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Original Article

Xihuang pill induces pyroptosis and inhibits progression of breast cancer cells via activating the cAMP/PKA signalling pathway

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Abstract: Xihuang pill (XHP), a traditional Chinese medicine, has been shown to be effective for breast cancer (BC) therapy in clinical trials. However, the molecular mechanism of XHP in BC remains unclear. The molecular mechanism of XHP in BC was investigated *in vivo* by generating murine mammary carcinoma 4T1 cell xenografts. Enzyme-linked immunosorbent assay (ELISA) and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) were used to detect pyroptosis-related indicators. Transcriptome sequencing was used to identify potential targets of XHP. Cell counting kit-8 (CCK-8), EdU, cell wound-healing, and transwell assays were performed to investigate the role of XHP in BC cells. Western blotting was performed to identify proteins related to the cAMP/PKA signalling pathway. XHP inhibited the growth of BC and induced pyroptosis. Western blotting confirmed the significant association between XHP and the cAMP/PKA signalling pathway revealed by Kyoto Encyclopedia of Genes and Genomes pathway analysis. XHP inhibited BC cell proliferation, migration, and invasion, and induced pyroptosis. Inhibiting the cAMP/PKA signalling pathway reversed the anti-cancer activity of XHP. XHP inhibits proliferation, migration, and invasion, and induces pyroptosis of BC cells via activating the cAMP/PKA signalling pathway.

Keywords: Breast cancer, Xihuang pill, pyroptosis, cAMP/PKA signaling pathway

Introduction

Breast cancer (BC) is one of the most prevalent malignant tumours, and has a relatively high incidence rate. Approximately 2.3 million cases of BC and 685,000 BC-related deaths were reported globally in 2020, making it the fifth most commonly diagnosed malignancy [1]. Surgical resection, radiation, chemotherapy, and endocrine therapy remain the mainstays in BC treatment [2]. Although these treatment options improve the overall survival rate of patients, they do not completely eliminate the disease and are often associated with adverse effects such as destroying the haematopoietic system, damaging the gastrointestinal system, and causing nausea and vomiting [3]. Therefore, there is an urgent need to identify alternate treatment options for BC.

Traditional Chinese medicines (TCMs) that can reduce the toxicity of chemo- and radiotherapy, alleviate the clinical symptoms caused by tumours, and reduce cancer pain represent good alternative and complementary therapeutic approach for BC [4]. Xihuang pill (XHP) is a well-known TCM formulation mainly composed of Xiang (olibanun), moyao (Myrrha), *Caryophyllus bamboo* (Moschus), and Niuhuang (cattle stone) [5]. It was recorded in the Surgical Quan Sheng Ji during the Qing Dynasty and was developed by Wang Weide [6]. XHP has been reported to have therapeutic effect in a variety of tumours, including gastric cancer [7], lung cancer [8], glioblastoma [9], and BC [10]. Rat serum containing XHP could decrease the proliferation of MDA-MB-435, MDA-MB 231, and MCF-7 cells [11]. Hs578t cell viability was also markedly reduced when treated with an aque-

ous extract of XHP. XHP inhibited the tumour growth of BC, possibly by promoting apoptosis of Treg cells in the tumour microenvironment [12]. However, studies on the role and mechanism of XHP in BC are rare, and whether XHP can affect pyroptosis and the cAMP/PKA signaling pathways in BC is still unknown.

Pyroptosis, also known as secondary necrosis, is a form of non-apoptotic programmed cell death closely associated with the inflammatory reaction [13]. It is characterised by cell swelling, formation of massive bubbles in the plasma membrane, and plasma membrane rupture [14]. Pyroptosis is correlated with the progression of multiple tumors, such as hepatocellular carcinoma [15], colorectal cancer [16], and lung cancer [17]. Pyroptosis activation has an essential role in BC development. Dihydroartemisinin induced pyroptosis in BC cells by activating the expression of deleted protein in caspase-3 and gasdermin E, leading to inhibition of tumour proliferation [18]. Metformin induces pyroptosis by enhancing Bax activation and cytochrome C release, leading to increased lactate dehydrogenase levels in BC [19]. However, the correlation between XHP and pyroptosis in BC is still unclear. Cyclic adenosine monophosphate (cAMP) mainly interacts with protein kinase A (PKA) and plays a key role in cell signalling [20]. The cAMP/PKA signaling pathway influences tumour progression. It can regulate the stemness and metastasis of colorectal cancer cells [21]. Blocking the cAMP/PKA signaling pathway enhances the inhibitory effect of aspirin on hepatocellular carcinoma [22]. In BC, the accumulation of lipid droplets is regulated by the activation of β-adrenergic receptors through the cAMP/PKA signaling pathway [23]. Carvedilol inhibits the progression of malignant breast cells by suppressing the cAMP/PKA signaling pathway [24]. However, whether XHP can regulate cAMP/PKA signaling pathway in BC is unknown.

In the present study, we performed *in vitro* and *in vivo* experiments to determine the role of XHP in BC and used transcriptome sequencing to identify the underlying molecular mechanism. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that XHP may regulate the cAMP/PKA signalling pathway, which has been demonstrated to play a significant role in the development of BC in previous studies. Moreover, flavonoids extracted from *Lonicera rupicola* Hook were found to induce pyroptosis in mononuclear phagocytes in mice by activating the cAMP/PKA signalling pathway [25]. Therefore, we hypothesised that XHP affects BC cell proliferation, migration, invasion, and pyroptosis through the cAMP/PKA signalling pathway and tested our hypothesis using *in vitro* experiments.

Materials and methods

Animal experiments

Female BALB/c mice (n = 18, aged 6-8 weeks, weighing 18-20 g, SPF Biotechnology Co. Ltd, Beijing, China) were used to generate the xenograft models. Murine mammary carcinoma 4T1 cells (1×10^6 cells/mL) were injected into the axilla of mice in a total volume of 100 μL. The experimental animals (six mice/group) were randomly assigned to three groups: the model group, low-dose XHP-treated group, and high-dose XHP-treated group. The XHP-treated groups were first gavaged on day 2 after the xenografts were established. The high-dose and low-dose XHP-treated groups were administered 0.3 and 0.2 mL of XHP (0.05 g/mL) per mouse. Mice in the model group were gavaged with the same volume of sterile water. Tumour width (W) and length (L) were measured every 5 days with a calipers to monitor the tumour growth. The tumour volume (V) was calculated as $V = (W^2 \times L)/2$. The mice were sacrificed 21 days following the injection, and their tumours were excised, weighed, and measured.

Hematoxylin-eosin (HE) staining

Paraffin sections of the tumour tissues were deparaffinised and rehydrated, and the slides were placed in haematoxylin staining solution for 5 min. The slides were soaked in a solution containing 1% acid ethanol (1% hydrochloric acid plus 70% ethanol) five times. The sections were then stained with eosin for 3 min before dehydration with graded alcohol and clearing in xylene. The stained sections were observed under a light microscope (Olympus, Japan) and imaged to examine the histopathological changes in the tumour.

Immunohistochemistry (IHC)

Following deparaffinisation, paraffin sections of the tumour tissues were hydrated in varying

concentrations of ethanol. Citrate antigen retrieval solution was added, followed by heating in a microwave (Boxun, Shanghai, China) to retrieve the antigens. The sections were blocked with 5% normal goat serum and incubated overnight at 4°C with a primary antibody against Ki67 (Abcam, Cambridge, UK). Immunohistochemical images were obtained with a light microscope (Olympus).

