SNS-032 combined with decitabine induces caspase-3/gasdermin E-dependent pyroptosis in breast cancer cells

YUXIN CHEN[1,2], DANYA ZHANG[1,2], JIE LI[1,2], YUE SUN[1,2], JING WANG[1,2] and LING XI[1,2]

[1]Department of Obstetrics and Gynecology, National Clinical Research Center for Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China; [2]Key Laboratory of Cancer Invasion and Metastasis, Ministry of Education, Hubei Key Laboratory of Tumor Invasion and Metastasis, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

Received September 5, 2024; Accepted January 13, 2025

DOI: 10.3892/ol.2025.14948

Abstract. SNS-032 is a synthetic compound that specifically inhibits cyclin-dependent kinases 2, 7 and 9. Its primary anti-cancer activity involves cell cycle arrest, which prevents tumor cell growth. However, there are limited reports on whether SNS-032 induces pyroptosis, a novel inflammation-mediated programmed cell death pathway in breast cancer (BC). The present study demonstrated that SNS-032 treatment decreased cell viability by inducing pyroptosis in BC cells. Typical morphological indications of pyroptosis were observed, including cell swelling and destruction of cell membrane integrity, leading the release of adenosine 5'-triphosphate and lactate dehydrogenase. Furthermore, the expression of caspase-3, the N terminus of gasdermin E (GSDME) and B-cell lymphoma-2 (BCL-2)-associated X protein increased, whereas expression of BCL-2 decreased. In addition, Z-DEVD-FMK, a specific caspase-3 inhibitor, markedly alleviated pyroptosis triggered by SNS-032. These findings suggested that SNS-032 induced caspase-3/GSDME-dependent pyroptosis. Furthermore, the present study demonstrated that decitabine (DAC), a DNA methyltransferase inhibitor, upregulated the expression of GSDME protein and enhanced SNS-032-induced caspase-3/GSDME-dependent pyroptosis in BC cells. In conclusion, these results suggest that caspase-3/GSDME-induced pyroptosis can be facilitated by SNS-032 treatment in BC cells, and DAC has the potential to enhance SNS-032-induced pyroptosis by increasing GSDME expression. This mechanistic insight indicates that SNS-032 is a promising therapeutic agent for BC treatment.

Correspondence to: Professor Ling Xi, Department of Obstetrics and Gynecology, National Clinical Research Center for Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Building 3, 1095 Jiefang Avenue, Wuhan, Hubei 430030, P.R. China
E-mail: lxi@tjh.tjmu.edu.cn

Abbreviations: ATP, adenosine 5'-triphosphate; BAX, Bcl-2-associated X protein; BC, breast cancer; BCL-2, B-cell lymphoma-2; CLL, chronic lymphocytic leukemia; DAC, decitabine; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; GSDM, gasdermin; GSDME, gasdermin E; GZM, granzyme; LDH, lactate dehydrogenase; ns, not significant; PBS, phosphate-buffered saline; PCD, programmed cell death; PI, propidium iodide; PVDF, polyvinylidene difluoride

Key words: SNS-032, pyroptosis, decitabine, BC, caspase-3/GSDME

Introduction

Breast cancer (BC) is the most prevalent malignant tumor in women worldwide. According to the World Health Organization, BC accounts for the majority of cancer related mortalities among women globally, which seriously impacts the physical and psychological health of patients (1-3). The 5-year survival rate of BC has reached 90% because of advances in drug therapy and the establishment of a precise diagnosis, with the treatment level of BC improving annually (4,5). Chemotherapy remains an essential component of the comprehensive treatment of BC because it can substantially increase the 5-year survival rate and improve prognosis (6). However, issues with treatment such as drug resistance, drug-related toxicity and poor tolerance to traditional chemotherapeutic drugs must be resolved. Therefore, the development of new drugs has become a priority.

Pyroptosis is a novel form of programmed cell death (PCD) mediated by members of the gasdermin (GSDM) family that occurs after autophagy and apoptosis (7). It is morphologically characterized by cell swelling, dissolution and vesicular protrusions, followed by cell membrane rupture, which leads to the release of proinflammatory cytokines and cell contents into the extracellular space. These include high mobility group B protein (HMGB)14, IL-18, IL-1, adenosine 5'-triphosphate (ATP) and HMGB1, all of which trigger an inflammatory response (7-9). This is closely associated with immune system activation (10). The GSDM family of proteins, which are effectors of pyroptosis, can be cleaved by the caspase family and granzyme (GZM) to release the active, membrane pore-forming N-terminal domain and split the junction domain between the N- and C-terminus (9). The N-terminus of GSDM family proteins forms large pores in the cell membrane, breaking osmotic pressure causing cell expansion and producing large vacuoles (11-13).

GSDME, a protein belonging to the GSDM family, is distinct from GSDMD and specifically cleaved by caspase-3 (14,15). Previously, GSDME has been found to serve a role in the occurrence and development of malignant tumors, including lung cancer, colorectal cancer and BC (16-18). In addition, paclitaxel accelerates pyroptosis in small cell lung cancer cells, and increasing the expression of GSDME can effectively enhance their sensitivity to paclitaxel (19). There appears to be an association between low survival rate and low expression of GSDME in patients with BC, indicating that GSDME might serve as a tumor suppressor in BC (20).

GSDME is highly expressed in normal cell lines; however, most tumor cell lines have methylated promoters that lower GSDME expression compared with that in normal cells, making it more difficult to activate pyroptosis in tumor cells (14,15). As a result, when treating malignant tumors, appropriate chemotherapy drugs could be chosen based on the expression level of GSDME. Drugs which can increase GSDME expression may increase the sensitivity of cells to chemotherapy drugs and achieve a more potent anticancer effect (21). The DNA methyltransferase inhibitor, decitabine (DAC; Fig. 1A), can induce functional re-expression of the abnormally silenced GSDME gene in cancer (22,23); therefore, DAC can be used to increase the expression of GSDME in BC cells with low GSDME expression, thereby enhancing the killing of tumor cells through pyroptosis. Therefore, the combination of DAC and GSDME-inducing pyroptosis agents for cancer treatment may be a promising strategy for BC treatment.

SNS-032 (formerly BMS-387032; Fig. 1A) was originally designed to obtain a selective cyclin-dependent kinase 2 (CDK2) inhibitor by the Bristol-Myers Squibb Pharmaceutical Research Institute (Stamford, USA) (24). Subsequent studies have found that the anticancer activity of this compound is mainly dependent on the inhibition of CDK7 and CDK9 (24-26), which effectively kill chronic lymphocytic leukemia (CLL) cells *in vitro* by blocking RNA polymerase II phosphorylation and inhibiting RNA synthesis (24). Several studies have demonstrated that SNS-032 has a strong anticancer effect on hematological malignancies (24-27) and solid tumors, such as uveal melanoma (25), cervical cancer (28) and non-small cell lung cancer (29). In addition, SNS-032 has been reported in phase I clinical studies in patients with metastatic refractory solid tumors (30), advanced CLL and multiple myeloma (31). However, to date, there have been no further reports of SNS-032 in clinical trials. SNS-032 not only inhibits tumor proliferation through the cell cycle, but also induces apoptosis, leading to tumor cell death in melanoma (25), BC (32) and esophageal squamous cell carcinoma (33). However, no studies have reported the activation of the pyroptosis pathway to inhibit tumors.

SNS-032 combined with the demethylating drug, DAC, is a promising treatment strategy for patients with BC. The present study aimed to establish the mechanism of SNS-032 to inhibit tumors and to determine whether the combination with DAC can improve inhibition of tumors, providing a new approach for BC treatment.

Materials and methods

Cell culture. MCF-7 cells, a human BC cell line from Procell Life Science & Technology Co., Inc., were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.). The mouse BC cell line 4T1, derived from BALB/c mice, were from cell bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences (Beijing, China) and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no G4003; Wuhan Servicebio Technology Co., Ltd.). All cell lines were cultured in a humidified incubator at 37˚C and 5% CO_2. The cells were seeded 24 h later. Trypsin/EDTA at 0.25% (w/v) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific, Inc.

Materials. SNS-032 (cat. no S1145) and DAC (cat. no S1200) were purchased from Selleck Chemicals and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM. The pan-caspase-specific inhibitor Z-VAD-FMK (cat. no HY-16658B), necroptosis inhibitor Necrostatin-1 (cat. no HY-15760) and specific cell caspase-3 inhibitor Z-DEVD-FMK (cat. no HY-12466) were purchased from MedChemExpress and dissolved in DMSO at concentrations of 10, 5 and 10 mM, respectively. The lactate dehydrogenase (LDH) Release Assay kit (cat. no C0017), bicinchoninic acid assay (cat. no P0009) and ATP Assay kit (cat. no S0027) were purchased from Beyotime Institute of Biotechnology. The Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Test kit (cat no. 556547) was purchased from BD Biosciences.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) was used to determine the viability of the BC cell lines. MCF-7 and 4T1 cells were pretreated with or without DAC (5 μmol/l) for 24 h at 37˚C seeded in 96-well plates at 3,000 cells/well and treated with different doses (0, 1, 2, 4, 8, 16 and 32 μmol/l) of SNS-032. According to the manufacturer's instructions, 100 μl working solution was added to each well after 24 h SNS-032 treatment and incubated for 2 h. The absorbance at 450 nm was measured using a Synergy 2 microplate reader (BioTek; Agilent Technologies, Inc.) and cell viability was determined using the following formula: Cell viability (%)=(experimental group OD_{450}-blank control OD_{450})/(control group OD_{450}-blank control OD_{450}) x100.

Colony formation assay. MCF-7 and 4T1 cells were seeded in six-well plates (1,000 cells/well), incubated overnight and treated with DAC (5 μmol/l) and SNS-032 (10 μmol/l in MCF-7 and 15 μmol/l in 4T1 cells) at 37˚C. The culture was allowed to continue for 7 days, with medium changes every 3 days. The cells were fixed with 4% paraformaldehyde for 10 min (cat. no S1101; Wuhan Servicebio Technology Co., Ltd.), stained with 0.1% crystal violet for 15 min and thoroughly washed at room temperature. Finally, the number of colonies (>50 cells/colony) in each well was counted using ImageJ (V1.8.0.112; National Institutes of Health) and the colony formation efficiency was calculated as a percentage relative to the control (100%).

