

# SZC017, a novel oleanolic acid derivative, induces apoptosis and autophagy in human breast cancer cells

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**Abstract** Oleanolic acid (OA) and its derivatives such as 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), CDDO-Me, and CDDO-Im show potent anticancer function. In this study, we elucidated the anticancer effect of SZC017, a novel OA derivative and identified the mechanisms by which SZC017 induces MCF-7 cell death. We found that SZC017 effectively decreased the cell viability of these breast cancer cells, but was less toxic to MCF10A mammary epithelial cells. Mechanisms underlying the inhibition of cell viability are apoptosis, autophagy induction, and G<sub>0</sub>/G<sub>1</sub> phase arrest. SZC017 treatment suppressed the levels of Akt, phosphorylated-Akt (p-Akt), p-IkB $\alpha$ , total p65, and total p-p65, in addition to p-p65 in both the cytoplasm and nucleus. Furthermore, the inhibition of p65 nuclear translocation was confirmed by immunofluorescence staining. Cell viability was increased after pretreatment with chloroquine, an inhibitor of autophagy, whereas the level of procaspase-3 was significantly decreased. A concentration-dependent increase in the intracellular reactive oxygen species (ROS) level was observed in both MCF-7 and MDA-MB-231 cells. Additionally, pretreatment with *N*-acetyl-L-cysteine (NAC), a ROS scavenger, increased cell viability and the expression of Akt and procaspase-3, but decreased the ratio of LC3-II/I. These data show that SZC017 is an effectively selective anticancer agent against breast cancer cells, highlighting

the potential use of this derivative as a breast cancer therapeutic agent.

**Keywords** SZC017 · Oleanolic acid derivative · Apoptosis · Autophagy · Breast cancer cells

## Introduction

Breast cancer is the most prevalent malignancy and the leading cause of cancer-related death in women worldwide. Approximately 1.38 million patients accounting for 23 % of the total new female cancer cases were newly diagnosed, and 458,400 accounting for 14 % of the total new female cancer deaths died in 2008. However, the 10-year relative survival rate has increased from 74.8 % in 1975 to 90.3 % in 2003 due to the improvements of treatment (i.e., chemotherapy, hormone therapy, and targeted drugs) and use of mammography screening [1, 2]. Patients diagnosed with early-stage may receive adjuvant treatments, including chemotherapy and radiation therapy, after breast-conserving surgery, while most patients with late-stage will receive chemotherapy. However, adverse effects of chemotherapy, including the possibility of cardiomyopathy, impaired fertility, premature menopausal, and congestive heart failure, will increase after treatments with HER-2 targeted drugs and anthracyclines [1]. Therefore, there is still urgent to develop novel therapeutic drugs.

Several natural products identified in plants have shown their chemopreventive and therapeutic potential on breast cancer [3]. Oleanolic acid (OA, 3 $\beta$ -hydroxyolean-12-en-28-oic acid), which has been isolated from more than 1600 plants and especially abundant in olive from which its name derives, is a multifunctional pentacyclic triterpenoid compound. OA exhibits many biological functions such as

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anti-inflammation, antiviral, anti-microbe, anti-parasitic and apoptosis induction in many cancer types [4–6]. Several OA derivatives such as CDDO, and its CDDO-Me and CDDO-Im show many biological activities, which are presently under evaluation in phase I studies. Other OA derivatives including di-CDDO (nitrile at C-17 position of CDDO) and various amides such as CDDO-MA (methyl amide), CDDO-EA (ethyl amide), and CDDO-TFEA (trifluoromethyl amide) also show potent anticancer function [5]. Thus, OA derivatives remain important fields of study in their synthesis and potential anticancer efficacy to human breast cancer.

In the present study, we aimed to explore the anticancer effect of SZC017 on human breast cancer cells in vitro and elucidate the underlying mechanisms. SZC017 effectively decreased the cell viability of both MCF-7 and MDA-MB-231 cell lines, but showed less cytotoxicity against MCF10A cells. Mechanistically, we then demonstrated that apoptosis and autophagy induction and the inhibition of the Akt/NF- $\kappa$ B signaling are mediated by excessive ROS in SZC017-treated MCF-7 cells. Herein, we firstly report that the potential anticancer effect of SZC017, a novel OA derivative, on human breast cancer cell line MCF-7.

## Materials and methods

### Chemicals

OA and SZC017 were kindly provided by Prof. Shisheng Wang (Dalian University of Technology), and 2 mg OA and SZC017 were dissolved by 186  $\mu$ l dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$  (Fig. 1a, b), respectively. Dulbecco's modified Eagle medium (DMEM), DMEM/F-12 medium, trypsin EDTA, horse serum, and fetal bovine serum (FBS) were purchased from GIBCO BRL (Gaithersburg, MD, USA). The Reactive Oxygen Species Assay Kit, Cell Cycle and Apoptosis Analysis Kit, and Nuclear and Cytoplasmic Extraction kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), penicillin, streptomycin, chloroquine (CQ) diphosphate salt, cholera toxin, insulin, hydrocortisone, human epidermal growth factor, and *N*-Acetyl-L-Cysteine (NAC) were obtained from Sigma-Aldrich (St Louis, MO, USA). The fluo-3/AM was procured from Dojindo Laboratories (Kumamoto, Japan). The Annexin V-FITC Apoptosis Detection Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The primary antibodies against  $\beta$ -actin, Histone H3, Akt, p-Akt, procaspase-9, procaspase-3, Bax, Bcl-2, Beclin 1, microtubule-associated protein 1 light

chain 3 (LC3) were purchased from Proteintech (Chicago, IL, USA). Antibodies against p65, p-p65 (Ser536), p-I $\kappa$ B $\alpha$  (Ser32/Ser36) and all the secondary antibodies were obtained from Abbkine (Redlands, California, USA).

### Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231, and human mammary epithelial cells MCF10A were obtained from Shanghai Institute of Biochemistry and Cell Biology. MCF-7 and MDA-MB-231 cells were maintained in high-glucose DMEM supplemented with 10 % FBS, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. MCF10A cells were maintained in DMEM/F-12 medium supplemented with 5 % horse serum, 0.1 mg/ml cholera toxin, 10 mg/ml insulin, 0.5 mg/ml hydrocortisone, 20 ng/ml human epidermal growth factor, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were incubated in a humidified atmosphere of 5 %  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

