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Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Internalization and transport mechanisms of the walnut-derived peptide in bEnd.3 cells

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ARTICLE INFO

Article history:

Received 11 March 2024

Received in revised form 29 March 2024

Accepted 1 August 2024

Keywords:

Walnut-derived peptides

Endothelial cells

Blood-brain barrier

Caveolin

Transport mechanism

ABSTRACT

The blood-brain barrier (BBB) is a major challenge in drug delivery for the treatment of central nervous system diseases. Walnut derived peptide TWLPLPR (TW-7) has been proved to promote neuronal mitochondrial autophagy and enhance hippocampal neuronal synaptic plasticity, thereby improving learning and memory abilities in mice. We investigated the internalization mechanism and intracellular transport pathway for the walnut-derived peptide, TW-7, using bEnd.3 cells in an *in vitro* BBB model system. TW-7 was taken up by the bEnd.3 cells in a concentration-, temperature-, and energy-dependent manner; this involved increases in caveolin-1 and caveolin-2 protein expression and phosphorylation and inhibition of P-glycoprotein-mediated efflux. Subcellular localization of TW-7 in bEnd.3 cells was observed, indicating that the plasma membrane, endoplasmic reticulum, Golgi apparatus, lysosomes, and mitochondria participated in intracellular trafficking and that the peptide escaped from lysosomes over time. Caveolae may be critical for TW-7 uptake by brain microvascular endothelial cells, assisting TW-7 to cross the BBB. The results of this study provide a theoretical basis for the mechanism of active peptide penetrating the BBB, and provide a reference for developing neuroprotective active peptide products.

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1. Introduction

Driven by increasing life expectancy and population aging, the prevalence of central nervous system (CNS) diseases is increasing globally; these diseases are responsible for approximately 12% of all deaths worldwide^[1]. This trend imposes a serious economic burden on society and mental stress on individuals. In recent decades, researchers have discovered the effective role of active peptides in the treatment of

central nervous system disorders. In particular, food-borne bioactive peptides have been shown to significantly protect neurons in the brain through a variety of mechanisms, including antioxidation, anti-inflammation, anti-apoptosis, and protection of lysosomal homeostasis^[2-4]. Due to the high physiological activity and low toxicity of food-borne bioactive peptides, some neuroprotective peptides have been recognized as potential drugs for the treatment of cognitive impairment, depression, and neurodegenerative diseases^[5]. However, the delivery of these peptides to the brain is limited, and one of the most significant impediments is the blood-brain barrier (BBB)^[6].

The BBB consists of highly specialized neurovascular units that strictly regulate the transport of substances between the blood and the brain^[7]. Brain microvascular endothelial cells and tight junctions (TJs) constitute a high trans-endothelial electrical resistance (TEER)

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Peer review under responsibility of Beijing Academy of Food Sciences.

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(1 000–2 000 $\Omega \cdot \text{cm}^2$), which limits the paracellular pathway transport of molecules in the blood^[8]. Early studies suggested that some small lipophilic molecules (< 500 Da) can cross the BBB through passive diffusion^[9]. Furthermore, the number of vesicles is 14-fold lower in brain microvascular endothelial cells (BMECs) than in peripheral vascular endothelial cells, which limits the ability of cells to pinocytosis^[10]. These properties of the BBB prevent the entry of neuroprotective molecules into damaged brain tissue, highly limiting the effective treatment of neurological diseases and the bioavailability of active peptides. Over the past decades, researchers have developed various techniques, including invasive and non-invasive methods, to facilitate BBB penetration. Invasive methods disrupt the integrity of the BBB, whereas non-invasive techniques do not and are, therefore, more promising^[11]. Bioactive peptides utilizing endogenous cellular transport mechanisms for uptake by BMECs and penetration of the BBB are of interest.

BBB-penetrating peptides refer to oligopeptide chains that can permeability into the BBB in both selective and non-selective ways^[12]. BBB penetrating peptides have attracted widespread attention due to their broad physiological activities such as neuroprotection, anti-tumor, and antiviral functions, especially as carrier systems for bioactive molecules^[13]. These peptides have been described with different transcytosis mechanisms such as receptor-dependent (adsorption-mediated transcytosis), receptor-dependent (receptor-mediated endocytosis), and carrier-mediated endocytosis mechanisms^[14]. At present, BBB penetration peptides are mostly derived from computer design, phage display technology, and endogenous peptides. However, the ability of food-born bioactive peptides to penetrate BBB and the transport mechanism are unclear. Developing foodborne bioactive peptides that directly penetrate the BBB through endogenous transmembrane mechanism has the advantages of high bioavailability, good safety, and no introduction of exogenous substances.

In a previous study, we demonstrated the remarkable ability of protein hydrolysate (< 3 kDa) from walnuts (*Juglans mandshurica* Maxim.) to ameliorate cognitive deficits in mice^[15]. Subsequently, the walnut-derived peptide TWLPLPR (TW-7) was identified, which was found to protect hippocampal neurons by promoting mitophagy and ameliorate memory impairments by preserving BBB integrity^[3,16]. However, the mechanism by which TW-7 penetrates the BBB remains unclear. Therefore, the objective of this study was to investigate the uptake and transport mechanisms of TW-7 in bEnd.3 cells. We also examined the intracellular trafficking of TW-7 in bEnd.3 cells through subcellular localization and further clarified the efflux transport pathway of TW-7. These results provide a theoretical basis for the development of bioactive peptides that penetrate the BBB.

2. Materials and methods

2.1 Materials

The peptides TW-7 and fluorescein isothiocyanate-labeled TW-7 (FITC-TW-7) (purity 97%) were synthesized by Jiangsu Ji Tai Peptide Industry Science and Technology Ltd. (Yancheng, China). The mouse brain microvascular endothelial cell line, bEnd.3, was purchased from Zhong Qiao Xin Zhou Biological Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium

(DMEM) were purchased from HyClone Co. (Logan, UT, USA). Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), and pancreatin with or without ethylenediaminetetraacetic acid (EDTA) were acquired from Meilun Biotechnology Co., Ltd. (Dalian, China). Primary antibodies against Cav-1 (#3267) and β -actin (13E5) were sourced from Cell Signaling Technology (Beverly, MA, USA), while those against Cav-2 (abs131510), phospho-caveolin-2 (p-Cav-2, Ser23; abs139933), and phospho-caveolin-1 (p-Cav-1, Tyr14, abs131011) were obtained from Absin Bioscience Inc. (Shanghai, China). P-glycoprotein (P-gp, 22336-1-AP) and breast cancer resistance protein (BCRP, 27286-1-AP) were purchased from Proteintech (Chicago, IL, USA). Zonula occludens-1 (ZO-1, A0659) and Claudin-5 (A10207) were obtained from ABclonal Biotechnology (Wuhan, China). Secondary antibodies were obtained from AbbKine (Wuhan, China). Endoplasmic reticulum (ER)-Tracker Red, Golgi-Tracker Red, Lyso-Tracker Red, DiI-cytomembrane Tracker Red, and MitoTracker Red CMXRos were purchased through Beyotime Biotechnology (Shanghai, China). Chlorpromazine and methyl- β -cyclodextrin (M- β -CD) were acquired from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). All reagents used were of analytical-grade purity.

2.2 Cell culture

The bEnd.3 cells were cultured in a 37 °C incubator with 5% CO₂ using DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution, and the medium was changed every 2 days. Upon reaching an approximate confluency of 80%–90%, the cells were digested using a solution containing 0.25% trypsin and then seeded into 12-well plates (5×10^4 cells/mL).

2.3 In vitro BBB model

The bEnd.3 cells (5×10^4 cells/mL; 500 μL) were seeding onto 12-well transwell plates (Corning Life Sciences, Tewkesbury, MA, USA) that had a pore size of 0.4 μm . Complete DMEM was added to the basolateral (BL) and apical (AP) sides at a volume of 1.5 and 0.5 mL, respectively. TEER values were measured using an epithelial volt- $\Omega \cdot \text{m}$ voltohmmeter (RE1600; Jingong Hongtai, Beijing, China) to assess the integrity of BBB. TEER values were measured every day; only TEER values that reached 200 $\Omega \cdot \text{cm}^2$ were used for the experiments. The DMEM was removed and replaced with 1.5 mL of HBSS on the BL side and 0.5 mL of HBSS containing 100 $\mu\text{mol/L}$ TW-7 on the AP side once the BBB model was established. After 2 h, the medium from the AP and BL sides was taken out, and the ability of TW-7 to cross the BBB was determined using the Waters Alliance e2695 HPLC system (Waters Alliance, Wilmslow, UK).