LDH assay. LDH release was detected using an LDH release Assay Kit. The supernatants were collected by centrifugation

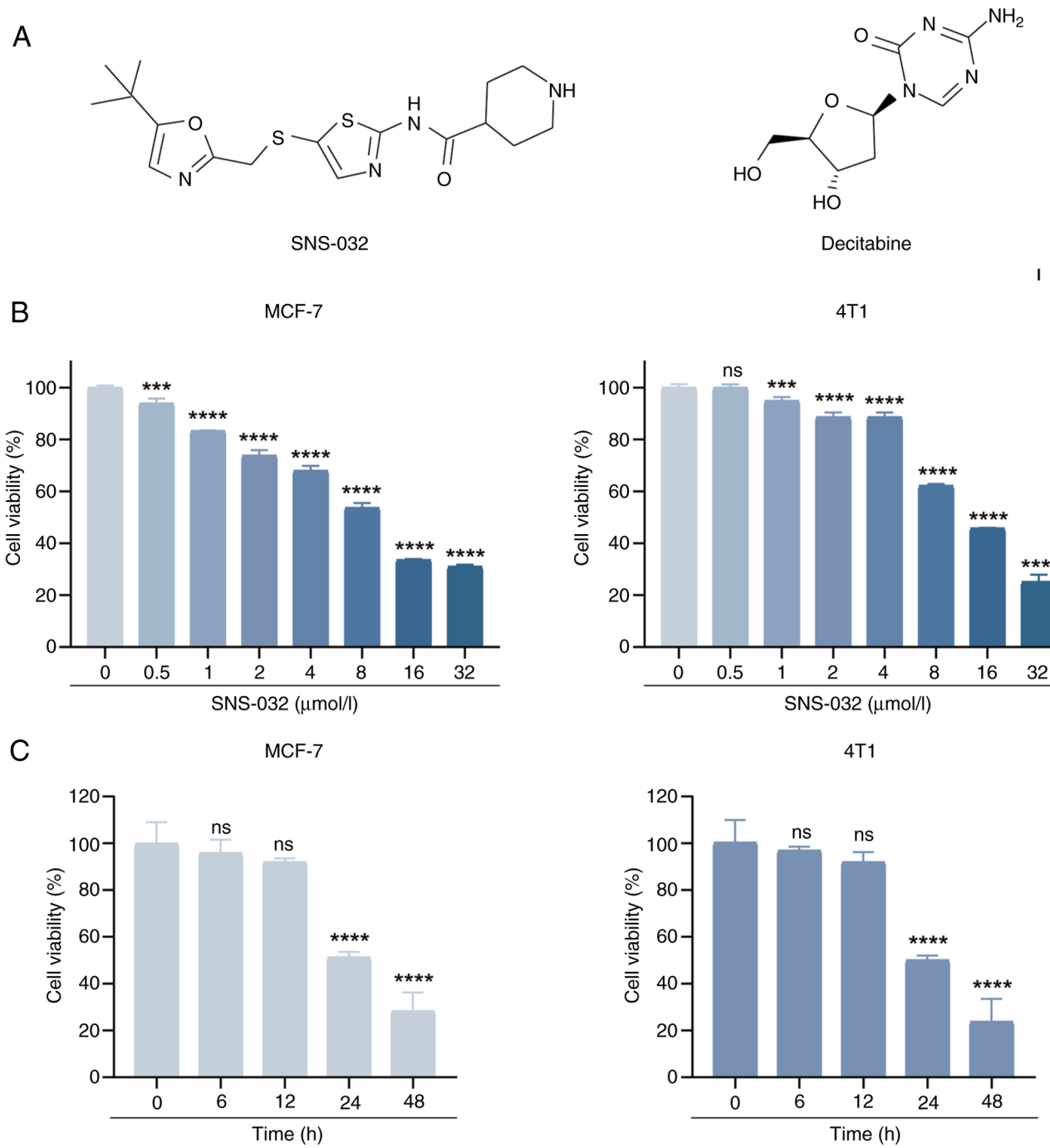

Figure 1. SNS-032 inhibits cell growth in breast cancer cells. (A) Chemical structures of SNS-032 and decitabine. (B) MCF-7 and 4T1 cells were treated with SNS-032 (0-32 μmol/l) for 24 h, and cell viability was analyzed by the CCK-8 assay. (C) MCF-7 and 4T1 cells were treated with SNS-032 (10 and 15 μmol/l, respectively) for different lengths of time, and cell viability was analyzed using the CCK-8 assay. The cells were exposed to 0.1% dimethyl sulfoxide as a control. Data are presented as mean ± standard deviation (n=3). ***P<0.001 and ****P<0.0001 vs. control group. ns, not significant; CCK-8, Cell Counting Kit-8.

of cells at 400 x g for 5 min at 4˚C after treatment with SNS-032 (10 μmol/l in MCF-7 and 15 μmol/l in 4T1 cells) for 24 h with or without DAC (5 μmol/l) pretreatment for 24 h at 37˚C. According to the manufacturer's protocol, after the working detection reagent was prepared, 60 μl LDH detection reagent and 100 μl sample supernatant were added into 96 wells quickly. After 30 min incubation, a Synergy 2 microplate reader was used to measure the absorbance at 490 nm in the dark and the data were processed according to the following formula: LDH release (%)=(experimental group OD_{490}-blank control OD_{490})/(maximum release group OD_{490}-blank control OD_{490}) x100.

ATP assay. An ATP Assay Kit was used for quantitative determination of ATP. The medium was discarded and the lysis buffer was added to the BC cells after treatment with SNS-032 (10 μmol/l in MCF-7 and 15 μmol/l in 4T1 cells) for 24 h with or without DAC (5 μmol/l) pretreatment for 24 h at 37˚C. The cell lysate was collected and centrifuged at 12,000 x g for 5 min at 4˚C to obtain the supernatants. According to the manufacturer's

protocol, 100 μl ATP detection reagent was added to a black 96-well plate and incubated at room temperature for 5 min. Subsequently, 20 μl each sample supernatant was added immediately into the black 96-well plate at room temperature. Finally, a Synergy 2 microplate reader was used to detect the luminescence of samples immediately and ATP levels were calculated as follows: ATP cell viability (%)=(experimental group luminescence/control group luminescence) x100.

Annexin V/PI double staining. BC cell lines, MCF-7 and 4T1, were seeded into 12-well plates overnight at a density of $1x10^5$ cells/well and treated with different concentrations of SNS-032 for 24 h in the presence or absence of DAC, Z-VAD-FMK (20 μmol/l), Necrostatin-1 (10 μmol/l) or Z-DEVD-FMK (20 μmol/l) at 37˚C. Cells were collected, centrifuged at 300 x g for 5 min at 4˚C and washed twice with cold PBS. The following staining procedure was performed according to the manufacturer's protocol. Resuspended cells were mixed with 200 μl 1x Binding Buffer and incubated with 5 μl Annexin V-FITC and 5 μl PI at room temperature for 10 min. SNS-032-induced cell death was monitored with Annexin V-FITC/PI and detected with CytoFLEX flow cytometer (Beckman Coulter, Inc.). All data were analyzed using FlowJo V10.8.1 (BD Biosciences).

Western blotting analysis. Total cell protein was extracted from BC cells after treatment with SNS-032 (10 μmol/l in MCF-7 and 15 μmol/l in 4T1 cells) for 24 h with or without DAC (5 μmol/l) pretreatment for 24 h or Z-DEVE-FMK (20 μmol/l) pretreatment for 3 h at 37˚C using radioimmunoprecipitation lysis buffer (cat. no G2002; Wuhan Servicebio Technology Co., Ltd.) for western blotting. The buffer contained a 1% protease inhibitor cocktail (cat. no G2006; Wuhan Servicebio Technology Co., Ltd.). The cell samples were incubated in cold lysis buffer for 20 min, centrifuged at 13,600 x g for 20 min at 4˚C. Protein concentration was measured using the bicinchoninic acid assay. Equal amounts of protein sample (30 μg) were loaded and separated by SDS-polyacrylamide gel electrophoresis (Dakewe Biotech Co., Ltd.) and transferred to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma). The PVDF membranes were blocked with 5% bovine serum albumin (cat. no GC305006; Wuhan Servicebio Technology Co., Ltd.) for 1 h at room temperature and incubated with the primary antibody at 4˚C overnight. The following antibodies were used: Anti-GSDME (1:1,000; cat. no ab215191; Abcam), anti-caspase-3 (1:1,000; cat. no #9662; Cell Signaling Technology, Inc.), anti-Bcl-2-associated X protein (BAX; 1:2,000; cat. no ab32503; Abcam), anti-B-cell lymphoma-2 (BCL-2; 1:2,000; cat. no ab182858; Abcam), anti-mouse cleaved N-terminal GSDMD (Asp275; 1:1,000; cat. no #36425; Cell Signaling Technology, Inc.), anti-cleaved N-terminal GSDMD (1:1,000; cat. no ab215203; Abcam) and anti-GAPDH (1:5,000; cat. no A19056; ABclonal Biotech Co., Ltd.). The PVDF membrane was exposed to anti-rabbit secondary antibody (1:20,000; cat. no ANT020; Wuhan Antejie Biotechnology Co., Ltd.) at room temperature for 1 h after washing three times with TBST (0.1% Tween-20 in Tris-HCl buffer). Protein bands were visualized using a multimode chemiluminescence system (Bio-Rad Laboratories, Inc.) and ImageJ software (version 1.5) for densitometry analysis.

Statistical analysis. All data presented were obtained from ≥3 independent experiments and are expressed as mean ± standard deviation. Data were analyzed using GraphPad Prism 8 (Dotmatics). The significance of the differences was assessed using one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference in all comparisons.

