a primary role in renal reabsorption, without mediation by other major renal transporters. The urine accumulation/excretion of carnosine changes correspondingly with different doses of inhibitors or gentamicin in injury models, which can sensitively reflect the status of tubular reabsorption function. While interpreting our findings, it is also crucial to contextualize the strength of the existing evidence that underpins the current PEPT2 research field. Several key studies cited here, although instrumental in establishing PEPT2's substrate profile and function, possess inherent methodological limitations. Many pivotal in vivo studies rely on knockout mouse models, which, while powerful for establishing function, may not fully recapitulate the complex physiology and compensatory mechanisms of the human kidney. Furthermore, some investigations may be constrained by factors such as small sample sizes, validation in single cell-line systems, or a lack of independent reproducibility. Our work strives to provide a more robust evidence chain for carnosine as a biomarker through the integration of clinical samples, multiple cell models, and animal experiments.

Furthermore, carnosine has a half-life of 5.5 hours, and its sample preprocessing is simple, with a sensitive method that is easy for clinical detection. Therefore, carnosine holds promise for evaluating the function of PEPT2 in renal tubular reabsorption and may provide a reference for determining or adjusting the dosage of its substrate medications. Our identification of carnosine as an endogenous biomarker for PEPT2 not only provides a new tool for assessing renal reabsorption function but also holds significant translational implications for PEPT2-based targeted drug development. For instance, when designing peptide or RNA therapeutics targeting the central nervous system or the kidneys, PEPT2 can be leveraged as a potential target to enhance drug enrichment in specific tissues (e.g., the kidney) or to aid its passage across the blood-brain barrier. However, this strategy faces several challenges: First, the precise delivery and retention of drugs in the target organ (e.g., crossing the blood-brain barrier or avoiding rapid excretion in urine) remains a significant hurdle. Second, given the broad tissue expression of PEPT2, potential off-target effects must be vigilantly considered. Third, especially for RNA or novel peptide therapeutics, their unique pharmacokinetic profiles, immunogenicity, and complex manufacturing processes will face stringent regulatory scrutiny. Therefore, systematically addressing these delivery, safety, and regulatory hurdles is crucial for translating PEPT2-targeting strategies into clinical practice.

Authors' Contribution

Conceptualization: Xinan Wu.

Data curation: Yile Li.

Formal analysis: Fenglin Ran.

Investigation: Xueyan Gou.

Project administration: Yile Li.

Resources: Kannan Yile Li.

Software: Ahmed Fenglin Ran.

Supervision: Fenglin Ran.

Visualization: Yile Li.

Writing—original draft: Yile Li.

Writing—review & editing: Fenglin Ran.

Ethical Approval

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Lanzhou University

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