2.4 Reversed-phase high-performance liquid chromatography (RP-HPLC)

TW-7 was analyzed using a Symmetry C₁₈ column (250 mm $\times$ 4.6 mm, 5 μm). The mobile phases consisted of solvent A, which contained 0.1% trifluoroacetic acid (TFA) in acetonitrile, and solvent B, which contained 0.1% TFA in water. The elution process involved a gradient of solvent B ranging from 76% to 51%, carried out over a period of 25 min at a flow rate of 1 mL/min. An absorbance detector, set at a wavelength of 220 nm, was used for detection, and the injection volume was set to 10 μL .

2.5 *In vitro* uptake of FITC-TW-7

Cells were treated with 100 $\mu\text{mol/L}$ FITC-TW-7 for 2, 4, 8, 12, and 24 h to determine the time dependence of uptake. Different concentrations of FITC-TW-7 (25, 50, 100, and 200 $\mu\text{mol/L}$) were incubated with cells for 4 h to study the concentration dependence. DAPI (2 g/mL) was used to label cell nuclei for 5 min at 37 °C after being washed 3 times with PBS. A LEICA SP8 confocal laser scanning microscope (LEICA Camera AG, Germany) was used to take the pictures.

2.6 Mechanism of TW-7 uptake by bEnd.3 cells

To evaluate the mechanisms of TW-7 uptake by bEnd.3 cells, cells were pretreated at 4 or 37 °C for 2 h and then incubated with FITC-TW-7 (100 $\mu\text{mol/L}$) for a further 4 h. Potential endocytic pathways were studied by pre-incubating cells with an endocytosis inhibitor, either 2 mmol/L M- β -CD or 25 $\mu\text{mol/L}$ chlorpromazine, for 2 h at 37 °C, followed by incubation with 100 $\mu\text{mol/L}$ TW-7 for 4 h. Qualitative and quantitative analyses were performed using confocal laser scanning microscope and flow cytometry, respectively, and images were captured using ImageJ software (NIH, Bethesda, MD, USA).

2.7 Flow cytometry analysis

For quantitative analysis, cells were seeded in 6-well plates at a concentration of 1×10^5 cells/mL and subjected to the same treatment described as *in vitro* uptake of FITC-TW-7 and mechanism of TW-7 uptake by bEnd.3 above. After collection and washing three times with PBS, the percentage of positive cells was determined by flow cytometry using an Accuri C6 instrument (BD Biosciences, San José, CA, USA).

2.8 Subcellular localization of TW-7

To evaluate the possible destinations of TW-7 within bEnd.3 cells, we investigated the co-localization of TW-7 with various subcellular components, including the Golgi complex, endoplasmic reticulum, lysosomes, cytomembranes, and mitochondria. Cultured bEnd.3 cells grown in 24-well plates at 5×10^4 cells/mL for 24 h and were exposed to FITC-TW-7 (100 $\mu\text{mol/L}$) for 4 h. Following three washes with PBS, the cells were stained with Golgi-Tracker Red (10 $\mu\text{mol/L}$), ER-Tracker Red (1 $\mu\text{mol/L}$), Lysosome-Tracker Red (50 nmol/L), Cytomembrane-Tracker Red (50 $\mu\text{mol/L}$), or Mitochondria-Tracker Red for 30 min. Subsequently, the cells underwent 3 additional washes with PBS before being examined under a fluorescence microscope. The degree of pixel-by-pixel covariance in the signal was quantified using ImageJ software by calculating Pearson's correlation coefficient (PCC). PCC values range from 0 to 1, where complete co-localization is indicated by a value of 1.

2.9 Immunofluorescence analysis

Cultured bEnd.3 cells were treated with 100 $\mu\text{mol/L}$ TW-7 for 4 h. The cells were fixed with 4% paraformaldehyde at 37 °C for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, washed 3 times

with precooled PBS, and then treated with PBS containing 3% goat serum for 30 min at 25 °C. Cells were incubated with antibodies against ZO-1 (1:200), claudin-5 (1:200), Cav-1 (1:200), Cav-2 (1:200), and P-gp (1:200) overnight (4 °C). After washing 3 times with PBS, the cells were incubated with AlexaFluor® 555 goat anti-rabbit IgG for 1 h at 25 °C in the dark and stained with DAPI for 5 min to label the nuclei. Samples were examined by fluorescence microscopy (Carl Zeiss Jena GmbH, Jena, Germany).

2.10 Western blotting analysis

Cell lysis buffer for Western and immunoprecipitation was used to extract protein, and BCA kit was used to determine protein concentration. Cell lysates were then mixed with sodium dodecyl sulfate (SDS) protein loading buffer and heated to 100 °C for 10 min. Samples (30 μg protein/well) were separated on 8%, 10%, and 12% SDS-polyacrylamide gels and subsequently electrophoretically transferred onto a polyvinylidene difluoride membrane at 0.8 mA/cm² of gel. After blocking with 5% skim milk liquid at 25 °C for 1 h, the membrane was washed three times with TBST and then probed overnight with the primary antibodies against P-gp (1:2 000), BCRP (1:2 000), Cav-1 (1:5 000), p-Cav-1 (1:2 000), Cav-2 (1:2 000), and p-Cav-2 (1:2 000). Following three washes with TBST, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 h at 25 °C. Finally, an ECL kit was used for detection, and the protein bands were evaluated by the Image Quant LAS 500 system. β -Actin was used as a loading control.

2.11 Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

bEnd.3 cells were seeded in 6-well plates at a concentration of 1×10^5 cells/mL, and treated with 100 $\mu\text{mol/L}$ TW-7 for 4 h. Cells were collected and total RNA was extracted using RNAiso Plus (TAKARA, Japan). Total RNA was then reverse transcribed into single-stranded cDNA using GoScript™ Reverse Transcription (Promega, USA) and subsequently amplified. ACTIN was used as a housekeeping gene. Samples were placed in a 96-well QuantStudio™ 5 Real-Time PCR system (ABI, USA) for qPCR analysis. Each sample was run in triplicates.

2.12 Statistical analysis

Each group of experiments was performed 3 times, and the data were presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using one-way ANOVA and followed by Tukey's test. $P < 0.05$ was considered statistically significant. Data were graphed using GraphPad Prism software (Boston, MA, USA).

3. Results

3.1 Ability of TW-7 to cross the BBB *in vitro*

The BBB maintains central nervous system homeostasis by regulating the exchange of substances between the blood and brain tissue, selectively absorbing nutrients from the bloodstream^[17]. To

evaluate the ability of TW-7 to cross the BBB, an *in vitro* model was established using bEnd.3 cells in a transwell system (Fig. 1A). Briefly, 100 $\mu\text{mol/L}$ TW-7 was added to the AP side (mimicking the vascular side), and the liquid was recovered from the BL side (mimicking the brain side) after incubation at 37 °C for 2 h. The peptide detected on the BL side was considered to have crossed the BBB. A linear relationship between peptide concentration and peak area was established to quantify the extent of TW-7 penetration into the BBB. As shown in Fig. 1B, the concentration and peak area of TW-7 showed a good linear relationship. Figs. 1C and D show that TW-7 appears on both the AP and BL sides of the cell layer, suggesting that the peptide could traverse the model BBB. After calculation, the apparent permeation coefficient value was 7.9×10^{-6} cm/s, indicating that a portion of TW-7 can cross the BBB. Claudin-5, a transmembrane protein highly expressed in endothelial cells, is involved in the formation of the BBB by restricting, limiting, and regulating intercellular permeability. ZO-1 binds TJ proteins to the cytoskeleton to form a stable TJ system. Alterations in ZO-1 and Claudin-5 levels were closely related to BBB integrity^[18]. Immunofluorescence analysis showed that after TW-7 treatment, ZO-1 and Claudin-5 were distributed along the intercellular junctions in bEnd.3 cell monolayers (Fig. 1E). The expression levels of ZO-1 and Claudin-5 proteins were subsequently measured by Western blotting. As shown in Fig. S1, the expression levels of ZO-1 and Claudin-5 protein were no significant difference in the TW-7 treated group than in the control group. These results suggest that TW-7 can cross the BBB without compromising TJ integrity.