### Cell viability analysis

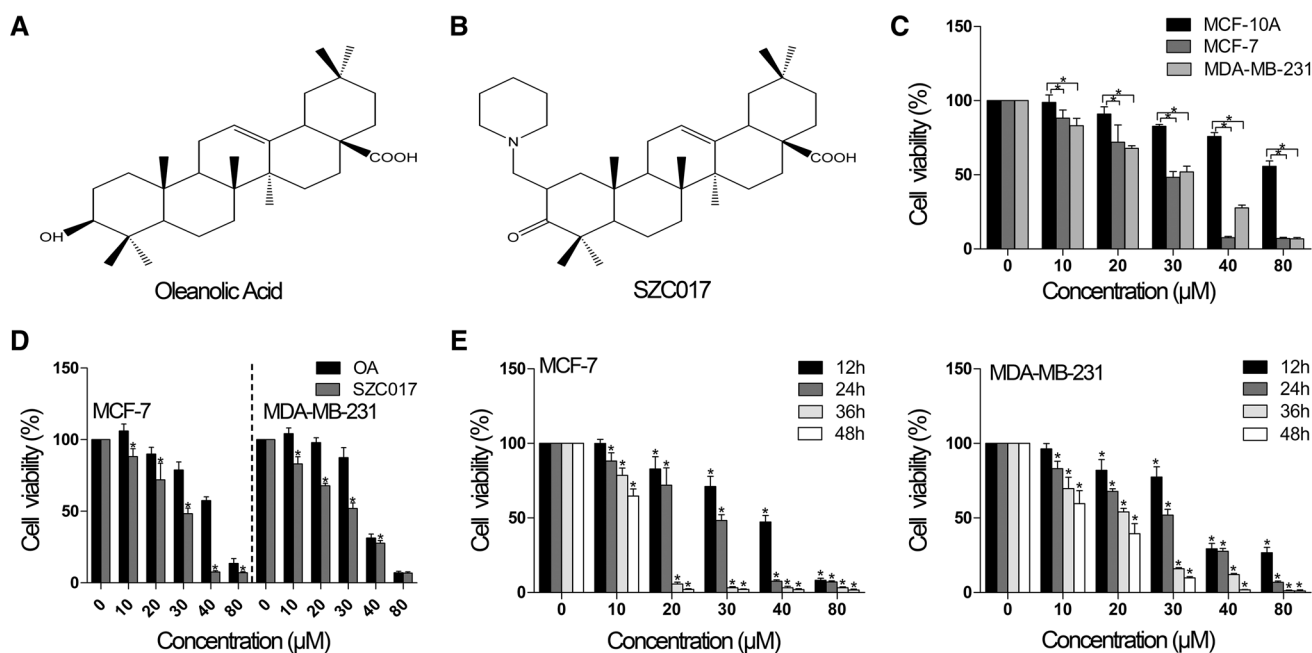
Cell viability was estimated using MTT assay as previously reported [7]. Briefly, cells were seeded into 96-well plates at density of  $10 \times 10^4$  cells/ml and further incubated for 24 h, and then cells were treated with SZC017 with different concentrations. After an exposure to SZC017, 15  $\mu$ l MTT stock solution (5 mg/ml) was added. Additional 4 h incubation later, 100  $\mu$ l SDS-isobutanol-HCl solution (10 % SDS, 5 % isobutanol and 12 mM HCl) was added and the plates were further incubated at  $37^{\circ}\text{C}$  overnight. Absorbance was measured at 570 nm with a microplate reader (Multiskan MK3; Pioneer Co; China).

### Cell apoptosis analysis

The Annexin V-FITC Apoptosis Detection Kit was used to determine whether apoptosis involves in the inhibition effect of SZC017 on cell viability in MCF-7 cells. We seeded cells in 6-well plates and cultured for 24 h. The cells were then collected and washed after treatment with SZC017. After being stained with Annexin V-FITC and propidium iodide (PI) for 30 min in the dark at room temperature according to instructions of manufacturer, the samples were then analyzed using FACScan flow cytometry (BD FACSAria II; BD Co; America).

### Cell cycle analysis

To determine whether the distribution of cell cycle is affected by SZC017, MCF-7 cells were cultured in 6-well plates, and then treated with SZC017 for 24 h. In order to get an accurate results of the distribution of cell cycle,



**Fig. 1** The selective inhibition effect of SZC017 on cell viability in breast cancer cells. **a** The chemical structure of OA. **b** The chemical structure of SZC017. **c** The inhibition effect of SZC017 on MCF10A, MCF-7, and MDA-MB-231 cell lines. Cells were treated with SZC017 at concentrations ranging from 10 to 80  $\mu\text{M}$ , and cell viability was measured by the MTT assay after treatment for 24 h. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from MCF10A cell lines, \* $p < 0.05$ . **d** The inhibition effect of SZC017 on cell viability in MCF-7 and MDA-MB-231 cell lines, respectively. Cells were treated with OA (10, 20,

30, 40, and 80  $\mu\text{M}$ ) and SZC017 (10, 20, 30, 40, and 80  $\mu\text{M}$ ) for 24 h, and then cell viability was assessed by the MTT assay. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from OA group, \* $p < 0.05$ . **e** The concentration and time-dependent effect of SZC017 on MCF-7 and MDA-MB-231 cell lines. Both MCF-7 and MDA-MB-231 cell lines were treated with SZC017 at concentrations ranging from 10 to 80  $\mu\text{M}$ , and cell viability was measured by the MTT assay at indicated time points, respectively. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control, \* $p < 0.05$

suspension containing cell debris was removed [8], and we carefully collected and fixed cells in 70 % cold ethanol overnight at 4  $^{\circ}\text{C}$ . According to instructions of manufacturer, the PI staining reagent (50 mg/ml PI and 1 mg/ml RNase in 1 ml of sodium citrate buffer, pH 7.4) was prepared and then incubated with samples in the dark at 37  $^{\circ}\text{C}$  for 30 min. Cell cycle distribution was determined by FACScan flow cytometry (BD FACSAria II; BD Co; America), and the data were analyzed using the multicycle program from Phoenix Flow Systems (San Diego, CA).

#### Measurement of intracellular ROS level

The level of intracellular ROS was measured by flow cytometry using Reactive Oxygen Species Assay Kit. Cells were cultured in 6-well plates, collected and washed with PBS for three times after treatment. Cells were then suspended with serum-free medium containing 10  $\mu\text{M}$  DCFH-DA for 30 min in the dark at 37  $^{\circ}\text{C}$ . After being washed with serum-free medium for three times, the samples were measured using FACScan flow cytometry (BD FACSAria II; BD Co; America).

#### Measurement of intracellular calcium level

To assess the effect of SZC017 on intracellular calcium level in MCF-7 cells, cells were loaded with 10  $\mu\text{M}$  of fluo-3/AM containing 0.05 % pluronic F-127 for 1 h at 37  $^{\circ}\text{C}$ . Then, cells were harvested by the Trypsin–EDTA and washed twice with HBSS and incubated with HBSS for 30 min at 37  $^{\circ}\text{C}$ . The intracellular calcium level was analyzed immediately by FACScan flow cytometry (BD FACSAria II; BD Co; America).

#### Immunofluorescence staining for p65 localization

The effect of SZC017 on the nuclear translocation of p65 was examined by means of immunofluorescence staining. Cells were cultured in chamber slides and then treated with different concentrations of SZC017 for 24 h. After then, cells were washed in phosphate-buffered saline (PBS) and fixed for 20 min at room temperature with 4 % paraformaldehyde. The samples were permeabilized with 0.4 % TritonX-100 for 10 min, and washed in PBS for three times. And then blocked with 2 % bovine serum albumin (BSA) in PBS for 1 h at 37  $^{\circ}\text{C}$ . Antibody against

p65 in the 1 % blocking solution was added into the samples and incubated for overnight at 4 °C. Following three 5-min washes with PBS, fluorescein-conjugated secondary antibody were added in 1 % blocking solutions and incubated for 1 h. At last, the stained samples were mounted with DAPI (1 µg/ml for 10 min) to stain cell nuclei. After three additional 10-min washes, the samples were examined and analyzed with a fluorescence microscope (Labophot 2; Nikon, Tokyo, Japan).

### Transmission electron microscopy

MCF-7 cells were seeded in 6-well plates, and treated with SZC017 for 24 h. Cells were harvested, collected and prefixed in 2.5 % glutaraldehyde overnight at 4 °C. The samples were then postfixed, dehydrated, embedded, sectioned, and stained as previously described [9]. Finally, the electron micrographs were captured using a Transmission Electron Microscope (JEM-2000EX; JEOL Co; Japan).

### Western blot analysis

The whole-cell lysates were prepared in an ice-cold lysis buffer containing 150 mM NaCl, 20 mM Tris–Cl (pH 7.5), 1 % Triton X-100, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM NaF, 1 % aprotinin, and 10 µg/ml leupeptin. Cytoplasmic and nuclear extracts were prepared using Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions. The protein extracts were separated by 12 % SDS–polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membrane. After being blotted with TBST buffer containing 5 % nonfat dry milk, the PVDF membrane was incubated with specific primary antibody (1:1000) diluted in TBST buffer at 4 °C. The next day, membranes were probed with secondary antibody (1:1000) diluted in TBST for 1 h at room temperature. Protein bands were visualized using the enhanced Chemiluminescence reagent with LabWorks software (UVP, Upland, CA).

### Statistical analysis

Data are presented as mean ± standard deviation (SD) of three replicates. One-way ANOVA test and Tukey's multiple comparison test were used to compare the statistical differences between test and treatment groups. SPSS 17.0 software was used to analyze all the data. *p* values <0.05 were regarded as significant.

