Identification and validation of active ingredient in cerebrotein hydrolysate-I based on pharmacokinetic and pharmacodynamic studies

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Abbreviations: AAs, amino acids; AUC, area under the curve; BBB, blood-brain barrier; CH, cerebrotein hydrolysate; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; I/R, ischemia/reperfusion; MRT, mean residence time; NSS, neurological severity scores; OA, occipital artery; OGD/R, oxygen-glucose deprivation/reperfusion; PCA, principal component analysis; Papp, apparent permeabilities; ROS, reactive oxygen species; TEER, transepithelial electrical resistance; TTC, Triphenyltetrazolium chloride
Abstract

Cerebrotein hydrolysate-1 (CH-1), a mixture of small peptides, polypeptides and various amino acids derived from porcine brain, has been widely used in the treatment of cerebral injury. However, the bioactive composition and pharmacokinetics of CH-1 are still unexplored because of their complicated composition and relatively tiny amounts in vivo. Herein, NanoLC-Orbitrap-Fusion-Lumos-Tribrid-MS/MS was firstly used to qualitatively analyze the components of CH-1. A total of 1347 peptides were identified, of which 43 peptides were characterized by high MS intensity and identification accuracy. We then innovatively synthesized 4 main peptides for activity verification, and the results suggested that Pep72 (NYEPPTVVPGGDL) had the strongest neuroprotective effect on both in vivo and in vitro models. Next, a quantitative method for Pep72 was established based on LC-MS/MS with the aid of Skyline software, and then used in its pharmacokinetic studies. The results revealed that Pep72 had a high elimination rate and low exposure in rats. In addition, hCMEC/D3 based in vitro model was built and firstly used to investigate the transport of Pep72. We found that Pep72 had extremely low blood-brain barrier (BBB) permeability, and was not a substrate of efflux transporters. The biotransformation of Pep72 in rat fresh plasma and tissues was investigated to explore the contradiction between pharmacokinetics and efficacy. A total of 11 main metabolites were structurally identified, with PGGDL and EPPTVPGGDL being the main metabolites of Pep72. Notably, metalloproteinase and cysteine protease were confirmed to be the main enzymes mediating Pep72 metabolism in rat tissues.

Keywords: Cerebrotein hydrolysate-1, Active composition, Pharmacokinetics, Blood-brain barrier permeability, Proteases
Significance Statement

NanoLC-Orbitrap-Fusion-Lumos-Tribrid-MS/MS was firstly applied to discover the components of CH-1. Four main peptides of CH-1 were then innovatively synthesized for pharmacodynamic comparison, and Pep72 (NYEPPTVPGGDL) was firstly proved to exert the highest neuroprotective effect. The pharmacokinetic and metabolic characteristics of Pep72 were systematically depicted on both in vivo and in vitro models. Therefore, the present study for the first time identified and validated the active ingredients of CH-1 from the perspectives of pharmacokinetics and pharmacodynamics, and provided a common technical platform and strategy for the study of active substances in other protein hydrolysates.
1. Introduction

Ischemic stroke, one of the common non-communicable diseases, was always caused by a sudden rupture or blockage of cerebral blood vessel, and led to the significant number of limbs paralysis, speech disorders, swallowing difficulties, cognitive impairments and even deaths worldwide (Traylor et al., 2012). This multi-factorial disease caused 80% of the incidence rate of stroke annually (Lee et al., 2021). As was well known, the onset of ischemic stroke could lead to excessive ERS, mtROS, Ca$^{2+}$ overload, and excitatory amino acid inducing neuronal toxicity in neurons (Zorov et al., 2014). Afterwards, neurons would raise the alarm of mitochondrial membrane potential decline, mitochondrial edema and other signs of mitochondrial permeability transition pore opening, ultimately driving mitochondrial contents to be released into the cytoplasm and triggering apoptosis events (Brenner and Moulin, 2012; Morciano et al., 2017). Inhibiting mitochondrial permeability transition pore induced apoptosis exerted significant efficacy in the treatment of ischemic stroke, and the drugs with multi-component or multi-target characteristics provided the possibility of treating stroke from the perspective of mitochondrial permeability transition pore opening (Li et al., 2020). However, intravenous thrombolysis with recombinant (r) tissue plasminogen activator (r-tPA or r-PLAT) was the only clinical scheme approved by the Food and Drug Administration so far, although stroke patients could only benefit from r-tPA within 4.5 h after cerebral ischemia (Liberale et al., 2018; Shen et al., 2023). In recent years, various neuroprotective agents have been tested for their therapeutic effects on stroke in animals, but have not yet been proven to work in a consistent manner in humans.

Protein is a crucial macronutrient of human body, and also a source of essential amino acid (AAs) and energy. Some food-borne proteins could also provide additional health benefits by releasing bioactive peptides encoded in their sequences, and had attracted great attention of food scientists worldwide (Chalamaiah et al., 2018). In recent years, it has been reported that protein hydrolysates or peptides from food sources have different health effects, including immune regulation and anticancer activity, blood pressure
lowering (ACE inhibition) effect, cholesterol lowering, antithrombotic and antioxidant activity, etc (Bhat et al., 2015). Several functional foods, health products and therapeutic drugs based on the biological activity of these peptides have been on the market or are being developed by food and pharmaceutical companies. For instance, cerebroprotein hydrolysate (CH) was developed as a neuropeptide preparation mimicking the effect of neurotrophin, which could regulate normal physiological function as well as the survival and regeneration of damaged neurological system (Brainin, 2018). In 2013, Lang et al. evaluated the efficacy and safety of CH (30 mL/d, lasting for 10 d) combined with r-IPA in 119 patients using a randomized controlled trial. The results suggested that the CH group showed a positive trend of accelerating recovery, and in terms of safety, there were no issues with the combination therapy regimen (Lang et al., 2013). Current findings also suggested that the effect of CH on neuroplasticity and nerve recovery was greater than that on neuroprotection, and might have the greatest impact in the rehabilitation stage (Mureșanu et al., 2022). In addition, there was a signal that CH had the potential to significantly reduce the economic burden of the national budget, which could be used as the standard treatment for patients with different severity, or combined with another drug treatment (Kulikov and Abdarashitova, 2015; Walter et al., 2015). In 2021, the guidelines of the European Academy of Neurology and European Federation of Neurorehabilitation Societies on drug support for early exercise rehabilitation after acute ischemic stroke recommended the use of two drugs, namely, CH and citalopram (Beghi et al., 2021; Mureșanu et al., 2022). Therefore, CH could be widely used without relevant restrictions, which might enrich the current drug library for stroke treatment.