Kastin et al.^[19] first demonstrated that peptides could penetrate the BBB. Monolayer bEnd.3 cells are often used to establish a BBB

model *in vitro* because they highly express TJ proteins, such as ZO-1 and Claudin-5. Li et al.^[20] used bEnd.3 cells to establish a BBB model *in vitro* and demonstrated the BBB penetration efficiency of WTW peptide. In this study, we used bEnd.3 cells to devise a BBB model *in vitro* and found that TW-7 could cross the BBB without disrupting TJs. In recent years, research has reported that certain exogenous bioactive peptides, including the bovine α_{s1} -casein peptide, PIGSENSEKTTMPLW, and the fish skin gelatin prolyl endopeptidase inhibitory peptide, PPL, can cross the BBB in *in vitro* models^[21–22]. The binding of cationic charges in BBB-penetrating peptides to the membrane of brain endothelial cells *via* electrostatic interactions facilitates their ability to cross the BBB. Ahlschwede et al.^[23] used cationic BBB-penetrating peptide to carry nanoparticles across the BBB. In the current study, we found that the arginine residue at the C-terminus of TW-7 imparted a positive charge to the peptide and was one of the factors facilitating the movement of TW-7 across the BBB.

Typically, only a small proportion of the added peptide penetrates the BBB and enters the CNS, but this is sufficient to elicit a physiological response. It is also necessary to consider the safety of exposing TW-7 to neurons. A previous study demonstrated that TW-7 showed significant antioxidant and neuroprotective activities, which protected HT-22 cells from H_2O_2 -mediated damage *via* PINK1-mediated mitophagy^[3]. Furthermore, TW-7 solution, when administered intragastrically (30 mg/kg body weight) to *D*-galactose-induced mice daily for 8 weeks, inhibited MMP-9-mediated BBB disruption and ameliorated *D*-galactose-induced memory impairments^[16]. Therefore, these results suggested that TW-7 did not damage the CNS after penetrating the BBB, eliciting a protective effect on hippocampal neurons.

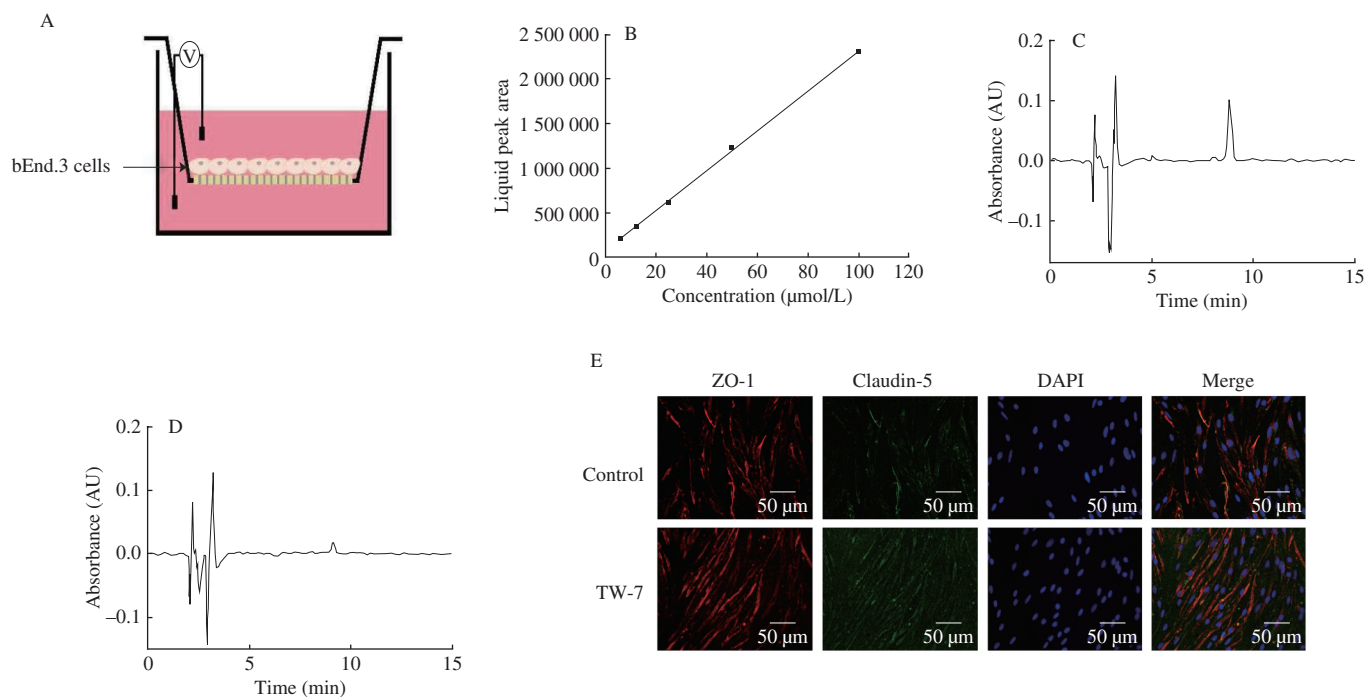


Fig. 1 Ability of TW-7 to cross the BBB *in vitro*. (A) Schematic diagram of the *in vitro* BBB model. (B) Linear relationship between concentration and peak area of the TW-7 solution. (C) RP-HPLC analysis of TW-7 on the apical side of the endothelial layer in the *in vitro* BBB model. (D) RP-HPLC analysis of TW-7 on the basolateral side of the endothelial layer in the *in vitro* BBB model. (E) Immunofluorescence staining pattern of ZO-1 (red) and Claudin-5 (green) of bEnd.3 cells grown on transwell membrane (scale bars = 50 $\mu\text{mol/L}$). All experiments were performed in triplicate ($n = 3$). Bars with different letters indicate statistically significant differences ($P < 0.05$).

3.2 Uptake characteristics of TW-7 by bEnd.3 Cells

To investigate characteristics of TW-7 uptake by bEnd.3 cells, we conducted experiments examining both time and concentration as variables. As shown in Fig. 2A, the intensity of green fluorescence in the cells increased as the concentration of FITC-TW-7 increased from 25 to 200 $\mu\text{mol/L}$. The mean fluorescence intensity was significantly different between each of the FITC-TW-7 concentrations tested reaching 20.15 ± 1.95 , 29.33 ± 2.68 , 48.38 ± 1.86 , and 52.30 ± 1.48 at 25, 50, 100, and 200 $\mu\text{mol/L}$ FITC-TW-7, respectively (Fig. 2B). Because fluorescence microscopy could only qualitatively analyze the cellular internalization of peptides and there may be artifacts, we quantified the degree of internalization of FITC-TW-7 by flow cytometry (Figs. 2C-E). The proportion of positive cells was $(52.67 \pm 1.20)\%$, $(65.5 \pm 0.1)\%$, $(71.2 \pm 1.3)\%$, and $(77.87 \pm 2.45)\%$ at 25, 50, 100, and 200 $\mu\text{mol/L}$ FITC-TW-7, respectively; moreover, the mean fluorescence intensity rose from 189.56 ± 78.49 at 25 $\mu\text{mol/L}$ FITC-TW-7 to 280.33 ± 186.93 at 200 $\mu\text{mol/L}$ FITC-TW-7. To investigate the time-dependent uptake of the peptide, bEnd.3 cells were exposed to 100 $\mu\text{mol/L}$ FITC-TW-7 for various durations (2, 4, 8, 12, and 24 h). As shown in Figs. 2F-G, the fluorescence intensity of the cells increased from 0 to 4 h but remained stable from 4 to 24 h ($P < 0.05$). Both results confirmed that the uptake of TW-7 by bEnd.3 cells was concentration- and time-dependent.