Results

SNS-032 can affect the cell viability of BC cells. To evaluate the anticancer effect of SNS-032 on BC cells, a CCK-8 assay was used to assess the effects of SNS-032 on the activity of human and mouse BC cell lines, MCF-7 and 4T1. Results from CCK-8 experiments (Fig. 1B) demonstrated that SNS-032 caused significant decreases in cell viability in a dose-dependent manner in BC cells compared with the control. The half-maximal inhibitory concentrations of the two BC cell lines were calculated to be 9 μmol/l (MCF-7) and 13.5 μmol/l (4T1). Based on these results, the following concentrations were used in the subsequent time-course investigations: 10 μmol/l for MCF-7 and 15 μmol/l for 4T1. SNS-032 significantly decreased the viability of BC cells in a time-dependent manner as shown by CCK-8 assays (Fig. 1C). These results indicate that the inhibitory effect of SNS-032 on BC cells was dose- and time-dependent, and the results were significantly different compared with the untreated control or 0 h timepoint ($P<0.001$), with the exceptions being the 0.5 μmol/l concentration in 4T1 cells, and the 6 and 12 h timepoints in both MCF-7 and 4T1 cells. To further support these results, colony formation experiments were performed on MCF-7 and 4T1 cells, as shown in Fig. 2B and C, which demonstrated that SNS-032 significantly inhibited the cloning of BC cells ($P<0.001$). In summary, these data show that SNS-032 treatment reduces the viability of BC cells.

Evaluation of the cell death pathways of SNS-032 in BC cells. To explore the cell death pathway of SNS-032 in BC cells, cells were pretreated for 3 h with pan-caspase inhibitor, Z-VAD-FMK (20 μmol/l), necroptosis inhibitor Necrostatin-1 (10 μmol/l) or specific cell caspase-3 inhibitor Z-DEVD-FMK (20 μmol/l). MCF-7 and 4T1 cells were treated with SNS-032 for 24 h to observe cellular changes. Annexin V-FITC and PI were used to stain the cells for apoptosis studies, and the efficacy of the three inhibitors in reducing cell death caused by SNS-032 was assessed, as shown in Figs. 3 and 4B. Based on the results of flow cytometry, Necrostatin-1 had little effect on inhibiting SNS-032 induced-BC cell death, Z-VAD-FMK could partially inhibit the cell death of BC cells induced by SNS-032, while Z-DEVD-FMK was able to more markedly inhibit the SNS-032-induced cell death of BC cells, indicating the effect of the caspase-3 specific inhibitor was superior. Therefore, the possibility that cell death induced by SNS-032 is due to necroptosis can be excluded. Additionally, the results suggest that the cell death triggers may be related to the caspase family and caspase-3.

SNS-032-induced pyroptosis is dependent on activation of caspase-3. MCF-7 and 4T1 cells were treated with four different treatments. During 24 h SNS-032 treatment,

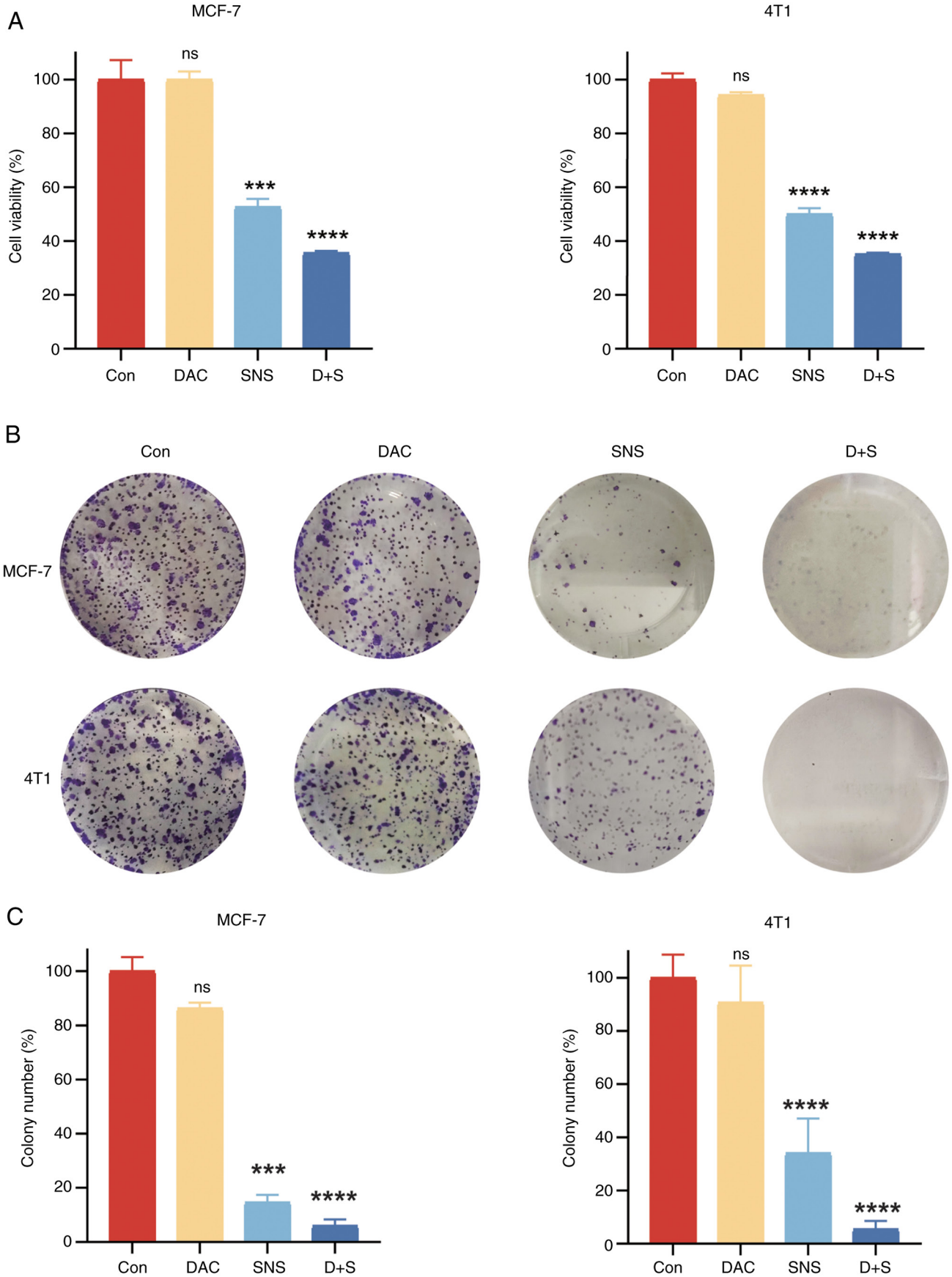

Figure 2. DAC can promote the anticancer effect of SNS-032. (A) After 24 h treatment with or without DAC (5 μmol/l), MCF-7 and 4T1 cells were treated with SNS-032 (10 and 15 μmol/l, respectively) for 24 h and cell viability was analyzed using a CCK-8 assay. (B) After 24 h treatment with or without DAC (5 μmol/l), the results of a colony formation experiment were obtained after adding SNS-032 (10 and 15 μmol/l, respectively) to MCF-7 and 4T1 cells for 24 h. Representative images are shown. (C) Quantification of colony formation in MCF-7 and 4T1 cells treated with SNS-032. The cells were exposed to 0.1% dimethyl sulfoxide as a control. Data are presented as mean ± standard deviation (n=3). ***P<0.001 and ****P<0.0001 vs. control. ns: not significant; DAC, decitabine; SNS, SNS-032; Con, control; D + S, decitabine + SNS-032; CCK-8, Cell Counting Kit-8.