## Results

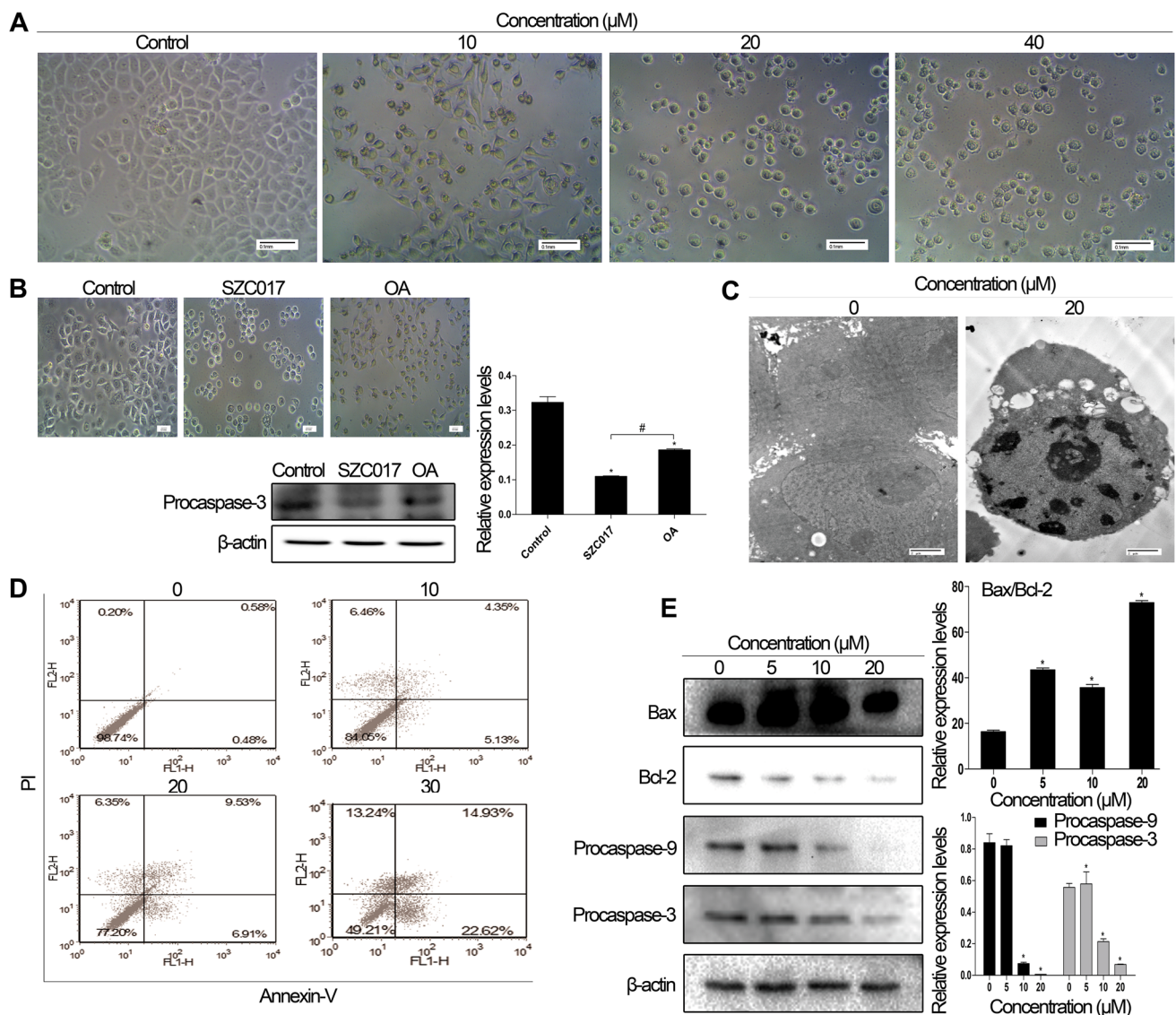
### SZC017 inhibits cell viability in breast cancer cells

To determine whether SZC017 is a potential selective anticancer agent, MCF10A mammary epithelial cell line, MCF-7 and MDA-MB-231 breast cancer cell lines were treated with different concentrations of SZC017 for 24 h and cell viability was measured by MTT assay. As shown in Fig. 1c, SZC017 significantly decreased the cell viability of both MCF-7 and MDA-MB-231 breast cancer cell lines, but showed less toxic to MCF10A mammary epithelial cell line, which suggest that SZC017 was a selective anticancer agent against breast cancer cells. Moreover, SZC017 treatment could cause a more significant reduction in cell viability in breast cancer cells, as compared with OA (Fig. 1d). Furthermore, we found that MCF-7 cell line was more sensitive than MDA-MB-231 cell line in response to SZC017 treatment. Although SZC017 was able to decrease the cell viability of both breast cancer cell lines, the half maximal inhibitory concentration value (IC<sub>50</sub>) after 24 h of SZC017 treatment was 26.47 µM for MCF-7 cells and 28.09 µM for MDA-MB-231 cells, respectively. Even under the same concentration, SZC017 caused a more obvious reduction in cell viability of MCF-7 cells than of MDA-MB-231 cells with the increase of incubation time (Fig. 1e). These data suggest that SZC017 is a potential selective anticancer agent against breast cancer cells, especially MCF-7 cells.

### SZC017 induces apoptosis in MCF-7 cells

To determine whether the inhibitory effect of MCF-7 cells on cell viability by SZC017 is due to the induction of apoptosis, we next performed some experiments as following. As shown in Fig. 2a, SZC017 treatment caused typical morphological changes of apoptosis such as plasma membrane blebbing, cell shrinkage and fragmentation of MCF-7 cells. A previous publication has pointed out that OA possesses the pro-apoptotic capacity against MCF-7 cells [10]. But compared with OA, the induction of apoptosis by SZC017 was more obvious in MCF-7 cells. As shown in Fig. 2b, MCF-7 cells treated with SZC017 became rounded, whereas cells treated with OA still maintained the irregular appearance. Moreover, although both SZC017 and OA were able to decrease the expression of procaspase-3, SZC017 treatment could almost completely inhibit the expression of procaspase-3 in MCF-7 cells. The ultrastructural changes were detected by TEM [11], subsequently showing that the morphological changes accompanying apoptosis were initiated by SZC017 treatment including chromatin condensation and nuclear





**Fig. 2** Apoptosis induction effect of SZC017 in MCF-7 cells. **a** Inverted contrast phase microscopy showed morphology changes of MCF-7 cells treated with SZC017 (10, 20, and 40  $\mu\text{M}$ ) for 24 h. **b** The effect of OA and SZC017 on apoptosis in MCF-7 cells. Differences of morphology changes of MCF-7 cells were observed after treatment with OA and SZC017 for 24 h. After treatment with OA (20  $\mu\text{M}$ ) and SZC017 (20  $\mu\text{M}$ ) for 24 h, Western blot analysis of procaspase-3 was evaluated of MCF-7 cells. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control,  $p < 0.05$  and #significantly different from SZC017 group,  $p < 0.05$ . **c** Transmission electron microscopy revealed the

fragmentation in MCF-7 cells. To confirm these findings associated with apoptosis, the percentage of cells undergoing apoptosis was measured by flow cytometry. We found that SZC017 induced apoptosis in MCF-7 cells in a concentration-dependent manner. For instance, after treatment with different concentrations of SZC017, the percentage of total apoptotic cells was increased from a baseline value of 1.06 % for control to 37.55 % for 20  $\mu\text{M}$

occurrence of apoptosis in MCF-7 cells after treatment with 20  $\mu\text{M}$  SZC017 for 24 h. **d** Flow cytometry analysis exhibited the apoptosis induction effect of SZC017 in MCF-7 cells. After treatment with SZC017 at concentrations of 10, 20, and 40  $\mu\text{M}$  for 24 h, samples were then stained with Annexin-V FITC and PI and analyzed by flow cytometry. **e** Western blot analysis was performed to determine apoptosis pathway induced by SZC017 in MCF-7 cells. Cells were treated with different concentrations of SZC017 (5, 10, and 20  $\mu\text{M}$ ) for 24 h. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control,  $p < 0.05$

group (Fig. 2d). Since procaspase-9 and procaspase-3 are essential for the intrinsic apoptotic pathway [11], we then evaluated the level of them. As shown in Fig. 2e, SZC017 significantly inhibited the expression of both procaspase-9 and procaspase-3. It was also discovered that SZC017 increased the ratio of Bax/Bcl-2 that is critical in determining the susceptibility to apoptosis via regulating mitochondrial function [12]. Taken together, our data indicate

that the inhibition of cell viability of MCF-7 cells by SZC017 is due to its capacity of induction of intrinsic apoptosis.