Extensive research has demonstrated that the cellular internalization of peptides follows a concentration- and time-dependent manner. Rodrigues et al.^[24] found that the FI-Angiopep-7 entry into cells was influenced by its concentration in rat BMECs and astrocytes. Specifically, they observed significantly higher uptake with 100 $\mu\text{mol/L}$ FI-Angiopep-7 than with 10 and 30 $\mu\text{mol/L}$ FI-Angiopep-7. Similarly, Rodrigues et al.^[25] showed that crotonamine peptide NrTPs (15 $\mu\text{mol/L}$) generated a higher percentage of positive cells than 5 $\mu\text{mol/L}$ concentrations in various cell types. Koppelhus et al.^[26] demonstrated that PNA-peptide conjugates showed maximum uptake within 2–4 h depending on the cell type. Chen et al.^[27] reported that CPP P1 was taken up by MCF7, A549, and BV2 cells in a concentration-dependent manner. However, the fluorescence decreased as the incubation time increased. In this study, we found that the proportion of positive cells and mean fluorescence intensity correlated with FITC-TW-7 concentration. Studies have shown that peptide adsorption to the cell membrane increases the flexibility of the membrane, leading to concentration-dependent uptake^[28]. In addition, the fluorescence in bEnd.3 cells was constant after 4 h, indicating that FITC-TW-7 taken up by cells reached a dynamic equilibrium within 4 h.

3.3 Mechanism of TW-7 Uptake in bEnd.3 cells

Internalization mechanisms are classified as energy-dependent and energy-independent. Temperature is considered a crucial factor in determining the internalization pathways^[29]. To investigate the mechanism by which TW-7 is internalized by bEnd.3 cells, the cells were incubated with 100 $\mu\text{mol/L}$ FITC-TW-7 at either 4 or 37 $^{\circ}\text{C}$. As illustrated in Fig. 3A, compared to incubation at 37 $^{\circ}\text{C}$, the penetration efficiency of TW-7 was significantly inhibited at 4 $^{\circ}\text{C}$. This was illustrated by both cell fluorescence imaging (Fig. 3A) and mean fluorescence intensity (Fig. 3B, $P < 0.05$). This finding indicated that TW-7 internalization into bEnd.3 cells consumed energy.

BMECs exhibit weak abilities for phagocytosis and micropinocytosis^[8]. Endocytosis inhibitors M- β -CD (a cholesterol binder that inhibits lipid raft/caveolae) and chlorpromazine (an inhibitor of clathrin assembly in the plasma membrane) were used to evaluate the internalization pathway of TW-7^[30]. Treatment with M- β -CD or chlorpromazine at 37 $^{\circ}\text{C}$ for 2 h significantly reduced the fluorescence intensity in bEnd.3 cells (Figs. 3C and D, $P < 0.05$). The cell mean fluorescence intensity of M- β -CD treated group was significantly lower than that of chlorpromazine treated group ($P < 0.05$). Flow cytometric analysis provided quantitative evidence to support the results. The uptake of FITC-TW-7 was significantly inhibited by chlorpromazine and M- β -CD, reducing the mean fluorescence intensity by 26.6% and 75.6%, respectively ($P < 0.05$). These results suggested the mechanisms of TW-7 uptake by bEnd.3 cells was mediated by both clathrin and caveolae, and that caveolae-mediated endocytosis played a major role in the internalization mechanism of TW-7.

Earlier studies suggested that peptides passively diffuse across the plasma membrane without the need for energy input or endocytosis. However, in recent years, an increasing number of studies have shown that endocytosis is also an important process for the internalization of peptides^[31]. Chen et al.^[27] found that peptide P1 was mainly taken up by cells *via* the receptor-mediated endocytic pathway and that its penetration efficiency was significantly reduced with chlorpromazine and M- β -CD treatment. Maraming et al.^[32] reported that the lysine/tryptophan-rich CPP, KT2, was internalized by HCT 116 cells *via* clathrin- and caveolae-mediated endocytosis mechanisms. Kim et al.^[33] revealed that internalization of TAT-M13 in HeLa, MDA-MB-231, and A375-SM cells were almost completely inhibited by chlorpromazine and M- β -CD. Similarly, our results indicated that TW-7 was internalized *via* an energy-dependent endocytic pathway associated with clathrin- and caveolae-mediated endocytosis and that the latter process plays a more important role.

3.4 TW-7 promotes the expression of caveolins

Caveolins are proteins play a crucial role in maintaining the structure and function of caveolae. To further investigate the involvement of caveolae-dependent uptake in TW-7, we analyzed the expression of the caveolae marker proteins, Cav-1 and Cav-2, in bEnd.3 cells. As shown in Fig. 3G, the expression of Cav-1 in the 50 and 100 $\mu\text{mol/L}$ TW-7 treatment group was significantly higher than that in the control group ($P < 0.05$). Meanwhile, the level of p-Cav-1/Cav-1 was also increased with increasing TW-7 concentration ($P < 0.05$). Similar trends were observed for Cav-2 and p-Cav-2/Cav-2 levels in TW-7 treated bEnd.3 cells (Fig. 3H). Cav-1 and Cav-2 are phosphorylated as substrates of sarcoma tyrosine kinase. As shown in Fig. S2, the expression of Src in the TW-7 treatment group was significantly higher than that in the control group ($P < 0.05$).

Co-localization of Cav-1 and Cav-2 is an important indicator of caveolae. Therefore, we assessed the co-localization of Cav-1 and Cav-2 through immunofluorescence in bEnd.3 cells (Fig. 3I). The PCC for Cav-1 co-localization with Cav-2 increased from 0.74 to 0.84 after treatment with 100 $\mu\text{mol/L}$ TW-7. Furthermore, TW-7 showed a high degree of co-localization with Cav-1, the PCC value was 0.9 (Fig. 3J).

The Cav family comprises Cav-1, Cav-2, and Cav-3, which serve as the primary structural proteins of the caveolae and play significant

