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Targeted intracellular delivery of BH3 mimetic peptide inhibits BCL-2 activity and prevents breast cancer development

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ABSTRACT

Breast cancer, as a malignant tumor with easy metastasis and poor prognosis, threatens the health of women around the world. Increasing studies have shown that the Bcl-2 family of apoptosis-related proteins is often expressed abnormally in breast cancer. The Bcl-2 homology 3 (BH3) mimetic peptide can bind and neutralize Bcl-2, preventing its binding to the apoptosis "effector" proteins Bak and Bax, thereby promoting the apoptosis process. However, there is a lack of effective intracellular delivery system for BH3 to exert its biological activity. Therefore, this study utilized an activatable supercharged polypeptide (ASCP) tumor-targeted delivery platform based on pH and protease response to achieve the targeted release of BH3 at the tumor site. Ultimately, intracellular delivery of BH3 was achieved and induced apoptosis of breast tumor cells, preventing the development of breast cancer.

KEYWORDS

Breast cancer; B-cell lymphoma 2 (Bcl-2) family; BH3; activatable supercharged polypeptide; peptide delivery

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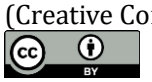

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1. Introduction

Breast cancer is the most common malignant tumor in women and one of the five most common cancers in the world. Clinically, breast cancer is mainly classified based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression (1,2). According to the data released in 2019, breast cancer accounted for 30 % of newly diagnosed malignant tumors in females, and led to 15 % of female deaths from cancer (3).

Over the past three decades, significant advances have been made in our understanding of the role of the B-cell lymphoma 2 (Bcl-2) family in apoptosis and cancer (4). The Bcl-2 family includes pro-apoptotic BH3 proteins (Bid, Bim, Puma, Noxa, Bad, Bmf, Hrk and Bik), anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1) and effector pro-apoptotic proteins (Bax, Bak and Bok) (5-7). Through structural analysis of Bcl-2 family proteins, it was found that the hydrophobic groove interaction formed between them through the BH domain (8, 9). Overexpression of anti-apoptotic proteins (such as Bcl-2) promotes its binding to pro-apoptotic proteins (such as Bax or Bak) and inhibits the polymerization of pro-apoptotic proteins on the mitochondrial membrane, thereby preventing the release of cytochrome c and Initiation of apoptosis (10, 11). Therefore, the Bcl-2 protein plays a central role in preventing apoptosis and helps breast cancer cells escape the apoptotic process.

Recently, an increasing number of Bcl-2 homology 3 (BH3) mimics have been developed, which bind and neutralize Bcl-2, preventing Bcl-2 binding to the apoptotic “effector” proteins Bak and Bax, thus promoting the apoptotic process (12). At the same time, since Bcl-2 is overexpressed in about 75 % of breast cancer, BH3 mimetic peptide are also considered as candidate drugs for the treatment of breast cancer (13, 14). Currently, there are many BH3 mimetics entering clinical research, among which Venotoclax/ABT-199 has been approved by the FDA for the treatment of chronic lymphocytic leukemia (CLL) (15, 16).

Despite the excellent efficacy of BH3 mimetics, there are still two issues that urgently need to be addressed (17). First, most of the BH3 mimics currently studied are one of the domains in pro-apoptotic effector proteins, which must cross the cell membrane to be active. Therefore, efficient intracellular delivery systems are needed to achieve transmembrane delivery of BH3. In addition, BH3 mimetics can only reduce systemic toxicity and increase the therapeutic window dose if they are active at the tumor site. Therefore, a tumor-targeted delivery system is needed to achieve targeted delivery of BH3.

In this study, we used activatable supercharged polypeptide (ASCP), a tumor-targeted intracellular delivery platform, to deliver the BH3 mimetic peptide (STKKLSECLKRIGDELDSNM), restore cell apoptosis, and prevent the development of breast tumors (17, 18). The delivery platform consists of a charge shielding sequence CSS, an MMP-2 protease cleavage site, and a cell penetration module SCP. By fusion and expression of ASCP and BH3 mimetic peptide into ASCP-BH3 protein, low pH & protease response was achieved while also completing intracellular delivery of BH3 mimetic peptide, ultimately inducing apoptosis of breast cancer cells. In summary, this study provides a new method for tumor-targeted delivery of BH3 mimetic peptides and provides a strategy for the treatment of breast cancer.