Improving understanding of the pharmacokinetics and metabolic fate of protein hydrolysate components would greatly contribute to the rational use of protein products in clinical settings, developing active peptide candidate drugs, especially in avoiding clinical drug interactions. However, to date, the composition of CH and its pharmacokinetics in vivo after administration are still unknown because of their complicated composition, short half-life, and relatively tiny amount in vivo, which seriously limits its further exploration. In recent years, some emerging interdisciplinary technologies and strategies based on
high-resolution biological mass spectrometry have been widely used for the identification and research of bioactive molecules, therapeutic targets, and potential mechanisms in traditional Chinese medicine (GE, 2019; Luan et al., 2023). These technologies and strategies provided noteworthy references for the pharmacokinetic and metabolic studies of protein extracts. Cerebroprotein hydrolysate-1 (CH-1), one kind of CH developed by Hebei Zhitong Biopharmaceutical Co., Ltd (Shijiazhuang, Hebei, China), was prepared from denatured proteins and had been proved to exert a neuroprotective role by MEK/ERK1/2 and JAK2/STAT3 pathways (Ren et al., 2021; Zhu et al., 2021). The present study aims to reveal the active peptides in CH-1, and then explore the pharmacological properties of the active peptides by examining their pharmacokinetic characteristics. In this process, a total of 1347 peptides were identified in CH-1 using NanoLC-Orbitrap-Fusion-Lumos-Tribrid-MS/MS system. Among them, 4 peptides with the highest exposure levels (HGGTIPIVPT, NLDIERPT, NVDLIPK, NYEPPTVVPGGDL) were synthesized and compared in pharmacodynamics to identify the active peptides of CH-1. NYEPPTVVPGGDL was confirmed to have the highest pharmacological activity, and was named as Pep72. Then the pharmacokinetics of Pep72 was systematically investigated on in vivo and in vitro models. Pep72 was found to have extremely low blood-brain barrier (BBB) permeability, and could be hydrolyzed into 11 metabolites by metalloproteinase and cysteine protease. PGGDL and EPPTVPGGDL were the main forms of Pep72 present in rat tissue, and might also be the main forms of Pep72 exerting neuroprotective effects.

2. Experimental methods

2.1 Chemicals and reagents

Cerebroprotein hydrolysate-1 (CH-1) was kindly supplied by Hebei Zhitong Biopharmaceutical Co., Ltd (Shijiazhuang, Hebei, China). Pep72 and decarboxylated-Pep72 (ND12, internal standard) were synthesized by Genscript Biotech Corporation (Nanjing, Jiangsu, China). Silicon coater momofilament nylon sutures were
purchased from Reward Life Technology Co., Ltd. (Shenzhen, Guangdong, China). ELISA kits of inflammatory factors (IL-6, IL-1β and TNF-α) were purchased from Excell Biotechnology Co., Ltd. (Suzhou, Jiangsu, China). Bovine serum albumin (BSA), TritonX-100, superoxide dismutase (SOD) activity detection kit, lipid oxidation (MDA) detection kit and TUNEL apoptosis detection kit were purchased from Shanghai Beyotime Biotechnology Co., LTD. (Shanghai, China). Anti-NeuN antibody and goat anti-rabbit IgG H&L were purchased from Abcam Inc. (Cambridge, UK).

2.2 Animals and treatments

Healthy male Sprague-Dawley (SD) rats (aged 8~9 weeks, weighing 180~220 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were raised under controllable temperature (25 °C) and humidity (55–60 %) conditions with access to food and water ad libitum. All the animal experiments were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

The ischemia/reperfusion (I/R) model rats were built by middle cerebral artery occlusion combined reperfusion as follows briefly: (i) rats were anaesthetized using 3% chloral hydrate, (ii) ligated the right proximal common carotid artery (CCA), external carotid artery (ECA) and occipital artery (OA) with silk thread, (iii) blocked the internal carotid artery (ICA) with microvascular clamp and coagulated the ECA, (iv) inserted a silicon coater monofilament nylon suture into the CCA, (v) induced reperfusion by pulling out the monofilament. In addition, the sham-operated rats underwent the same procedures except the ligation of ICA, ECA and OA. Then the I/R model rats were randomly divided into I/R, I/R + Pep72 and I/R + CH-1 groups. The I/R + Pep72 rats were given Pep72 at a dose of 10 mg/kg for 7 days by intraperitoneal injection after I/R modeling. The I/R + CH-1 treatment rats were intraperitoneally administered with a dose of 5 mL/kg for 7 consecutive days after I/R modeling.

2.3 Evaluation Indexes
Neurological severity scores (NSS)

To eliminate the rats with failed surgery, neurological function was evaluated after reperfusion. The NSS includes motor function testing, autonomous motor testing, sensory testing, balance testing, reflex and abnormal movement testing, etc. The higher the NSS, the more severe the nerve damage. The specific scoring assay was as follows: 0 point for no neurological impairment, 1 point for mild focal neurological impairment (absolute inability to extend the front paw), 2 points for moderate focal neurological impairment (left rotation), 3 points for severe focal neurological impairment (left inversion), 4 points for rats’ inability to walk automatically and loss of consciousness.

Rotarod test

Before I/R modeling, rats were trained on a rotating rod for 3 days (3 times per day), accelerating their rotation speed from 0 to 40 rpm within 300 s, while recording the latency. Under the same conditions, failed modeling rats were screened on the first day after I/R modeling. The test was performed on the 7th day after I/R modeling and drug administration. The latency of the descent on the rod was recorded.

Triphenyltetrazolium chloride (TTC) staining

Rat brain tissues were collected and freeze for about 20 min to facilitate cutting into 2 mm thick coronal slices. Then the slices were incubated in 0.1% TTC solution at 37 °C for 20 min, and then immobilized overnight in 4% paraformaldehyde. Finally, the brain sections were imaged with the infarct area in white and non-infarcted tissue in red. The infarct size of brain was calculated by Image J software.

Immunofluorescence labeling of neuron and apoptosis

Intact brains were collected, immobilized, dehydrated and cut into 30 μm thick slices using cryo slicer. After sealing in 0.3% Triton X-100 and 5% BSA for 20 min, the slices were incubated with mouse anti NEUN antibodies overnight. Next, the slices were incubated with secondary antibody for 60 min after washing 3 times with PBS. Then the TUNEL staining solution was added into slices and incubated 60 min at 37 °C. Finally, the slices
were imaged with confocal microscope and counted the apoptotic neurons by Image J.