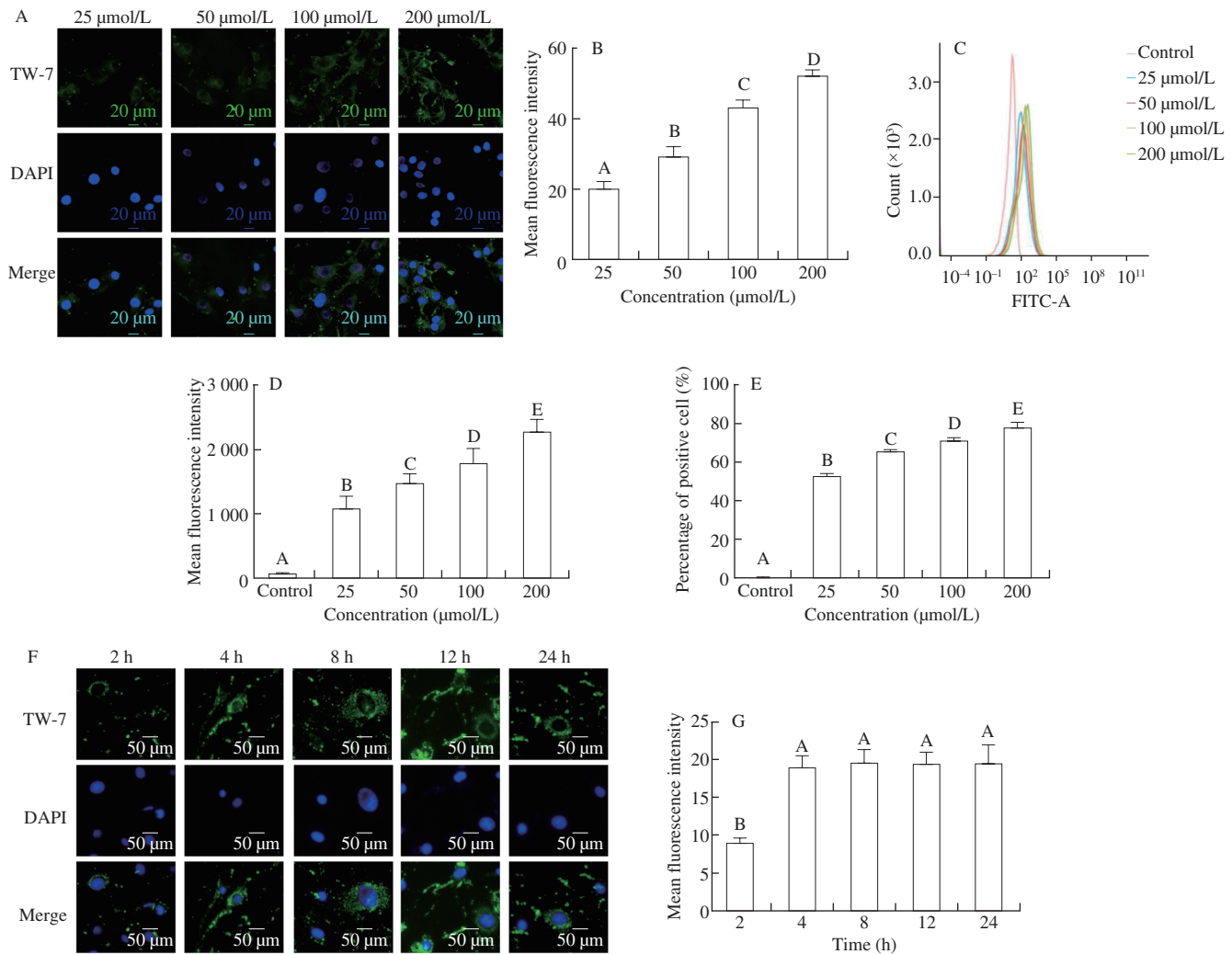


Fig. 2 Characteristics of uptake of TW-7 by bEnd.3 cells. (A) Fluorescence images of FITC-TW-7 at concentrations with 25, 50, 100, 200 μmol/L for 4 h (scale bars = 20 μm). (B) Quantitative fluorescence intensity of FITC-TW-7 at concentrations with 25, 50, 100, 200 μmol/L for 4 h. (C) Effect of incubation concentration on the internalization of FITC-TW-7 detected by flow cytometer. (D) Mean fluorescence intensity and (E) percentage of positive cells detected by flow cytometer. (F) Fluorescence images of FITC-TW-7 (100 μmol/L) with 2, 4, 8, 12, 24 h. (G) Quantitative fluorescence intensity of FITC-TW-7 with 2, 4, 8, 12, 24 h (scale bars = 50 μm). Data were presented as means ± SD. All experiments were performed in triplicate ($n = 3$). Different uppercase letters indicate statistically significant differences ($P < 0.05$).

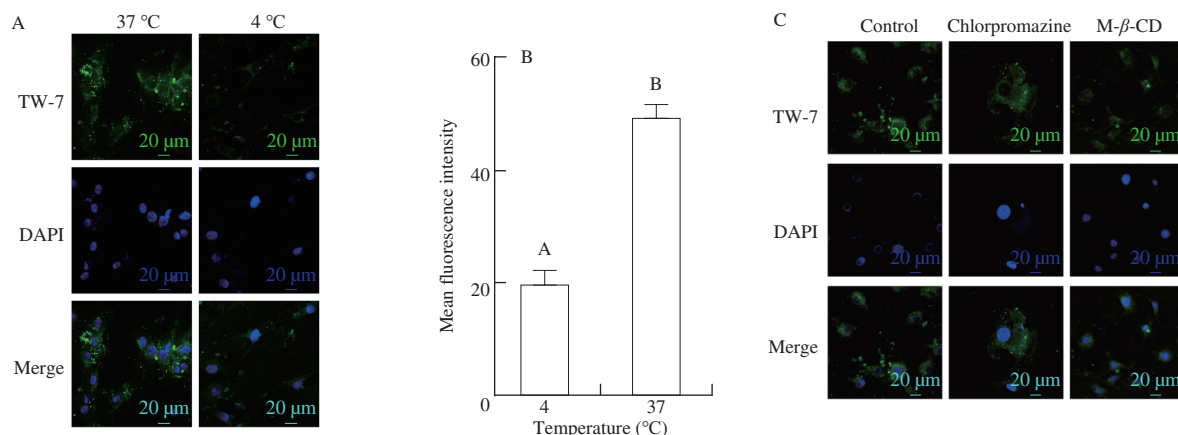


Fig. 3 Mechanism of TW-7 internalization into bEnd.3 cells. (A) Fluorescence images of FITC-TW-7 (100 μmol/L) incubated at 4 and 37 °C (scale bars = 20 μm). (B) Quantitative fluorescence intensity of FITC-TW-7 (100 μmol/L) incubated at 4 and 37 °C. (C) Fluorescence images of FITC-TW-7 (100 μmol/L) incubated with M-β-CD and chlorpromazine (scale bars = 20 μm). (D) Quantitative assays of fluorescence intensity. (E) The effect of incubation with M-β-CD and chlorpromazine on the internalization of FITC-TW-7 detected by flow cytometer. (F) Mean fluorescence intensity detected by flow cytometer. (G) Protein expression of Cav-1 and p-Cav-1. (H) Protein expression of Cav-2 and p-Cav-2. (I) Representative images of the immunofluorescence staining pattern of the co-localization of Cav-1 and Cav-2. (J) Co-localization images of TW-7 and Cav-1 (scale bars = 50 μm). Data are presented as means ± SD. All experiments were performed in triplicate ($n = 3$). Different uppercase letters indicate statistically significant differences ($P < 0.05$).

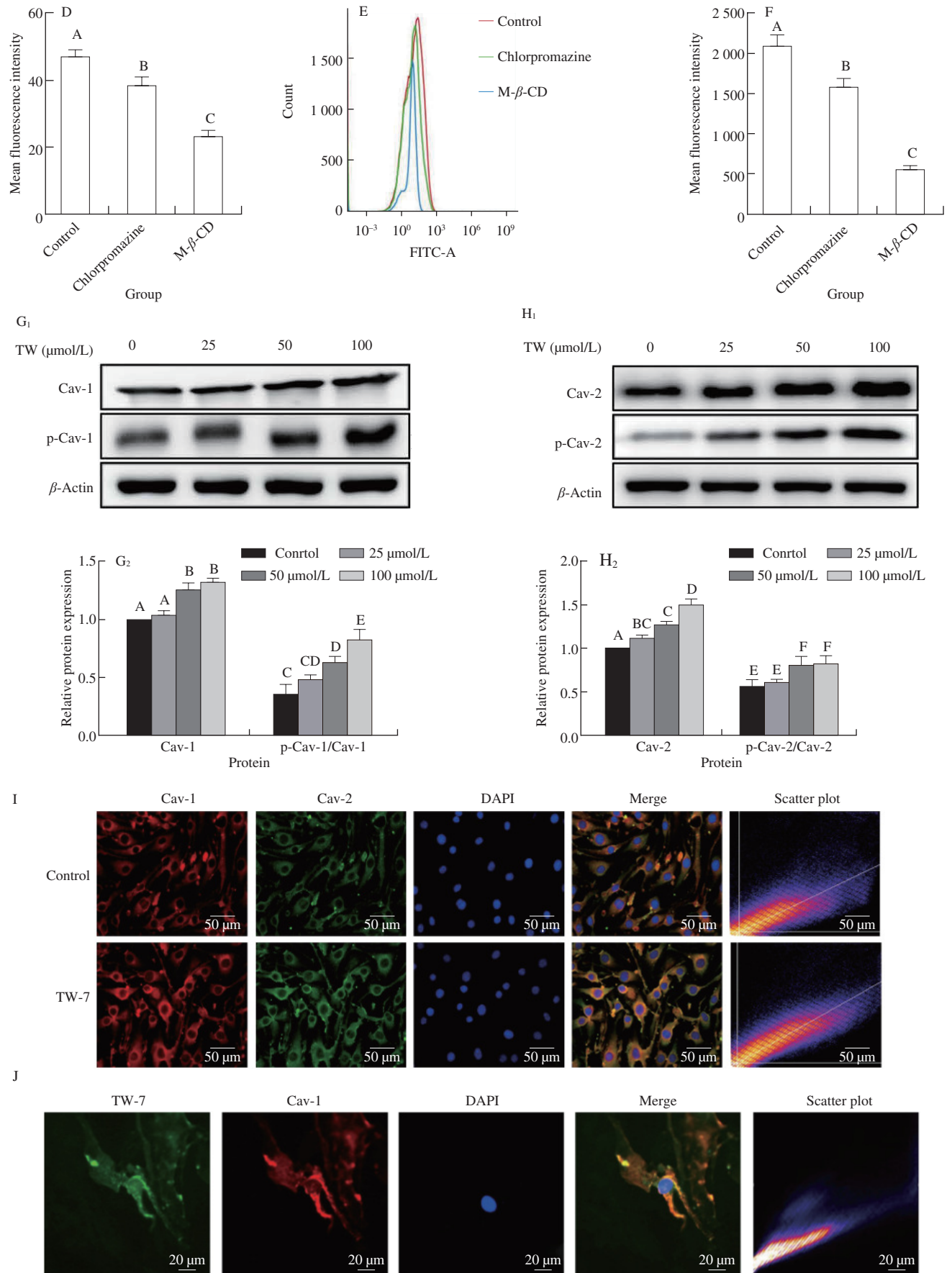


Fig. 3 (Continued)

roles in regulating caveolar dynamics and endocytic functions in endothelial cells. However, Cav-3 is predominantly expressed in the skeletal musculature and cardiac myocytes^[29]. Cav-1 plays a vital role in the endocytic processes of the BBB, whereas Cav-2 has been implicated in the formation of deep caveolae attached to the plasma membrane^[32]. Deng et al.^[34] demonstrated that increasing the expression level of Cav-1, and subsequently the number of caveolae, can enhance BBB permeability. More importantly, the regulation of endocytosis by Cav-1 and Cav-2 relies heavily on their phosphorylation status. Src kinase initiates intercellular transport by regulating the phosphorylation of Cav-1, and the physical association of Cav-2 with Cav-1 raises the possibility that Cav-2 may also be a cellular target for tyrosine phosphorylation by Src family kinases^[35].