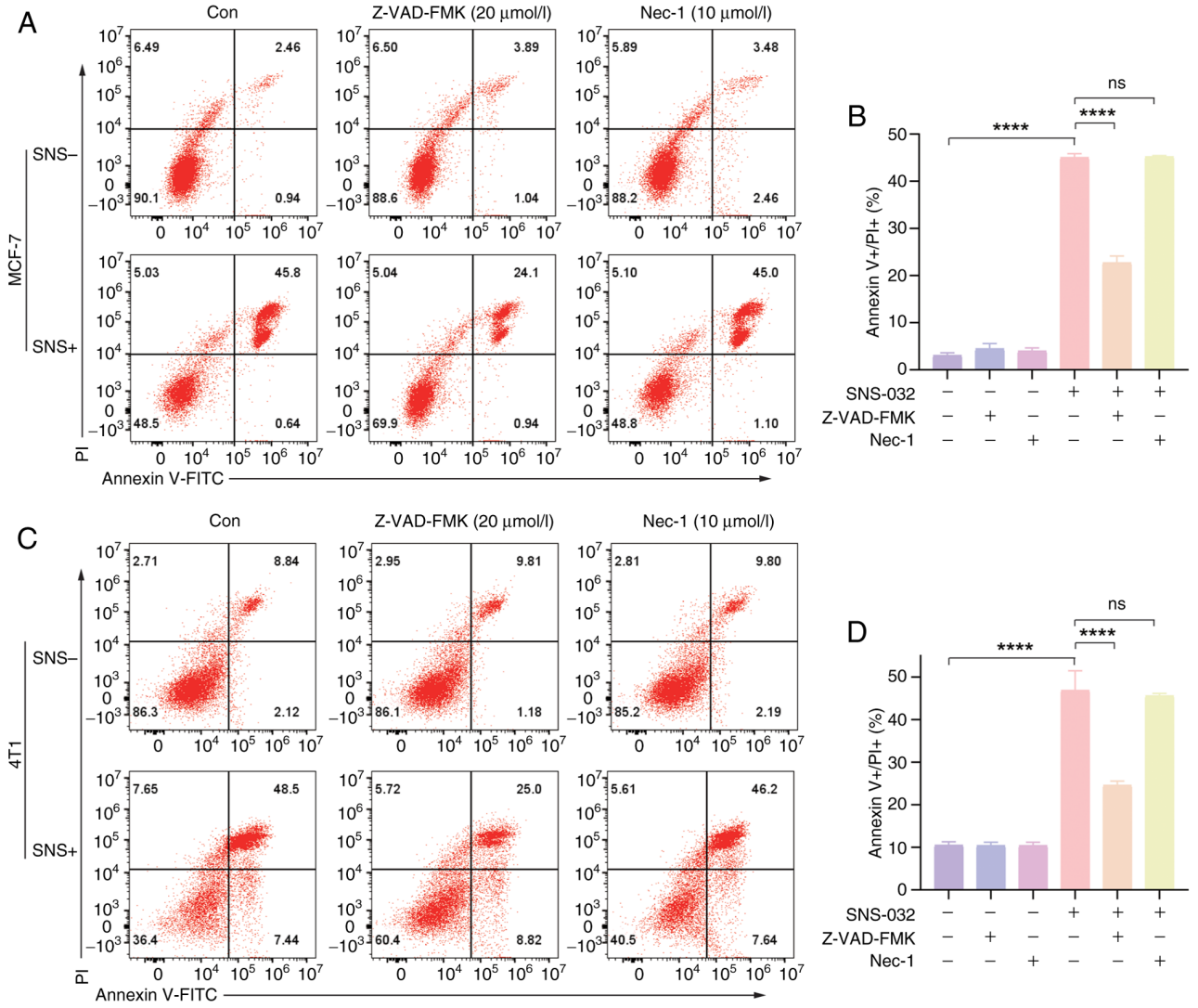

Figure 3. Different cell death inhibitors dissimilarly inhibit SNS-032-induced BC cell death. Annexin V-FITC/PI assay was performed to analyze pyroptosis and apoptosis of cells after pretreatment with SNS-032 for 24 h with or without Z-VAD-FMK (20 μmol/l) and Nec-1 (10 μmol/l) for 3 h in (A) MCF-7 and (B) 4T1 cells. Representative images are shown. The percentage of late-stage cell apoptosis in (C) MCF-7 and (D) 4T1 cells was quantified with SNS-032 treatment with or without Z-VAD-FMK and Nec-1. The cells were exposed to 0.1% dimethyl sulfoxide as a control. Data are presented as mean ± standard deviation (n=3). ****P<0.0001 vs. control. ns, not significant; SNS, SNS-032; Con, control; Nec-1, Necrostatin-1; FITC, fluorescein isothiocyanate.

swelling, plasma membrane fragmentation and morphological alterations were observed (Fig. 4A). There were no discernible morphological changes in the control group, indicating that SNS-032 acts as an anticancer agent by inducing pyroptosis in BC cells. These morphological changes were consistent with the typical morphological changes of pyroptosis rather than with the classical changes observed during apoptosis, including shrinkage, rounding, volume reduction, deformation and maintenance of membrane integrity. As pyroptosis results in the destruction of the cell membrane, which is comparable to that of late-stage apoptotic cells, the potential of SNS-032 treatment to promote pyroptosis was evaluated by detecting the percentage of Annexin V-FITC+/PI+ cells using flow cytometry in BC cells. The results demonstrated that following exposure to SNS-032, the populations of Annexin V+/PI+ cells in MCF-7 and 4T1 cells significantly increased, resulting in a notably higher rate of late-stage apoptosis (Figs. 3A, C, 4B). These results demonstrate that SNS-032 is associated with BC cell death.

Previous studies have reported that pyroptosis is a caspase-dependent PCD pathway in which GSDME is cleaved by active caspase-3, releasing its N-terminal domain to penetrate the cell membrane, leading to cell swelling, rupture and ultimately cell death (11,12). To assess this, BC cells were treated with the caspase-3 inhibitor Z-DEVD-FMK. Subsequently, western blotting and semi-quantitative analysis were performed to evaluate the changes of GSDME full-length (GSDME-FL) and N-terminus domain (GSDME-N) expression levels in BC cells treated using different approaches (Fig. 4C). Western blotting revealed that the N-terminus of GSDME could barely be detected after Z-DEVD-FMK inhibited caspase-3 activation (Fig. 4C-E). SNS-032 treatment alone significantly increased cleavage and activation of caspase-3, with a corresponding significant increase in GSDME-N compared with those in the control and Z-DEVD-FMK treatment groups. However, after SNS-032 treatment of BC cells pretreated with Z-DEVD-FMK, activated caspase-3-induced GSDME-N production was significantly reduced compared

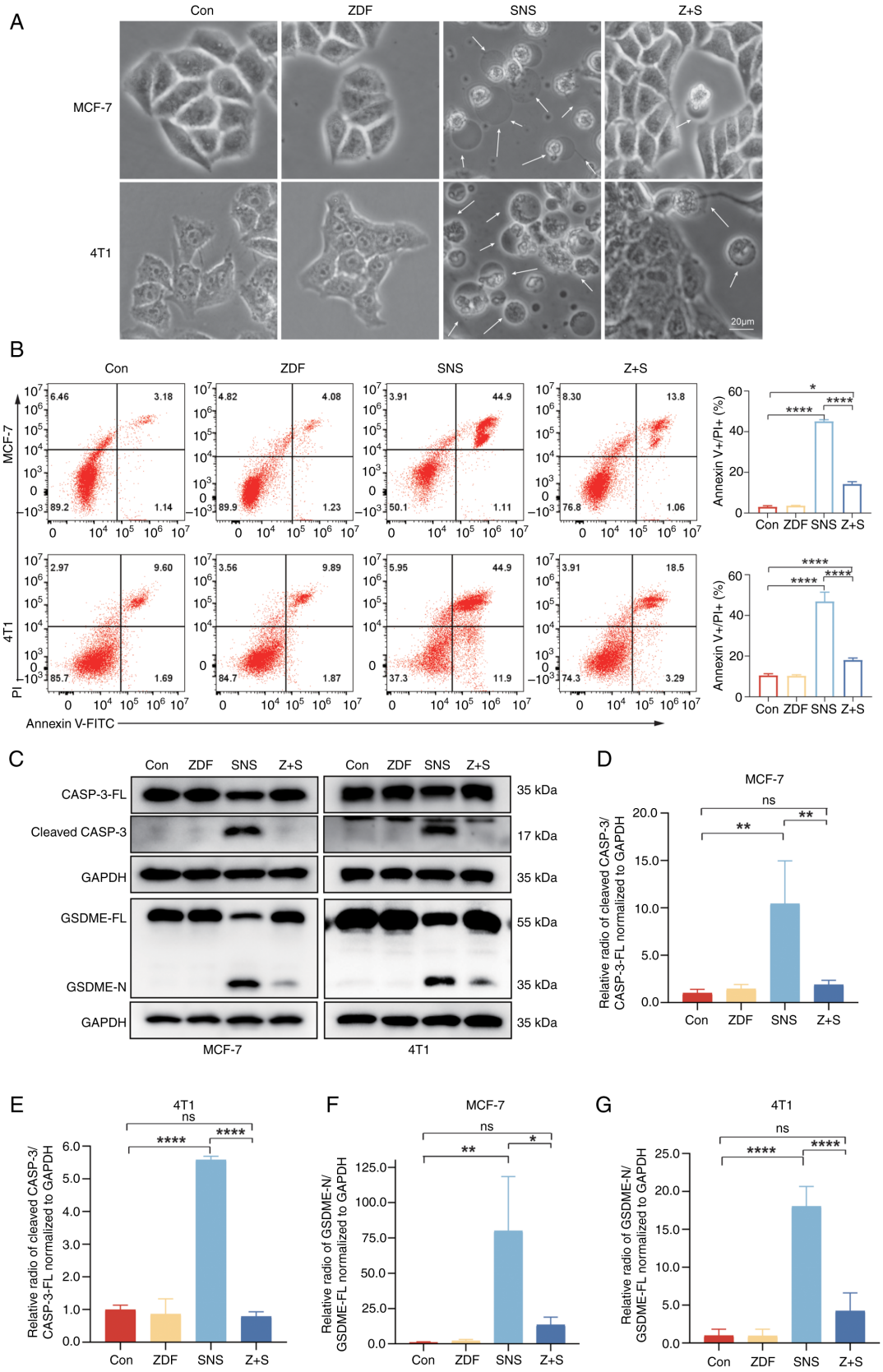

Figure 4. Caspase-3 is required for SNS-032-dependent pyroptosis. (A) Representative microscopic images of MCF-7 and 4T1 cells pretreated with SNS-032 (10 and 15 μmol/l, respectively) with or without Z-DEVD-FMK (20 μmol/l) for 3 h. The white arrows indicate pyroptotic cells. Scale bar, 20 μm. (B) Annexin V-FITC/PI assay was performed to analyze pyroptosis and apoptosis of cells after pretreatment with SNS-032 for 24 h with or without Z-DEVD-FMK (20 μmol/l) for 3 h. The percentage of late-stage cell apoptosis in cells was quantified. Representative images are shown. (C) Cleaved-caspase-3 and GSDME proteins were detected after SNS-032 pretreatment with or without Z-DEVD-FMK (20 μmol/l) for 3 h by western blotting in MCF-7 and 4T1 cells. Semi-quantitative western blot analysis of cleaved caspase-3 in (D) MCF-7 and (E) 4T1 cells and GSDME-N in (F) MCF-7 and (G) 4T1 cells. The cells were exposed to 0.1% dimethyl sulfoxide as a control. Data are presented as mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ****P<0.0001 vs. control. ns, not significant; FITC, fluorescein isothiocyanate; Con, control; ZDF, Z-DEVD-FMK; SNS, SNS-032; Z + S, Z-DEVD-FMK + SNS-032; GSDME, gasdermin E; CASP-3, caspase-3; FL, full-length; N, N-terminus.