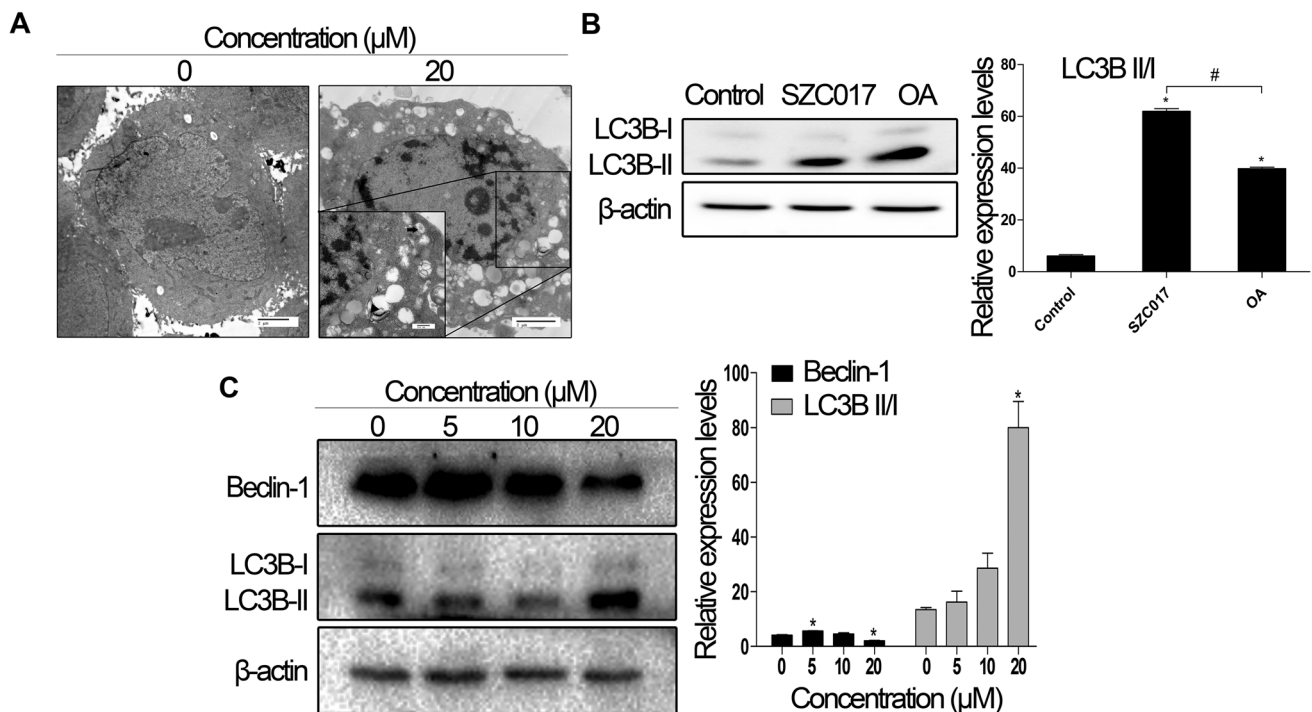
### SZC017 induces autophagy in MCF-7 cells

Autophagy can be detected through many ways such as the TEM, which is the most convincing and standard method to verify autophagy, and the level determination of LC3 and Beclin 1, which could be confirmed by Western blot assay [13]. As shown in Fig. 3a, many small vesicles (arrows) and huge vacuoles (arrowheads) were presented in cytoplasm, especially inside these compartments were cellular organelles or cytosol that was confirmed by ultrastructural morphology observations of SZC017-treated MCF-7 cells by the higher magnification. The TEM result revealed the occurrence of autophagy that these typical morphological changes clearly reflect the classical autophagy characteristics [14–16]. A previous study has demonstrated that OA can initiate autophagy in cancer cells [17]. As compared with OA, the induction of autophagy was more obvious in MCF-7 cells by SZC017. As shown in Fig. 3b, both SZC017 and OA increased the expression of LC3-II, but

showed difference in the conversion of LC3-I to LC3-II which is a hallmark of autophagy [13]. After calculation, we found that the ratio of LC3-II/I of SZC017 treatment was higher than that of OA. Furthermore, our data showed that SZC017 induced autophagy in MCF-7 cells in a concentration-dependent manner. SZC017 increased the ratio of LC3-II/I in MCF-7 cells with the increase of concentration (Fig. 3c). The data showed that 5  $\mu$ M SZC017 could increase the expression of Beclin 1, whereas both 10 and 20  $\mu$ M SZC017 could decrease the expression of Beclin 1 in MCF-7 cells.

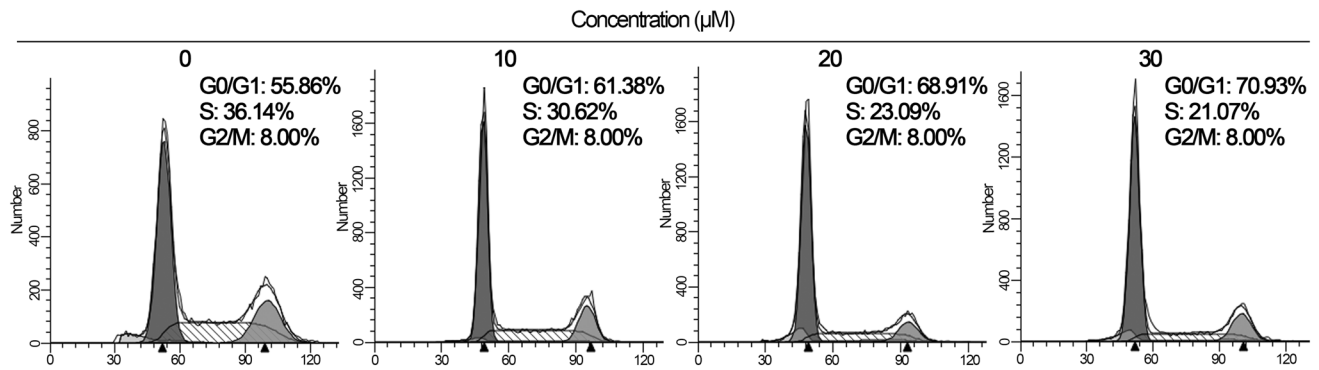
### SZC017 induces G<sub>0</sub>/G<sub>1</sub> cell cycle arrest

To determine whether cell cycle arrest is induced by SZC017, which is considered an important mechanism in inhibiting cell viability of cancer cells [18], we then performed PI staining to measure cell cycle distribution. As shown in Fig. 4, SZC017 could induce an obvious accumulation of cells in G<sub>1</sub> phase in a concentration-dependent manner and a reduction of cell numbers in S phase. In the control group, 55.86 % of total cells were distributed in G<sub>0</sub>/G<sub>1</sub> phase, while 36.14 % were in S phase. However, the



**Fig. 3** Autophagy induction effect of SZC017 in MCF-7 cells. **a** Transmission electron microscopy showed the occurrence of autophagy that was induced by SZC017 in MCF-7 cells after treatment with 20  $\mu$ M SZC017 for 24 h. **b** The induction effect of OA and SZC017 on autophagy in MCF-7 cells. Western blot analysis of LC3 was evaluated after treatment with SZC017 (20  $\mu$ M) for 24 h. The values are expressed as mean  $\pm$  SD of three replicates.