Transcriptome sequencing and analysis of the differentially expressed genes (DEGs)

Total RNA was extracted from tumour tissues from the model and high-dose XHP-treated groups using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Poly (A) + mRNA was isolated from the total RNA using the poly (A) mRNA magnetic isolation module. Transcriptome sequencing libraries were prepared and sequenced on the Illumina Novaseq 6000 platform. The transcripts were quantified using the StringTie software and DEGs were then determined using DESeq2. The filtering criteria for the significant DEGs were as follows: FDR < 0.05 and |Log2FC| > 1. DEGs from the Database for Annotation (http://david.abcc.ncifcrf.gov/) were subjected to both gene ontology (GO) and KEGG analyses.

Cell culture

BC MCF-7 cells were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and grown in Rosewell Park memorial institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Experimental animals were separated into three groups: control group, XHP-treated group, and XHP + H-89 (cAMP/PKA signalling pathway inhibitor)-treated group. To investigate the effect of XHP on BC cells, stable passaged MCF-7 cells were pretreated with extracted drug-containing mouse serum (20%) and then continuously treated with XHP for 7 days. XHP-treated MCF-7 cells were further treated with H-89 (30 μm) for 1 h to examine the effect of XHP on the cAMP/PKA signalling pathway. VX-765 (Sigma, Shanghai, China), caspase-1 inhibitor, was dissolved in DMSO at a concentration of 10 μm. XHP-treated MCF-7 cells, were further treated with VX-765 for 1 h to examine the effect of XHP on the pyroptosis in BC.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (Esebio, Shanghai, China) were used to determine the levels of IL-1β, IL-6, TNF-α, and cAMP in mouse serum and cell culture supernatant. Absorbance was read at 450 nm on a microplate reader (DALB, Shanghai, China).

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using the TRIzol® Reagent (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference gene. The RT-qPCR amplification reaction conditions were set as follows: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 20 s, and the number of cycles is 40. Relative expression of the target genes was computed using the $2^{-\Delta\Delta CT}$ method. The primer sequences used in the PCR reactions are listed in **Table 1**.

Western blot analysis

Whole cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Solarbio, Beijing, China). Whole cell extracts were collected and the protein content was measured using a bicinchoninic acid kit (BCA) (Solarbio). The proteins were separated on a sodium dodecyl sulphate (SDS)-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membrane was blocked at room temperature with 5% fat-free dry milk and then incubated with the following primary antibodies: anti-GSDMD (1:2000, Abcam), anti-caspase-1 (1:2000, Abcam), anti-caspase-4 (1:2000, Abcam), anti-NLRP3 (1:2000, Abcam), and anti-β-actin (1:2000, Abcam). The membranes were then incubated with the secondary antibody (1:10000, Abcam) at room temperature for 2 h and rinsed with TBS + Tween four times for 10 min each. Protein bands were visualised using enhanced chemiluminescence reagents (Amersham, Little Chalfont, UK).

Cell counting kit-8 (CCK-8) assay

MCF-7 cells (1×10^4 cells/well) were plated in a 96-well plate. Then, 10 μL CCK-8 reagent (Solarbio) was added to each well following

Table 1. The primer sequences included in the study

Primer name	Sequences (5'-3')
M-GAPDH-F	CTCATGACCACAGTCCATGC
M-GAPDH-R	TTCAGCTCTGGGATGACCTT
M-Caspase-1-F	CACAGCTCTGGAGATGGTGA
M-Caspase-1-R	TCTTTCAAGCTTGGGCACTT
M-Caspase-4-F	ACAATGCTGAACGCAGTGAC
M-Caspase-4-R	CTGGTTCCTCCATTTCCAGA
M-GSDMD-F	TGCGTGTGACTCAGAAGACC
M-GSDMD-R	ATAAAGCTCCAGGCAGCGTA
M-NLRP3-F	ATGCTGCTTCGACATCTCCT
M-NLRP3-R	AACCAATGCGAGATCCTGAC

treatment with XHP or the cAMP/PKA pathway inhibitor H-89 at 0, 24, 48 and 72 h. The absorbance was read at 450 nm using a microplate reader (DALB).

EdU assay

MCF-7 cells (1×10^4 cells/well) were plated in a 96-well plate and cultured overnight. The cells were fixed in 4% formaldehyde for 30 min and then permeabilised using 0.1% Triton X-100 (Sigma, Shanghai, China) for 20 min. A fluorescent microscope was used to take images of the EdU staining.

Wound-healing assay

When MCF-7 cell density reached 80% or higher in the wells, a straight line was drawn vertically with a pipette tip on the monolayer. The damaged and dead cells were removed by washing the cells thrice with phosphate buffered saline. The wound areas were measured with ImageJ software 0 and 24 h after wounding. The wound-healing rate was calculated as: (original area - final area)/initial area × 100%.

Transwell assay

MCF-7 cells were resuspended at a density of 5×10^5/mL in serum-free media. The upper compartment of the Transwell chamber received 100 μL of the cell suspension, whereas the lower chamber received 600 μL medium containing 10% FBS. The lower chamber cells were removed from the growth medium, fixed in 4% paraformaldehyde for 30 min, and then treated with 0.2% Triton X-100 (Sigma) solution for 15 min. The cells were stained with 0.1% crystal violet for 30 min and examined under a microscope (Olympus).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, USA), and the outcomes are displayed as the means ± standard deviation (SD). The Student's *t*-test was used to compare two groups, and one-way analysis of variance (ANOVA) and Turkey's test were used to compare multiple groups. Statistical significance was set at $P < 0.05$.

Results

XHP inhibits the growth of 4T1 xenograft

First, we investigated the effect of XHP on the growth of BC using a xenograft model. Tumour volume and weight were considerably reduced in the high-dose XHP-treated group compared with those in the model and low-dose XHP-treated groups, indicating that XHP inhibits the growth of BC in a dose-dependent manner ($P < 0.05$, **Figure 1A-C**). HE staining revealed that the mammary gland structure and cell arrangement in the model group mice were slightly disordered, with concentrated and solidified nuclei and interstitial oedema. Following XHP treatment, tumour necrosis was apparent, cell hierarchy was clear, and the cells were arranged in a neat and orderly manner, especially following high-dose XHP treatment (**Figure 1D**). In addition, IHC staining revealed a substantial reduction in Ki-67 positive cells in the XHP-treated group compared with that in the model group, with the inhibitory effect increasing with increasing XHP concentration (**Figure 1E**).