Research has demonstrated that phosphorylation at tyrosine 14 of Cav-1 is essential for vesicle endocytosis^[36]. Furthermore, phosphorylation of serine 23 in Cav-2 can regulate the formation of Cav-1-dependent vesicles, and this process is influenced by Cav-1 and its subcellular localization^[37]. Li et al.^[38] reported that endothelial monocyte-activating polypeptide-II increased the number of pinocytotic vesicles in BMECs, which correlated with the phosphorylation and upregulation of Cav-1 and Cav-2. Our results are consistent with these findings. We observed that the walnut-derived peptide, TW-7, influenced the expression levels of Cav-1 and Cav-2. Furthermore, we found

phosphorylation patterns in pTyr14-Cav-1 and pSer23-Cav-2 similar to those observed in the expression of Cav-1 and Cav-2. Based on these findings, we hypothesized that TW-7 was internalized by bEnd.3 cells through a Cav-dependent transcellular pathway, in which both the phosphorylation and upregulation of Cav-1 and Cav-2 play a role.

3.5 Effect of TW-7 on efflux transport of bEnd.3 cells

BMECs have high expression of multiple efflux transporters on the apical membrane^[39]. While these efflux transporters remove harmful substances from the brain, they also block therapeutic substances from entering the CNS. To investigate the involvement of P-gp and BCRP, we examined the impact of TW-7 on the expression of these proteins in bEnd.3 cells. Compared to the control, TW-7 treatment had no significant effect on the expression of BCRP (Fig. 4A), but significantly decreased the expression of P-gp protein ($P < 0.05$; Fig. 4B). The immunofluorescence staining results (Fig. 4C) showed that the P-gp fluorescence intensity of bEnd.3 cells treated with 100 $\mu\text{mol/L}$ TW-7 was significantly decreased when compared to the control group, and the degree of co-localization with Cav-1 was reduced (the PCC value decreased from 0.67 to 0.36). As shown in Fig. S3, the gene expression levels of P-gp were significantly reduced by treatment with 100 $\mu\text{mol/L}$ TW-7.

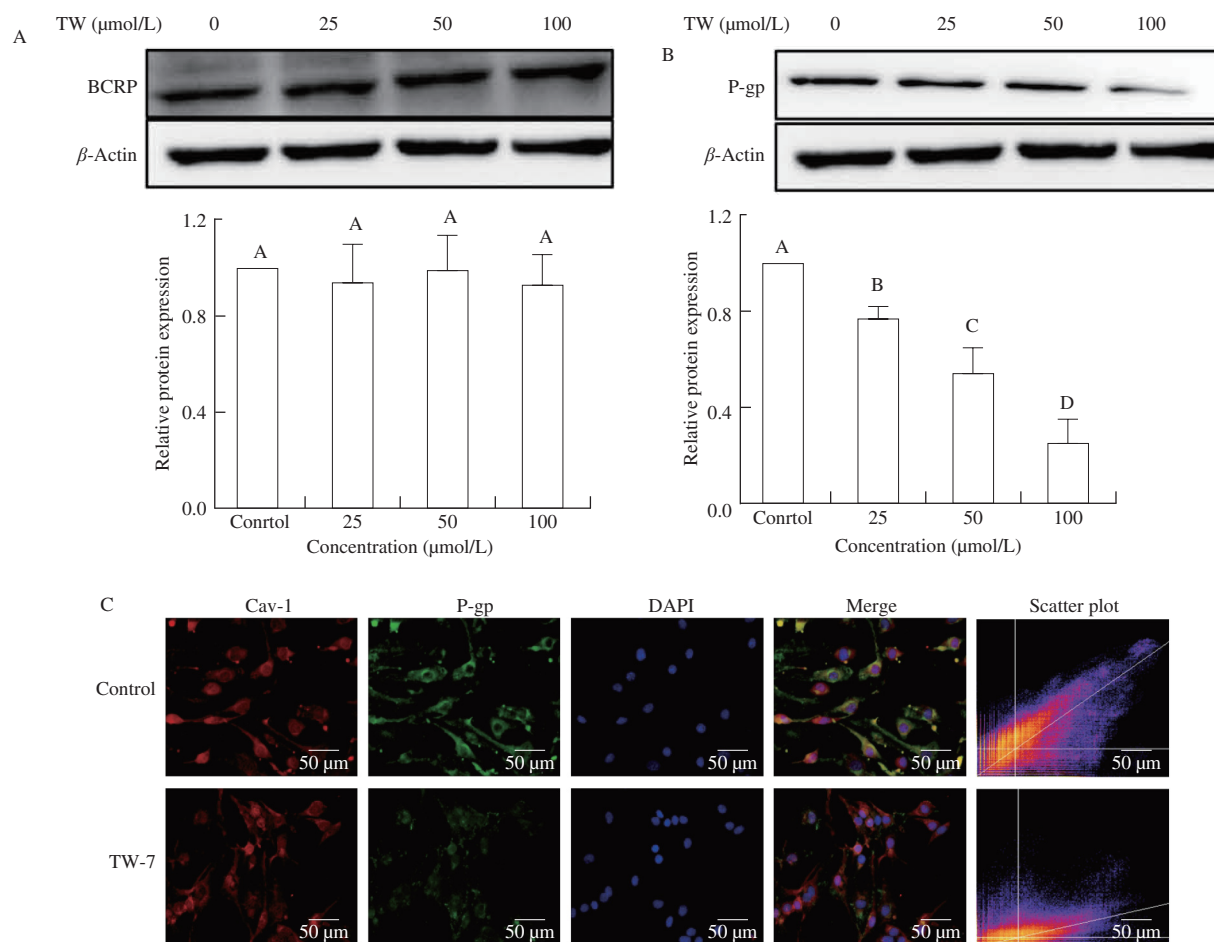


Fig. 4 Efflux mechanisms of TW-7. Protein expression of (A) BCRP and (B) P-gp. (C) Representative images of the immunofluorescence staining pattern of the colocalization of P-gp and Cav-1 (scale bars = 50 μm). Data are presented as means $\pm$ SD. All experiments were performed in triplicate ($n = 3$). Different uppercase letters indicate statistically significant differences ($P < 0.05$).