\*Significantly different from control, \* $p < 0.05$  and # significantly different from SZC017 group, # $p < 0.05$ . Western blot analysis was also performed to determine the expression of autophagy-related proteins (Beclin 1 and LC3) after 20  $\mu$ M SZC017 treatment for 24 h in MCF-7 cells. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control, \* $p < 0.05$



**Fig. 4** Analysis of the cell cycle distribution of SZC017-treated MCF-7 cells. Cells were treated with different concentrations of SZC017 (10, 20, and 30 μM) for 24 h, and cell cycle distribution was then analyzed by flow cytometry

number of cells in G<sub>0</sub>/G<sub>1</sub> phase was increased to 70.93 %, while cells in S phase was decreased to 21.07 %, respectively. These data indicate that the inhibition of cell viability by SZC017 is associated with the cell cycle arrest at G<sub>1</sub> phase.

#### The Akt is the target of SZC017 in MCF-7 cells

Akt is a major anti-apoptotic signaling pathway, and previous studies have demonstrated that chemotherapeutic agents induce cancer cell apoptosis via inhibiting the Akt pathway [18–21]. As we expected, SZC017 could significantly suppress the expression of both Akt and p-Akt which is an active form of the Akt in a concentration-dependent manner (Fig. 5a). These data suggest that Akt pathway might be an effective target for SZC017 in MCF-7 cells.

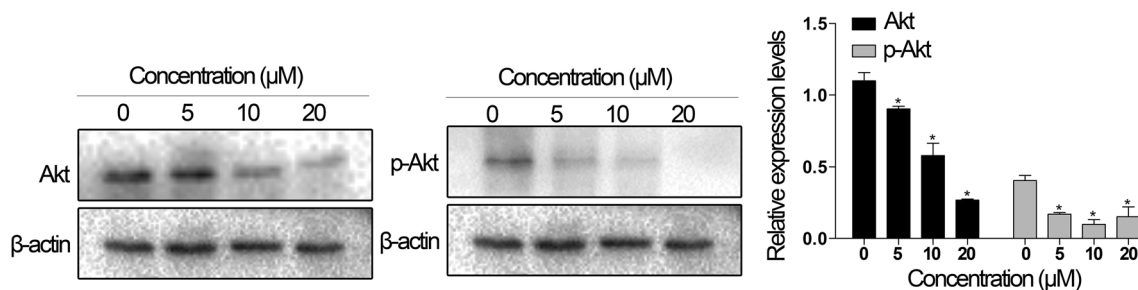
#### The NF-κB signaling pathway is the target of SZC017 in MCF-7 cells

NF-κB signaling is an important downstream pathway of Akt signaling and also involved in regulating cell survival and apoptosis [22]. Therefore, we focused our attention on assessing the level of several NF-κB pathway-related

proteins namely p-IκBα, total p65, total p-p65, and p-p65 in both cytoplasm and nucleus. The results showed that the NF-κB pathway was obviously suppressed by SZC017 in MCF-7 cells. As shown in Fig. 6a, SZC017 significantly suppressed the expression of p-IκBα, total p65, and total p-p65. Moreover, both p-p65 which is a critical molecule in modulating NF-κB p65 nuclear translocation and improving its DNA binding function [23, 24] in cytoplasm and nucleus were also suppressed by SZC017. To determine whether p65 nuclear translocation could be inhibited by SZC017, immunofluorescence analysis was performed. The data indicated that p65 nuclear translocation was inhibited by SZC017 with the increase of concentration (Fig. 6c). Treatment with 5 μM SZC017 exhibited little suppression on p65 nuclear translocation, but 20 μM SZC017 obviously inhibited p65 nuclear translocation. These results indicate that the NF-κB pathway is effectively suppressed by SZC017 in MCF-7 cells.

#### SZC017 inhibits cell viability and apoptosis in MCF-7 cells via the induction of autophagy

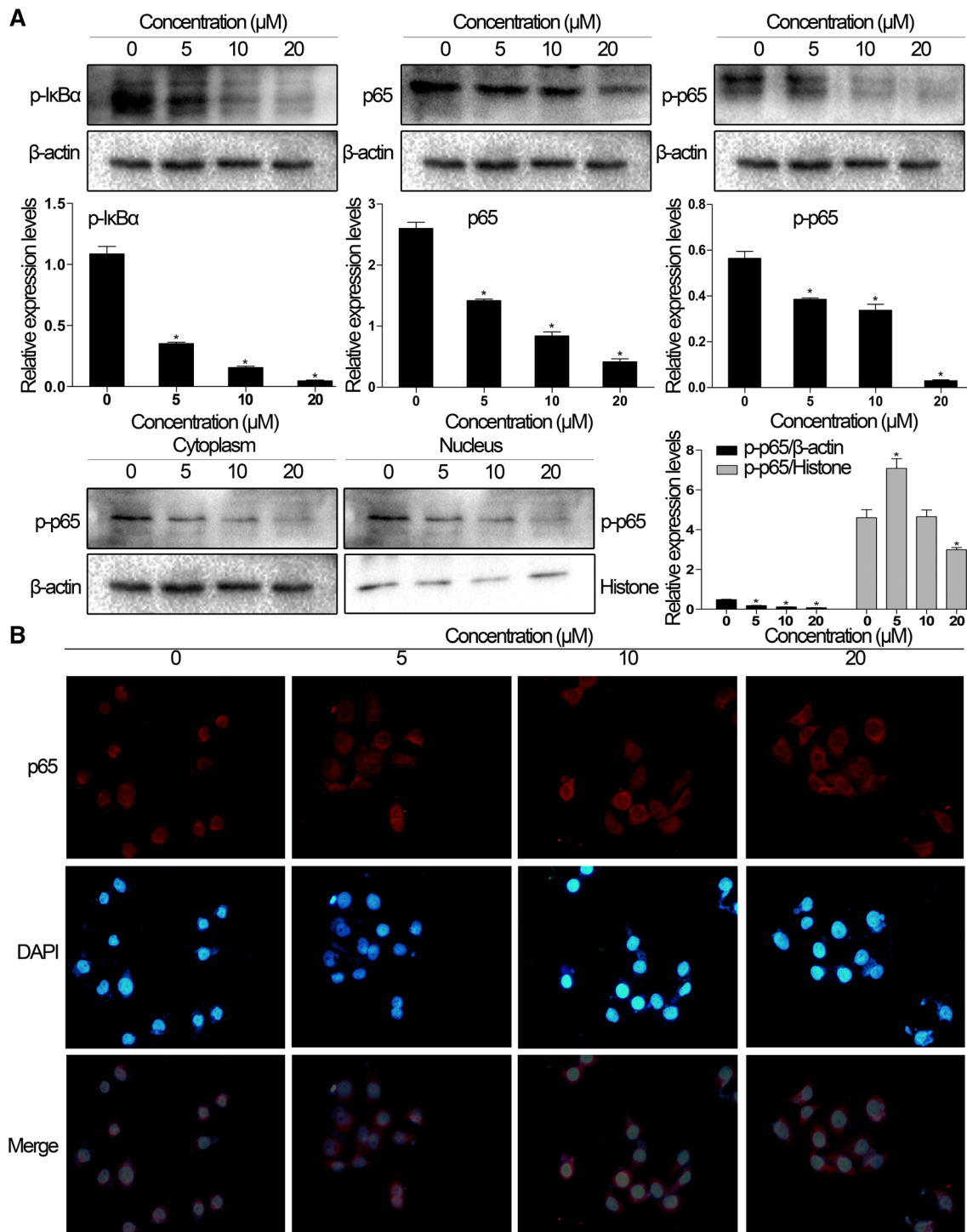
To determine whether the autophagy induced by SZC017 is associated with the inhibition of cell viability and the



**Fig. 5** Western blot analysis of Akt and p-Akt in response to SZC017 treatment in MCF-7 cells. Treatment (24 h) of MCF-7 cells with SZC017 (5, 10, and 20 μM) resulted in suppression of both Akt and

p-Akt. The values are expressed as mean ± SD of three replicates. \*Significantly different from control, \*p < 0.05