XHP promotes pyroptosis of BC in vivo

Next, we examined the role of XHP in pyroptosis of BC *in vivo*. ELISA revealed that the concentrations of inflammatory factors, including IL-1β, IL-6, and TNF-α, were lower in the model group compared with those in the control group. Addition of XHP markedly increased the release of these inflammatory factors. The concentration of IL-1β and TNF-α was considerably higher in the high-dose XHP-treated group compared with that in the low-dose XHP-treated group (**Figure 2A**, $P < 0.05$). Moreover, mRNA expression of pyroptosis-related factors, such as GSDMD, caspase-1, NLRP3, and caspase-4,

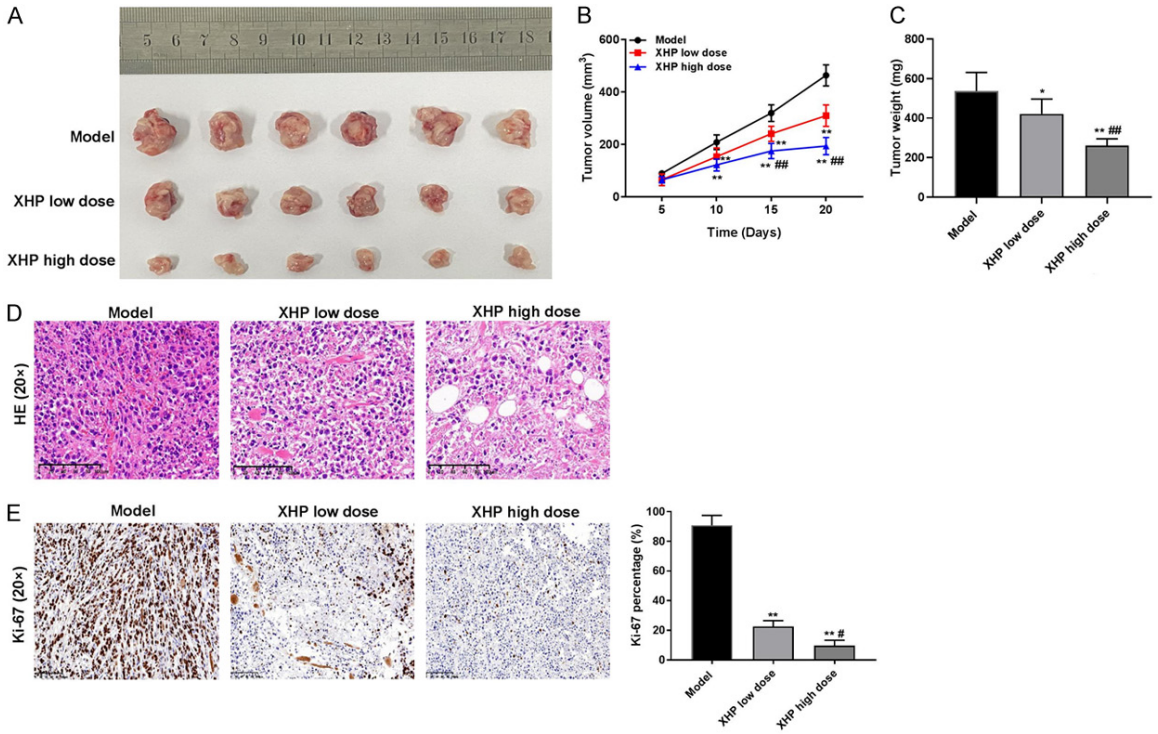


Figure 1. XHP inhibits the growth of BC. A-C. The morphology, tumour volume and tumour weight of the xenograft in different groups. D. HE staining to observe histopathological changes. E. Ki-67 immunohistochemical staining assay to detect cell proliferation. The number of samples containing mice in each group was six. Scale bars for the IHC and pathological figures were 100 μm and the magnification of the image is 200×. Compared with the model group, $^{*}P < 0.05$, $^{**}P < 0.01$. Compared with the low-dose XHP-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

was substantially lower in the model group compared with that in the control group. In addition, the mRNA expression of GSDMD, NLRP3, and caspase-4 was significantly higher in the high-dose XHP-treated group compared with that in the low-dose XHP-treated group (**Figure 2B**, $P < 0.05$). These results suggest that XHP may trigger BC cell pyroptosis *in vivo*.

XHP regulates the cAMP/PKA signalling pathway

To study the molecular mechanisms of XHP in BC, RNA samples of tumour tissues from the model and high-dose XHP-treated groups were subjected to transcriptome sequencing. In total, 28 significantly upregulated DEGs and 83 significantly downregulated DEGs were identified (**Figure 3A** and **3B**). The top ten upregulated and downregulated DEGs are shown in **Table 2**. GO analysis revealed that, in the biological processes, the DEGs were abundant in muscle system process (GO: 0003012), muscle contraction (GO: 0006939), and muscle cell differentiation (GO: 0042692); in cellular processes, the DEGs were enriched in plasma membrane protein complex (GO: 0098797) and caveola (GO: 0005901); and in molecular processes, the DEGs were primarily associated with plasma membrane protein complex components (GO: 0098797), channel activity (GO: 0015267), and passive transmembrane transporter activity (GO: 0022803) (**Figure 4A**). KEGG analysis revealed that the DEGs were mainly enriched in the cAMP/PKA signalling pathway, neuroactive ligand-receptor interactions, and insulin secretion (**Figure 4B**).

XHP activates the cAMP/PKA signalling pathway in BC cells

Based on the KEGG functional enrichment analysis results, we investigated whether XHP affects BC development by regulating the cAMP/PKA signalling pathway. Western blotting results indicated that the relative protein levels of p-CREB/CREB and p-PKA/PKA were markedly lower in the model group compared with

