Cancer Letters 448 (2019) 94–104

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Dual inhibition of Akt and ERK signaling induces cell senescence in triple-negative breast cancer

Qiang He[a], Shuyin Xue[d], Yiqing Tan[a], Ling Zhang[b], Qing Shao[c], Lei Xing[b], Yunhai Li[a], Tingxiu Xiang[a], Xinrong Luo[b,**], Guosheng Ren[a,b,*]

[a] Chongqing Key Laboratory of Molecular Oncology and Epigenetics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[b] Department of Endocrine and Breast Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[c] Breast Center, Chongqing University Cancer Hospital & Chongqing Cancer Institute & Chongqing Cancer Hospital, Chongqing, China
[d] Rytime Dental, Chongqing, China

ARTICLE INFO

Keywords:
Akt
ERK
Cell senescence
Triple-negative breast cancer
Norcantharidin

ABSTRACT

Activated Akt and ERK signaling pathways are closely related to breast cancer progression, and Akt or ERK inhibition induces cell senescence. However, the crosstalk between the Akt and ERK signaling pathways in cell senescence and how to simultaneously suppress Akt and ERK signaling in triple-negative breast cancer (TNBC) are undefined. In this study, we found that norcantharidin (NCTD) effectively induced cell senescence and cell cycle arrest in TNBC *in vitro*, which was accompanied by a decline in phosphorylated Akt and ERK1/2 and a rise in p21 and p16. The inhibitors LY294002 and U0126 imitated the effect of NCTD when these two inhibitors were combined regardless of crosstalk between these two signaling pathways. In addition, NCTD inhibited the growth of xenografts via downregulation of phosphorylated Akt and ERK1/2 and upregulation of p21 *in vivo*. However, NCTD upregulated the level of soluble signaling factors of the senescence-associated secretory phenotype (SASP) in a NF-κB-independent manner. Collectively, these findings demonstrate that NCTD induced cell senescence and cell cycle arrest mainly by simultaneously blocking Akt and ERK signaling in TNBC, suggesting that NCTD may be used as a potential adjuvant therapy in TNBC.

1. Introduction

Breast cancer is the leading malignant tumor in women worldwide [1]. It has been demonstrated that there are multiple hallmarks in cancer cells, including sustained proliferation signaling, especially in triple-negative breast cancer (TNBC) [2]. TNBC, is one of the molecular types of breast cancer, and does not respond to endocrine therapy and anti-human epidermal growth factor receptor 2 (HER2) therapy, as it does not express the estrogen receptor (ER), progesterone receptor (PR) or HER2. As a result, TNBC has the highest risk of recurrence of all breast cancers; therefore, the identification of potential adjuvant therapy for TNBC is essential [3].

It is well known that Akt and ERK signaling are dysregulated or mutated in breast cancers, especially in TNBC [4–7]. Downregulation of phosphorylated Akt or ERK levels induce cell apoptosis, cell cycle arrest and cell senescence in several cancers, including breast cancer [8–13]. However, the crosstalk between these two signaling pathways may lead to attenuation of the effect of single inhibition [14]. This suggests that dual inhibition of Akt and ERK signaling may achieve greater induction of cellular senescence and tumor suppression than single inhibition [15].

Norcantharidin (NCTD), the demethylated analog of cantharidin, retains the antitumor features of cantharidin with less toxicity [16]. It was demonstrated that NCTD suppresses tumor cell proliferation, cell migration and invasion, and induces cell apoptosis and cell cycle arrest by inhibiting the Akt or ERK signaling pathways in several carcinomas [17–19]. Moreover, NCTD inhibits tumor growth and angiogenesis by abrogating the ERK signaling pathway, but has little effect on the phosphorylation of Akt [20]. Furthermore, NCTD induces cell senescence by repressing YAP signaling in non-small lung cancer cells [21]. However, there is little research concerning the effect of NCTD on TNBC. In addition, crosstalk between the Akt and ERK signaling pathwaysin cell senescence and how to simultaneously suppress Akt and ERK signaling are unclear in TNBC. In this study, we demonstrate

* Corresponding author. Chongqing Key Laboratory of Molecular Oncology and Epigenetics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China.
** Corresponding author.
E-mail addresses: xinrongluo@126.com (X. Luo), rengs726@126.com (G. Ren).

https://doi.org/10.1016/j.canlet.2019.02.004
Received 28 October 2018; Received in revised form 5 January 2019; Accepted 1 February 2019