2. Materials and methods

2.1 Purification of ASCP-BH3 fusion protein

GenScript (China) was entrusted to complete the gene synthesis of ASCP-BH3. After the plasmid derived from the pET-28a (+) vector were transformed into *Escherichia coli* BL21(DE3) cells (Cat# C504-02, Vazyme, China), protein expression was induced using 1 mM IPTG (Cat# ST098-1g, Beyotime, China) at 26 ℃ for 12 h. ASCP-BH3 was separated by Ni^{2+} affinity chromatography column (Cat# 17524801, Cytiva, USA). Unbound protein was

removed by removal solution (20 mM Tris, 500 mM Nacl, 20 mM Imidazole), while ASCP-BH3 was eluted by elution solution (20 mM Tris, 500 mM Nacl, 150 mM Imidazole). The buffer system of ASCP-BH3 was replaced with PBS through a desalting column (Cat# 29048684, Cytiva, USA).

2.2 Characterization of ASCP-BH3 fusion protein

Western Blot: HIS antibody was used as the primary antibody (dilution of 1:10000, Cat# 66005-1-Ig, ProteinTech, China) and HRP-goat anti-mouse antibody (dilution of 1:10000, Cat# SA00001-1, ProteinTech, China) was used as the secondary antibody. After incubating the secondary antibody, immediately clean the fiber membrane with TBST, use DAB as the substrate in a dark environment, and develop color for 5 min. Finally, after washing with PBS, record the photos of the color development.

RP-HPLC: The C18 chromatographic column was used to detect the purity of ASCP-BH3 fusion protein. The detection wavelength of the liquid chromatograph was set to 214 nm, and the flow rate was 1 ml/min. The mobile phases were double distilled H_2O and acetonitrile, and were added with 0.065 % (v/v) TFA. The elution gradient was from 5 % (v/v) acetonitrile to 70 % (v/v) acetonitrile in 30 min.

2.3 Cell lines

MCF-7, human breast cancer cell was obtained from American type culture collection (ATCC, Cat# HTB-22ATCC, USA). MCF-10A, human normal breast cell was obtained from American type culture collection (ATCC, Cat# CRL-10317, USA). MCF-7 and MCF-10A cells were cultured in high-glucose DMEM (Cat# 12100046, Gibco, USA) with 10 % fetal bovine serum (FBS) (Cat# 10099141C, Gibco, USA). MCF-7 and MCF-10A cells were cultured in a cell culture incubator (5 % CO_2, 37 °C).

2.4 Cellular uptake of ASCP-BH3 fusion protein

ASCP-BH3 was incubated with fluorescein isothiocyanate (FITC, Cat# HY-66019, MedChemExpress, China) for 24 h (4 °C) and free FITC was removed through a desalting column. After incubating ASCP-BH3 (5 μM) and MCF-7 cells for 24 h, the average fluorescence intensity of the cells was detected by flow cytometry. Before using confocal microscopy for detection, we need to culture 1×10^5 MCF-7 cells in a small dish and incubate ASCP-BH3 (5 μM) with the cells for 24 h. After cells were fixed with paraformaldehyde, using DAPI (dilution of 1:10000, Cat# C1002, Beyotime, China) as the nuclear dye.

Lysosomal staining: ASCP-BH3 (5 μM) and MCF-7 cells were co-incubated for 24 h. Then, add Lyso-Tracker (dilution of 1:10000, Cat# C1046, Beyotime, China) and incubate for 30 min. Finally, Lyso-Tracker was removed and MCF-7 cells were observed using a laser confocal microscope.

2.5 Cell proliferation assay

After MCF-7 or MCF-10A were cultured in a 96-well plate for 12 h, different concentrations of ASCP-BH3 and MCF-7/MCF-10A cells were incubated for 24 h. Add CCK-8 detection solution (dilution of 1:20, Cat# C0037, Beyotime, China) to each well, react at 37 °C for 30 min, and detect the absorbance of each well at 450 nm.

2.6 Clonogenic assay

MCF-7 cells were cultured in 6-well plates (500 cells/well), and subsequent experiments were performed 3 days later. Add BH3 peptide (5 μM, 2 days) and ASCP-BH3 (5 μM, 2 days) to the 6-well plate respectively. After removing the cell culture supernatant, polymethylmethacrylate (Cat# P0099, Beyotime, China) was added to fix the

cell spots. Use crystal violet staining solution (Cat# C0121, Beyotime, China) to stain cell spots.