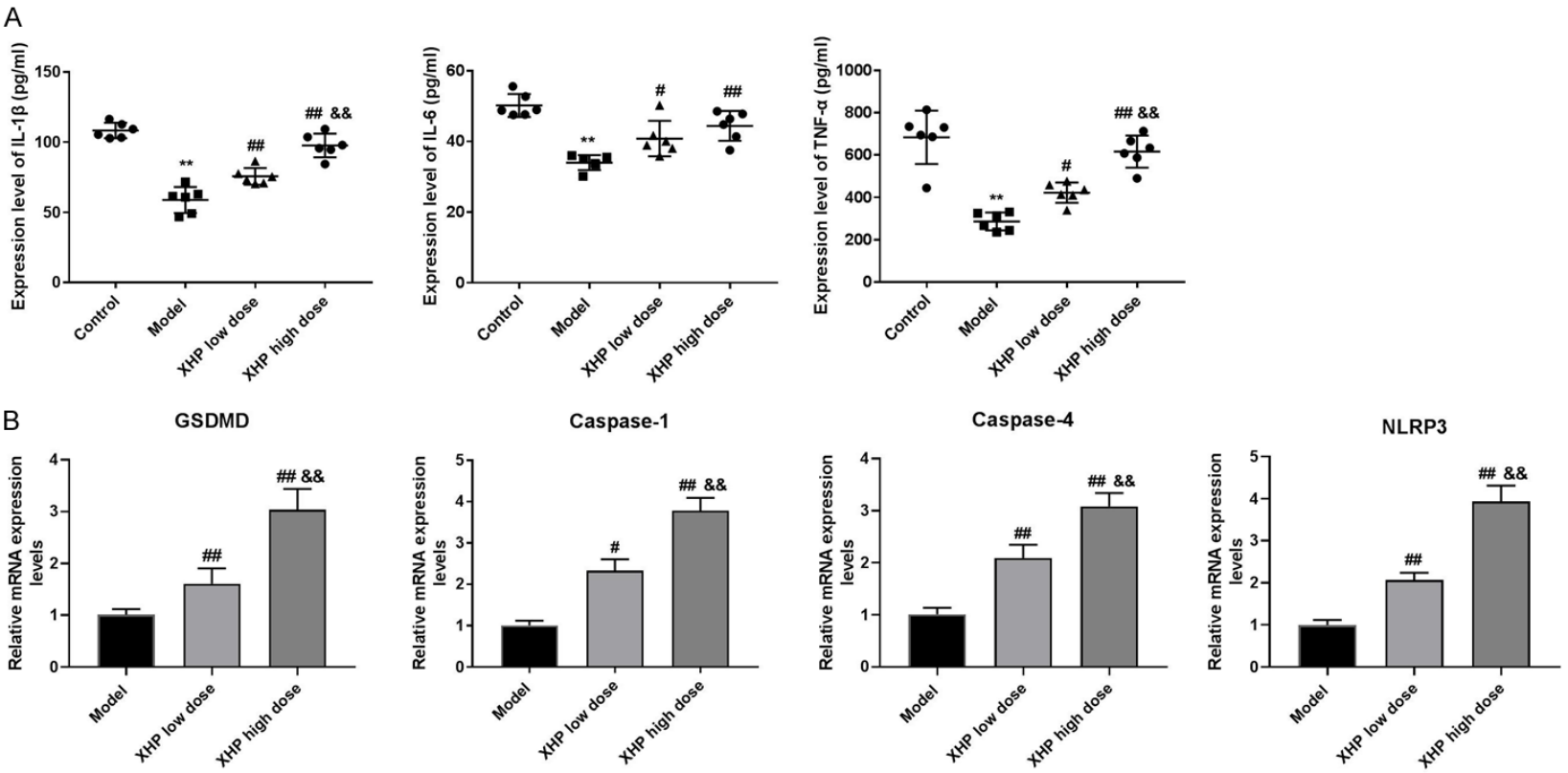


Figure 2. XHP can promote the pyroptosis of BC cancer in vivo. A. Concentrations of inflammatory factors IL-1β, IL-6 and TNF-α in tumour tissues of BC were detected by ELISA. B. RT-qPCR estimated the mRNA expression levels of GSDMD, Caspase-1, Caspase-4 and NLRP3. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$. The number of samples containing mice in each group was six. Compared with the low-dose XHP-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

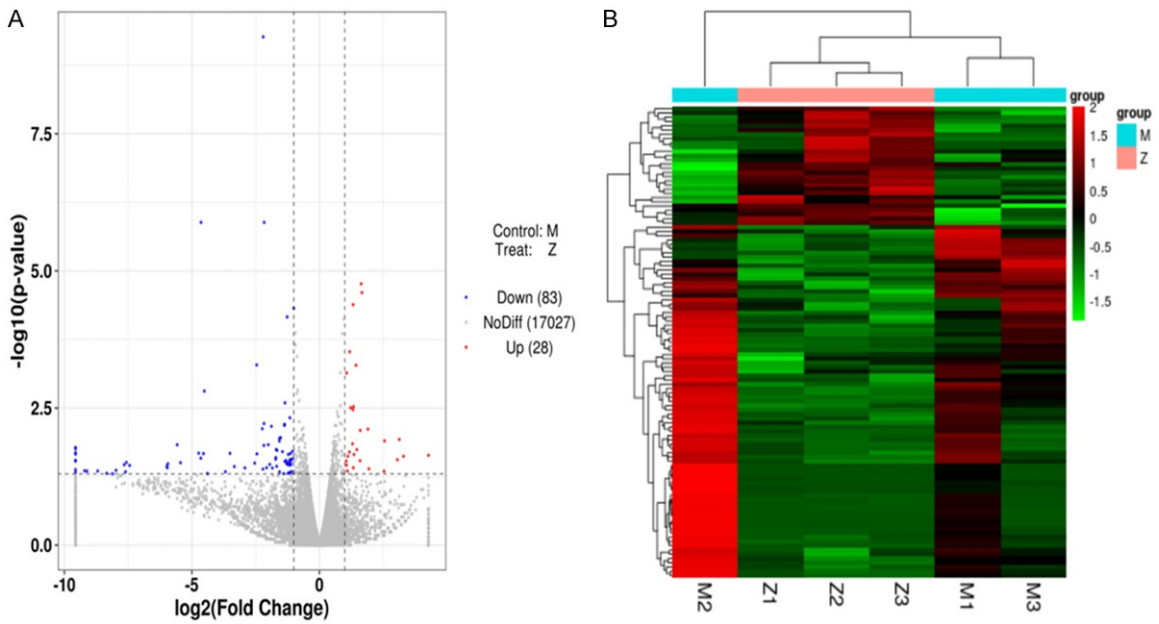


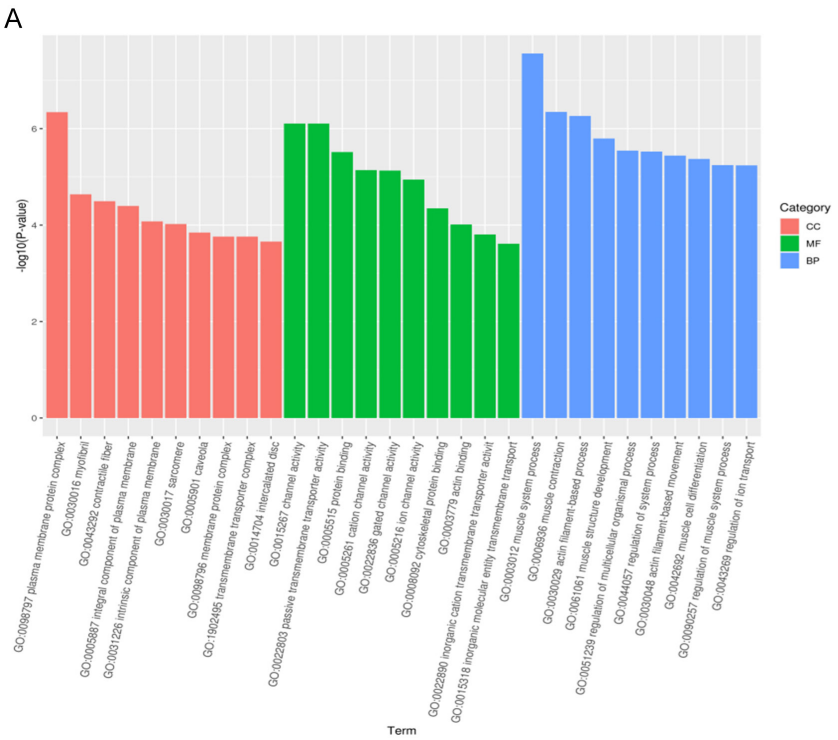
Figure 3. Transcriptome sequencing to analyze the molecular mechanism of XHP in BC. A. Volcano plots of the DEGs were drawn with the R language ggplots2 package. B. The heat map of the DEGs from mouse tumour tissues treated with or without XHP (control group) was generated using the pheatmap package in R language software.

Table 2. The up-regulated genes and down-regulated genes ranked in the top 10

Genes	Description	Pval	Up/Down
OLFR805	Olfactory receptor 805	1.71E-05	Up
SAA3	Serum amyloid A 3	2.48E-05	Up
PROKR2	Prokineticin receptor 2	4.13E-05	Up
TNN	Tenascin N	2.98E-04	Up
RUFY4	RUN and FYVE domain containing 4	5.25E-04	Up
NNAT	Neuronatin	7.25E-04	Up
STRA6L	STRA6-like	2.98E-03	Up
ADAMDEC1	ADAM-like, decysin 1	3.13E-03	Up
HHIP	Hedgehog-interacting protein	3.37E-03	Up
RETNLG	Resistin like gamma	7.68E-03	Up
AKAP6	A kinase (PRKA) anchor protein 6	5.4E-10	Down
MYOG	Myogenin	1.3E-06	Down
CDH15	Cadherin 15	1.31E-06	Down
PDE4B	Phosphodiesterase 4B, cAMP specific	4.78E-05	Down
C3	Complement component 3	6.9E-05	Down
6430571L13Rik	RIKEN cDNA 6430571L13 gene	5.19E-04	Down
TRPC3	Transient receptor potential cation channel, subfamily C, member 3	1.55E-03	Down
MEOX1	Mesenchyme homeobox 1	2.54E-03	Down
IGFBP6	Insulin-like growth factor binding protein 6	4.76E-03	Down
ACKR2	Atypical chemokine receptor 2	6.07E-03	Down

those in the control group ($P < 0.01$), whereas XHP treatment increased p-CREB/CREB and p-PKA/PKA levels in a dose-dependent manner (**Figure 5**, $P < 0.05$). These findings suggest that XHP likely activates the cAMP/PKA pathway in BC cells.