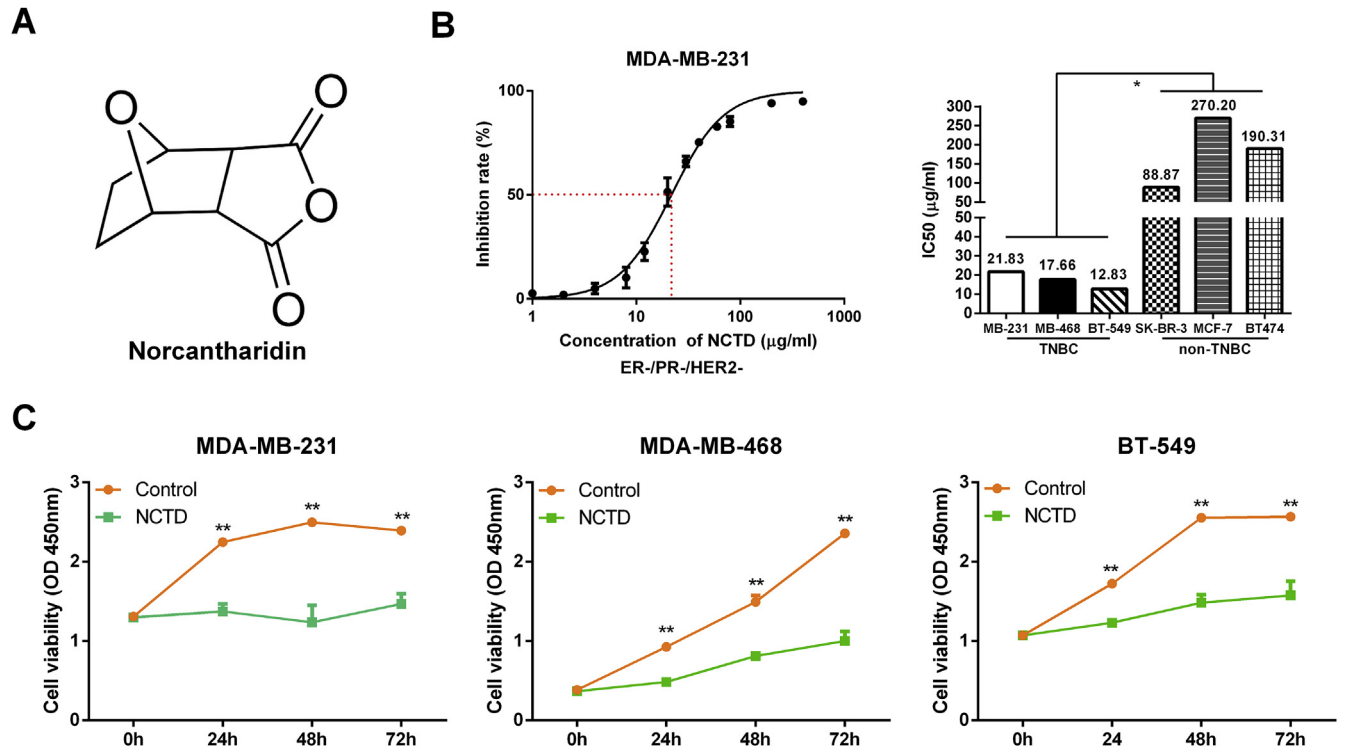


Fig. 1. ***Norcantharidin inhibits cell proliferation in TNBC cells.*** (A) The chemical structure of norcantharidin. (B) TNBC cells and non-TNBC cells were treated with NCTD at different concentrations for 24 h, and cell viability was detected using the CCK-8 assay. The IC50 in MDA-MB-231, MDA-MB-468, BT-549, SK-BR-3, MCF-7 and BT-474 cells, calculated with Graphpad Prism 7, was 21.83, 17.66, 12.83, 88.87, 270.2 and 190.31 μg/ml, respectively. (C) TNBC cells, MDA-MB-231, MDA-MB-468 and BT-549, were treated with NCTD at concentrations corresponding to the IC50 for 0 h, 24 h, 48 h or 72 h, and cell viability was measured using the CCK-8 assay. Mean ± SD, n = 3, $^{**}p < 0.01$.

that NCTD induces cell senescence, cell apoptosis and cell cycle arrest by simultaneously down-regulating the Akt and ERK signaling pathways *in vitro* and *in vivo*. However, NCTD upregulates the level of soluble signaling factors of the senescence-associated secretory phenotype (SASP) in a NF-κB-independent manner. These findings suggest that NCTD may be used as a potential adjuvant therapy for TNBC.

2. Materials and methods

2.1. Reagents and cell lines

NCTD was purchased from Sigma-Aldrich (St. Louis, MO, USA). The inhibitor of PI3K (LY294002) and inhibitor of MEK1/2 (U0126) were purchased from Cell Signaling Technology (#9901 and #9903; Danvers, MA, USA). Breast cancer cell lines, MDA-MB-231, MDA-MB-468, BT-549, SK-BR-3, MCF-7 and BT474 were cultured in RPMI 1640 medium (Gibco-BRL, Karlsruhe, Germany) with 10% fetal bovine serum (FBS) (Gibco-BRL) and 100 U/ml of penicillin and streptomycin (Gibco-BRL) at a constant temperature of 37 °C with 5% CO_2.

2.2. Cell viability assay

Cells were cultured in 96-well plates at a density of 5,000 cells per well. Different concentrations of NCTD were then added to replace the old medium. After 24 h, the Cell Counting Kit (CCK-8) (Beyotime Institute of Biotechnology, Jiangsu, China) was used to measure optical density (OD) values with a microplate reader (Infinite 200 PRO, TECAN, Männedorf, Switzerland) at the absorbance of 450 nm according to the manufacturer's instructions. The formula used to calculate the inhibition rate was as follows:

$$\text{Inhibition rate} = (1 - OD_{NCTD}/OD_{control}) \times 100\%$$

The IC50 values were calculated using Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.3. Cell proliferation assay

Cells were cultured at a density of 5,000 cells per well in 96-well plates. Then medium with the corresponding NCTD IC50 was added to each well to replace the old medium. At 24 h, 48 h and 72 h after adding NCTD, OD values were measured as described above. Data were obtained from three independent wells and experiments were repeated three times.

2.4. Flow cytometry analysis of the cell cycle and apoptosis

Cells were cultured at a density of 400,000 cells per well. Then medium with the corresponding NCTD IC50 was added to each well to replace the old medium. After 24 h and 48 h, cells were trypsinized with pancreatin, washed once with PBS, fixed with 75% ethanol (4 °C) and stained with propidium iodide (PI). The cell cycle distribution was analyzed with Cell Quest (BD Bio-sciences, San Jose, CA, USA). To assess apoptosis, cells were resuspended with 100 μl binding buffer after washing with PBS and incubated with Annexin V-FITC and PI (Sigma-Aldrich) at room temperature for 30 min. Cell apoptosis was then analyzed with Cell Quest (BD Bio-Sciences). Acridine orange/ethidium bromide (AO/EB) fluorescence staining was performed to measure early and late apoptosis. Cells treated with or without NCTD were washed with PBS and stained with AO/EB solution (100 μg/ml, 1:1 Solarbio) for 5 min at room temperature. The stained cells were then observed with a fluorescence microscope (Leica, Hilden, Germany). All experiments were repeated three times.