**Measurement of proinflammatory cytokines**

Fresh tissues in ischemic cortex were homogenized with ice PBS at a proportion of 1:9 (w/v). After centrifugation, the supernatant was taken to measure the levels according to the ELISA kits instructions of IL-1β, IL-6 and TNF-α, MDA and SOD kits. The measured data were calibrated using the protein concentrations determined by the BCA protein assay kit.

**Metabolomic analysis of rat brain tissue**

Briefly, 30 mg of rat cerebral cortex on the left side were homogenized with 900 μL methanol containing 13C-glutamine (15 μg/mL) as an internal standard. After extraction, transfer the supernatant and dry it, then dissolve the dried sample again with ultrapure water until analysis by LC-Q-TOF/MS (LC-Triple TOF 5600, SCIEX, Foster City, CA, USA). Chromatographic separation was carried out on a Waters XBridge BEH Amide column (100 mm × 4.6 mm, 3.5 μm) under a gradient elution procedure. The Triple TOF 5600 MS parameters used were as follows: source temperature, 550 °C; ion spray voltage, -4500 V; ion source gas 1, 33 psi; curtain gas, 25 psi; ion source gas 2, 33 psi; collision gas, medium; MS1 scan, m/z 50-1000; information-dependent acquisition, m/z 50-900; collision energy, -30 V. The raw data were imported into the Master view 2.0 and Multiquant 2.0 (Sciex, Canada) software for data processing. Finally, principal component analysis (PCA) and differential metabolite screening were performed on Metaboanalyst 5.0 ([http://www.metaboanalyst.ca/](http://www.metaboanalyst.ca/)). In addition, heatmap drawing and pathway enrichment were also conducted using Metaboanalyst 5.0.

**2.4 Cell culture and treatments**

Cell culture

The human cerebral microvascular endothelial cells (hCMEC/D3) were purchased from the ATCC (Manassas, VA, USA), and cultured in the Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin).
The hCMEC/D3 were maintained in a humidified incubator (5% CO2) at 37 °C for cultivation.

Establishing an oxygen-glucose deprivation/reperfusion (OGD/R) model for hCMEC/D3 cells

The normal high-glucose DMEM medium of hCMEC/D3 cells were replaced with glucose-free DMEM, and maintained for 24 h in an anoxic chamber with continuous addition of 95% N2, 5% CO2, and less than 1% oxygen. For reoxygenation, the glucose-free medium was changed to high glucose DMEM and the cell plates were transferred to the normal incubator (95% air and 5% CO2) for 1 h. The control cells were cultured in normal DMEM and normal conditions.

Cell treatment

Firstly, Pep72 was dissolved in ddH2O at an original concentration of 100 mg/mL, and then gradually diluted to 5, 1 and 0.2 μg/mL using culture medium. During OGD/R process, hCMEC/D3 cells of the Pep72 treatment group were treated with Pep72 at doses of 0.2, 1, 5 μg/mL, respectively.

Cell viability and scratch assays

After cultivation, the medium was replaced with CCK-8 and incubated at 37 °C for 2 h. The OD value was read at 450 nm to calculate cell viability. In the scratch test, hCMEC/D3 cells were inoculated into 6-well plates. When the confluence exceeded 90%, the cells were scraped straight and then gently washed twice with serum-free culture medium. Continuously cultivate cells as required, and the images of cells were taken at 0 and 24 h to calculate cell migration. When analyzing the migrations, we imported images into the software of Image J, adjusted the threshold value to pitch on the scratch regions, measured the areas of scratch regions and compared the areas of the same region at 0 h and 24 h. The migration rates were calculated as following: Migration rate = (scratch area at 0 h - scratch area at 24 h) / scratch area at 0 h* 100%.

Measurement of reactive oxygen species (ROS) in cells
DCFH-DA fluorescent probes diluted with serum-free culture medium were added to hCMEC/D3 cells. After co-incubating with the cells at 37 °C for 30 min, no fluorescent DCFH was oxidized by reactive oxygen species to fluorescent DCF. Then the cells are washed 3 times with serum-free medium to remove extracellular DCFH-DA. Fluorescence intensities were measured by reading the plate at excitation λ488 nm and emission λ525 nm.

2.5 Quantitative analysis of Pep72 in biological matrix using LC-MS/MS

Fresh rat tissues (liver, brain, kidney) were homogenized with cold PBS at 1:9 (w:v). In 40 μL homogenate, 150 μL of methanol containing 4 μL internal standard (1 μg/mL of ND12) was added and vortex mixed at 2000 g for 5 min. After centrifugation, the supernatant was evaporated and reconstituted in 40 μL of H2O. Then the Pep72 was measured using LC-MS/MS system. Chromatographic separation was carried out on a Prevail organic column (2.1×100mm, 3 μm) under a gradient elution program. The mobile phase A was H2O containing 0.2% formic acid and 5 mM ammonium formate. Mobile phase B was acetonitrile. The gradient program was as follows: 0 min, 10% B → 1.0 min, 10% B → 1.2 min, 30% B → 6 min, 54% B → 6.1 min, 95% B → 6.6 min, 95% B → 6.8 min, 10% B → 9.8 min, 10% B. The MS parameters were as follows: ion spray voltage, 5500 V; source temperature, 550 °C; ion source gas 1, 70 psi; curtain gas, 55 psi; ion source gas 2, 40 psi; collision gas, 6 psi.

2.6 Pharmacokinetic studies of Pep72 in rats

Pharmacokinetics of Pep72 in vivo

A total of 12 rats were injected with Pep72 intravenously at a dose of 25 mg/kg. Blood samples were collected from the posterior orbital venous plexus at 2, 5, 10, 20, 40, 60, 90, and 120 min after administration. Plasma was obtained by centrifugation at 8000 g for 5 min, and then stored at -80 °C before use. Pharmacokinetic parameters of Pep72 were calculated by Phoenix WinNonlin software (Pharsight Inc., Mountain View, CA).

Tissue distribution of Pep72 in vivo
Rats were given Pep72 intravenously at a dose of 25 mg/kg. The tissues including heart, liver, spleen, lung, kidney, cortex, hippocampus and striatum were collected at 2 min and 15 min after administration. Rinse the tissue with physiological saline to remove residual blood, and then store at -80 °C until analysis.