2.7 Detection of apoptosis by flow cytometry and JC-1

Flow cytometry: After MCF-7 cells were cultured in a 24-well plate for 12 h, ASCP-BH3 (5 μM, 24 h) was added to the wells. Subsequently, the cell supernatant was removed and all cells were collected. Then, the Annexin V-FITC/PI Apoptosis Detection Kit (Cat# A211-01, Vazyme, China) was used to determine apoptosis. 5 μL Annexin V-FITC and 5 μL PI were incubated for 15 min at 4 °C. Add Binding Buffer, and the sample was detected in a flow cytometer within 1 h.

JC-1: MCF-7 cells were treated using ASCP-BH3 (5 μm) for 24 h. Then, cells were treated 30 min under 37 °C using JC-1 (10 μg/mL, Cat# C2005, Beyotime, China). After washing with PBS, red and green fluorescence was observed by laser confocal microscope.

2.8 Animal Care

All nude mice were housed in individual ventilated cages (IVCs) and kept in a 12 h light-dark cycle, at a temperature of 25 °C and a relative humidity of 50%.

2.9 In Vivo Anti-Tumor Efficacy of ASCP-BH3

MCF-7 cells (1×10^6/mouse) were injected subcutaneously into mice, and they were randomly divided into groups when they grew to 100 mm^3. Saline, BH3 peptide, and ASCP-BH3 (once every two days, 0.25 mg/mouse) were injected into mice through the tail vein (100 μL/mouse). During this period, mouse tumor volume ($V = 1/2 \times L \times W^2$ (L, length; W, width of tumor)) and mouse body weight were recorded every two days. The mice were sacrificed on day 21, and tumor tissues were obtained. AiFang Biology (China) was commissioned to carry out Ki67 staining analysis of tumors.

2.10 ASCP-BH3 Biological Toxicity Assay

Saline, BH3 peptide and ASCP-BH3 (once every two days, 0.25 mg/mouse, 100 μL/mouse) were injected into mice, respectively. After three consecutive injections, the blood and liver tissues of the mice were obtained. Liver tissues were fixed in 4 % paraformaldehyde and embedded in paraffin and stained with Hematoxylin-eosin staining (H&E) for immunohistochemical analysis (Servicebio Inc, China). Blood Biochemical Indexes: The red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), and hemoglobin count (HGB) commissioned Servicebio Inc (China) to conduct testing. Hepatorenal toxicity was evaluated by aminotransferase (ALT) and aspartate aminotransferase (AST) kits.

2.11 Data and Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.1. The data are presented as the means ± SD (n≥3). Statistical analysis was performed using a one-way ANOVA.

3. Result

3.1 Construction and Characterization of ASCP-BH3

Tumor is an extremely heterogeneous tissue with multiple proteases within it and an acidic microenvironment

(19). In view of the above characteristics, we designed the protease MMP-2 response site and the shielding sequence (CSS) that can achieve charge conversion in an acidic environment, and cooperated with the cell penetration module (SCP)to achieve targeted release of active substances at the tumor site (this system is called ASCP) (18). Next, we fused BH3 and ASCP and expressed them in *Escherichia coli* BL21(DE3). ASCP-BH3 was purified through Ni^{2+} affinity chromatography column, and a highly pure fusion protein was obtained (Figure 1A & Figure 1B). Its properties were identified by Western Blot, and the results showed that the fusion protein contained the expected purified His tag (Figure 1C). The results of RP-HPLC showed that ASCP-BH3 with higher purity (>90 %) was obtained through purification (Figure 1D).