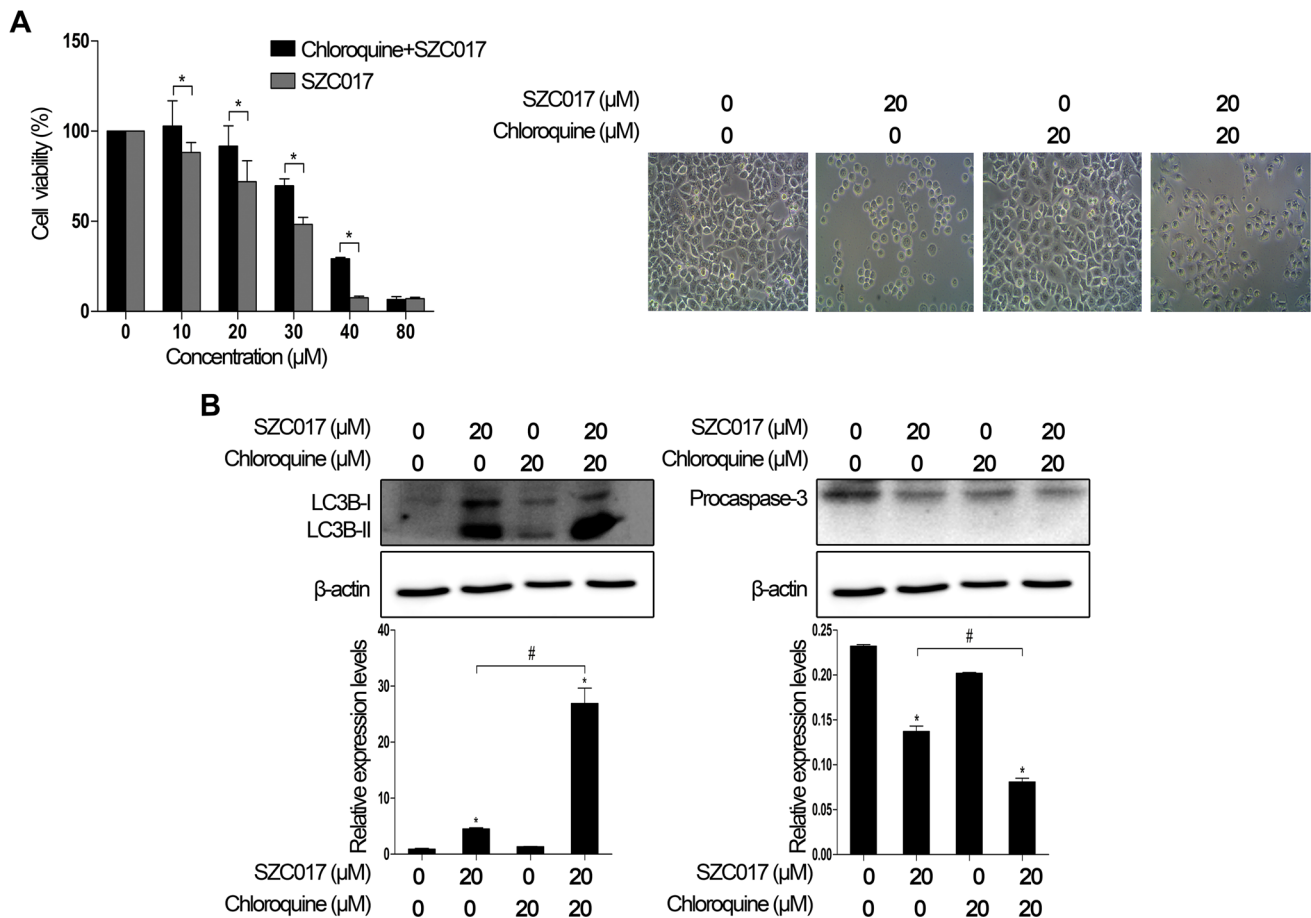
**Fig. 6** Inhibition effect of SZC017 on the NF- $\kappa$ B pathway. **a** Western blot analysis of several NF- $\kappa$ B pathway related proteins, including p-I $\kappa$ B $\alpha$ , total p65, total p-p65, and p-p65 in both cytoplasm and nucleus. Treatment (24 h) of MCF-7 cells with SZC017 (5, 10, and 20  $\mu$ M) resulted in the expression change of these proteins. The

values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control, \* $p < 0.05$ . **b** Immunofluorescence staining analysis of p65 localization. MCF-7 cells treated with SZC017 (5, 10, and 20  $\mu$ M) for 24 h were labeled for p65 (red), and nuclei were stained with DAPI (blue) (Color figure online)

induction of apoptosis, the CQ, an autophagy inhibitor, was utilized owing to the inhibition of autophagy by it at a later stage [13]. Interestingly, pretreatment with CQ increased

the cell viability of MCF-7 cells treated with SZC017. Except for the control and 80  $\mu$ M groups, CQ caused a significant induction in the cell viability of MCF-7 cells





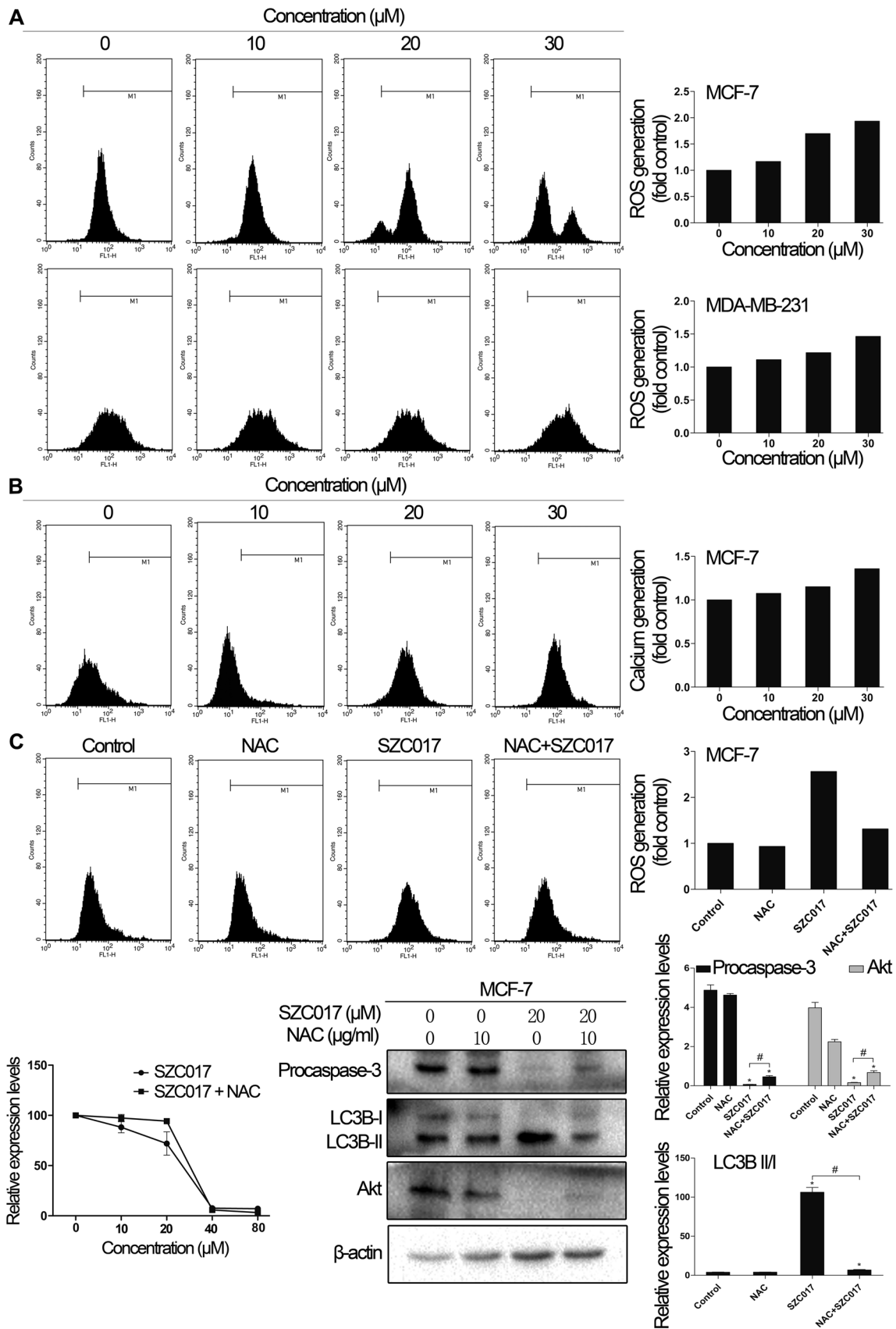
**Fig. 7** Effect of the autophagy on the apoptosis in SZC017-treated MCF-7 cells. **a** Pretreatment with CQ prevented cells from SZC017-induced inhibition of cell viability. MCF-7 cells were pretreated with CQ (20 μM) for 3 h and then treated with various concentrations of SZC017 (10, 20, 30, 40, and 80 μM) for 24 h. The cell viability was measured by MTT assay, and the morphology observations were recorded by inverted phase contrast microscopy. **b** Western blot