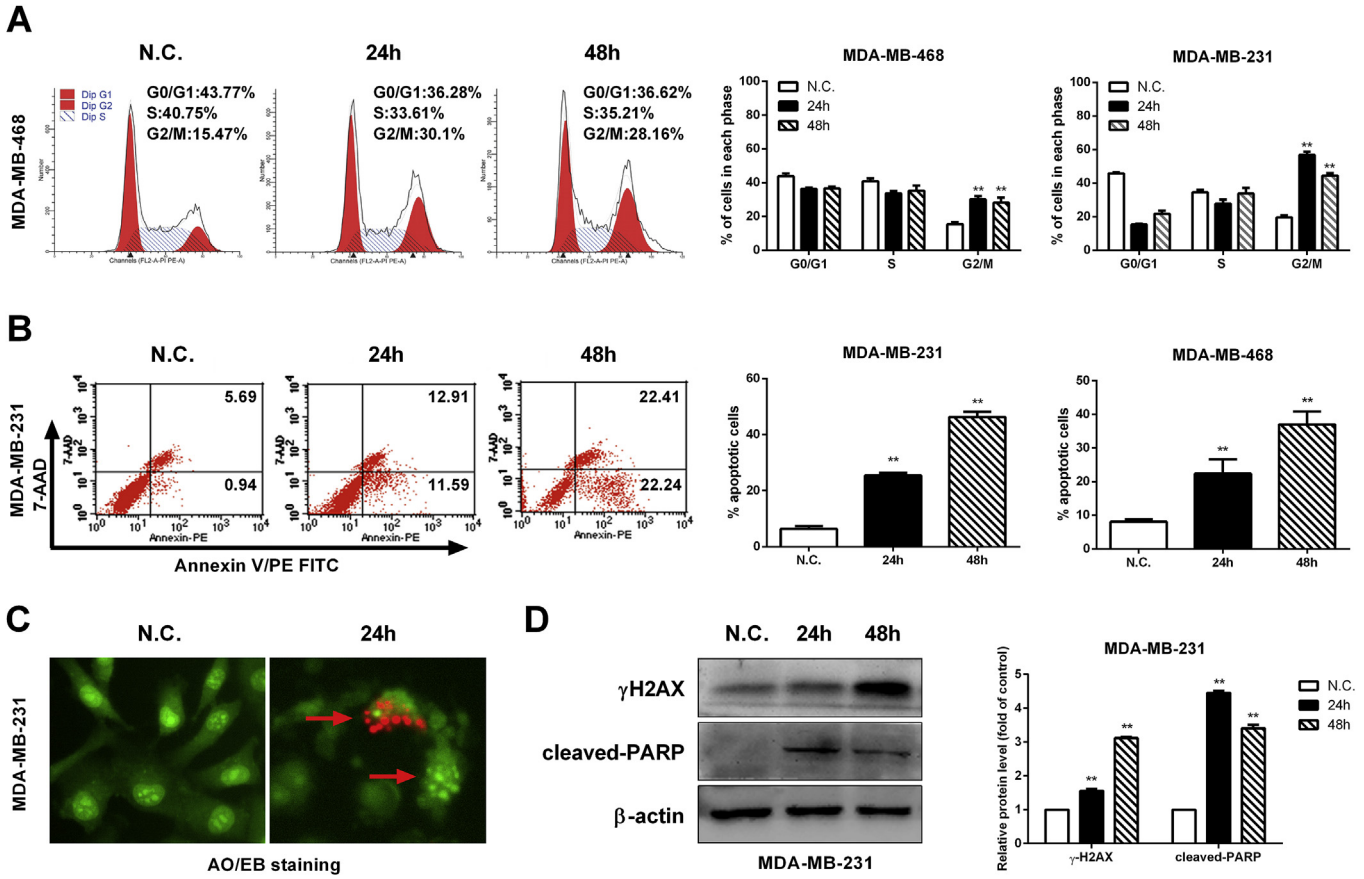


Fig. 2. ***NCTD induces cell cycle arrest and cell apoptosis in TNBC cells.*** (A) MDA-MB-468 and MDA-MB-231 cells were treated with NCTD at concentrations corresponding to the IC50 for 0 h, 24 h or 48 h, and the cell cycle was examined by flow cytometry analysis. The histogram shows the percentage of cells in different cell cycle phases. Mean ± SD, n = 3, $^{**}p < 0.01$. (B) Cell apoptosis was detected by flow cytometry analysis of annexin V/PE-FITC/7-AAD, and the histogram shows the percentage of apoptotic cells (annexin V/PE FITC positive cells) in the different groups. Mean ± SD, n = 3, $^{**}p < 0.01$. (C) NCTD induced early and late apoptosis in MDA-MB-231 cells detected by AO/EB staining. The arrows indicate apoptotic bodies. (D) The protein levels of γ-H2AX and cleaved-PARP after NCTD treatment for 24 h or 48 h were examined by immunoblotting with β-actin as a control. The histogram shows the grayscale values of the immunoblotting results. Mean ± SD, n = 3, **p < 0.01.

2.5. SA-β-gal staining assay

Cells were cultured at a density of 400,000 cells per well. Then medium with the corresponding NCTD IC50 was added to each well to replace the old medium. After 24 h and 48 h, senescence-associated β-galactosidase (SA-β-gal) within the cell was stained with the Senescence β-Galactosidase Staining Kit (Beyotime, C0602) following the manufacturer's instructions and the stained cells were observed using an optical microscope. All experiments were repeated three times.

2.6. Immunoblot

As described previously, total proteins were extracted using protein extraction buffer (4% sodium dodecyl sulfate-polyacrylamide, 20% glycerol, 120 mM Tris-HCl, pH 6.8) [22]. Forty micrograms of proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. After blocking with 5% milk in Tris-buffered saline (TBS), the membranes were incubated with corresponding primary antibodies against pAKT (#4060; 1:1000 dilution, Cell Signaling Technology), pERK1/2 (#4370; 1:1000 dilution, Cell Signaling Technology), p21 (#2947; 1:1000 dilution, Cell Signaling Technology), cleaved-PARP (#5625; 1:1000 dilution, Cell Signaling Technology), p16 (ab51243; 1:1000 dilution, Abcam), γ-H2AX (ab11175; 1:1000 dilution, Abcam) and β-actin (#3700; 1:1000 dilution, Cell Signaling Technology) overnight at 4 °C and then incubated with the relevant secondary antibody (1:3000, Cell Signaling Technology). TBS buffer was used to wash the membranes after probing with the primary and secondary antibodies. The protein bands were then detected with Electro-Chemi-Luminescence (ECL) (Invitrogen, Carlsbad, CA, USA). All experiments were performed three times. The intensity of the protein bands was measured using ImageJ 1.52 version (NIH, Bethesda, MD, USA) (Java 1.8.0_172).