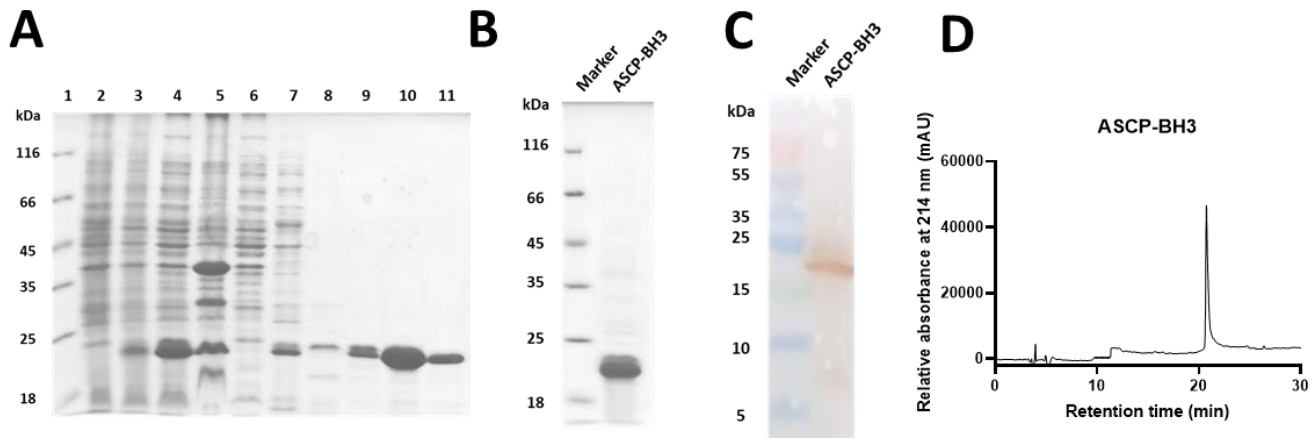

Figure 1. Expression and characterization of ASCP-BH3 fusion protein. (A) SDS-PAGE identified ASCP-BH3 through Ni^{2+} affinity chromatography column purification process. 1-5: Marker, Control, *E.coli* after IPTG processing, Ultrasonic disruption of supernatant, Ultrasonic disruption of precipitate; 6-8: Unbound protein; 9-11: Target protein. (B) SDS-PAGE identified desalted ASCP-BH3 fusion protein. (C) Western Blot identified the properties of the fusion protein ASCP-BH3. (D) RP-HPLC identified the purity of fusion protein ASCP-BH3.

3.2 Cellular uptake and microenvironmental response of ASCP-BH3

In order to verify the tumor microenvironment responsiveness of ASCP-BH3, we first measured the cellular uptake efficiency of ASCP-BH3 (FITC label) under the conditions of pH 7.4, pH 6.5, and pH 6.0 through flow cytometry. The results showed that in MCF-7 breast cancer cells, the cellular uptake efficiency of ASCP-BH3 at pH 6.0 was much greater than that at pH 7.4 (Figure 2A). At the same time, with the addition of MMP-2 enzyme, the cellular uptake efficiency of ASCP-BH3 further increased (Figure 2B). In summary, ASCP-BH3 can efficiently achieve transmembrane transport under MMP-2 enzyme and acidic environment.

In addition, the results taken by laser confocal microscopy showed that ASCP-BH3 was localized in the cytoplasm after entering the cells (Figure 2C&D). At the same time, lysosomes were stained by Lyso-Tracker, and ASCP-BH3 showed a significant localization difference from lysosomes (Figure 2E&F). This shows that after ASCP-BH3 enters the cytoplasm, it can achieve effective lysosomal escape and help BH3 exert its biological activity.