2.7 Pharmacokinetic studies of Pep72 in hCMEC/D3 cells

In vitro uptake of Pep72 on hCMEC/D3 cells

To investigate the time-dependence of Pep72 uptake, hCMEC/D3 cells were seeded in 24-well plates. When the cells fully fuse, wash the cells three times with pre-heated PBS, then add 1 mL PBS containing 100 μg/mL Pep72 to each well and incubate at 37 °C for 10, 20, 40, 60, 120, 360, 480 min. Cell uptake was stopped by removing drug solution and washing the cells 3 times with pre-cooled PBS. Cellular protein concentrations were determined with BCA protein assay kit. To investigate the concentration-dependence of Pep72 uptake, hCMEC/D3 cells were seeded in 24-well plates. When the cells fully fused, wash the cells 3 times with pre-heated PBS. Then 1 mL PBS containing 2, 5, 20, 50, 100 μg/mL Pep72 were added to each well. After incubating for 2 h at 37 °C, cell uptake was stopped by removing drug solution.

In vitro transport of Pep72 on hCMEC/D3 cells

To establish an in vitro BBB permeability research model, hCMEC/D3 cells were grown on the PET membrane in the upper of transwell chamber (Millicell cell culture inserts, 0.4 μm pore size, 12 mm diameter; Millipore Corporation) at a density of 4×10^5 cells/mL. A total of 1 mL complete medium was added to the lower chamber and incubated at 37 °C for culture. The integrity of the BBB was characterized by in vitro transdermal resistance (TEER), 4-h leakage test, and sodium fluorescein penetration test. PBS containing Pep72 (20 or 100 μg/mL) or sodium fluorescein (100 μg/mL) was then loaded into both apical (AP) and basolateral (BL) chambers. After incubation at 37 °C for 30, 60, 90 and 120 min, PBS was taken out from the opposite side for analysis, and the same volume of PBS was added as the initial volume. The concentration of Pep72 in PBS was analyzed by
LC-MS/MS. Then the Papp and efflux ratio were calculated according to the concentrations of Pep72 in AP and BL chambers. Fluorescence intensities of sodium fluorescein were measured by reading the plate at excitation λ428 nm and emission λ534 nm.

2.8 Biotransformation of Pep72 in rat plasma, liver, kidney and brain

Pep72 (500 μg/mL) was added to pre-heated rat fresh plasma, liver, kidney, and brain homogenates. After incubating for 0, 2, 5, 10, 20, 40, 60, 90 and 120 min at 37 °C, 40 μL of reaction solution was quickly removed from the system, and 150 μL pre-cooled methanol was added to terminate the reaction. The reaction was eddied for 5 min at 2000 rpm and remained stationary for another 5 min. After centrifuging at 18000 g for 10 min, 130 μL supernatant was collected to dry. The residue was re-constituted in 40 μL of distilled water.

A hybrid quadrupole time-of-flight tandem MS (AB SCIEX Triple TOFs 5600 LC-triple-TOF/MS, Foster City, CA) coupled with a Shimadzu Prominence HPLC system was used to qualitatively analyze the metabolites of Pep72. Chromatographic separation was carried out on a Prevail Organic Acid (3 μm, 100 × 2.1 mm) using a gradient elution program. The MS parameters were optimized as follows: TOF MS Scan: 100-2000 m/z; ion spray voltage, 4500 V; ion source gas 1, 50 psi; curtain gas, 20 psi; ion source gas 2, 50 psi; collision gas, medium; source temperature, 500 °C. A typical information dependent acquisition (IDA) was used to carry MS/MS experiment.

2.9 Qualitative and quantitative analysis of amino acids (AAs) in biological matrix

In 100 μL metabolic incubation system, 500 μL ice-cold acetonitrile containing 200 ng/mL of 2,5-dihydroxybenzoic acid (DHB, IS) was added to terminate the reaction and precipitate proteins. After centrifuging at 18000 g for 10 min, the supernatant was collected to dry. The residue was re-constituted in 50 μL of borate buffer and 50 μL of benzoylchloride for AA derivatization. The supernatant was injected into the LC-MS/MS system to measure the concentrations of AAs. In this process, chromatographic
separation was carried out on a Waters XBridge Amide (3.5 μm, 4.6 × 100 mm) under a gradient elution program as previously reported (Shen et al., 2021). The MS parameters were as follows: ion spray voltage, 5000 V; ion source gas 1, 55 psi; curtain gas, 20 psi; ion source gas 2, 50 psi; source temperature, 550 °C.

2.10 Identification of specific proteases mediating Pep72 metabolism

In the fresh plasma or tissue homogenates, 1 and 10 mM protease inhibitors, including AEBSF, EDTA-4Na, N-ethylmaleimide, PMSF, aprotinin, E-64, leupeptin, bestatin and pepstatin, were added to inhibit protease activity, respectively. After incubation at 37 °C for 10 min, Pep72 (100 μg/mL) was also added to the reaction system, and continued to incubate for another 1 h. Ice methanol (150 μL) was used to terminate the reaction, and the concentrations of Pep72 in the incubation system were measured by LC-MS/MS.

2.11 Statistical analysis

All data were analyzed by Student t test in 2 groups or one-way analysis of variance (ANOVA) in 3 or more groups. GraphPad Prism software (version 8.0, San Diego, CA, USA) was used to calculate statistical significance. Statistically significant difference was considered for \( P<0.05 \).

3 Results

3.1 Identification of active peptides in CH-1

We are currently committed to developing peptide analysis technology for protein hydrolysates based on MS system. In this process, desalting from CH-1 solution was accomplished by Oasis HLB cartridges. The Nano-LC system was used for chromatographic separation, and data was acquired on the NanoLC-Orbitrap-Fusion-Lumos-Tribrid-MS/MS system (Thermo Fisher, Waltham, MA, USA). Proteins were identified by comparing with database of UniProtKB/Swiss-Prot pig (Sus scrofa) in Peaks Studio 10.5 software (Bioinformatics Solution Inc., Waterloo, Canada). Trypsin/P was designated as cleavage enzyme, with the false discovery rate
threshold 5% and log P 56.76. The results were exported in "*.CSV" file format. A total of 1347 peptides were identified in the CH-1, and 43 peptides were characterized by high MS intensity and identification accuracy. We then established a LC-MS/MS quantitative analysis assay for these 43 peptides aided by Skyline software. The LC-MS/MS parameters are shown in Supplemental materials Table S1. Among these peptides, the MS intensity of 4 peptides (HGGTIPIVPT, NLDIERPT, NVDLIPK and NYEPPTVVPGGDL) was much higher than other peptides, which might be the main candidate active ingredients of CH-1. Therefore, we synthesized these 4 main peptides for activity verification. By comparing the neuroprotective effects of these 4 peptides (2.5 μg/mL) on OGD/R-PC12 cells, NYEPPTVVPGGDL was confirmed to have the highest pharmacological activity and named as Pep72 (Fig. 1A). The neuroprotective effect of Pep72 was then further verified on OGD/R-hCMEC/D3 cells. In this process, oxidative stress levels were evaluated by measuring intracellular ROS using DCFH-DA probes. The fluorescence intensity in OGD/R model cells was substantially greater than in controls. Pep72 administration reduced intracellular ROS levels in a dose dependent manner (Fig. 1B). In addition, OGD/R modeling significantly reduced cell viability, and exogenous Pep72 enhanced the viability at a dose range of 0.2-5 μg/mL (Fig. 1C). Scratch assay was conducted to measure the migration and damage repair capability of OGD/R-hCMEC/D3 cells. The migration rate of cells in each group was compared according to the scratch area at 0 and 24 h calculated by Image J software. The results demonstrated that Pep72 significantly increased the migration rate of OGD/R cells (Fig. 1D and 1E).