A


-log10(P.value)
Term
Category
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MF
BP
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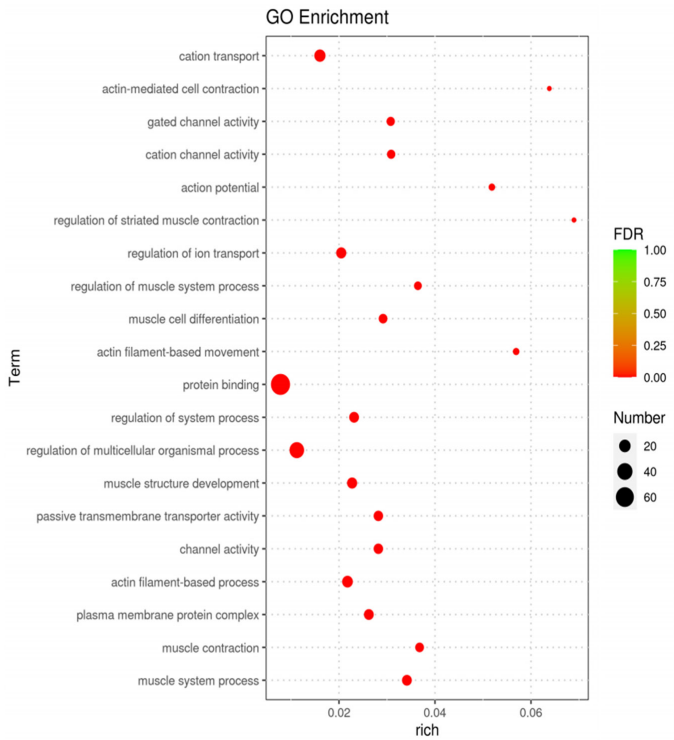
GO:0030016 myofibril
GO:0043292 contractile fiber
GO:0005887 integral component of plasma membrane
GO:0031226 intrinsic component of plasma membrane
GO:0030017 sarcomere
GO:0005901 caveola
GO:0098796 membrane protein complex
GO:1902495 transmembrane transporter complex
GO:0014704 intercalated disc
GO:0022803 passive transmembrane transporter activity
GO:0015267 channel activity
GO:0005515 protein binding
GO:0005261 cation channel activity
GO:0022836 gated channel activity
GO:0005216 ion channel activity
GO:0008092 cytoskeletal protein binding
GO:0022890 inorganic cation transmembrane transporter activit
GO:0015318 inorganic molecular entity transmembrane transporter activit
GO:0003012 muscle system process
GO:0006936 muscle contraction
GO:0030029 actin filament-based process
GO:0061061 muscle structure development
GO:0051239 regulation of multicellular organismal process
GO:0044057 regulation of system process
GO:0030048 actin filament-based movement
GO:0042692 muscle cell differentiation
GO:0090257 regulation of muscle system process
GO:0043269 regulation of ion transport


GO Enrichment
Term
rich
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1.00
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Number
20
40
60
cation transport
actin-mediated cell contraction
gated channel activity
cation channel activity
action potential
regulation of striated muscle contraction
regulation of ion transport
regulation of muscle system process
muscle cell differentiation
actin filament-based movement
protein binding
regulation of system process
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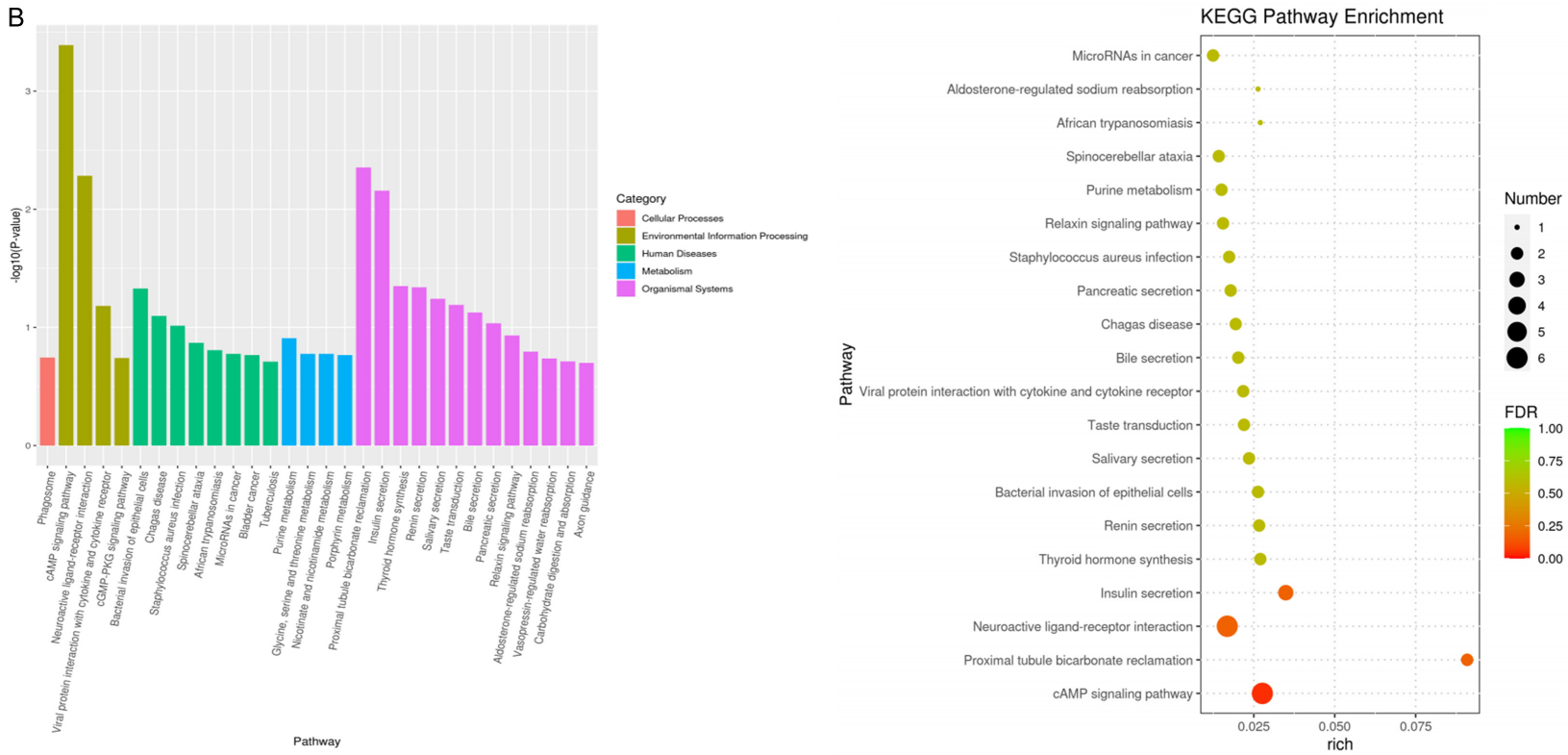


Figure 4. The DEGs were subjected to GO and KEGG functional enrichment analysis. A. GO functional annotation analysis of the DEGs. B. KEGG functional enrichment analysis of the DEGs.