2.7. In vivo tumor model

All our animal experiments were conducted in accordance with guidelines approved by the Institute Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Nude mice were purchased from Beijing HFK Bioscience Co., Ltd., China. Ten female nude mice were equally and randomly divided into two groups, the NCTD group and the control group. Then 2×10^6 of MDA-MB-231 cells were injected into the subcutaneous tissue. Two weeks later, tumor formation was confirmed by measurement with a Vernier caliper and the IVIS Bioluminescence and Fluorescence Imaging System (PerkinElmer, Waltham, MA, USA). The data showed that there was no significant difference in the tumor size between the NC and NCTD groups [23–25]. After tumor formation, NCTD was injected intraperitoneally into mice in the NCTD group at a concentration of 28 mg/kg (a dose of 1/5 LD_{50}) every 3 days for 4 weeks. Tumor size and body weight were measured each week. Tumor volume was calculated using the following formula: V = a*bˆ2/2 (a and b represent the long

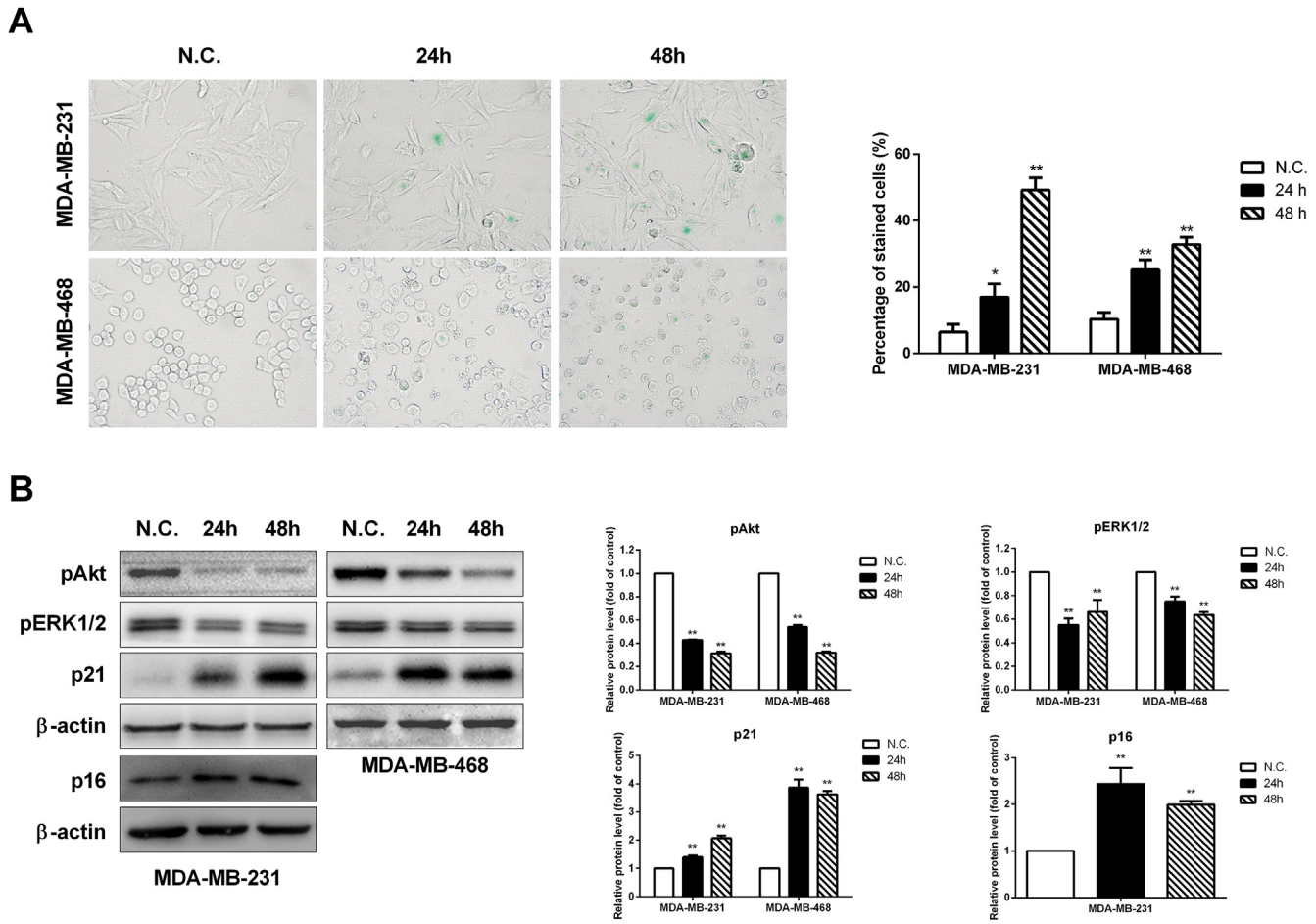


Fig. 3. ***NCTD induces cell senescence in TNBC cells***. (A) MDA-MB-468 and MDA-MB-231 cells were treated with NCTD at concentrations corresponding to the IC50 for 0 h, 24 h or 48 h, and cell senescence was observed by β-galactosidase staining. The histogram shows the percentage of stained cells in each group. Mean ± SD, n = 3, $*p < 0.05$ and $**p < 0.01$. (B) The protein levels of pAkt, pERK1/2, p21 and p16 after NCTD treatment for 24 h or 48 h were examined by immunoblotting with β-actin as a control. The histogram shows the grayscale values of the immunoblotting results. Mean ± SD, n = 3, $**p < 0.01$.

and short diameter of the tumor) [26]. Luminescent images were obtained with the IVIS Bioluminescence and Fluorescence Imaging System (PerkinElmer, Waltham, MA, USA).

2.8. Immunohistochemistry

Tumors dissected from nude mice were fixed in 4% paraformaldehyde for 48 h and dehydrated overnight. After embedding in paraffin, the tumor samples were sectioned. A two-step method was used for immunostaining. Firstly, tumor samples were deparaffinized by soaking in formaldehyde and dehydrated in various concentrations of ethanol. The sections then underwent sodium citrate antigen retrieval and were cooled to room temperature. Hydrogen peroxide (3%) was used to block endogenous peroxidase activity and goat serum was used to block non-specific antigens. The relevant primary antibody against pAKT (#4060; 1:200 dilution), pERK1/2 (#4370; 1:250 dilution) and p21 (#2947; 1:250 dilution) (Cell Signaling Technology) were then incubated with the tumor samples at 4 °C overnight. The following day, the tumor samples were incubated in polyperoxidase-*anti*-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and horseradish peroxidase-labeled streptavidin for 30 min each at 37 °C. After staining with DAB and hematoxylin, the sections were dehydrated in ethanol and transparentized in formaldehyde. ImageJ 1.52 version (NIH) (Java 1.8.0_172) was used to analyze the staining intensity of the sections.