3.2 Therapeutic effect of Pep72 on I/R rats

After I/R modeling, rats were injected intraperitoneally with Pep72 at a dose of 10 mg/kg or saline (vehicle) for 7 consecutive days. In this process, CH-1 was used as a positive drug to determine the therapeutic effect of Pep72. As shown in Fig. 2A, the weight of I/R model rats decreased continuously, and this weight loss could be significantly reversed by administration of Pep72 or CH-1. We then conducted a rotarod test to investigate the therapeutic effect of Pep72 on neuromuscular coordination in I/R rats. As shown in Fig.
I/R modeling considerably reduced the residence time, while both Pep72 and CH-1 administration could significantly prolong rats’ time remaining on the rotary rod. TTC staining was then used to determine the size of cerebral infarction. As shown in Fig. 2C and 2D, significant cerebral infarction appeared after I/R surgery, and the cerebral infarction area of rats treated with Pep72 or CH-1 significantly decreased from 12% to less than 6%. On the 7th day after I/R modeling, there were significant differences in NSS among I/R model, Pep72-treated and CH-1-treated rats. Clearly, both Pep72 and CH-1 could significantly improve the NSS of I/R rats (Fig. 2E). The levels of pro-inflammatory cytokines were also measured to further confirm the pharmacological activity of Pep72. As illustrated in Fig. 2F - 2H, the pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β, in I/R model rats were much higher than those in the sham-operated controls. Pep72 or CH-1 treatment could significantly reduce the levels of intracerebral pro-inflammatory cytokines of I/R rats.

To further confirm the neuroprotective effect of Pep72, we used immunofluorescence to perform TUNEL and NeuN double labeling staining on paraffin sections of the cortical infarct region. Obviously, there were serious neuron loss and a large number of apoptotic cells in the infarcted area of the cerebral cortex in I/R model rats. Both Pep72 and CH-1 administration could reduce neuronal loss and inhibit cell apoptosis (Fig. 3A and 3B). In addition, we also investigated the antioxidant stress effect of Pep72. The results suggested that I/R modeling led to a significant increase of MDA and a decrease of SOD level, indicating oxidative stress in the brain of I/R rats. Pep72 or CH-1 administration could significantly reduce intracerebral oxidative stress via adjusting the MDA and SOD to normal level (Fig. 3C and 3D). More importantly, metabonomics was used to investigate the regulation of intracerebral small molecule metabolites of I/R modeling, Pep72 and CH-1 administration. The results of principal component analysis (PCA) showed significant differences in metabolites between sham-operated and I/R model rats. Both Pep72 and CH-1 treatments could promote the small molecule metabolites of I/R model rats to approach those of the sham-operated rats (Fig. 3E). Compared with the controls, at least 25 different metabolites in the I/R rats exhibited significant changes, mainly
including thymidylic acid, acetoacetic acid, ketoacid, hypoxanthine, AAs, etc (Fig. 3F).

Notably, I/R modeling could lead to significant accumulation of amino acids, and Pep72 or CH-1 administration could regulate these differential metabolites towards the sham-operated group. In addition, the results of KEGG pathway analysis showed that pentose phosphate, aminoacyl-tRNA and arginine biosynthesis were significantly enriched (Fig. 3G and 3H).

3.3 Establishment and validation of quantitative method for Pep72 in biological matrices

The therapeutic effect of Pep72 on I/R was confirmed on both *in vitro* and *in vivo* models. We then attempted to investigate the pharmacokinetic characteristics of Pep72 in rats and hCMEC/D3 cells. Firstly, it was necessary to establish a robust quantitative assay for Pep72 in biological matrices. Here, Skyline software was used to predict precursor ions and product ions of Pep72, exported them as file.csv, and then imported the file.csv into AB SCIEX 6500 to construct the acquisition method. After using LC-MS/MS for signal acquisition based on the acquisition parameters calculated by Skyline, the raw data was re-imported into the Skyline software to select the optimal MRM and optimize its CE value. As shown in Fig. 4A and 4B, the most intensive parent ion and product ion of Pep72 were at *m/z* 679.33 and 458.22, respectively. Then the selective reaction monitoring transition (679.33→458.22) was used to optimize CE, and the optimum CE was confirmed as 32.0 V (Fig. 4C). To increase the accuracy and reproducibility of quantification, structural analogue of Pep72 (decarboxylated Pep72, MRM 623.00→345.00) was synthesized and used as an internal standard. By optimizing the chromatographic separation conditions, the retention times of Pep72 and internal standard were adjusted to 5.21 and 5.03 min, respectively, so that the analytes were not interfered by endogenous impurities (Fig. 4D and 4E). The calibration curves of Pep72 in rat plasma were linear over the concentration range of 1.0-1000 ng/mL with correlation coefficient *r*>0.99 (Fig. 4F). By evaluating the intra-day and inter-day accuracy, precision, and matrix effects of Pep72 in QC samples with different concentrations, it was found that this quantitative assay could meet the
requirements of biological sample analysis (Table S2 and Table S3).