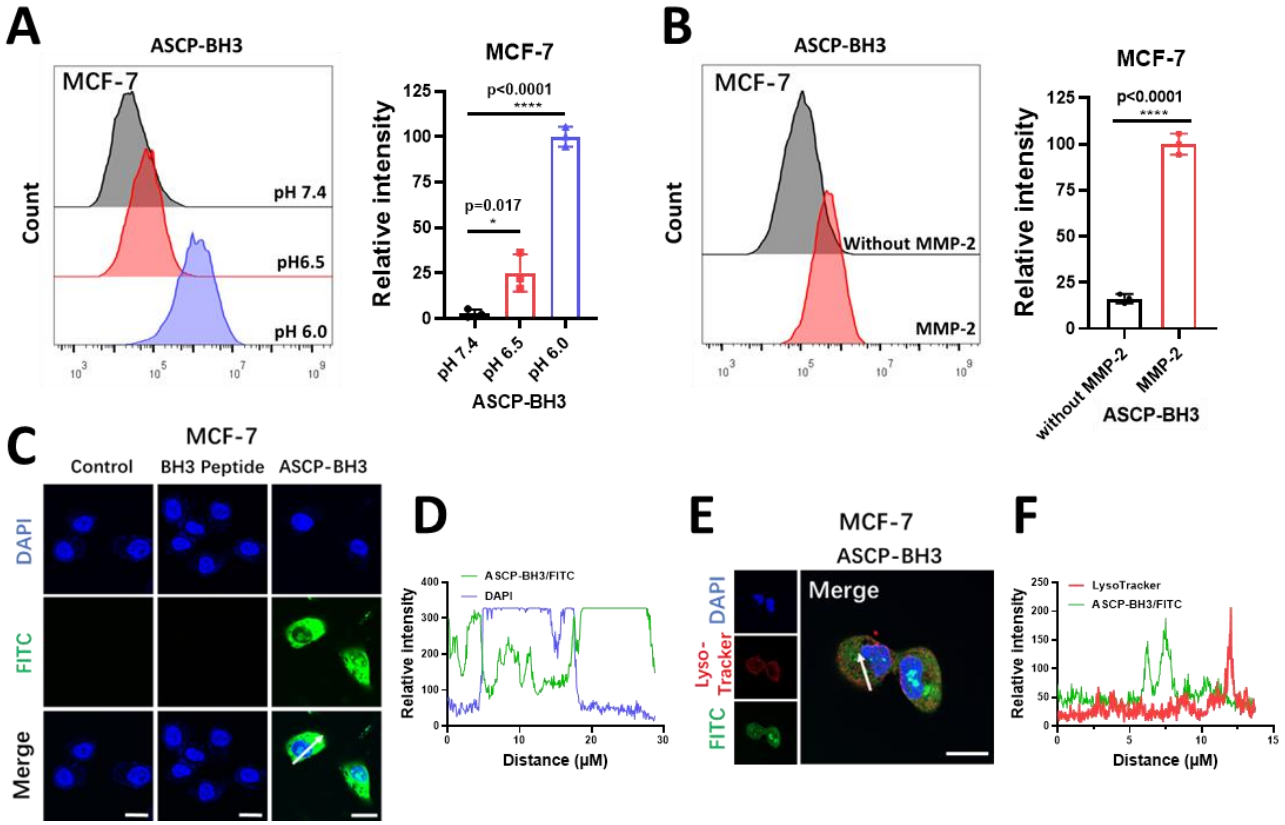

Figure 2. Cell uptake efficiency and Intracellular localization of ASCP-BH3 fusion protein. (A) Cellular uptake efficiency of ASCP-BH3 fusion protein under three additions (pH 6.0, pH 6.5, pH 7.4). (B) Cellular uptake efficiency of ASCP-BH3 after adding MMP-2 enzyme. (C) Confocal laser observation of intracellular localization of ASCP-BH3; scale bars, 10 μm. (D) Intracellular distribution fluorescence curve of ASCP-BH3. (E) Laser confocal observation of the co-localization of ASCP-BH3 and lysosomes; scale bars, 10 μm. (F) Colocalization curve of ASCP-BH3 and lysosome.

3.3 ASCP-BH3 can effectively induce apoptosis

Because the Bcl-2 protein in tumor cells can bind to Bax and other proteins, it prevents tumor apoptosis (20-22). The BH3 mimetic peptide can bind to Bcl-2 and prevent the binding of Bcl-2 to Bax, thereby initiating cell apoptosis. Therefore, in order to verify whether ASCP-BH3 can cause cell apoptosis after entering cells, we co-incubated ASCP-BH3 with MCF-7 cells for 24 hours. Through the cell viability measurement of MCF-7 cells, the results showed that ASCP-BH3 can start to produce activity at a concentration of 1.25 μM and completely kill tumor cells at a concentration of 20 μM (IC_{50}=4.25 μM) (Figure 3A). In addition, ASCP-BH3 has no obvious toxicity to normal breast cells MCF-10A at the same concentration (Figure 3B).

In addition, since phosphatidylserine will turn the cell membrane from inside to outside during early apoptosis, we examined this process by flow cytometry. The results show that compared with the BH3 peptide group, ASCP-BH3 can effectively cause the eversion of phosphatidylserine (PS). At the same time, PI dye confirmed the cell death process after apoptosis (Figure 3C&D). The changes of mitochondrial membrane potential in MCF-7 cells were measured by JC-1(Figure 3E). The results showed that ASCP-BH3 could effectively reduce mitochondrial membrane potential (Green) and induce mitochondria-mediated apoptosis (Figure 3E). In addition, clonogenic assay showed that ASCP-BH3 can effectively inhibit the growth of MCF-7 tumor cells (Figure 3F&G).