2.9. TUNEL analyses

The sections undergoing immunohistochemistry analysis were deparaffinized by soaking in formaldehyde and then dehydrated in various concentrations of ethanol. To block endogenous peroxidase activity, the tumor samples were incubated with 3% hydrogen peroxide for 10 min at room temperature. The sections were then incubated with Proteinase K (Gibco BRL) in 20 μg/ml of 10 mM Tris/HCl (pH 8) for 10 min at room temperature, and incubated with the TUNEL reaction mixture which contained TdT and fluorescein-dUTP (Roche Applied Science, Pleasanton, CA, USA) for 60 min at 37 °C in the dark. After washing with PBS, the tumor samples were incubated with DAPI for 5 min at room temperature in the dark. Fluorescence was observed with a Leica LSM 400 laser scanning microscope. ImageJ 1.52 version (NIH) (Java 1.8.0_172) was used to quantify the stained nuclei.

2.10. NF-κB reporter gene assay

As described previously [27], MDA-MB-231 was cultured in 24-well plates and transfected with NF-κB luciferase reporter plasmid and Renilla luciferase reporter plasmid using Lipofectamine 2000 reagent (Invitrogen). Then medium with the corresponding NCTD IC50 or Akt/ERK inhibitors was added to the NCTD or inhibitors group to replace the old medium. After 24 h, cells were lysed and luciferase activity was detected using the Dual-Luciferase Reporter Assay (Promega Inc., Madison, WI, USA) following the manufacturer's instructions. Renilla luciferase activity was normalized to that of the firefly luciferase activity.

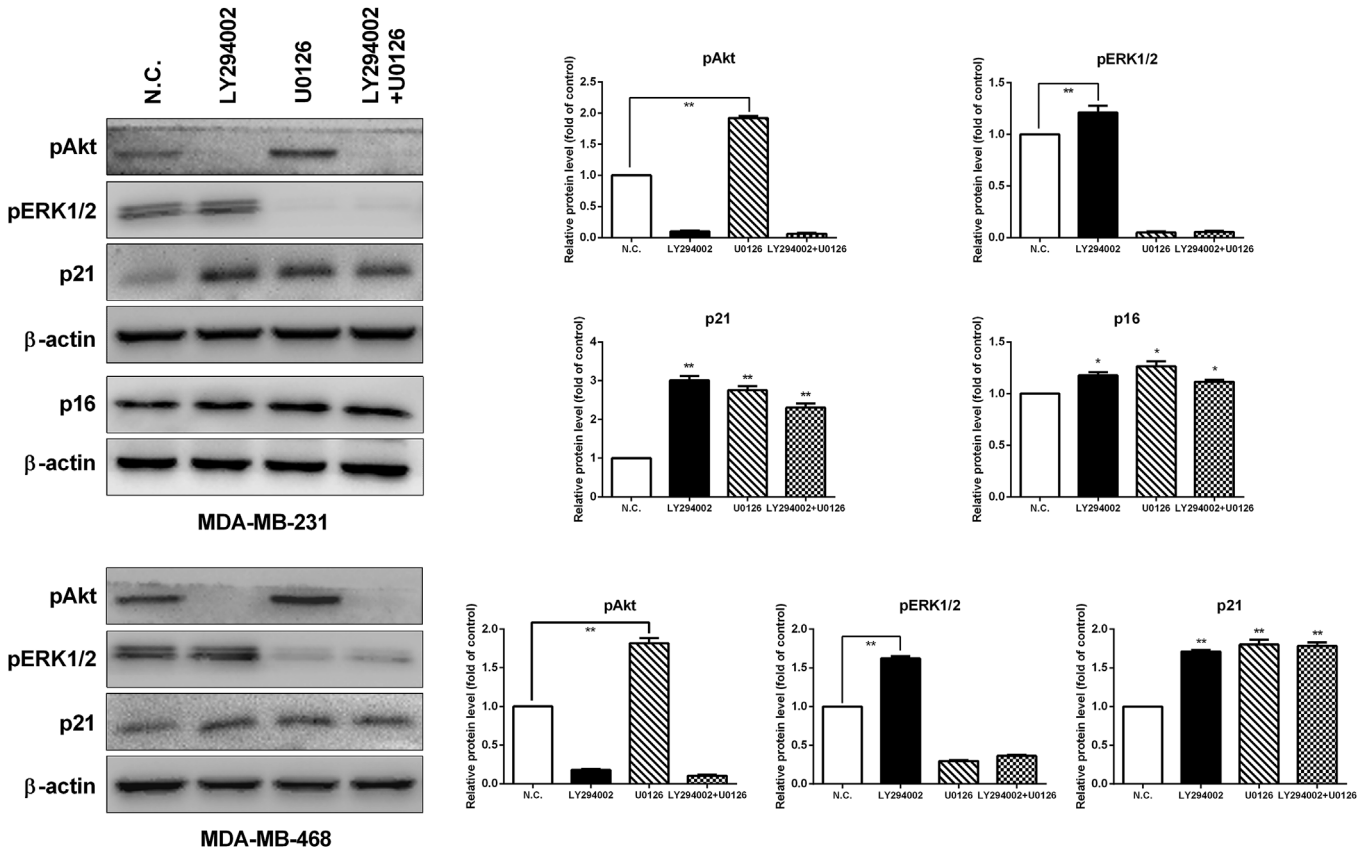


Fig. 4. ***The crosstalk between Akt and ERK signaling in TNBC cells***. MDA-MB-468 and MDA-MB-231 cells were treated with LY294002 and/or U0126 for 24 h, and the protein levels of pAkt, pERK1/2, p21 and p16 were detected by immunoblotting with β-actin as a control. The histogram shows the grayscale values of the immunoblotting results. Mean ± SD, n = 3, $^{**}p < 0.01$.