### 3.4 Pharmacokinetic characteristics of Pep72 in vivo and in vitro

The characterization of the pharmacokinetics and distribution of therapeutic peptides is a hot topic in the pharmaceutical industry, especially with the increasing clinical demand for biopharmaceuticals. Herein, the validated LC-MS/MS was used to evaluate the pharmacokinetics of Pep72. As shown in Fig. 5A, Pep72 was rapidly eliminated in rats after injection administration. The pharmacokinetic parameters were calculated and listed in the Table S4. The results showed that the pharmacokinetic behavior of male and female rats tended to be consistent. The half-life of Pep72 in male and female rats was 15.58 ± 1.09 and 16.04 ± 1.05 min, respectively. After 2 h of administration, the Pep72 concentrations in plasma were below the minimum quantification limit. Meanwhile, the distribution of Pep72 was also investigated in rats, and the results suggested that the exposure level of Pep72 in the rat kidney was significantly higher than other tissues at 2 min after administration (Fig. 5B). In rat heart, liver, spleen, and lung, the concentrations of Pep72 at 15 min was significantly lower than those at 2 min after administration, which further suggested that Pep72 had a high elimination rate in rats. In addition, we also measured the distribution of Pep72 in different brain regions, and the result indicated that Pep72 could permeate the BBB and be distributed in the rat brain. The concentrations of Pep72 in cortex, hippocampus and striatum at 15 min were similar to those at 2 min, and the elimination rate of intracerebral Pep72 was much slower than that in other tissues.

Furthermore, hCMEC/D3 based in vitro BBB model was used to investigate the uptake and transport of Pep72. Firstly, we investigated the cytotoxicity of Pep72 on hCMEC/D3 cells, and the result suggested that Pep72 did not cause cell damage within the concentration range of 0.2 to 1000 μg/mL. The uptake results demonstrated the uptake of Pep72 by hCMEC/D3 cells increased in a concentration dependent manner (Fig. 5C). To investigate the time-dependent uptake of Pep72, PBS containing 50 μg/mL Pep72 was incubated at 37 °C for 10, 20, 40, 60, 120, 360, 480 min. Clearly, the uptake of Pep72 by hCMEC/D3 cells continued to increase over time within 0-4 h (Fig. 5D). We next assessed
the transport characteristics of Pep72 on hCMEC/D3 cells. Transepithelial electrical resistance (TEER, Millicell-ERS epithelial volt-ohmmeter, Millipore Corporation) was used to evaluate the integrity of hCMEC/D3 monolayers. The permeability experimental device is shown in Fig. 5E. The measurement results suggested that the TEER value of the hCMEC/D3 cell monolayer model gradually increased after inoculation, and reached a peak of 118.17 ± 6.11 Ω• cm² on the 6th day, which indicated that the hCMEC/D3 monolayer on the 6th day of inoculation was the densest and could be used for drug permeability research (Fig. 5F). Leakage experiments were conducted on hCMEC/D3 cells on the 6th day of cultivation. Serum free culture media were added to the upper and lower chambers of Transwell to form a liquid level difference greater than 0.5 cm. After incubation at 37 °C for 4 h, significant liquid level differences were observed, indicating that the hCMEC/D3 monolayer cell model formed a barrier function (Fig. 5G). The apparent permeabilities (Papp, cm/s) in each direction (A→B and B→A) were calculated based on previously reported methods (Liang et al., 2013). Sodium fluorescein could hardly penetrate the blood-brain barrier, so it was selected as a negative control. The Papp value of sodium fluorescein was about 4×10⁻⁶ cm·s⁻¹, which was consistent with the literature reports. After incubation with hCMEC/D3 in Millicell for 30, 60, 90, and 120 min, the Papp_A→B and Papp_B→A of Pep72 were all less than 4×10⁻⁶ cm·s⁻¹, indicating that Pep72 was difficult to penetrate the BBB (Fig. 5H). In addition, the efflux ratio of Pep72 was below 2, which suggested that Pep72 was not a substrate of efflux transporters.

3.5 Biotransformation of Pep72 in plasma and tissue homogenate

Pep72 (20 μg/mL) was incubated in fresh plasma, liver, kidney, and brain homogenate at 37 °C. The residual amount of Pep72 was measured after 0, 2, 5, 10, 20, 40, 60, 90, and 120 min of incubation to investigate the metabolic stability of Pep72. As shown in Supplemental Fig. S1, Pep72 rapidly degraded in liver, kidney and brain homogenate, and had completely degraded after incubation for 20 min. In addition, the degradation rate of Pep72 in rat plasma was significantly lower than that in tissues. A total of 11 metabolites, including GG, DL, EPPTVPGGDL, TVVPGGDL, VPGGDL, EPPTVVP,
YEPTVVPGGDL, PGGDL, PPTVVPGGDL, VVPG and TVVP, were identified in rat plasma and tissue homogenate. The metabolic pathways of Pep72 and the structures of the metabolites have been shown in Fig.6A. Among these metabolites, VVPG and TVVP were only appeared in rat kidney, and the metabolic types of Pep72 in plasma, liver and brain were the same (Fig. 6B to 6E). In fresh plasma, PGGDL, TVVPGGDL, EPPTVVPGGDL and VPGGDL were the main metabolites. Pep72 could be rapidly hydrolyzed into VPGGDL in plasma, and the amount of VPGGDL gradually decreased with increasing incubation time. In the contrast, the amounts of PGGDL, TVVPGGDL and EPPTVVPGGDL greatly increased with increasing incubation time. In fresh liver homogenate, the exposure levels of PGGDL and EPPTVVPGGDL were much higher than other metabolites, and the highest exposure level occurred at 10 min after incubation. Subsequently, the levels of these two metabolites gradually decreased with the prolongation of incubation time. Similar to that in rat liver, Pep72 could be rapidly hydrolyzed into PGGDL and EPPTVVPGGDL in fresh kidney homogenate, and these metabolites rapidly decreased from 10 min. VVPG and TVVP were unique metabolites in rat kidneys and were also the main metabolites in kidney homogenate after 20 min of incubation. In rat brain homogenate, PGGDL and EPPTVVPGGDL were also the main metabolites, and EPPTVPGGDL would further hydrolyze into EPPTVP with the prolongation of incubation time.

Peptides are composed of AAs, and may be hydrolyzed into AAs under the action of hydrolytic enzymes. Since AAs are endogenous substances, we investigated the ability of Pep72 to transform into AAs by comparing the amount of AAs without and with Pep72 incubation. In rat plasma incubation system, glutamic acid, proline, valine and tryptophan were significantly enhanced in the Pep72 incubation system (Supplemental Fig. S2A). Asparagine, aspartic acid, proline and threonine significantly increased in liver homogenate (Supplemental Fig. S2B), and no AA increased obviously in kidney homogenate (Supplemental Fig. S2C). In brain homogenate, only aspartic acid and leucine significantly increased in the Pep72 incubation system (Supplemental Fig. S2D). Therefore, the main metabolites of Pep72 should be PGGDL and EPPTVPGGDL, which
might be active ingredients for Pep72 to exert neuroprotective effects.