analysis of the expression of LC3 and procaspase-3. MCF-7 cells were pretreated with CQ (20 μM) for 3 h and then 20 μM SZC017 was incubated with cells for further 24 h. The values are expressed as mean ± SD of three replicates. \*Significantly different from control, \* $p < 0.05$  and #significantly different from SZC017 only group, # $p < 0.05$

(Fig. 7a). The pictures taken by inverted phase contrast microscopy confirmed the result of MTT assay above (Fig. 7a). Cells only treated with 20 μM SZC017 became fragmented and rounded, but cells pretreated with CQ had irregular appearance and were still alive. As shown in Fig. 7b, since pretreatment with CQ significantly increased the ratio of LC3II/I suggesting that CQ was able to effectively inhibit the autophagy induced by SZC017. To determine the relationship between the autophagy and apoptosis, we then evaluated the expression of procaspase-3 after pretreatment with CQ. Figure 7b clearly revealed that pretreatment with CQ dramatically suppressed the level of procaspase-3, as compared with only SZC017 group. These data suggest that the autophagy induced by SZC017 is a promoter of cell death and an inhibitor of the apoptosis induced by SZC017 in MCF-7 cells.

### Excessive ROS generation is required for SZC017-induced apoptosis, autophagy, and Akt inhibition

Mitochondrial dysfunction results in an increase in intracellular ROS generation which in turn will induce cancer cell apoptosis via mitochondrial stress signaling pathway [25]. Our result showed that SZC017 increased the intracellular ROS generation in both breast cancer cell lines in a concentration dependent manner, especially in MCF-7 cell line (Fig. 8a). Besides, the intracellular calcium is an important molecule in inducing apoptosis, therefore, we used the calcium-sensitive dye fluo-3/AM to assess whether the exposure of SZC017 could increase the calcium accumulation in MCF-7 cells. Our result showed that the fluo-3/AM fluorescence intensity was increased in response to SZC017 treatment in a concentration-dependent manner (Fig. 8b). To determine the role of ROS in SZC017-treated



**Fig. 8** Effect of SZC017 on intracellular ROS generation. **a** SZC017 increased the intracellular ROS generation in both MCF-7 and MDA-MB-231 cells. Cells were treated with SZC017 at concentrations of 10, 20, and 30  $\mu$ M for 24 h, and then the level of intracellular ROS was detected by flow cytometry. **b** SZC017 increased the intracellular calcium generation in MCF-7 cells. MCF-7 cells were treated with SZC017 at concentrations of 10, 20, and 30  $\mu$ M for 24 h, and then the level of intracellular calcium was detected by flow cytometry. **c** Effect of excessive ROS generation in SZC017-treated MCF-7 cells. Cells were pretreated with 10  $\mu$ g/ml NAC for 1 h and then treated with 20  $\mu$ M SZC017 for further 24 h. Pretreatment with NAC (10  $\mu$ g/ml) for 1 h increased the cell viability of SZC017-treated MCF-7 cells. Western blot analysis of procaspase-3, LC3, and Akt after pretreatment with 10  $\mu$ g/ml NAC for 1 h. The values are expressed as mean  $\pm$  SD of three replicates. \*Significantly different from control,  $^{\#}p < 0.05$  and  $^{\#}p < 0.05$

MCF-7 cells, NAC, an antioxidant ROS scavenger, was utilized to clean up the ROS induced by SZC017. As we expected, pretreatment with NAC prevented the intracellular ROS generation, as compared with only SZC017 group (Fig. 8c). Moreover, except the control, 40 and 80  $\mu$ M groups, pretreatment with NAC could effectively increase the cell viability which was inhibited by SZC017 (Fig. 8c). Considering some reports that ROS is an important molecule in regulating apoptosis, autophagy, and the Akt pathway [13, 26, 27]. Thus, we next investigated into the effect of NAC on the changes of associated proteins with apoptosis, autophagy, and the Akt pathway. The results showed that pretreatment with NAC significantly increased the level of procaspase-3 and the Akt in SZC017-treated MCF-7 cells, but decreased the ratio of LC3-II/I (Fig. 8c). In a word, our data suggest that excessive ROS is a critical upstream factor in regulating apoptosis, autophagy, and inhibition the Akt pathway.

## Discussion

The present study demonstrated that SZC017, a novel derivative of OA, selectively killed breast cancer cells and showed more obvious pharmacological effects, including cell viability inhibition and the induction of apoptosis and autophagy, as compared with OA. Moreover, our data indicated that SZC017 caused excessive intracellular ROS generation, leading to Akt/NF- $\kappa$ B signaling suppression and subsequent induction of apoptosis and autophagy in MCF-7 cells. Furthermore, we proved that SZC017-induced autophagy promoted cell viability rate and inhibited apoptosis induced by SZC017.

Apoptosis is a tightly regulated process that maintains the balance of cell numbers in multicellular organisms in an orderly, non-inflammatory way, while the disruption of this balance commonly exists in human cancers [13, 28]. A

previous study showed that OA exhibited an inhibitory effect on hepatocellular carcinoma by inducing apoptosis through the mitochondrial pathway [29]. Our findings also showed that SZC017 induced apoptosis in a concentration-dependent manner (Fig. 2d) and exhibited a more obvious induction of apoptosis, as compared with OA in MCF-7 cells (Fig. 2b). Apoptosis is mediated through two main pathways, namely the intrinsic pathway (characterized by mitochondrial stress and involving Bcl-2 family proteins, caspase-9, and effector caspase-3) and the extrinsic pathway (characterized by ligand-based activation of cell-surface death receptors following the activation of certain caspases). Recent studies have shown that derivatives of OA induce apoptosis in different cancer cell types mainly by the intrinsic apoptosis pathway [30–33]. Therefore, we focused on evaluating the apoptotic mechanism of several intrinsic apoptosis-related molecules. As we expected, Bcl-2, an apoptosis inhibitor, was significantly suppressed and the ratio of Bax/Bcl-2 was increased (Fig. 2e), indicating that the mitochondria was severely damaged by SZC017. As the hallmarks of intrinsic apoptosis, the activity of procaspase-9 and procaspase-3 was subsequently suppressed following the induction of mitochondrial damage by many stimulants [12, 34]. ROS can be generated in various organelles such as the mitochondria (considered the main source of intracellular oxidant production), the endoplasmic reticulum, and peroxisomes [35]. A higher level of ROS was generated by SZC017 in MCF-7 cells than in MDA-MB-231 cells (Fig. 8a), and our results confirmed that ROS was also a critical regulator of apoptosis induced by SZC017 (Fig. 8c), suggesting that ROS might be critical in regulating apoptosis in breast cancer cells. Excessive ROS generation leads to mitochondrial permeability transition pore (MPTP) formation and mitochondrial depolarization, which is dependent on a high level of intracellular calcium, and thus causes apoptotic cell death involving the inhibition of procaspase-3 in cancer cells [36].