2.11. Statistical analysis

GraphPad 7.0 software was used for statistical analysis. The two-tailed Student's t-test was used to determine the *p*-value, and a $p < 0.05$ was considered statistically significant. * represents $p < 0.05$ and ** represents $p < 0.01$.

3. Results

3.1. NCTD suppresses cell proliferation in TNBC cells in vitro

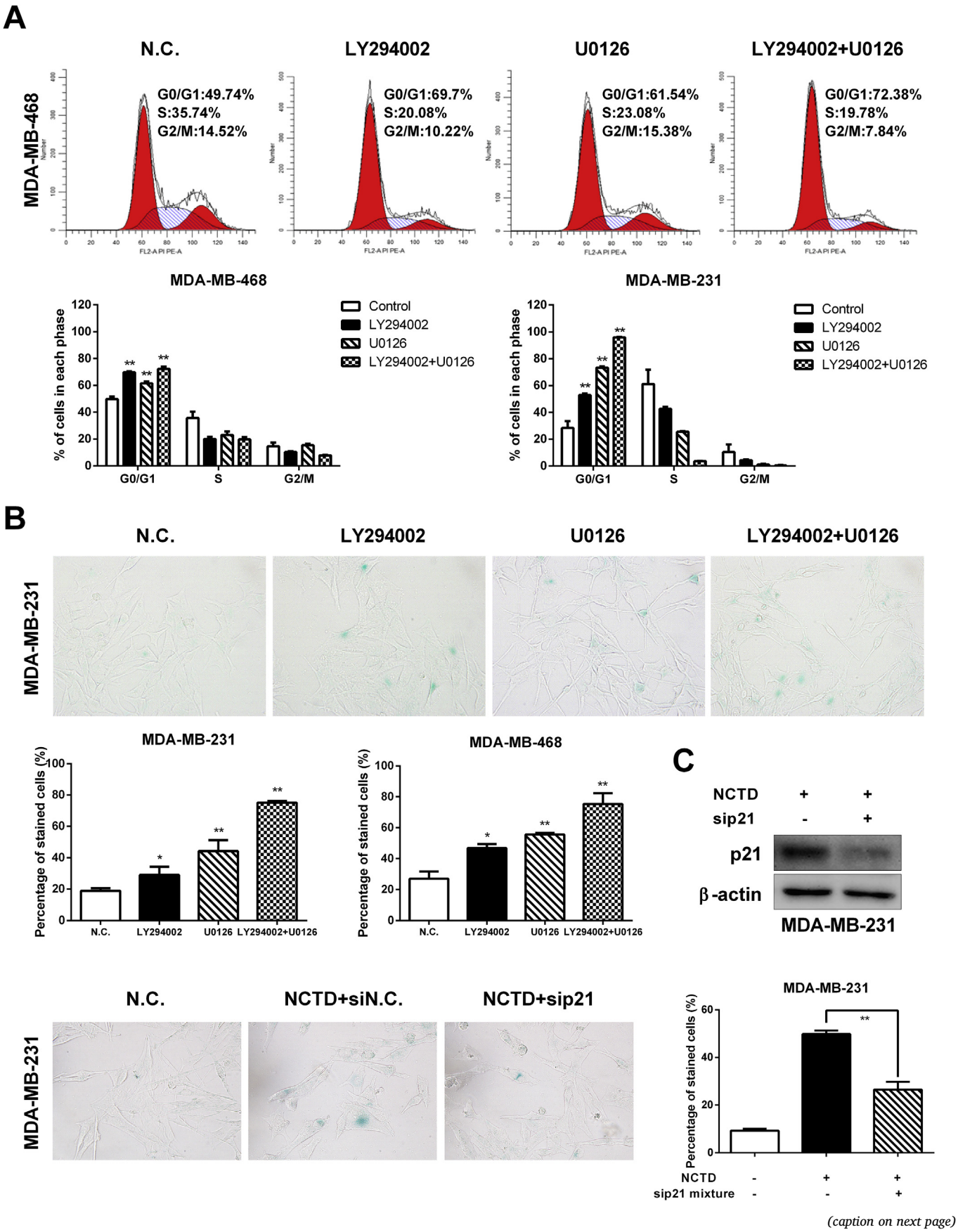
It was shown that NCTD induces cell apoptosis and cell cycle arrest in MDA-MB-231 cells, a triple-negative (ER-/PR-/HER2-) breast cancer (TNBC) cell line [28]. We assumed that NCTD may significantly inhibit the growth of TNBC cells. Firstly, we set the concentration range of NCTD for different breast cancer cell lines and the CCK8 assay was performed to measure the number of viable cells after culture with NCTD at various concentrations for 24 h. The results showed that the optical density (OD) values of MDA-MB-231, MDA-MB-468, BT-549, SK-BR-3, MCF-7 and BT474 cells markedly decreased in a dose-dependent manner and the IC50 value was calculated (Fig. 1B). The results were consistent with our hypothesis. Therefore, we treated TNBC cell lines, MDA-MB-231, MDA-MB-468 and BT-549, with NCTD at corresponding concentrations of IC50 and the results of the CCK8 assay showed that the OD values in the NCTD group were significantly reduced compared with the control group at 24 h, 48 h and 72 h (Fig. 1C). These data indicate that NCTD significantly inhibits the proliferation of TNBC cell lines *in vitro*.

3.2. NCTD induces cell cycle arrest and apoptosis in TNBC cells

It has been demonstrated that cell cycle arrest and apoptosis contribute to inhibition of cell proliferation in breast cancer [29,30]. It has also been proved that NCTD induces cell cycle arrest and apoptosis in various cancers [17,18]. Therefore, we treated MDA-MB-231 and MDA-MB-468 cells with NCTD at relevant IC50 concentrations for 24 h or 48 h to determine its effect on cell cycle arrest and apoptosis. Flow cytometry was conducted and the representative results showed that the number of cells in the G2/M phase was significantly increased in the NCTD group compared with the control group, accompanied by a decrease in the G0/G1 and S phase (Fig. 2A). In addition, the pro-apoptotic effect of NCTD was evaluated by the annexin V/PE-FITC/7-AAD staining assay and the representative results showed that the number of apoptotic cells was significantly increased in the NCTD group compared with the control group (Fig. 2B). Moreover, we performed AO/EB staining and the results showed that the number of early and late apoptotic cells was obviously increased in the NCTD group compared with the control group (Fig. 2C). More importantly, the results showed that the protein levels of γ-H2AX and cleaved-PARP were significantly increased in the NCTD group compared with the control group (Fig. 2D). Thus, these data confirm that NCTD effectively induces cell cycle arrest and cell apoptosis by upregulating γ-H2AX and cleaved-PARP in TNBC cells.