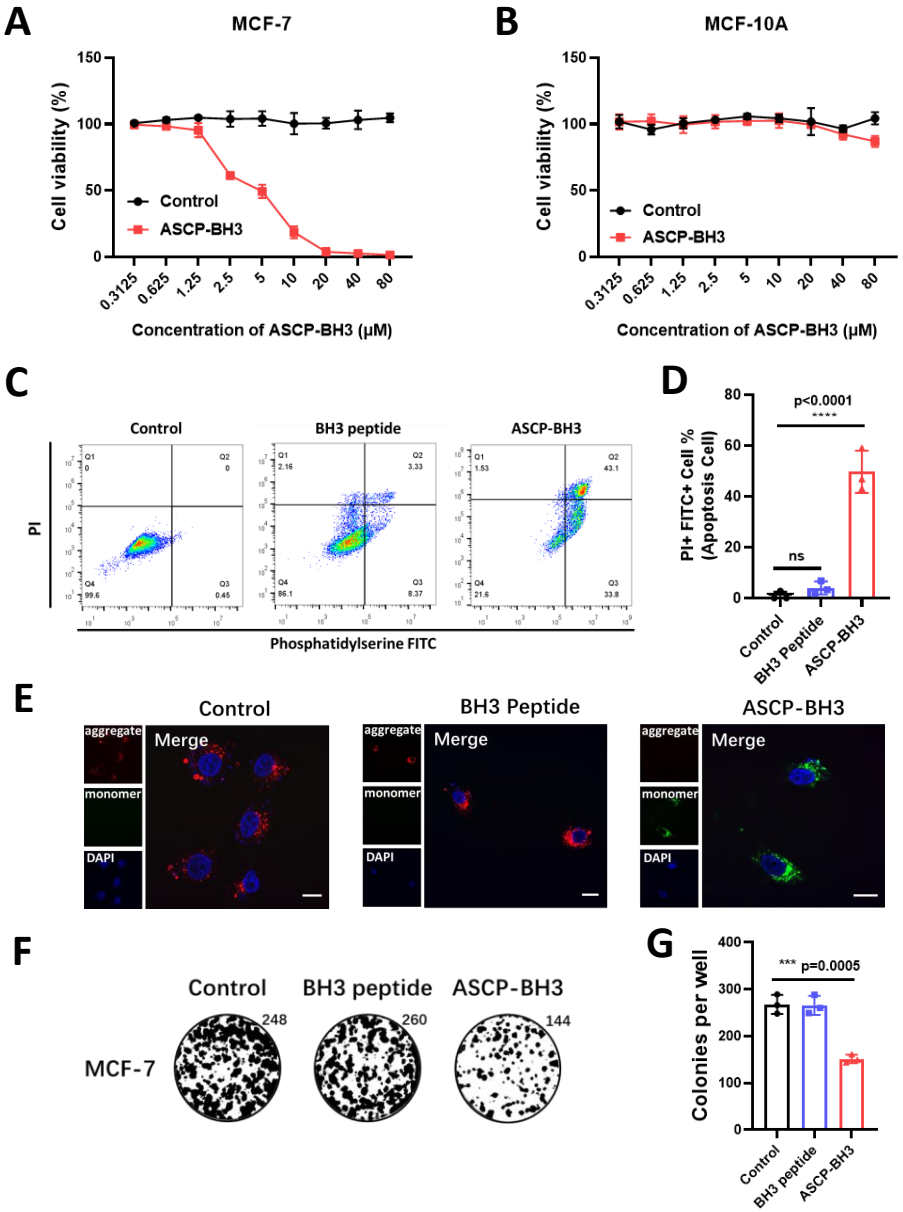

Figure 3. ASCP-BH3 can induce cell apoptosis. (A) CCK-8 was used to determine the killing activity of ASCP-BH3 at different concentrations against MCF-7 and (B) MCF-10A. (C) Flow cytometry was used to measure cell apoptosis induced by ASCP-BH3. (D) Proportion of PI^+ $FITC^+$ cells. (E) Changes in mitochondrial membrane potential (red: JC-1 aggregates, normal; green: JC-1 monomers apoptosis); scale bars, 10 μm. (F) Clonogenic assay was used to measure the proliferation inhibition of MCF-7 by ASCP-BH3 (5 days). (G) Counting of cell clone spots.

3.4 ASCP-BH3 can safely inhibit tumor growth

3.4.1 ASCP-BH3 can inhibit tumor growth in MCF-7 tumor-bearing mice

The anti-tumor activity of ASCP-BH3 *in vivo* was further studied through the MCF-7 subcutaneous transplantation tumor model. After the mice were randomly divided into groups, they were injected with physiological saline, BH3 peptide, and ASCP-BH3 (once every two days, 0.25 mg/mouse) through the tail vein. By detecting the tumor growth curve, the results showed that ASCP-BH3 can effectively inhibit the growth of MCF-7

tumors. On day 21, tumor volume was reduced by 70 % compared with the control group (1422.47 mm^3 vs 414.56 mm^3, P<0.0001) (Figure 4A). After the mouse tumors were peeled off, the measurement of the tumor weight further showed that ASCP-BH3 can effectively inhibit the growth of tumors (Figure 4B&C). Furthermore, there was no significant difference in mouse body weight between groups (Figure 4D).