Akt/NF- $\kappa$ B signaling is an important pathway that modulates multiple biological activities including cell survival, differentiation, and apoptosis induction [37, 38]. Hyperactivation of the Akt/NF- $\kappa$ B signaling pathway, which is considered to be an effective therapeutic target for cancers, has already been reported in a variety of human cancers such as breast cancer, non-small cell lung cancer, and hematological malignancies [20, 39, 40]. Consistent with previous reports, we also observed that the activity of both Akt and p-Akt was significantly suppressed by SZC017 (Fig. 5), suggesting that because Akt is an effective target for SZC017, its inhibition may result in cancer cell death. In recent publications, several plant-derived compounds were considered to be highly effective in suppressing the Akt pathway as a result of killing cancer

cells in vitro via ROS-dependent mechanisms [41–43]. Our results show that the excessive ROS generation induced by SZC017 was effectively blocked by NAC (Fig. 8c). Subsequently, we also discovered that the level of Akt was dramatically increased after blocking ROS generation (Fig. 8c) and that a decrease in ROS could rescue the survival rate of cancer cells. These data indicate that ROS is an upstream molecule in the inhibition of Akt activity, and thus contributes to cell death in SZC017-treated MCF-7 cells.

NF- $\kappa$ B is considered to be a very important signaling complex that interacts with the Akt pathway, and targeting Akt/NF- $\kappa$ B signaling is now an attractively effective strategy for cancer therapy [44]. Alternatively, suppression of the NF- $\kappa$ B pathway will trigger apoptosis [45]. It also appears that the NF- $\kappa$ B pathway has a dual role in regulating autophagy [46]. NF- $\kappa$ B transcription complexes can be any of a variety of homo- and heterodimers formed by the subunits c-Rel, p65, RelB, p50/p105, and p52/p100, with the most common complex being the heterodimer p50/p65. Under unstimulated conditions, NF- $\kappa$ B dimers are located in the cytoplasm in an inactive form because of their association with I $\kappa$ B $\alpha$ , an inhibitor protein. In response to stimulation, I $\kappa$ B kinase (IKK) will be activated, leading to I $\kappa$ B $\alpha$  phosphorylation, ubiquitination, and degradation by the 26S proteasome. The released NF- $\kappa$ B dimer is further activated by posttranslational modifications and is then translocated to the nucleus where it binds to  $\kappa$ B DNA elements and modulates gene expression [47]. There are two I $\kappa$ B $\alpha$ -related ways to suppress NF- $\kappa$ B signaling. Bortezomib, an inhibitor of proteasomes, induces cell apoptosis via the suppression of the NF- $\kappa$ B pathway by suppressing I $\kappa$ B $\alpha$  degradation. On the other hand, 3,5-diethyl-1,3,5-thiadiazinane-2-thione (DETT) suppresses I $\kappa$ B $\alpha$  phosphorylation, preventing I $\kappa$ B $\alpha$  from being degraded by proteasomes [48, 49]. As observed in the current study, the level of p-I $\kappa$ B $\alpha$  was significantly suppressed by SZC017 (Fig. 6a); therefore, we propose that the I $\kappa$ B $\alpha$  phosphorylation inhibition pathway is responsible for the NF- $\kappa$ B signaling suppression in SZC017-treated MCF-7 cells. Phosphorylation of p65 at residue Ser536 functions to facilitate p65 nuclear translocation and its DNA binding function, release p65 from HDAC1 and HDAC3, and recruit p300 to the p65 complex which in turn modulates downstream gene expression [23, 24]. A recent publication has demonstrated that p65 phosphorylation-dependent NF- $\kappa$ B activation plays an important role in cancer cell survival [48]. In our observations, we discovered that the level of total p65 and p-p65 were suppressed (Fig. 6b), suggesting that both p65 and p-p65 might be involved in NF- $\kappa$ B pathway suppression. Thus, we began to focus on the role of p-p65 in SZC017-treated MCF-7 cells. Similar to bortezomib and DETT, SZC017 was able

to suppress the level of p-p65 in both the cytoplasm and nucleus (Fig. 6b), indicating that the nuclear translocation inhibition of p-p65 might be involved in NF- $\kappa$ B p65 suppression. Furthermore, nuclear translocation of NF- $\kappa$ B p65 was confirmed by immunofluorescence staining (Fig. 6c). Taken together, these data suggest that the Akt/NF- $\kappa$ B signaling pathway is a very effective target for SZC017 in MCF-7 cells.

Many natural products show anticancer activity in several different cancer types, mainly via autophagy-dependent mechanisms [50–52]. Autophagy is a highly conserved process with a self-protective function under particular conditions, while excessive autophagy may induce cell death and thus exhibit anticancer activity in eukaryotic cells [27]. Recent publications have demonstrated that OA treatment permits both normal and cancer cells to undergo autophagy, illustrating its anticancer functions [17, 53]. In order to compare the autophagy induction activity between OA and SZC017, we evaluated the level of LC3B expression, a hallmark of autophagy, by Western blot [13]. Our findings suggested that SZC017 showed a more obvious autophagy induction activity than OA in MCF-7 cells. Autophagy serves a dual function in regulating the fate of cancer cells; it functions as a pro-survival mechanism in response to stimuli such as nutrient deprivation, hypoxia, and metabolic-induced stress, whereas excessive autophagy may function as an inducer of non-apoptotic cell death [27]. Consistent with previous studies [17, 53], excessive autophagy induced by SZC017 was also a promoter of cell death, as evidenced by the significant increase in cell viability via the blocking of autophagy by CQ, an autophagy inhibitor [13]. Despite the complex relationship between autophagy and apoptosis [26], our observations supported that apoptosis triggered by SZC017 was significantly enhanced after blocking autophagy (Fig. 7b), suggesting that autophagy induced by SZC017 serves as an inhibitor of apoptosis in MCF-7 cells. Although apoptotic cell death could be suppressed by autophagy, autophagy induced by SZC017 still served as a promoter of cell death, suggesting that autophagic cell death could be induced by SZC017 in MCF-7 cells.

ROS is considered an important upstream molecule in starvation-induced autophagy. Atg4 being inactivated by an oxidative signal will promote lipidation of Atg8, and thus promote autophagy [54]. In our study, autophagy induction activity was suppressed after ROS generation was blocked by NAC, indicating that ROS, consistent with a previous study, could be the upstream molecule of autophagy induction in SZC017-treated MCF-7 cells. Moreover, Beclin 1 plays a critical role in modulating autophagy and would be increased in response to many stimuli [55]. However, recent publications have reported that several natural products can trigger autophagic cell



death via various mechanisms through Beclin 1-dependent and Beclin 1-independent routes of autophagy [56]. Our results confirmed that excessive autophagy could be caused by SZC017 in MCF-7 cells, as evidenced by a significant increase in the LC3-II/I ratio and an accumulation of autophagosomes in SZC017-treated MCF-7 cells, while Beclin 1 expression decreased after treatment with a higher concentration of SZC017.

Mechanistically, autophagy can be induced by some stimuli via a Beclin 1 suppression-dependent mechanism. Inactivation of the NF- $\kappa$ B pathway is thought to make a significant contribution to Beclin 1 transcription suppression, which then induces autophagy [57]. The NF- $\kappa$ B pathway serves as a negative modulator of autophagy induced by ROS and starvation in many cell lines. After being stimulated, p65 will translocate to the nucleus and directly binds the Beclin 1 promoter, thereby upregulating its protein levels [58, 59]. Taken together, we propose that SZC017 induces autophagy in MCF-7 cells by an NF- $\kappa$ B-dependent Beclin 1 suppression mechanism.

Therefore, in the present study, we demonstrated that SZC017, a novel OA derivative, is a potential selective anticancer agent against breast cancer cells *in vitro*. Our findings indicated that SZC017-induced excessive ROS is the upstream inhibitory molecule of Akt/NF- $\kappa$ B signaling and thereby triggers apoptosis and autophagy, which is not only a promoter of cell survival, but also an inhibitor of apoptosis in MCF-7 cells. However, the effect of SZC017 in other breast cancer cell lines and *in vivo*, as well as its role in other underlying mechanisms, remains to be further studied.

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#### Compliance with ethical standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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