4. Discussion

According to previous reports, protein hydrolysates possess a wide range of bioactivities and are promising ingredients for developing therapeutic drugs and functional foods (Bhat et al., 2015). In recent years, the research on protein hydrolysates or bioactive peptides used to produce value-added food ingredients or drugs has attracted the attention of food scientists and pharmaceutical scientists all around the world. As a mixture of small peptides, polypeptides and AAs hydrolyzed from brain tissue of pigs, CH has been widely used in the treatment of neurodegenerative diseases, acute ischemic stroke, and traumatic brain injuries. It is a fact that CH has shown to be safe and well tolerated, and there is no strict time window for clinical application (Ziganshina and Abakumova, 2015). It has been well acknowledged that for protein hydrolysates, the identification of active components contained is of great significance for their quality control and revealing the secrets of its effectiveness. Unfortunately, due to the complex composition, short half-life, and extremely low concentration in vivo of protein hydrolysates, their active ingredients and pharmacokinetics have not yet been explored. In recent years, some emerging interdisciplinary technologies and strategies based on high-resolution MS have been widely applied in the study of traditional Chinese medicine pharmacokinetics, and these technologies and strategies provided crucial references for the pharmacokinetic and metabolic studies of protein hydrolysates (GE, 2019; Luan et al., 2023).

In the present study, NanoLC Orbitrap Fusion Lumos Tribrid MS/MS system was used to systematically screen the components of CH-1, and a total of 1347 peptides were identified, and 43 peptides were characterized by high MS intensity and identification accuracy. We then innovatively synthesized 4 main peptides for activity verification. After comparing the neuroprotective effects of the peptides on OGD/R-PC12 cells, Pep72 was confirmed to have the strongest pharmacological activity compared to other peptides. In addition, Pep72 was found to enhance the viability, reduce intracellular ROS level and increase the migration rate of OGD/R-hCMEC/D3 cells in a dose-dependent manner.
More importantly, the therapeutic effect of Pep72 on ischemic stroke was then confirmed in I/R model rats. The results suggested that both Pep72 and CH-1 treatments could significantly improve the motor coordination ability, neurological function score, and reduce the cerebral infarction and levels of pro-inflammatory factors in I/R rats. Pep72 or CH-1 administration could also significantly reverse the aberrant metabolism of small molecules caused by I/R modeling while reducing neuronal loss, apoptosis, and oxidative stress levels. In sum, Pep72 was proved to exert significantly neuroprotective effect on in vitro and in vivo models. However, the low oral bioavailability and limited penetration efficiency of peptide/protein drugs significantly hinder the development of such drugs (Tong et al., 2020). As a peptide composed of 13 amino acids, what are the pharmacokinetic characteristics and active form of Pep72 after administration to rats or cells? To clarify these questions the characterization of the pharmacokinetics and distribution of Pep72 were investigated on in vivo and in vitro models. In the first step, a quantitative method for Pep72 was established and validated using LC-MS/MS with the aid of Skyline software. By measuring the concentration of Pep72 in rat plasma and tissues after administration, it was found that Pep72 had a high elimination rate in rats, and the exposure level of Pep72 in the rat kidney was significantly higher than other tissues. In addition, the concentrations of Pep72 in mouse cortex, hippocampus and striatum were much lower than that in other tissues, and the elimination rate of Pep72 in rat brain was also slower than that in other tissues.

The BBB is a huge obstacle to the delivery of candidates related to the central nervous system, which blocks the penetration of most molecules, thus posing a major challenge for researchers to find effective treatments for central nervous system disease (Daneman and Prat, 2015). In this study, hCMEC/D3 based in vitro BBB model was built and firstly used to investigate the uptake and transport of Pep72. The results suggested that Pep72 could be taken in by hCMEC/D3 cells, while its \( P_{app_{A\rightarrow B}} \) and \( P_{app_{B\rightarrow A}} \) were both less than \( 4 \times 10^{-6} \, \text{cm} \cdot \text{s}^{-1} \), indicating that Pep72 was difficult to penetrate the BBB. In addition, the efflux ratio of Pep72 was below 2, which meant that Pep72 was not a substrate of efflux transporters. Therefore, Pep72 had a significant neuroprotective effect, but its exposure in
the brain was low and the permeability of BBB was poor. To understand this contradiction between pharmacokinetics and efficacy, we investigated the biotransformation of Pep72 in rat fresh plasma and tissues. We found that Pep72 rapidly degraded in rat plasma and tissue homogenate, and the degradation rate in rat plasma was significantly lower than that in tissues. In addition, 11 main metabolites including GG, DL, EPPTVPGGDL, TVVPGGDL, VPGGDL, EPPTVVP, YEPTVPGGDL, PGGDL, PPTVPGGDL, VVP and TVVP, were identified in rat plasma and tissue homogenate. Among these metabolites, PGGDL and EPPTVPGGDL had the highest exposure levels, and with the extension of culture time, EPPTVPGGDL further hydrolyzed into EPPTVP. Peptides are composed of amino acids (AAs), and the physicochemical environment is a major barrier for peptides, involving enzymatic and acid catalyzed degradation (Hunter et al., 2012). Peptides could be hydrolyzed into AAs under the action of hydrolytic enzymes in tissues. Herein, Pep72 could significantly enhance the exposure of asparagine, aspartic acid, proline and threonine in rat liver, but had no significant effect on AA levels in the kidney and brain. Therefore, the main metabolites of Pep72 should be PGGDL and EPPTVPGGDL, which may be active ingredients for Pep72 to exert neuroprotective effects.