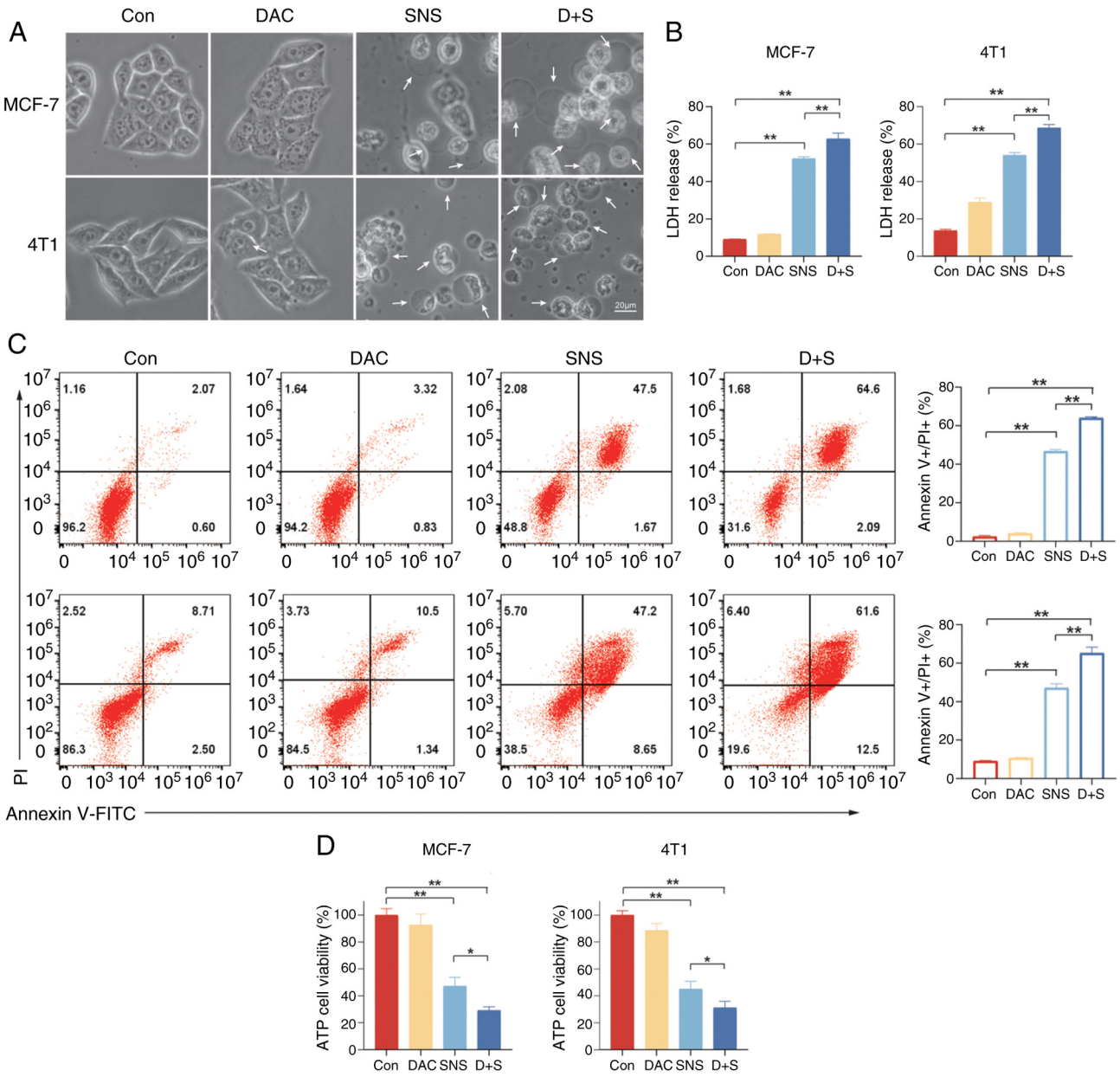

Figure 5. SNS-032 can cause the release of breast cancer cell contents and DAC can enhance the effect. (A) Representative microscopic images of MCF-7 (10 μmol/l) and 4T1 (15 μmol/l) cells treated with SNS-032 for 24 h with or without pretreatment with DAC (5 μmol/l). White arrowheads indicate the large vacuoles, cell membrane ruptures and organelle edema. Scale bar, 20 μm. (B) Quantification of the release of LDH after treatment with SNS-032 for 24 h with or without DAC (5 μmol/l) pretreatment for 24 h. (C) An Annexin V-FITC/PI assay was performed to analyze pyroptosis and apoptosis of cells after treatment with SNS-032 for 24 h with or without pretreatment with DAC (5 μmol/l) for 24 h in MCF-7 (10 μmol/l) and 4T1 (15 μmol/l) cells. Representative images are shown. (D) Quantification of ATP levels to assess cell viability after treatment with SNS-032 with or without DAC (5 μmol/l) pretreatment for 24 h. Data are presented as mean ± standard deviation (n=3). The cells were exposed to 0.1% dimethyl sulfoxide as a control. *P<0.05 and **P<0.01 vs. control. DAC, decitabine; SNS, SNS-032, D + S, decitabine + SNS-032; Con, control; ATP, adenosine 5'-triphosphate; LDH, lactate dehydrogenase; FITC, fluorescein isothiocyanate.

with SNS-032 treatment alone, providing further evidence that activated caspase-3 is required for GSDME cleavage (Fig. 4C-G). BC cells pretreated with Z-DEVD-FMK also showed significantly fewer FITC/PI double-positive cells compared with SNS-032 treatment alone (Fig. 4B). In addition to pyroptosis, the caspase family serves an important role in cell apoptosis (34-36). Z-VAD-FMK alone had no effect on cell death compared with the control in either of the cells and could inhibit BC cell death induced by SNS-032 in the present study (Fig. 3A and C). These results suggest that SNS-032 induces pyroptosis by activating caspase-3 cleavage and GSDME in BC cells. This indicates that caspase-3 serves a crucial role in the process of BC cell death induced by SNS-032 and provides an important basis for further exploration of the mechanism of cell death and the development of associated therapeutic targets.

DAC can enhance the pyroptosis induced by SNS-032 in BC cells. DAC, a DNA methyltransferase inhibitor that exhibits anticancer activity in various types of cancer (37), can demethylate BC cells and increase GSDME expression (38,39). In the present study, in combination with the CDK inhibitor

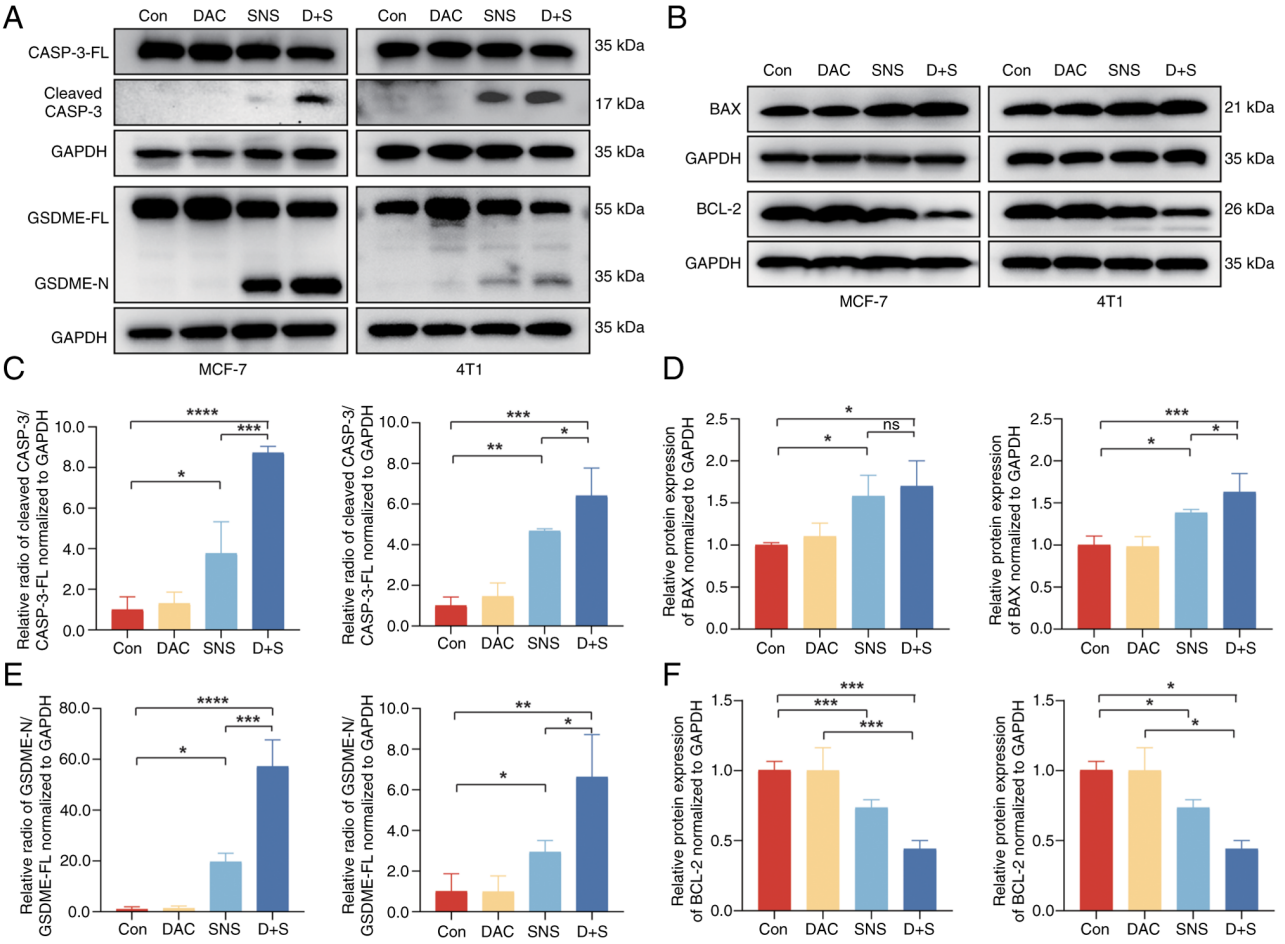

Figure 6. DAC enhances the protein expression changes of SNS-032-treated breast cancer cells. (A) Western blotting of cleaved-caspase-3 and GSDME proteins after SNS-032 treatment in MCF-7 (10 μmol/l) and 4T1 (15 μmol/l) cells with or without DAC (5 μmol/l) pretreatment for 24 h. (B) BAX and BCL-2 proteins were detected after SNS-032 treatment with or without DAC (5 μmol/l) pretreatment for 24 h by western blotting in MCF-7 (10 μmol/l) and 4T1 (15 μmol/l) cells. (C) Semi-quantitative western blot analysis of cleaved caspase-3 in MCF-7 and 4T1 cells. (D) Semi-quantitative western blot analysis of BAX in MCF-7 and 4T1 cells. (E) Semi-quantitative western blot analysis of GSDME-N in MCF-7 and 4T1 cells. (F) Semi-quantitative western blot analysis of BCL-2 in MCF-7 and 4T1 cells. The cells were exposed to 0.1% dimethyl sulfoxide as a control. Representative images are shown. Data are presented as mean ± standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. ns, not significant; DAC, decitabine; SNS, SNS-032; D + S, decitabine + SNS-032; BCL-2, B-cell lymphoma-2; BAX, Bcl-2-associated X protein; CASP-3, caspase-3; GSDME, gasdermin E; FL, full-length; N, N-terminus; Con, control.