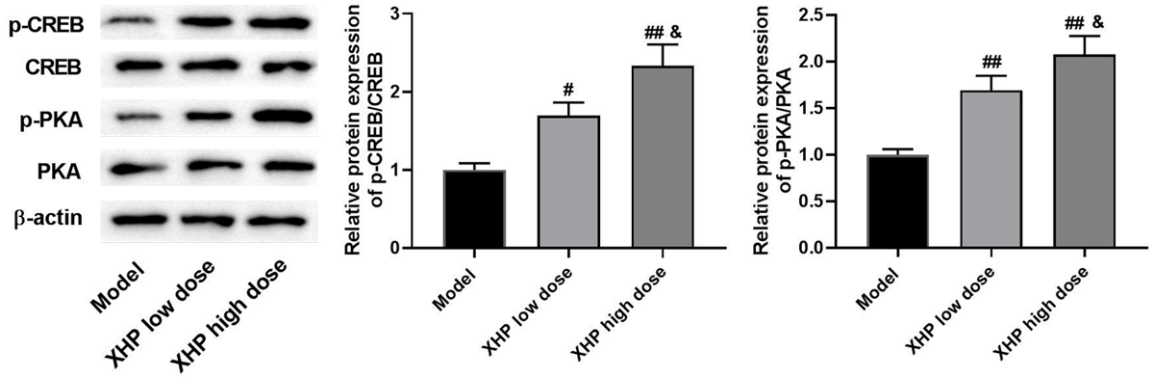


Figure 5. XHP can affect the expression of related proteins in the cAMP/PKA signaling pathway. Analysis of the cAMP/PKA pathway-related proteins by Western blot. The number of samples containing mice in each group was six. Compared with the model group, $^{*}P < 0.05$, $^{**}P < 0.01$. Compared with the low-dose XHP- treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

XHP inhibits BC cell proliferation, migration, and invasion by activating the cAMP/PKA signalling pathway and pyroptosis

To further understand the molecular mechanism of XHP in BC, we performed *in vitro* experiments. XHP increased the concentration of cAMP in MCF-7 cells, while H-89 and XHP+ VX-765 were able to reverse this trend (**Figure 6A**, $P < 0.05$). CCK-8 and EdU experiments showed that MCF-7 cell proliferation was reduced following XHP treatment in contrast to the control group, whereas the results were reversed to a certain extent following addition of H-89, (PKA inhibitor) as well as VX-765 (pyroptosis inhibitor) (**Figure 6B** and **6C**, $P < 0.05$). The wound-healing assay confirmed that XHP-treated MCF-7 cells showed significantly slower wound-healing than the control group. However, both in the XHP+H-89-treated group and the XHP+VX-765-treated group, the ability of the cells to heal the wound was significantly enhanced compared with that in the XHP-treated group (**Figure 6D**, $P < 0.05$). Similar results were observed in the transwell assay (**Figure 6E**, $P < 0.05$). In conclusion, we found that XHP activates the cAMP/PKA signalling pathway and pyroptosis, thereby inhibiting the proliferation, migration, and invasion of MCF-7 cells.

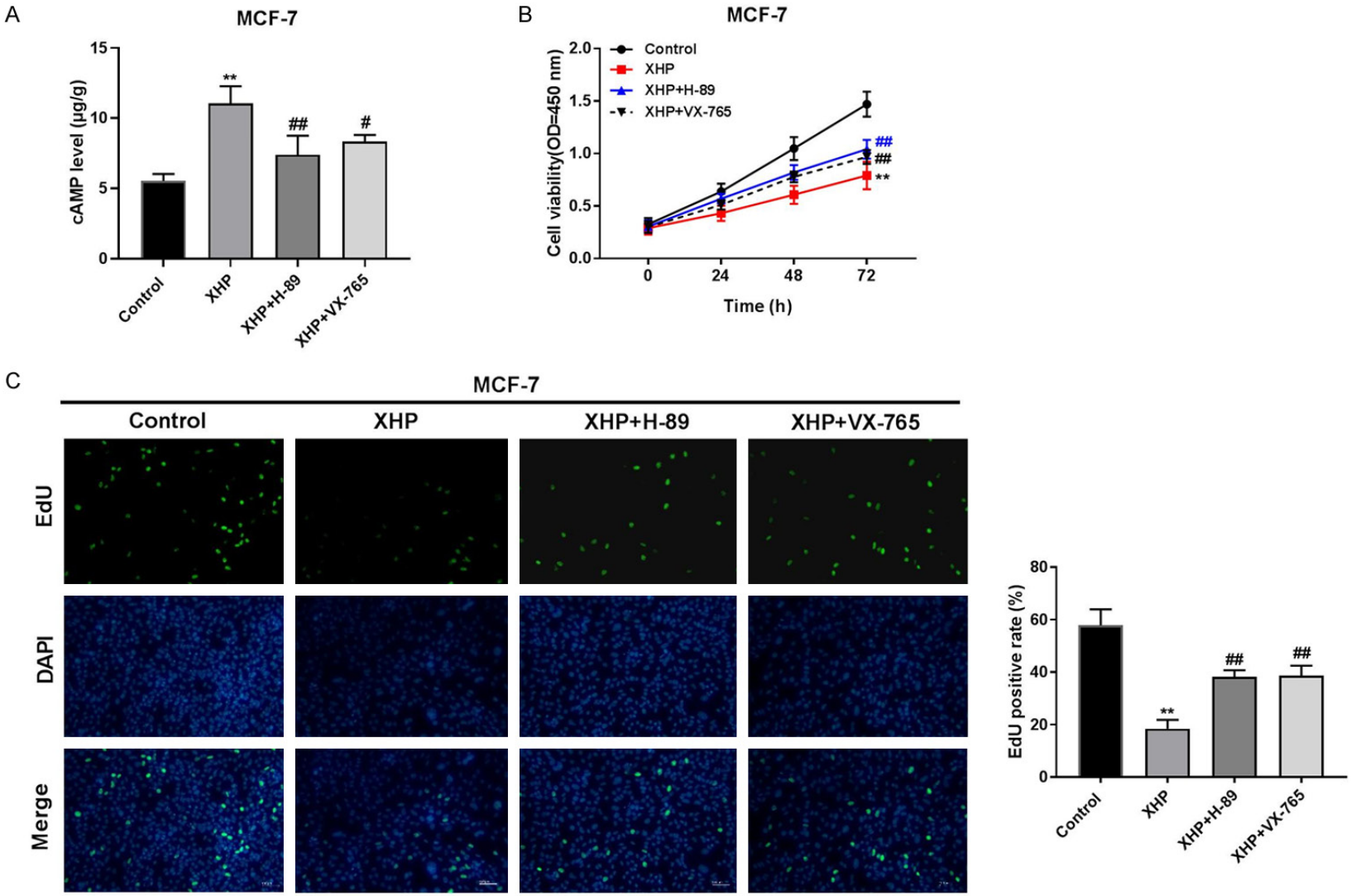
XHP induces pyroptosis of BC cells by activating the cAMP/PKA signalling pathway

Next, we investigated whether XHP promotes the pyroptosis of BC by modulating the cAMP/PKA pathway. The results showed that the concentration of inflammatory factors, such as IL-1β, IL-6, and TNF-α, was higher in the cell supernatant of the XHP-treated group compared with that in the control group. In contrast, the concentrations of these inflammatory factors were significantly reduced when H-89 or VX-765 was added to the XHP-treated cells (**Figure 7A**, $P < 0.05$). In addition, the protein levels of GSDMD, caspase-1, caspase-4, and NLRP3 were considerably higher in the XHP-treated group compared with those in the control group. However, the expression levels of these pyroptosis-related proteins in the XHP+H-89 group and the XHP+VX-765-treated group were much lower than those in the XHP-treated group (**Figure 7B**, $P < 0.05$). Taken together, these data suggest that XHP induces pyroptosis of BC by activating the cAMP/PKA signalling pathway.