Finally, the Ki67 immunohistochemistry results of mouse tumor tissues showed that the tumor proliferation of mice in the ASCP-BH3 group was significantly retarded (Figure 4E&F). In summary, compared with the control group, ASCP-BH3 can effectively target tumor tissue and prevent tumor development.

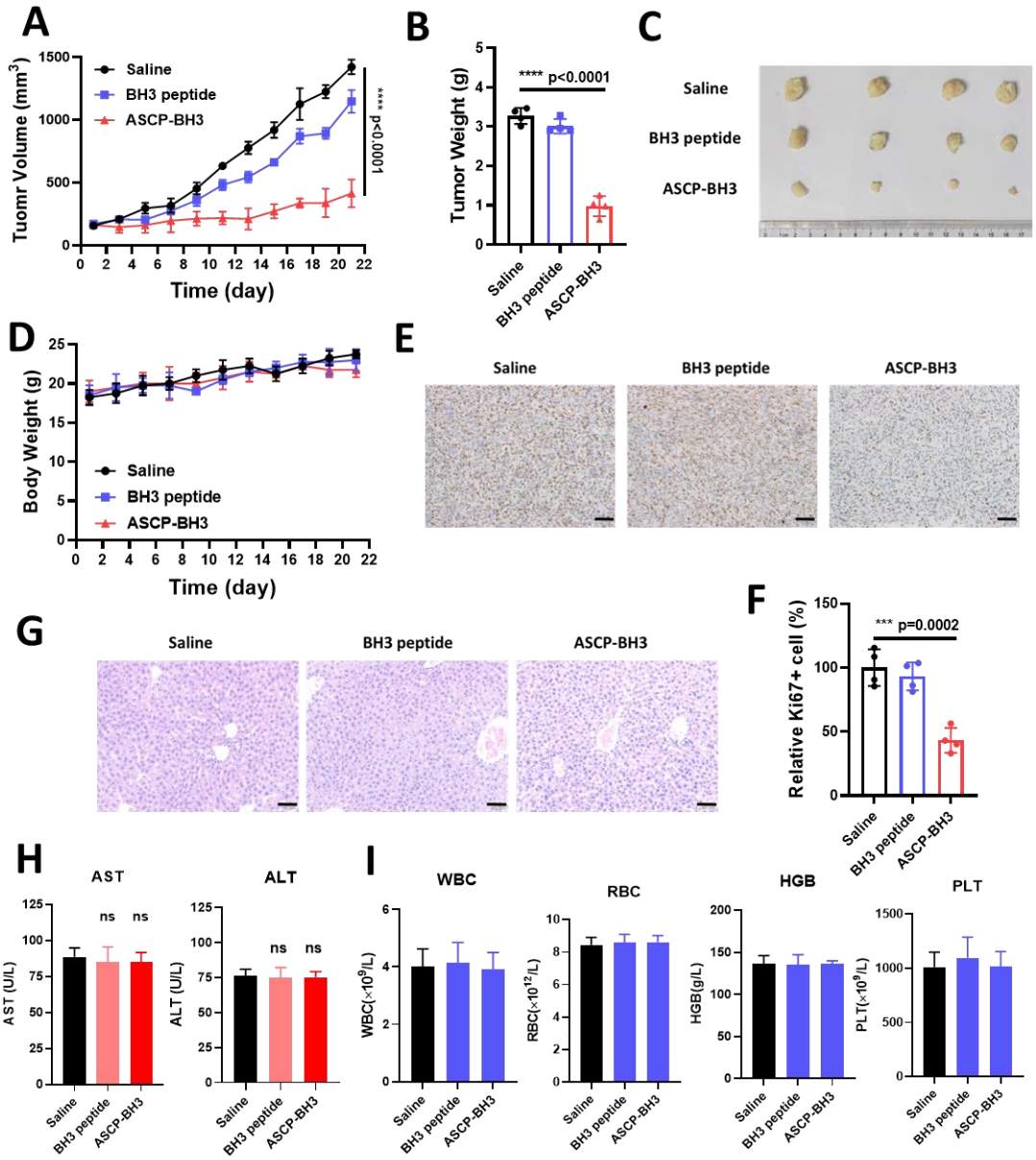

Figure 4. ASCP-BH3 can inhibit tumor growth in MCF-7 tumor-bearing mice. (A) Changes in tumor volume. (B) Tumor weight. (C) Photos of the tumor after dissection. (D) Body weight changes in mice. (E) Proliferation (Ki67) of the tumors were analyzed; scale bars, 50 μm. (F) Relative number statistics of Ki67-positive cells. (G) H&E staining of mouse liver tissue; scale bars, 50 μm. (H) Contents of AST&ALT in mouse serum after administration of different drugs. (I) Blood indicators (red blood cell count RBC, white blood cell counts WBC, platelet count PLT, and hemoglobin count HGB) of the mice.