The AP membrane of BMECs is characterized by high expression of various multidrug resistance transporters, and the ATP-binding cassette gene family, including P-gp and BCRP, has been extensively studied^[40]. P-gp, which exhibits significant overlap with BCRP, is localized within the caveolae in BMECs^[41]. P-gp is involved in the unidirectional efflux transport of various lipid drugs from the brain to blood. Recent studies have primarily focused on P-gp inhibitors. Han et al.^[30] showed that P-gp inhibition significantly enhanced the retention of a D-peptide ligand of the nicotinic acetylcholine receptors (nAChRs) in the brain, leading to improved therapeutic effects in glioma mouse models. Jablonski et al. demonstrated that blocking P-gp and BCRP enhanced riluzole penetration into the CNS of mice and significantly restored CNS function when treatment was initiated at the onset of symptoms of neurodegenerative disease^[42]. Previous studies have shown that Cav-1 strongly co-localizes with and negatively regulates P-gp. Barakat et al.^[43] demonstrated that Cav-1 overexpression inhibited p-gp activity, and the interaction between p-gp and Cav-1 was decreased when tyrosine-14 was mutated. Blouin et al.^[44] reported that the phosphorylated Cav-1, in conjunction with dynamin, could direct cell surface endocytosis, resulting in reduced P-gp efflux. Similarly, we found that TW-7 significantly increased Cav-1 expression and Tyr14 phosphorylation (Fig. 3G). Therefore, TW-7 may be a potential P-gp inhibitor

3.6 Intracellular Trafficking of TW-7 in bEnd.3 Cells

Following peptide uptake by caveolae, the subsequent and pivotal process is intracellular trafficking, which involves the directional movement of vesicular structures^[45]. Therefore, we investigated the co-localization of FITC-TW-7 with various subcellular

compartments in bEnd.3 cells. We employed several trackers that target specific cellular structures, including the cell membrane, ER, Golgi complex, lysosomes, and mitochondria. As shown in Fig. 5A, FITC-TW-7 exhibited varying degrees of overlap with the aforementioned organelles after 4 h of incubation. Our findings revealed a high degree of co-localization between TW-7 and the Golgi complex, cell membrane, and ER, with PCC values of 0.85, 0.90 and 0.78, respectively. There are 2 transport routes for caveola-mediated endocytosis, the conventional route through endosomes and lysosomes, and the alternative route through the ER and Golgi apparatus^[46]. Extensive research has established that the Golgi complex and ER are the major destinations for material transport during Cav-mediated endocytosis^[47]. Notably, studies conducted by Reilly et al.^[48] reported the accumulation of histone H3 tail peptide/PEI-pDNA complexes in the Golgi complex and ER, indicating a close association between caveolar uptake and transport. These findings strongly support the notion that caveolae are a probable source of polyplexes localized within the Golgi complex and ER. Degradation is another pathway of intracellular transport involving the transport of peptides to lysosomes^[47]. Our research provides further insights by demonstrating the co-localization of FITC-TW-7 with lysosomes, suggesting that TW-7 may undergo degradation into amino acids that can be utilized by cells.

Mitochondria are closely associated with oxidative stress^[49]. Previous studies demonstrated the remarkable effects of TW-7 on mitochondrial function, including the restoration of mitochondrial membrane potential, inhibition of mitochondrial production of reactive oxygen species, and preservation of mitochondrial morphology and function through JNK-regulated PINK1-mediated mitophagy^[24]. Based on the findings of this study, we hypothesized that TW-7 enters the mitochondria where it can reduce mitochondrial oxidative damage.

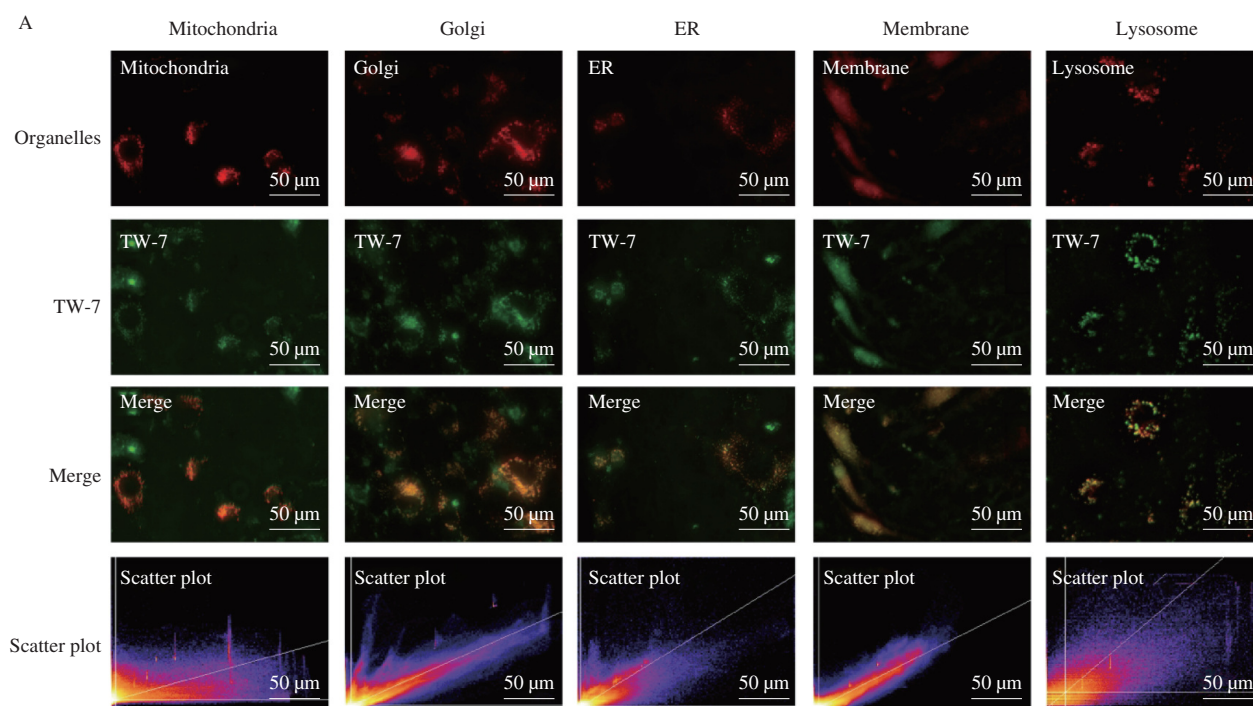


Fig. 5 Intracellular trafficking for TW-7. (A) Subcellular localization and quantification of FITC-TW-7 within the cell membrane, endoplasmic reticulum, Golgi, lysosomes, and mitochondria. (B) After incubation with FITC-TW-7 at 37 °C for 2, 4, 8, and 12 h, the colocalization within lysosomes was observed using fluorescence microscopy (scale bars = 50 μm).

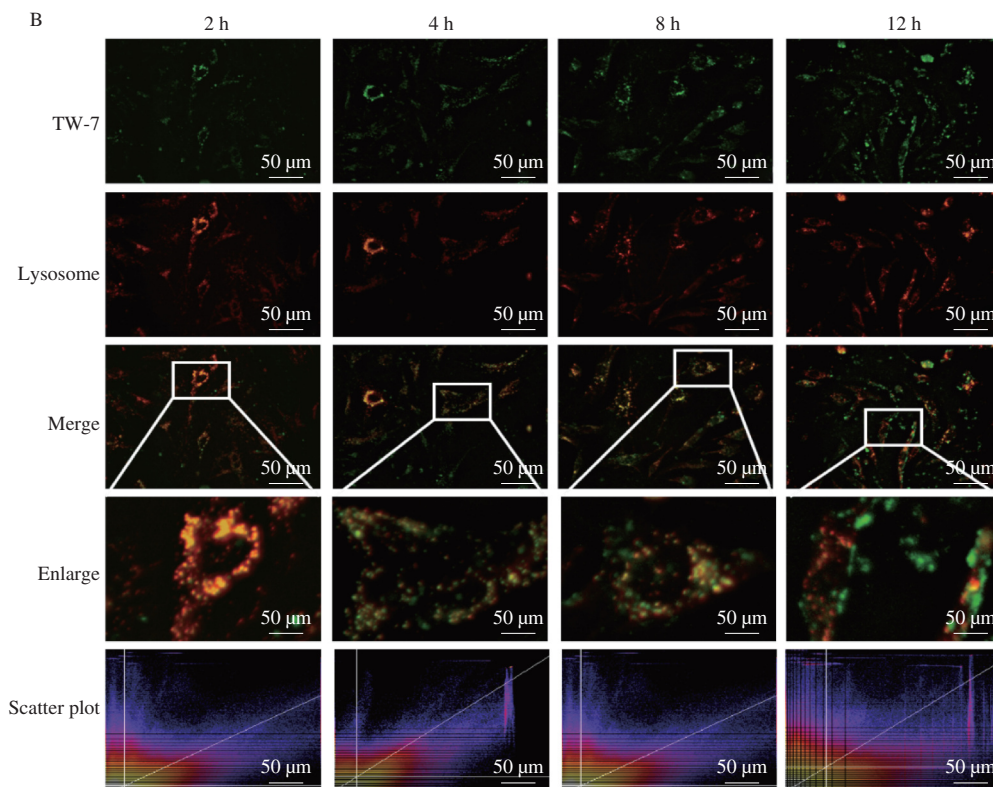


Fig. 5 (Continued)

Based on the aforementioned results, we hypothesized that TW-7 undergoes an intracellular transport pathway in bEnd.3 cells. It was likely that a fraction of TW-7 was sorted through the ER and Golgi complex and subsequently reached the plasma membrane for transcellular transport. Another portion of TW-7 was transported to lysosomes for degradation and cellular utilization. Importantly, TW-7 does not induce damage to mitochondria, and its localization within these organelles is crucial for preserving mitochondrial function and integrity.