SNS-032, DAC was used as a pretreatment agent in BC cells. According to the results shown in Fig. S1A, the expression of GSDME-FL was assessed in two BC cell lines following a 48 h treatment with DAC at concentrations of 2.5, 5 and 7.5 μmol/l. GSDME-FL expression was significantly increased when the cells were treated with 5 μmol/l DAC. Therefore, pretreatment with DAC at 5 μmol/l in the dark for 24 h was chosen before adding SNS-032. To evaluate the effects of selected concentrations of DAC on BC cells, cell viability was assessed using CCK-8 and colony formation assays. The cell viability of BC cells was not significantly affected with DAC treatment compared with the control group, as shown in Fig. 2A-C. Compared with SNS-032 monotherapy, the combination of DAC and SNS-032 demonstrated significantly reduced viability of BC cells (P<0.001). Marked pyroptotic morphological changes, such as large vacuoles and membrane balloon-like changes, occurred more frequently in BC cells following combination treatment with DAC and SNS-032 (Fig. 5A) compared with morphological changes in cells treated with SNS-032. Compared with the control group and single-drug treatment groups, BC cells treated with DAC and SNS-032 exhibited a significantly greater proportion of late-stage apoptotic cells (>60%), as illustrated in Fig. 5C.

During pyroptosis, cells disintegrate and lose membrane integrity, releasing large quantities of ATP and LDH. As illustrated in Fig. 5B and D, upon exposure to SNS-032, intracellular ATP levels were significantly decreased and LDH release levels were significantly increased (P<0.01) in BC cells compared with those in the control group. Furthermore, Fig. 5B and D demonstrate that there was a significant decrease in intracellular ATP levels and a significant increase in LDH release levels in MCF-7 and 4T1 cells with DAC and SNS-032 treatment compared with the control and SNS-032 treatment alone. By contrast, the DAC group demonstrated little effect compared with that of the control group, corroborating the suggestion that combined DAC treatment could accelerate SNS-032-triggered cell pyroptosis. A similar pattern was observed in subsequent experiments.

Previously, the process of pyroptosis has been divided into GSDMD-mediated classical and non-classical pathways, and other GSDME-mediated pathways (7). As shown in Fig. S1C, the expression of the N-terminus domain of GSDMD was

evaluated in BC cells after exposure to SNS-032 treatment to determine whether SNS-032-triggered pyroptosis. The results demonstrated that no GSDMD N-terminus domain expression was observed in SNS-032-treated BC cells. Western blotting was performed on MCF-7 and 4T1 BC cells to assess the expression of specific proteins triggered by pyroptosis. As shown in Fig. 6A, GSDME-FL was visibly increased in the DAC treatment group compared with the control group. The levels of cleaved GSDME-N were significantly increased in the drug combination group compared with SNS-032 alone (Fig. 6E). Consequently, the expression of apoptosis-related proteins in MCF-7 and 4T1 cells was assessed (Fig. 6B). As shown in Fig. 6D and F, following SNS-032 treatment, the expression of pro-apoptotic protein, BAX, was significantly increased whereas the expression of the anti-apoptotic protein, BCL-2, was significantly decreased. This suggested that SNS-032 also induces apoptosis in MCF-7 and 4T1 BC cells. There were no significant changes in the expression levels of cleaved caspase-3, BAX or BCL-2 between the DAC and control groups. The pro-apoptotic proteins, BAX and cleaved caspase-3, were significantly increased in SNS-032 treated groups and the anti-apoptotic protein BCL-2 was significantly decreased. Treatment with DAC and SNS-032 increased the expression of cleaved caspase-3 further and significantly decreased the expression of BCL-2 compared with SNS-032 alone (Fig. 6C and F). Based on the aforementioned results, it can be concluded that BC cells exposed to DAC demonstrate increased susceptibility to pyroptosis induced by SNS-032, due to the upregulated expression of GSDME. These results indicated that SNS-032 has anticancer effects on BC cells by inducing pyroptosis and that GSDME, but not GSDMD, is involved in pyroptosis induced by SNS-032 through caspase-3 activation.

Discussion

Over the past several decades, progress has been achieved in the prevention, detection and treatment of cancer. In women, BC is the most commonly diagnosed cancer and the leading cause of cancer-associated mortalities (1,3). Despite the development of several targeted drugs, effective BC therapeutic drugs are still limited (2,4). As reported in previous studies, more CDK-specific inhibitors are being developed with encouraging results and potential to become available therapeutic targets for malignant tumors (40,41).

The present study reported the effects of the CDK inhibitor, SNS-032, on BC cells. SNS-032 is a specific and potent anticancer drug that inhibits CDKs 2, 7 and 9 (24,25), blocks RNA polymerase II phosphorylation and inhibits RNA synthesis (26,27). SNS-032 is a promising anticancer drug for a number of cancers (25-29), nevertheless its anticancer mechanisms remain unclear. In the present study, it was experimentally validated that SNS-032 may induce GSDME-mediated pyroptosis, which is activated via caspase-3 pathway, in BC cell lines.

Previously, pyroptosis has been implicated in inflammatory PCD (8,42). Pyroptosis has emerged as a promising research topic in the context of traditional chemotherapy resistance. In cancers that develop resistance to conventional chemotherapy, pyroptosis represents a novel cell death pathway. Conventional chemotherapy relies predominantly on inducing apoptosis; however, when tumor cells acquire resistance to apoptosis, the pyroptotic pathway may still be effective. For instance, multiple drug-resistant tumor cells are sensitive to drugs that induce pyroptosis (43), thereby offering new insights for overcoming resistance. Moreover, studying different drug-induced pyroptosis mechanisms can further optimize the design of drugs and enhance their specificity and efficacy. Pyroptosis-inducing drugs can be used in combination with existing therapies such as chemotherapy drugs, to overcome tumor cell resistance (44), and immunotherapy drugs, to strengthen the immune response (45). In addition, the tumor cells of different patients vary in their gene expression and signaling pathways, leading to different sensitivities to pyroptosis-inducing drugs (46). Analyzing patient tumor samples to determine the expression of pyroptosis-related proteins and potential targets is beneficial for improving the accuracy of treatments and reducing drug side effects.

In eukaryotic cells, pyroptosis is defined as the creation of 10-20 nm pores in the cell membrane, followed by nuclear concentration, cell enlargement and the release of cytoplasmic contents into the extracellular environment. The formation of pores in the cell membrane (47,48) promotes the release of several components, including the inflammatory cytokine IL-18 (49). Pyroptosis enhances the immune response against cancer cells. When cancer cells undergo pyroptosis, they release certain damage-associated molecular patterns (DAMPs) (10). These DAMPs can activate antigen-presenting cells in the immune system, such as dendritic cells, which subsequently trigger the activation and recruitment of immune cells, such as T cells. This process strengthens the immune surveillance and killing ability of the body towards cancer cells. This property is crucial in the current context of the rapid development of immunotherapy, particularly in cancer types such as BC, and in the application of stereotactic body radiotherapy in metastatic disease. Inducing pyroptosis in combination with immunotherapy could improve the therapeutic effect in clinical trials.

Pyroptosis occurs through four signaling pathways: Typical and atypical inflammasome pathways, GZM-based systems and caspase-mediated pathways of apoptosis (13). The GSDM family is the final enforcer of these signaling cascades, which require upstream caspases or GZMs to cleave them (9). Most studies to date have focused on the roles of GSDMD and GSDME in pyroptosis. A previous study demonstrated caspase-3 can cleave GSDME during chemotherapy to generate GSDME-N, which then penetrates the plasma membrane and leads to pyroptosis (14). Pyroptosis stimulation is challenging in BC cells because of the markedly decreased expression of GSDME compared with expression in normal cells (14,15,22). Endogenous GSDME expression was profiled in 57 cancer cells from the National Cancer Institute-60 cell lines screen, which includes numerous BC cell lines. And MCF-7 cells exhibited the highest level of endogenous GSDME expression, indicating a higher probability of inducing GSDME-mediated pyroptosis (14). Therefore, this cell line was chosen as the human BC cell line for the present study.

It has been reported that DAC is capable of augmenting the expression of GSDME protein in 4T1 cells (50). Pyroptosis has the capacity to activate the body's immune system, which potentially responds to the tumor-killing effect of the drugs. To optimize the anticancer effect of SNS-032-induced

pyroptosis in BC, the BC cell line 4T1 was chosen, which is derived from BALB/c mice. The 4T1 cells can be utilized to construct immunocompetent BC mouse models in BALB/c mice, thereby paving the way for further investigations into the anticancer efficacy of SNS-032 *in vivo*. In addition, the DNA methyltransferase inhibitor, DAC, was utilized to induce functional re-expression of the abnormally silenced GSDME gene in cancer (22,23), and elevate the expression of GSDME in BC cells with low GSDME expression, thus promoting the anticancer ability through cell pyroptosis. Based on previous research (39,51) and the results of the present study, the optimal DAC concentration was determined to be 5 μmol/l. The optimal concentration of SNS-032 was determined to be 10 μmol/l in MCF-7 cells and 15 μmol/l in 4T1 cells for the subsequent experiments in the present study.