3.4.2 Biosafety verification of ASCP-BH3

Compared with small molecule drugs, protein drugs are more biologically safe. Therefore, we also measured the biological toxicity of ASCP-BH3 in terms of liver toxicity, tissue toxicity, and blood biochemistry. After intravenously injecting ASCP-BH3 into the mice, the blood and liver tissues of the mice were obtained 3 days later. HE staining results showed that ASCP-BH3 did not induce obvious liver inflammation (Figure 4G). By measuring the levels of AST/ALT in serum, the results showed that ASCP-BH3 did not cause obvious liver damage (Figure 4H). At the same time, there were no obvious abnormalities in various blood indicators (red blood cell count RBC, white blood cell counts WBC, platelet count PLT, and hemoglobin count HGB) of the mice (Figure 4I). In summary, ASCP-BH3 is a safe and effective drug for the treatment of breast cancer.

4. Discussion

Approximately 75 % of primary breast cancers express high levels of Bcl-2. Bcl-2 is highly expressed in approximately 85 % of ER-positive tumors, 50 % of HER2-positive tumors, and 40 % of triple-negative breast cancer (TNBC) (1). Therefore, the search for new, more effective drugs, either as monotherapy or in combination with standard treatments, remains an important direction in the treatment of patients with aggressive breast cancer.

In cells, the balance between survival and death is controlled by three members of the Bcl-2 family: multi-domain anti-apoptotic proteins (Bcl-2, Bcl-XL, etc.), multi-domain pro-apoptotic proteins (Bax, Bak, etc.), and the pro-apoptotic BH3-only protein group (Bid, Bim, etc.). Among them, pro-apoptotic proteins are apoptosis effectors, pro-apoptotic BH3-only proteins are the initiating factors of apoptosis, and anti-apoptotic proteins promote cell survival by inhibiting pro-apoptotic proteins (5-7). In healthy cells, anti-apoptotic proteins attach to and inhibit the effector proteins Bax or Bak, blocking their polymerization on the mitochondrial surface and preventing the initiation of apoptosis (10). Increasing evidence now suggests that Bcl-2, which is overexpressed in most ER-positive tumors, is an excellent therapeutic target (23). Currently, therapies using Bcl-2 inhibitors are attracting more and more attention from researchers. One of them is to use BH3 mimetic peptide to inhibit the anti-apoptotic activity of Bcl-2 (24-26). However, BH3-bound Bcl-2 is located in the cytoplasm, therefore, BH3 requires an efficient intracellular transport carrier (1).

In recent years, people have developed a series of technologies to mediate the entry of proteins or polypeptides into cells. One type is to directly transport proteins into cells through physical delivery methods such as microinjection, electroporation, ultrasound, and mechanical deformation. However, most of these methods are invasive and require special equipment (27, 28). In addition, the instantaneous cell penetration produced by these methods can also allow other proteins and biomolecules to enter cells, causing uncontrollable side effects. The other type is carrier-based protein delivery. Currently commonly used carriers include cationic liposomes (29), high molecular polymers (30, 31), nanoparticles (32), cationic peptides (33) and supercharged proteins (34) etc. Among them, peptide/protein carriers have received widespread attention due to their good biocompatibility and biodegradability.

In this study, we constructed an anion shielding sequence CSS that can neutralize the cationic peptide SCP (18,35). Moreover, CSS can complete charge conversion at the tumor site and, under the action of MMP-2 enzyme, remove the charge shielding of SCP. Ultimately, SCP can carry BH3 into the cytoplasm and complete the biological process of promoting cell apoptosis. At the same time, *in vivo* experiments showed that ASCP-BH3 can effectively inhibit the growth and proliferation of MC38 tumors. More importantly, the detection of AST/ALT levels in serum showed that ASCP-BH3 had no significant toxicity to mice while killing tumors. In summary, the successful design of ASCP-BH3 provides a new option for the treatment of breast cancer.

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This research received no external funding.

Conflict of interest

The authors claim that the manuscript is completely original. The authors also declare no conflict of interest.

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