3.7 TW-7 release from the lysosomal pathway

The lysosomal pathway of TW-7 was further investigated by conducting co-culture experiments with bEnd.3 cells. The intracellular distribution of FITC-TW-7 (green) and lysosomes labeled with LysoTracker Red (red) were observed by fluorescence microscopy at different times (2, 4, 8, and 12 h) after treatment. A substantial amount of yellow fluorescence was observed in the cells after 2 h, resulting from the overlap between green and red fluorescence signals. Co-localization was supported by a high PCC of 0.82, indicating a strong association between FITC-TW-7 and lysosomes at this time point. However, as the incubation time increased, the yellow fluorescence signal decreased, and the green and red fluorescence signals became more distinct at 4, 8, and 12 h. The PCC value decreased to 0.61 at 4 h, indicating a reduction in the degree of overlap between FITC-TW-7 and the lysosomes. Similar trends were observed at 8 and 12 h, with further separation between the red and green fluorescence signals and a significant weakening of the yellow fluorescence signal. The PCC values decreased to 0.47 and 0.31 at 8 and 12 h, respectively. These results indicated that TW-7 were internalized by cells and gradually dissociate from the lysosomes over time.

Endocytosis is a common mechanism through which peptides enter cells. However, the release of peptides appears to be a critical step that limits the efficiency of endocytic uptake. This process determines the ability of the peptides to reach the cytoplasm^[29]. Nonetheless, Cav-dependent endocytosis offers an alternative pathway that bypasses lysosomal fusion and degradation. Numerous studies have established that peptides and their cargo can escape lysosomes and access the cytosolic space^[50]. Chang et al.^[51] designed a transferrin cell-penetrating peptide-sterically stabilized liposome (i.e., TF-CPP-SSL) that successfully evaded lysosomal entrapment, leading to the release of liposomal contents into the cytosol and enabling the delivery of therapeutic agents across the BBB and into glioma cells. Similarly, Zheng et al.^[52] developed a neuron-specific delivery system composed of 2 D-peptides (CGN and QSH) able to escape from lysosomes and deliver siRNA into the cytoplasm of neurons. Research has shown that the lysosomal environment of cells is acidic, and the structure of peptides changes from irregular curls to α -spiral structure. This phenomenon leads to peptide escape from lysosomes, protecting the peptide chain from lysosomal hydrolysis^[53]. Consistent with these previous studies, our findings indicate that TW-7 can escape from lysosomes over time, potentially facilitating its penetration into the BBB. However, the precise mechanisms underlying the lysosomal escape require further investigation.

3.8 Internalization and trafficking mechanisms of TW-7 in bEnd.3 cells

Although numerous studies have elucidated the transport mechanisms of peptides across BBB, the current study primarily focused on understanding how foodborne cell-penetrating peptides are

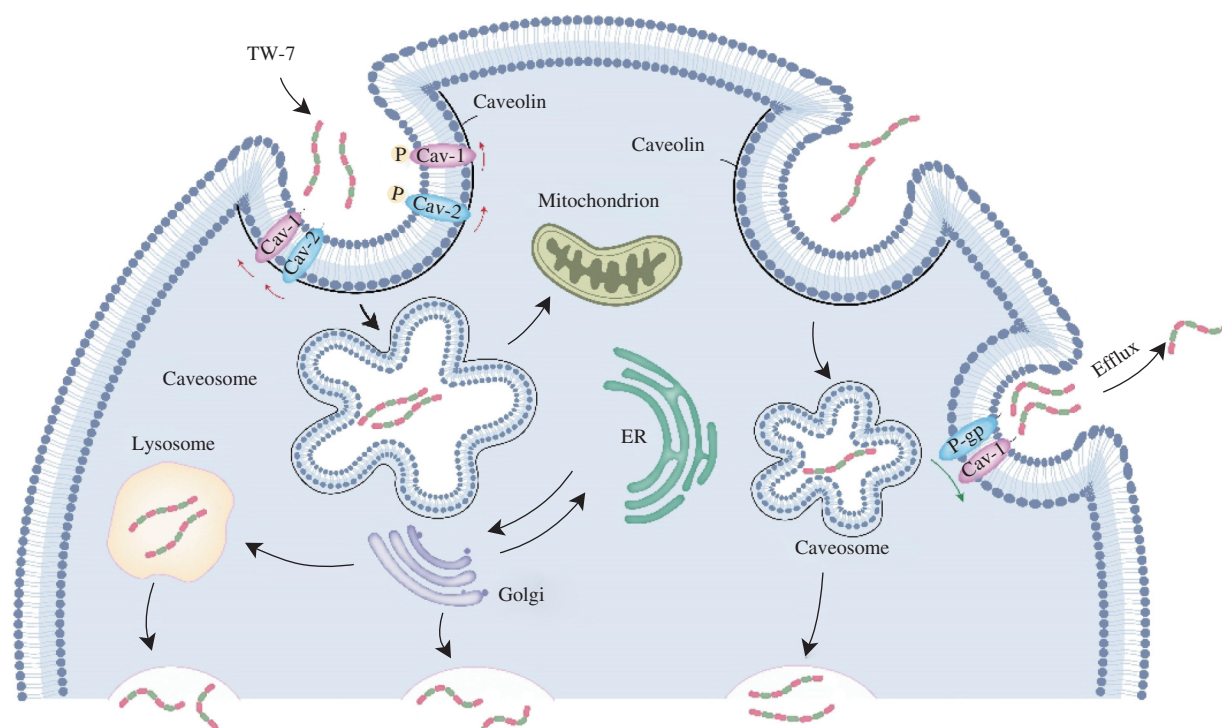


Fig. 6 Schematic representation of TW-7 internalization and transport in bEnd.3 cells.

transported across BMECs. There is a dearth of research investigating whether exogenous neuroprotective bioactive peptides can be taken up by BMECs and the mechanisms underlying transendothelial transport. In this study, bEnd.3 cells were used to investigate the molecular mechanisms underlying the trafficking of TW-7 across an *in vitro* BBB model. These findings offer valuable insights into the trafficking routes of exogenous bioactive peptides into bEnd.3 cells (Fig. 6). The transport of TW-7 is primarily mediated by a Cav-dependent pathway, which is facilitated by the upregulation of Cav-1 and Cav-2 expression and an increase in their phosphorylation levels. Additionally, TW-7 inhibited P-gp expression, leading to its accumulation within cells. Analysis of endocytic transport pathways revealed that TW-7 follows an intracellular trafficking pathway that localizes to the cell membrane, Golgi apparatus, endoplasmic reticulum, cell membrane, and mitochondria; this suggested that TW-7 underwent intracellular trafficking and has the potential to protect mitochondrial function. Further investigation revealed that TW-7, after entering the lysosome, separates from it over time, thereby avoiding degradation. This mechanism may contribute to the transmembrane transport.

4. Conclusion

In summary, the uptake and trafficking mechanisms of walnut-derived peptides in bEnd.3 cells were investigated for the first time. Our results show that TW-7 can penetrate the BBB *in vitro*. The mechanism of penetration was further analyzed using a bEnd.3 cell model. TW-7 was taken up by bEnd.3 cells in a concentration- and energy-dependent manner. The internalization mechanism of TW-7 is closely related to the Cav-mediated pathway and inhibits the efflux protein, P-gp. Moreover, the intracellular trafficking of TW-7 is

closely associated with the cell membrane, Golgi complex, ER, cell membrane, and mitochondria. Most importantly, we demonstrated that TW-7 escapes from the lysosomes, avoiding degradation. This study laid the foundation for the development of food-borne neuroprotective brain-targeted peptides and provided a theoretical basis for their movement across the BBB.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (22378368)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250354>.

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