3.3. NCTD induces cell senescence in TNBC cells

It was previously reported that cell proliferation inhibition induced cell senescence in cancers [31]. The inhibitory effect of NCTD on cell proliferation and the cell cycle inspired us to further study its impact on cell senescence. Hence, senescence associated β-galactosidase (SA-β-



(caption on next page)

Fig. 5. ***Synergistic action of Akt and ERK inhibition on cell cycle arrest and cell senescence.*** (A) MDA-MB-468 and MDA-MB-231 cells were treated with LY294002 and/or U0126 for 24 h, and the cell cycle was examined by flow cytometry analysis. The histogram shows the percentage of cells in different cell cycle phases. Mean ± SD, n = 3, $^{**}p < 0.01$. (B) Cell senescence was examined using β-galactosidase staining. The histogram shows the percentage of stained cells in each group. Mean ± SD, n = 3, $^{*}p < 0.05$ and $^{**}p < 0.01$. (C) MDA-MB-231 cells were treated with NCTD at concentration corresponding to the IC50 for 48 h after transfection with siRNA mixture of p21. The protein level of p21 was detected by immunoblotting with β-actin as a control. Cell senescence was observed by β-galactosidase staining. The histogram shows the percentage of stained cells in each group. Mean ± SD, n = 3, $^{**}p < 0.01$.

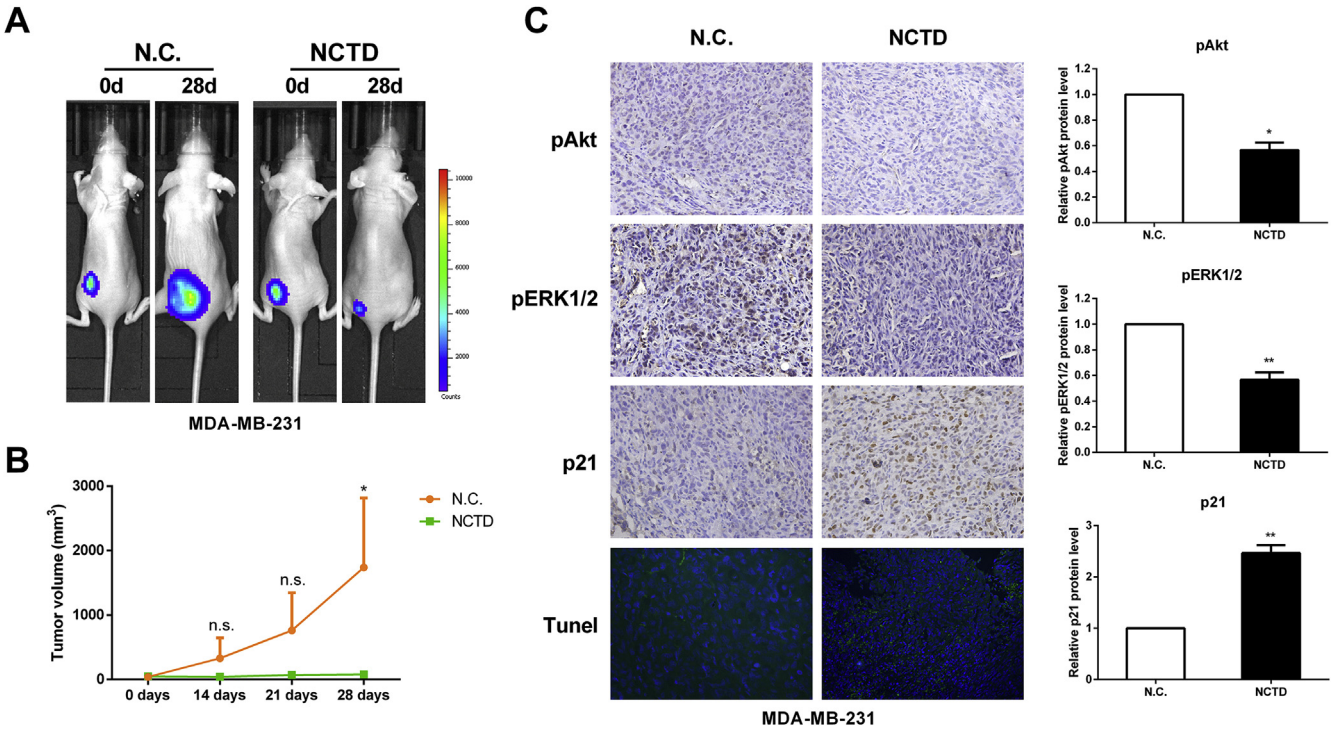


Fig. 6. ***NCTD inhibits tumor growth in vivo.*** (A) A tumor model was established with fluorescent MDA-MB-231 cells and NCTD was injected intraperitoneally into nude mice every 3 days for 4 weeks. The fluorescence intensity was detected with the In Vivo Imaging System. (B) The tumor growth curve. Mean ± SD, n = 5, $^{*}p < 0.05$. (C) The protein levels of pAkt, pERK1/2 and p21 were determined by immunohistochemistry (original magnification: 400×), and apoptotic cells were examined with the TUNEL Apoptosis Assay Kit. The histogram shows the intensity of immunostaining. Mean ± SD, n = 5, $^{*}p < 0.05$ and $^{**}p < 0.01$.

gal) staining was conducted on MDA-MB-231 and MDA-MB-468 cells which were treated with NCTD for 24 h and 48 h. The results showed that the number of stained cells was significantly increased at 24 h and 48 h (Fig. 3A). In addition, the percentage of stained cells at 48 h was higher than that at 24 h.These data indicate that NCTD increased the percentage of SA-β-gal-positive TNBC cells in a time-dependent manner.