Initially, SNS-032 was shown to inhibit in the viability of BC cells using CCK-8 and colony formation assays, and the combination of DAC and SNS-032 further enhanced this effect. Furthermore, the morphology of MCF-7 and 4T1 cells following SNS-032 treatment revealed swelling and generation of large vacuoles on the cell membrane, which is a characteristic morphological manifestation of pyroptosis.

Previous studies have shown that pyroptosis and apoptosis can be differentiated by the positivity of Annexin V/PI staining. Generally, pyroptosis occurs more rapidly than apoptosis. For double-staining with Annexin V and PI, in the early stage of pyroptosis, the integrity of the cell membrane is compromised, leading to the externalization of phosphatidylserine, to which Annexin V can specifically bind. Concurrently, due to the increased membrane permeability, PI can enter the cell through pores formed on the cell membrane and stain the nucleus (34). In flow cytometric analysis, a cell population that is positive for both Annexin V and PI will be detected. In the early stage of apoptosis, although phosphatidylserine also externalizes on the cell membrane, the integrity of the cell membrane remains intact initially. At this point, cells exhibit Annexin V-positive and PI-negative staining. In the present study, BC cells were pretreated with Z-VAD-FMK, Necrostatin-1 or Z-DEVD-FMK, and the proportion of cell death was detected using Annexin V/PI staining. The findings of present study suggested that cell death induced by SNS-032 is not due to necroptosis. However, the pan-caspase inhibitor, Z-VAD-FMK, significantly inhibited late-stage apoptosis induced by SNS-032, while Z-DEVD-FMK almost completely inhibited its anticancer effect. Meanwhile, SNS-032 combined with DAC could further increase the proportion of late-stage apoptotic cells. In addition, in BC cells, SNS-032 significantly increased LDH release and decreased intracellular ATP, and these effects were markedly enhanced in combination with DAC. Z-DEVD-FMK was used to inhibit the activation of caspase-3 in BC cells treated with SNS-032. Consequently, the characteristic morphological features of pyroptosis in MCF-7 and 4T1 cells were notably alleviated and the protein expression levels of cleaved caspase-3 and GSDME-N were markedly decreased compared with SNS-032 treatment alone. Therefore, pretreatment with Z-DEVD-FMK effectively reduced pyroptosis in BC cells treated with SNS-032.

Western blotting was used to evaluate caspase-3 and GSDME protein expression in BC cells during treatment with SNS-032. The results demonstrated that caspase-3 was activated, GSDME was cleaved and GSDME N-terminus protein was significantly increased compared with the negative control group. Furthermore, DAC treatment enhanced GSDME-FL expression and when combined with SNS-032, the expression of GSDME-N was higher compared with the SNS-032 group. These findings suggest that SNS-032 triggers pyroptosis in MCF-7 and 4T1 cells via the caspase-3/GSDME signaling pathway.

The study indicated that SNS-032 decreased the expression of BCL-2, and increased the expression of BAX, and those changes became more apparent when combined with DAC. These data demonstrate that SNS-032 can trigger the pyroptosis in MCF-7 and 4T1 cells through the caspase-3/GSDME pathway, and DAC can boost the expression of GSDME protein by methylation, thus enhancing pyroptosis induced by SNS-032 in BC cells. In conclusion, the present article demonstrates that the CDK2/7/9 inhibitor SNS-032 can induce pyroptosis and apoptosis of human BC MCF-7 cells and mouse BC 4T1 cells through the caspase-3/GSDME pathway *in vitro*, thereby reducing the viability of cancer cells, providing a potential new strategy for the treatment of BC.

SNS-032 as a CDK inhibitor, may have potential off-target effects such as the inhibition of other kinases with similar structures or functions. This might lead to unintended consequences in cellular processes which are not associated with the expected target. Another effect of SNS-032 could be its impact on transcriptional regulation which might affect the expression of genes not directly associated with its primary mechanism of action. In addition, it might potentially alter the overall cellular gene expression profile and lead to unforeseen physiological changes or cytotoxicity in cells that are not the target of treatment. Furthermore, there are considerable clinical limitations regarding CDK inhibitors. As low-dose CDK inhibitors are ineffective, highly selective CDK inhibitors require higher doses, making the side effects of CDK inhibitors in clinical trials a limiting factor (52).

CDK inhibitors are tyrosine kinase inhibitors, with a main concern being potential acquired resistance during treatment (53). Future research should utilize a number of strategies to address these potential off-target impacts. Firstly, high-throughput screening techniques, such as proteomics and genomics assays, can be utilized to comprehensively map the interactome of SNS-032. By identifying proteins and genes with which it interacts, on-target and off-target effects can be differentiated more precisely. In order to improve clinical treatment efficacy and prevent needless drug exposure that leads to drug resistance, biomarkers associated with CDK inhibitor resistance should be assessed in patient blood or tumor tissues. These could then be used to screen patient groups that are less likely to develop resistance and are most likely to benefit from SNS-032 treatment. Secondly, structure-activity relationship studies should be conducted. By implementing chemical modifications to SNS-032, analogs with improved selectivity for the intended target may be developed, thereby minimizing interactions with off-target molecules and delaying the emergence of resistance. In addition, SNS-032 can be combined with drugs that have different mechanisms of action, such as targeting tumor angiogenesis, to treat the tumor from multiple directions to

diminish the possibility of developing drug resistance (54). Immunomodulatory drugs, for instance immune checkpoint inhibitors, can be used to stimulate the body's immune system, synergizing with the inflammatory response induced by SNS-032-induced pyroptosis, to attack tumor cells that are resistant to treatment. Thirdly, it is imperative to utilize more physiologically relevant *in vitro* and *in vivo* models. For instance, patient-derived organoids or genetically engineered mouse models can offer a more precise evaluation of off-target effects, particularly those that are tissue-specific or disease-specific. Finally, long-term follow-up studies in both preclinical and clinical settings are crucial. Monitoring treated subjects over an extended duration can facilitate the detection of any delayed or cumulative off-target effects that may not be immediately evident. This approach allows for the optimization of dosage and treatment regimens aimed at reducing off-target toxicity while maximizing therapeutic efficacy.

The present study established that SNS-032 can cause apoptosis and pyroptosis of BC cells through caspase-3. Further research is required to establish the detailed mechanism underlying SNS-032-induced pyroptosis. Additionally, whether the antitumor activity of DAC in combination with SNS-032 could be utilized in *in vivo* experiments should be studied in the future.

Acknowledgements

Not applicable.

Funding

The present work was supported by the National Natural Science Foundation of China (grant no 82172717).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

The present study was proposed and designed by YC and LX. YC and DZ performed the experiments. Data collection and analysis were performed by JL, JW and YS. The manuscript was drafted by YC and checked by LX. YC and DZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. World Health Organization: Global breast cancer initiative implementation framework: assessing, strengthening and scaling up of services for the early detection and management of breast cancer: executive summary. World Health Organization, 2023
2. Xia C, Dong X, Li H, Cao M, Sun D, He S, Yang F, Yan X, Zhang S, Li N and Chen W: Cancer statistics in China and United States, 2022: Profiles, trends, and determinants. Chin Med J (Engl) 135: 584-590, 2022.
3. Britt KL, Cuzick J and Phillips KA: Key steps for effective breast cancer prevention. Nat Rev Cancer 20: 417-436, 2020.
4. Allemani C, Matsuda T, Di Carlo V, Harewood R, Matz M, Nikšić M, Bonaventure A, Valkov M, Johnson CJ, Estève J, *et al*: Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet 391: 1023-1075, 2018.
5. Kerr AJ, Dodwell D, McGale P, Holt F, Duane F, Mannu G, Darby SC and Taylor CW: Adjuvant and neoadjuvant breast cancer treatments: A systematic review of their effects on mortality. Cancer Treat Rev 105: 102375, 2022.
6. Anampa J, Makower D and Sparano JA: Progress in adjuvant chemotherapy for breast cancer: An overview. BMC Med 13: 195, 2015.
7. Frank D and Vince JE: Pyroptosis versus necroptosis: Similarities, differences, and crosstalk. Cell Death Differ 26: 99-114, 2019.
8. Shi J, Gao W and Shao F: Pyroptosis: Gasdermin-mediated programmed necrotic cell death. Trends Biochem Sci 42: 245-254, 2017.
9. Kovacs SB and Miao EA: Gasdermins: Effectors of pyroptosis. Trends Cell Biol 27: 673-684, 2017.
10. Du T, Gao J, Li P, Wang Y, Qi Q, Liu X, Li J, Wang C and Du L: Pyroptosis, metabolism, and tumor immune microenvironment. Clin Transl Med 11: e492, 2021.
11. Liu X, Xia S, Zhang Z, Wu H and Lieberman J: Channelling inflammation: Gasdermins in physiology and disease. Nat Rev Drug Discov 20: 384-405, 2021.
12. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, Sun H, Wang DC and Shao F: Pore-forming activity and structural autoinhibition of the gasdermin family. Nature 535: 111-116, 2016.
13. Rao Z, Zhu Y, Yang P, Chen Z, Xia Y, Qiao C, Liu W, Deng H, Li J, Ning P and Wang Z: Pyroptosis in inflammatory diseases and cancer. Theranostics 12: 4310-4329, 2022.
14. Wang Y, Gao W, Shi X, Ding J, Liu W, He H, Wang K and Shao F: Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. Nature 547: 99-103, 2017.
15. Rogers C, Fernandes-Alnemri T, Mayes L, Alnemri D, Cingolani G and Alnemri ES: Cleavage of DFNA5 by caspase-3 during apoptosis mediates progression to secondary necrotic/pyroptotic cell death. Nat Commun 8: 14128, 2017.
16. Xia X, Wang X, Cheng Z, Qin W, Lei L, Jiang J and Hu J: The role of pyroptosis in cancer: Pro-cancer or pro-'host'? Cell Death Dis 10: 650, 2019.
17. Chen L, Weng B, Li H, Wang H, Li Q, Wei X, Deng H, Wang S, Jiang C, Lin R and Wu J: A thiopyran derivative with low murine toxicity with therapeutic potential on lung cancer acting through a NF-kappaB mediated apoptosis-to-pyroptosis switch. Apoptosis 24: 74-82, 2019.
18. Zhou CB and Fang JY: The role of pyroptosis in gastrointestinal cancer and immune responses to intestinal microbial infection. Biochim Biophys Acta Rev Cancer 1872: 1-10, 2019.
19. Zeng H, Yang H, Song Y, Fang D, Chen L, Zhao Z, Wang C and Xie S: Transcriptional inhibition by CDK7/9 inhibitor SNS-032 suppresses tumor growth and metastasis in esophageal squamous cell carcinoma. Cell Death Dis 12: 1048, 2021.
20. Zhang Z, Zhang Y, Xia S, Kong Q, Li S, Liu X, Junqueira C, Meza-Sosa KF, Mok TMY, Ansara J, *et al*: Gasdermin E suppresses tumour growth by activating anti-tumour immunity. Nature 579: 415-420, 2020.
21. Thompson DA and Weigel RJ: Characterization of a gene that is inversely correlated with estrogen receptor expression (ICERE-1) in breast carcinomas. Eur J Biochem 252: 169-177, 1998.
22. Akino K, Toyota M, Suzuki H, Imai T, Maruyama R, Kusano M, Nishikawa N, Watanabe Y, Sasaki Y, Abe T, *et al*: Identification of DFNA5 as a target of epigenetic inactivation in gastric cancer. Cancer Sci 98: 88-95, 2007.