Discussion

BC has a high incidence of metastasis and recurrence, making it one of the deadliest malignancies in women that still lacks effective treatments [26]. XHP is a TCM that has been shown to have anti-cancer properties. XHP has been validated in clinical practice for the treatment of numerous malignancies. In this study, we found that XHP inhibited BC growth and triggered pyroptosis. *In vitro* studies further revealed that XHP inhibited the proliferation, migration, and invasion of MCF-7 cells and induced pyroptosis by stimulating the cAMP/PKA signalling pathway.

Pyroptosis is associated with infectious diseases, cardiovascular diseases, autoimmune diseases, and malignancies [27]. The association


A
MCF-7
cAMP level (μg/g)
15
10
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##
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Control
XHP
XHP+H-89
XHP+VX-765
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MCF-7
Cell viability(OD=450 nm)
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Control
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XHP+VX-765
EdU
DAPI
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EdU positive rate (%)
80
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40
20
0
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Control
XHP
XHP+H-89
XHP+VX-765

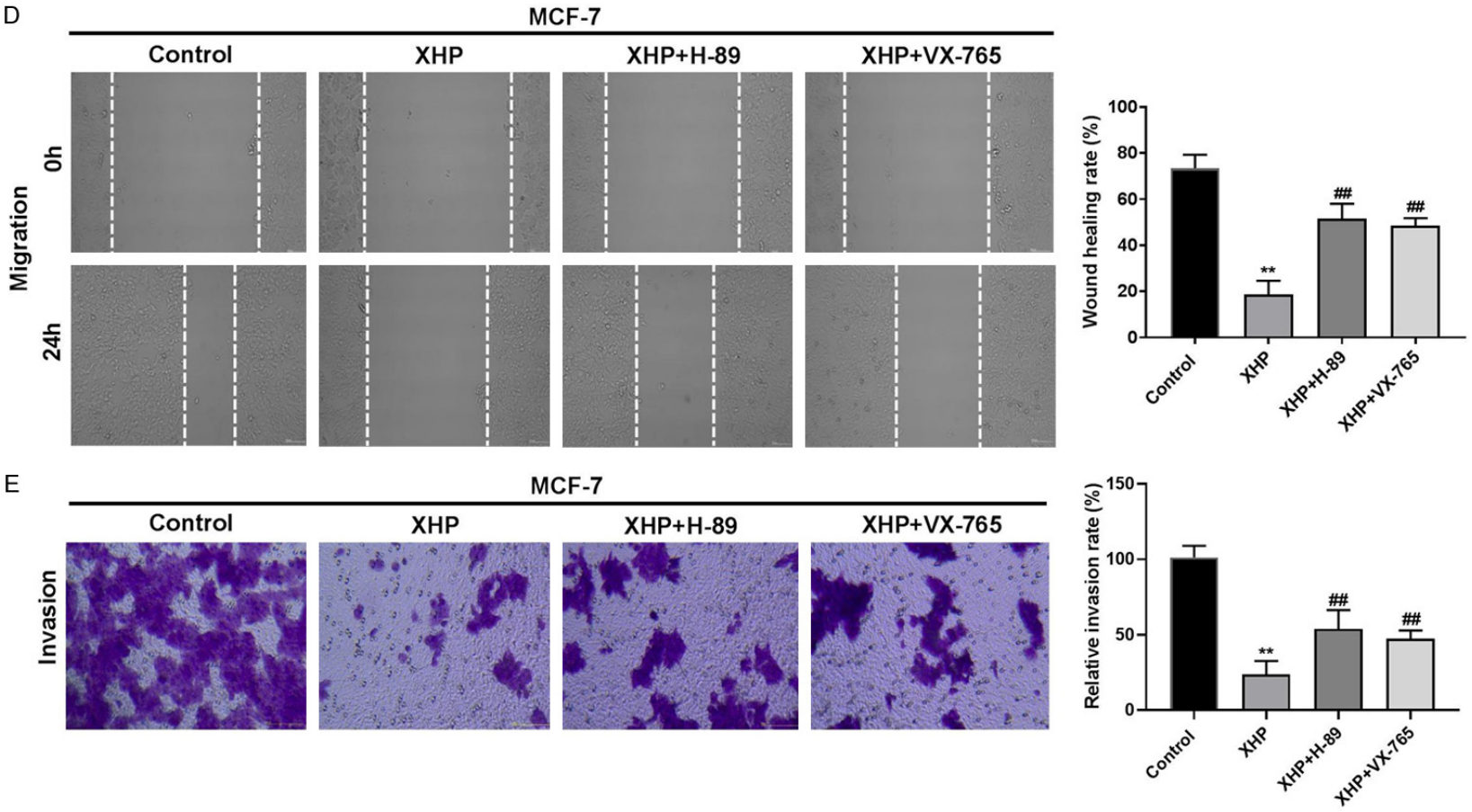

Figure 6. XHP regulated proliferation, migration, and invasion of BC cells by activating the cAMP/PKA signaling pathways in vitro. A. The cAMP concentrations were measured by ELISA assay. B. The proliferation of MCF-7 cells was measured by CCK-8 assay. C. The proliferation of MCF-7 cells was determined by EdU assays. D. The migration ability of MCF-7 cells was detected by cell wound healing assay. E. Transwell assay was performed to analyze cell invasion ability. The number of samples containing cells in each group was three. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$. Compared with the XHP-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