The types of proteases involved in protein and peptide hydrolysis mainly include serine protease, metalloproteinase, cysteine protease, aspartic protease, etc. According to previous reports, AEBSF, PMSF and aprotinin were the inhibitors of serine protease (Powers et al., 2002; Dorai et al., 2011). EDTA·4Na and bestatin were the inhibitors of metalloproteinase. N-ethylmaleimide and E-64 were the inhibitors of cysteine protease (Zhou et al., 2014). Leupeptin was the inhibitor of serine/cysteine protease, and pepstatin was the inhibitor of aspartic protease (Rey et al., 2016). Herein, the effects of 9 protease inhibitors on the metabolic stability of Pep72 in fresh plasma and liver homogenate were investigated, with no protease inhibitor as the controls (Fig.7). In fresh plasma, AEBSF, EDTA-4Na, N-ethylmaleimide and PMSF had no obvious influence on the stability of Pep72, while aprotinin, leupitin, bestatin and pepstatin could significantly reduce the degradation of Pep72. Therefore, serine protease, metalloproteinase, aspartic protease were the main
proteases that mediated the rapid metabolism of Pep72 in rat plasma. In liver homogenate, EDTA-4Na, N-ethylaleimide and bestatin could significantly inhibit the hydrolysis of Pep72 in a dose-dependent manner. When 10 mM EDTA-4Na, N-ethylmaleimide or bestatin were added to the incubation system, the residual amount of Pep72 increased by 5-fold. AEBSF, PMSF, E-64, leupitin and pepstatin could also inhibit the hydrolysis of Pep72 at 10 mM, but their inhibitory ability was significantly lower than the above 4 inhibitors. In addition, aprotinin and leupitin had no obvious effect on the stability of Pep72, and 1 mM E-64, aprotinin and pepstatin could promote the hydrolysis of Pep72, which might be mainly due to the reaction heat generated by the addition of these inhibitors to the incubation system. Therefore, metalloproteinase and cysteine protease were the main proteases that mediated the rapid metabolism of Pep72 in rat liver. In sum, the types of hydrolases in plasma and tissues were different, and the activity of hydrolases in the liver was significantly higher than that in plasma. This result was consistent with the above research on the metabolic rate of Pep72 in plasma and tissue homogenate. Thus, the present study is the first time to identify and verify the active ingredient of CH from the perspective of pharmacokinetics and pharmacodynamics, and provides a systematic technical platforms and strategies for the active substance research of other protein hydrolysates.
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Data Availability Statement

The authors claim that the publication contains all of the data supporting the study's conclusions.

Authorship Contributions

Participated in research design: Yan Liang, Guangji Wang, Huimin Guo, Huizhu Yang

Conducted experiments: Huimin Guo, Huizhu Yang, Hong Sun, Yexin Xu, Huafang Liu, Linlin Wu, Ke Ding, Tingting Zhang

Contributed new reagents or analytic tools: Chanjuan Di, Feng Xu, Lin Xie

Performed data analysis: Huimin Guo, Yan Liang

Wrote or contributed to the writing of the manuscript: Yan Liang, Huimin Guo, Huizhu Yang
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for Ischemic Stroke Care.


Footnotes

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Conflict-of-interest Statement

No author has an actual or perceived conflict of interest with the contents of this article.
**Figure Captions**

**Fig.1** Identification of active peptides in CH-1. (A) Cell viability of PC12 cells, ***$P<0.001$ with respect to control group, **$P<0.01$, # $P<0.05$ with respect to model group, (B) ROS of hCMEC/D3 cells, ****$P<0.0001$, **$P<0.01$, (C) Cell viability of hCMEC/D3 cells, **#$P<0.01$, # $P<0.05$ with respect to model group, (D) Migration images of hCMEC/D3 cells, (E) Migration rate of hCMEC/D3 cells, **$P<0.01$, * $P<0.05$.

**Fig.2** Investigation of the therapeutic effect of Pep72 on I/R in rats. (A) Weight loss of rats, (B) Latency of fall in rotarod test, (C) TTC staining of cerebral infarction, (D) Cerebral infarction area, (E) Neurological severity score, (F) Levels of IL-6 in infarct area, (G) Levels of IL-1β in infarct area, (H) Levels of TNF-α in infarct area.

**Fig.3** Investigation of the therapeutic effect of Pep72 on I/R in rats. (A) Dual immunofluorescent staining of NEUN and TUNEL in infarct area of cortex, (B) Positive cells of TUNEL+/NEUN+ in infarct cortex, (C) Levels of MDA, (D) Levels of SOD, (E) PCA analysis of metabolomics, (F) Heatmap of metabolites, (G-H) KEGG pathway analysis of metabolomics.

**Fig.4** Establishment and validation of quantitative method for Pep72 in biological matrices. (A) Signal intensities of Pep72 under different parent ions, (B) Signal intensities of Pep72 under different product ions, (C) CE value optimization with the aid of Skyline, (D) Representative mass chromatogram of rat blank plasma, (E) Representative mass chromatogram of rat plasma collected at 2 min after injection of Pep72, (F) Calibration curves of Pep72 in rat plasma at the concentration range of 1–1000 ng/ml.

**Fig.5** Pharmacokinetic characteristics of Pep72 in vivo and in vitro. (A) Plasma
concentration–time profiles of Pep72 in rats following intravenous administration, (B) Concentrations of Pep72 in the rat heart, liver, spleen, lung, kidney and brain at 2 min and 15 min after administration, (C) Cellular uptake of Pep72 in hCMEC/D3 cells at different concentrations, (D) Cellular uptake of Pep72 in hCMEC/D3 cells at different time, (E) Permeability experimental device, (F) TEER value of the hCMEC/D3 cell monolayer model, (G) Leakage experiments on hCMEC/D3 cells, (H) Apparent permeabilities (Papp) and efflux ratio of Pep72.

**Fig.6** Biotransformation of Pep72 in plasma and tissue homogenate. (A) The metabolic pathways of Pep72 and the structures of the metabolites, (B) Peak area of Pep72 metabolites in plasma, (C) Peak area of Pep72 metabolites in liver homogenate, (D) Peak area of Pep72 metabolites in kidney homogenate, (E) Peak area of Pep72 metabolites in brain homogenate.

**Fig.7** Metabolic stability of Pep72 after treatment with 9 protease inhibitors in fresh plasma and liver homogenate.
Figure 1

A

Cell Viability (% Control)

Control  | I/R  | HGGTIPVPT  | NLIDRPT  | NQOLIPK  | NVPEPTVPQGGDL  | CH-1

2.5 µg/mL

B

OGD/R  | Pep72 (1.0 µg/mL)  | Pep72 (5.0 µg/mL)

C

Cell Viability (% Control)

Control  | I/R  | 0.2  | 1.0  | 5.0

D

OGD/R  | Pep72

0.2 µg/mL  | 1.0 µg/mL  | 5.0 µg/mL

E

Migration ratio (%)

Control  | I/R  | 0.2  | 1.0  | 5.0

OGD/R  | Pep72(µg/mL)
Figure 7

**AEBSF**

**EDTA-4Na**

**N-ethylmaleimide**

**PMSF**

**Aprotinin**

**E-64**

**Leupeptin**

**Bestatin**

**Pepstatin**

Residual amount of Pep72 (%)