23. Kim MS, Chang X, Yamashita K, Nagpal JK, Baek JH, Wu G, Trink B, Ratovitski EA, Mori M and Sidransky D: Aberrant promoter methylation and tumor suppressive activity of the DFNA5 gene in colorectal carcinoma. Oncogene 27: 3624-3634, 2008.
24. Chen R, Wierda WG, Chubb S, Hawtin RE, Fox JA, Keating MJ, Gandhi V and Plunkett W: Mechanism of action of SNS-032, a novel cyclin-dependent kinase inhibitor, in chronic lymphocytic leukemia. Blood 113: 4637-4645, 2009.
25. Zhang J, Liu S, Ye Q and Pan J: Transcriptional inhibition by CDK7/9 inhibitor SNS-032 abrogates oncogene addiction and reduces liver metastasis in uveal melanoma. Mol Cancer 18: 140, 2019.
26. Wu Y, Chen C, Sun X, Shi X, Jin B, Ding K, Yeung SC and Pan J: Cyclin-dependent kinase 7/9 inhibitor SNS-032 abrogates FIP1-like-1 platelet-derived growth factor receptor alpha and bcr-abl oncogene addiction in malignant hematologic cells. Clin Cancer Res 18: 1966-1978, 2012.
27. Meng H, Jin Y, Liu H, You L, Yang C, Yang X and Qian W: SNS-032 inhibits mTORC1/mTORC2 activity in acute myeloid leukemia cells and has synergistic activity with perifosine against Akt. J Hematol Oncol 6: 18, 2013.
28. Kang MA, Kim W, Jo HR, Shin YJ, Kim MH and Jeong JH: Anticancer and radiosensitizing effects of the cyclin-dependent kinase inhibitors, AT7519 and SNS-032, on cervical cancer. Int J Oncol 53: 703-712, 2018.
29. Kodym E, Kodym R, Reis AE, Habib AA, Story MD and Saha D: The small-molecule CDK inhibitor, SNS-032, enhances cellular radiosensitivity in quiescent and hypoxic non-small cell lung cancer cells. Lung Cancer 66: 37-47, 2009.
30. Heath EI, Bible K, Martell RE, Adelman DC and Lorusso PM: A phase 1 study of SNS-032 (formerly BMS-387032), a potent inhibitor of cyclin-dependent kinases 2, 7 and 9 administered as a single oral dose and weekly infusion in patients with metastatic refractory solid tumors. Invest New Drugs 26: 59-65, 2008.
31. Tong WG, Chen R, Plunkett W, Siegel D, Sinha R, Harvey RD, Badros AZ, Popplewell L, Coutre S and Fox JA: Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. J Clin Oncol 28: 3015-3022, 2010.
32. Xie G, Tang H, Wu S, Chen J, Liu J and Liao C: The cyclin-dependent kinase inhibitor SNS-032 induces apoptosis in breast cancer cells via depletion of Mcl-1 and X-linked inhibitor of apoptosis protein and displays antitumor activity in vivo. Int J Oncol 45: 804-812, 2014.
33. Zeng H, Yang H, Song Y, Fang D, Chen L, Zhao Z, Wang C and Xie S: Transcriptional inhibition by CDK7/9 inhibitor SNS-032 suppresses tumor growth and metastasis in esophageal squamous cell carcinoma. Cell Death Dis 12: 1048, 2021.
34. Miao EA, Rajan JV and Aderem A: Caspase-1-induced pyroptotic cell death. Immunol Rev 243: 206-214, 2011.
35. Ai Y, Meng Y, Yan B, Zhou Q and Wang X: The biochemical pathways of apoptotic, necroptotic, pyroptotic, and ferroptotic cell death. Mol Cell 84: 170-179, 2024.
36. Kesavardhana S, Malireddi RKS and Kanneganti TD: Caspases in cell death, inflammation, and pyroptosis. Annu Rev Immunol 38: 567-595, 2020.
37. Marino G, Niso-Santano M, Baehrecke EH and Kroemer G: Self-consumption: The interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 15: 81-94, 2014.
38. Wang B, Li H, Yang R, Zhou S and Zou S: Decitabine inhibits the cell growth of cholangiocarcinoma in cultured cell lines and mouse xenografts. Oncol Lett 8: 1919-1924, 2014.
39. Mi D, Li J, Wang R, Li Y, Zou L, Sun C, Yan S, Yang H, Zhao M and Shi S: Postsurgical wound management and prevention of triple-negative breast cancer recurrence with a pryoptosis-inducing, photopolymerizable hydrogel. J Control Release 356: 205-218, 2023.
40. Parua PK and Fisher RP: Dissecting the Pol II transcription cycle and derailing cancer with CDK inhibitors. Nat Chem Biol 16: 716-724, 2020.
41. Swaffer MP, Jones AW, Flynn HR, Snijders AP and Nurse P: CDK substrate phosphorylation and ordering the cell cycle. Cell 167: 1750-1761 e1716, 2016.
42. Yang F, Bettadapura SN, Smeltzer MS, Zhu H and Wang S: Pyroptosis and pyroptosis-inducing cancer drugs. Acta Pharmacol Sin 43: 2462-2473, 2022.
43. Chen W, Yang KB, Zhang YZ, Lin ZS, Chen JW, Qi SF, Wu CF, Feng GK, Yang DJ, Chen M, *et al*: Synthetic lethality of combined ULK1 defection and p53 restoration induce pyroptosis by directly upregulating GSDME transcription and cleavage activation through ROS/NLRP3 signaling. J Exp Clin Cancer Res 43: 248, 2024.
44. Su L, Chen Y, Huang C, Wu S, Wang X, Zhao X, Xu Q, Sun R, Kong X, Jiang X, *et al*: Targeting Src reactivates pyroptosis to reverse chemoresistance in lung and pancreatic cancer models. Sci Transl Med 15: eabl7895, 2023.
45. Zhang S, Zhang Y, Feng Y, Wu J, Hu Y, Lin L, Xu C, Chen J, Tang Z, Tian H and Chen X: Biomineralized two-enzyme nanoparticles regulate tumor glycometabolism inducing tumor cell pyroptosis and robust antitumor immunotherapy. Adv Mater 34: e2206851, 2022.
46. Pan J, Li Y, Gao W, Jiang Q, Geng L, Ding J, Li S and Li J: Transcription factor Sp1 transcriptionally enhances GSDME expression for pyroptosis. Cell Death Dis 15: 66, 2024.
47. Broz P, Pelegrin P and Shao F: The gasdermins, a protein family executing cell death and inflammation. Nat Rev Immunol 20: 143-157, 2020.
48. Zhang Y, Chen X, Gueydan C and Han J: Plasma membrane changes during programmed cell deaths. Cell Res 28: 9-21, 2018.
49. Man SM, Karki R and Kanneganti TD: Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunol Rev 277: 61-75, 2017.
50. Xie B, Liu T, Chen S, Zhang Y, He D, Shao Q, Zhang Z and Wang C: Combination of DNA demethylation and chemotherapy to trigger cell pyroptosis for inhalation treatment of lung cancer. Nanoscale 13: 18608-18615, 2021.
51. Gong W, Fang P, Leng M and Shi Y: Promoting GSDME expression through DNA demethylation to increase chemosensitivity of breast cancer MCF-7/Taxol cells. PLoS One 18: e0282244, 2023.
52. Bose P, Simmons GL and Grant S: Cyclin-dependent kinase inhibitor therapy for hematologic malignancies. Expert Opin Investig Drugs 22: 723-738, 2013.
53. Bixby D and Talpaz M: Seeking the causes and solutions to imatinib-resistance in chronic myeloid leukemia. Leukemia 25: 7-22, 2011.
54. Uzhachenko RV, Bharti V, Ouyang Z, Blevins A, Mont S, Saleh N, Lawrence HA, Shen C, Chen SC, Ayers GD, *et al*: Metabolic modulation by CDK4/6 inhibitor promotes chemokine-mediated recruitment of T cells into mammary tumors. Cell Rep 35: 108944, 2021.