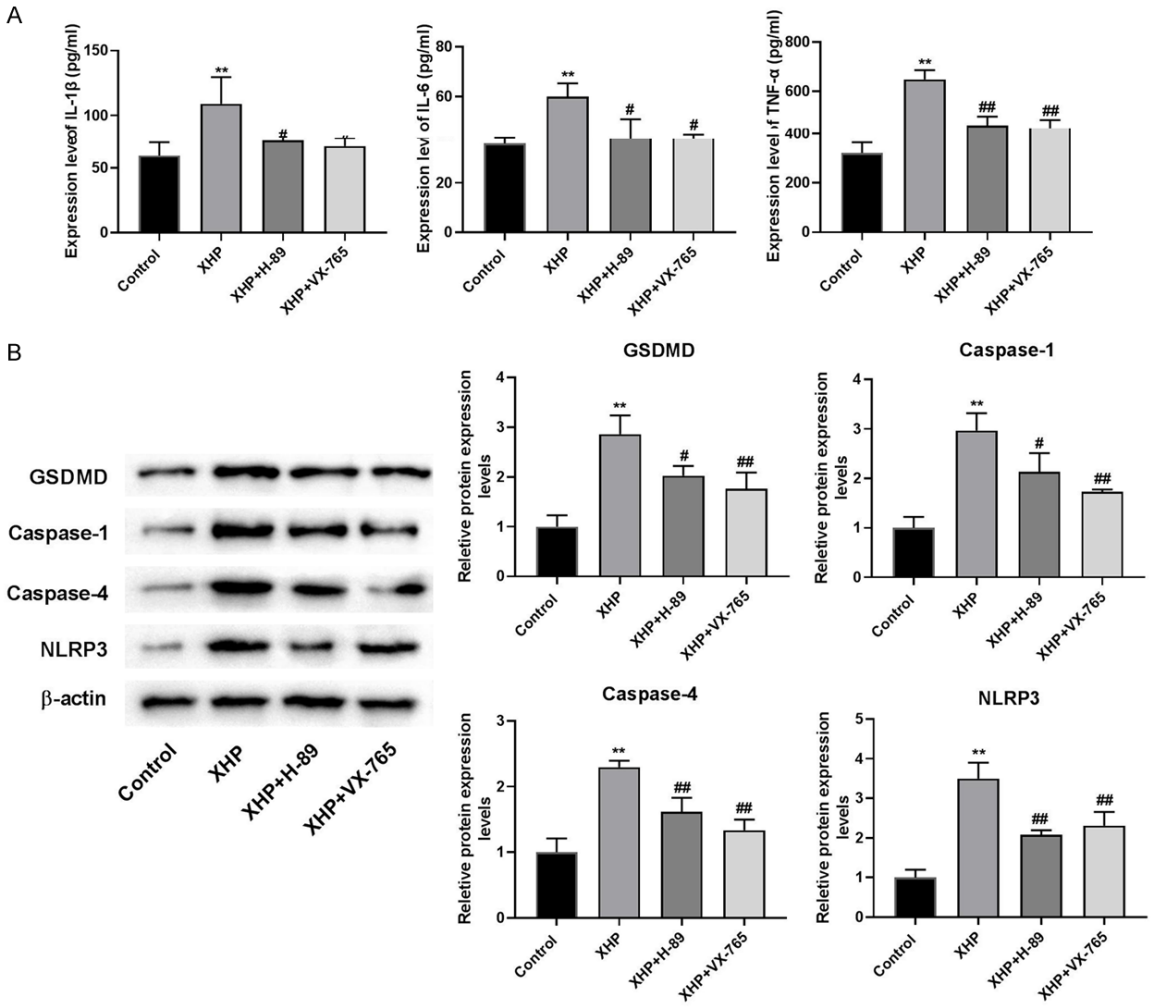

Figure 7. XHP induced the pyroptosis of BC cells by regulating the cAMP/PKA signaling pathway in vitro. A. ELISA experiment to measure the concentrations of inflammatory factors, including IL-1β, IL-6 and TNF-α. B. The protein expression levels of GSDMD, Caspase-1, Caspase-4, and NLRP3 were determined by Western blot. The number of samples containing cells in each group was three. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$. Compared with the XHP-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

between pyroptosis and malignancies is becoming increasingly apparent owing to ongoing research and offers potential therapeutic possibilities. Several studies have shown that TCM inhibits BC development by regulating pyroptosis. Nobiletin has shown to induce pyroptosis in BC cells through regulation of the miR-200b/JAZF1 axis and thereby inhibit the proliferation and growth of BC cells [28]. Percolated extract of *Spatholobus suberectus Dunn* exhibited activity against triple-negative breast cancer (TNBC) via pyroptosis [29]. Polydatin was found to regulate pyroptosis in TNBC cells, which in turn inhibited tumour growth [30]. In addition, pyroptosis is closely associated with inflammation [31]. Numerous inflammatory molecules are engaged in the pyroptosis process, including the caspases, gasdermin (GSDM) protein family, interleukin (IL) series molecules, and NOD-like receptors (NLRs), among others [32]. In this study, we found that high-dose XHP promoted the release of inflammatory factors IL-1β, IL-6 and TNF-α and significantly increased the expression of GSDMD, caspase-1, caspase-4 and NLRP3 in BC. GSDMD, caspase-1, caspase-4 and NLRP3 are core proteins of pyroptosis, and their abnormal expression affects the progression of many tumors. For example, Cisplatin triggeres pyroptosis in TNBC by activating the MEG3/NLRP3/caspase-1/GSDMD pathway to produce antitumour effects [33]. In malignant human breast cells, capsai-

cin can continuously activate caspase-4, leading to a delay in cell death [34]. In conclusion, we found that XHP could activate pyroptosis in BC.

To investigate the molecular mechanism of XHP in BC, we performed transcriptome sequencing and found that XHP may regulate cAMP/PKA signalling pathway. Subsequently, western blotting revealed that XHP markedly elevated the relative protein levels of p-CREB/CREB and p-PKA/PKA. Some studies have reported that inhibiting the cAMP/PKA signalling pathway may inhibit BC progression. A previous study showed that phosphodiesterase 3A promotes the invasive-metastatic cascade response in BC by inhibiting the cAMP/PKA signalling pathway [35]. Activation of the cAMP-PKA signalling pathway reduced Ca(2+) entry and inhibited the migration of BC cells [24]. Therefore, we investigated whether XHP regulates the cAMP/PKA signalling pathway to affect BC cell proliferation, migration, and invasion. Our results showed that the proliferation, migration, and invasion of MCF-7 cells was significantly enhanced following addition of H-89, indicating that inhibition of the cAMP/PKA signalling pathway reversed the anti-tumour effect of XHP. Therefore, our data suggest that XHP inhibits the development of BC by activating the cAMP/PKA signalling pathway.

Several studies have shown that multiple signalling pathways can regulate pyroptosis in BC and thereby influence tumour progression. Transfection of poly I: C, a synthetic dsRNA analogue, promotes pyroptosis by suppressing the TGF-β signalling pathway in TNBC [36]. The tumour suppressor DRD2 acts by limiting NF-κB signalling, triggering BC cells pyroptosis, which in turn inhibits BC tumourigenesis [37]. The correlation between the cAMP/PKA signalling pathway and pyroptosis has been studied in diseases such as sepsis [38], and in neurological deficits [39], but rarely in BC. In this study, we found that XHP could activate the cAMP/PKA pathway, leading to the induction of pyroptosis, which in turn inhibited the proliferation, invasion and migration of BC cells.

There are also some shortcomings in this study. For example, we found that XHP could regulate 8 significantly up-regulated genes and 83 significantly down-regulated genes by RNA sequencing, while only the correlation between XHP and the cAMP/PKA signaling pathway was investigated. In addition, our study referred to the relevant literature [33, 40] and only detected changes in the expression of the core protein of pyroptosis, but did not use transmission electron microscope to detect due to limited scientific conditions.

Conclusion

We found that XHP inhibits the growth of BC and induces pyroptosis. Transcriptome sequencing analysis revealed that XHP may regulate the cAMP/PKA signalling pathway. Activation of the cAMP/PKA signalling pathway was identified as the underlying mechanism by which XHP suppresses BC cell proliferation, migration, and invasion *in vivo*. XHP induces pyroptosis by stimulating the cAMP/PKA signalling pathway. The results of this study provide mechanistic insights into the role of XHP in BC and experimental basis for the clinical application of XHP in BC therapy.

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Disclosure of conflict of interest

None.

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