It has been reported that Akt and ERK signaling are closely related to cell senescence, however, there is still some controversy regarding this issue [32–35]. To understand the relationship between NCTD-induced cell senescence and the Akt and ERK signaling pathways, the levels of Akt and ERK1/2 phosphorylation were measured by immunoblot in the NCTD-treated and control groups. The results showed that the protein levels of pAkt and pERK1/2 were significantly decreased at 24 h and 48 h in the NCTD group compared with the control group. By contrast, the level of p21 and p16, which reflects the state of cell senescence, was significantly increased in the NCTD group compared with the control group. Therefore, these data indicate that NCTD-induced cell senescence maybe targeting pAkt and pERK1/2 signaling, at least partly.

3.4. Synergistic action of Akt and ERK inhibition on cell cycle arrest and cell senescence

The above data indicate that inhibition of pAkt and pERK1/2 signaling could be involved in NCTD-induced cell cycle arrest and cell senescence. It has been demonstrated that crosstalk exists between the pAkt and pERK1/2 signaling pathways, which means that when one pathway is suppressed, the other will be enhanced to compensate for the weakened function [14]. To evaluate the interaction between pAkt and pERK1/2, cells were treated with LY294002, an inhibitor of phosphoinositide 3-kinase (PI3Ks), and/or U0126, a highly selective inhibitor of both MEK1 and MEK2. It was found that the level of pERK1/2 was elevated in cells treated with LY294002, while the level of pAkt was upregulated in cells treated with U0126. In addition, the levels of pAkt and pERK1/2 were simultaneously decreased when LY294002 and U0126 were combined, and the level of p16 was elevated when inhibitors were used (Fig. 4). Thus, pAkt and pERK1/2 may interact with each other when one of them is inhibited. Therefore, there is potential crosstalk between pAkt and pERK1/2 and we assumed that inhibition of both may imitate the action of NCTD to induce cell cycle arrest and cell senescence.

In order to validate this hypothesis, LY294002 and U0126 were used to treat MDA-MB-231 and MDA-MB-468 cells, and the cell cycle and cell senescence were analyzed. The results of flow cytometry showed that the G0/G1 phase in both cell lines was blocked when LY294002 and U0126 were used separately or in combination (Fig. 5A). Furthermore, the results of SA-β-gal staining showed that the number of stained cells was significantly elevated when either LY294002 or U0126 was used, and the percentage of stained cells was much higher when LY294002 and U0126 were combined (Fig. 5B). In order to identify how NCTD induced cell senescence, MDA-MB-231 was transfected with siRNA mixture of p21 and treated with NCTD at concentration corresponding to the IC50 for 48 h. The results of SA-β-gal staining showed

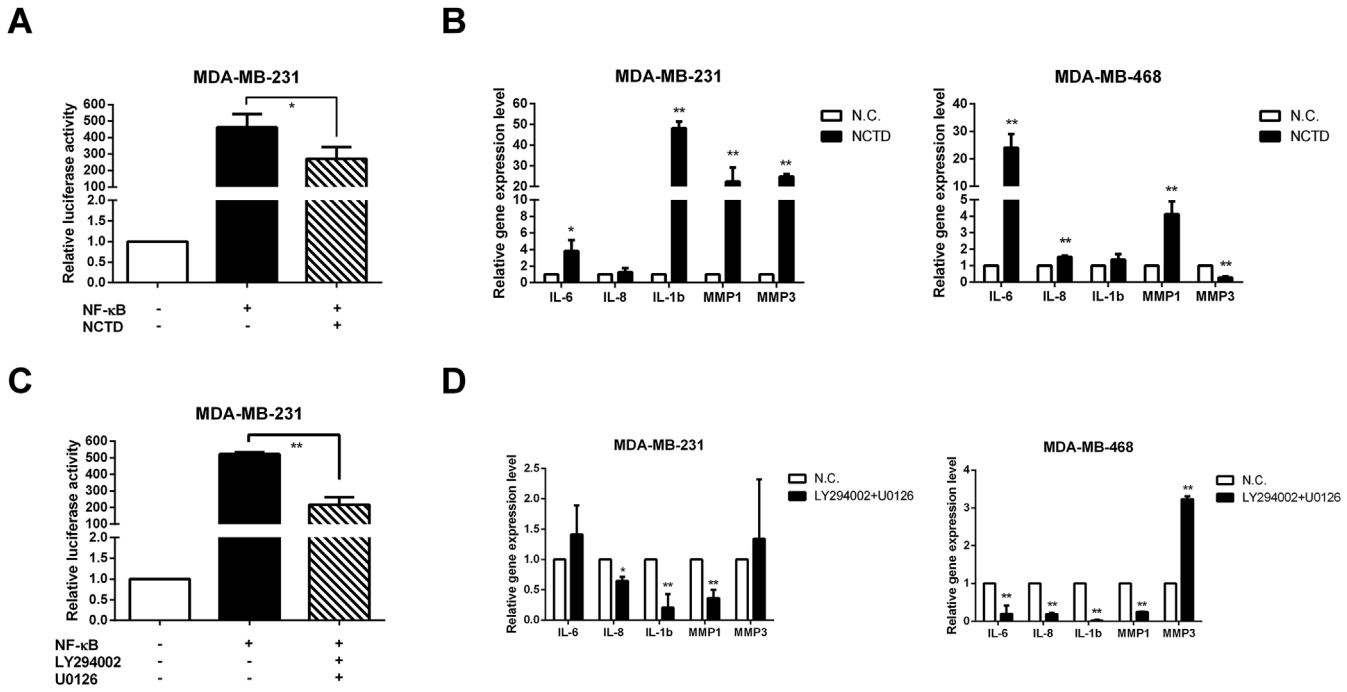


Fig. 7. ***NCTD suppresses NF-κB signaling but increases markers of senescence-associated secretory phenotype.*** (A) MDA-MB-231 was treated with NCTD for 24 h, and luciferase activity was measured using the luciferase reporter assay. The histogram shows the relative luciferase activity of each group. Mean ± SD, n = 3, $*p < 0.05$. (B) MDA-MB-231 and MDA-MB-468 were treated with NCTD for 24 h, and the mRNA levels of *IL-6*, *IL-8*, *IL-1β*, *MMP1* and *MMP3* were detected by RT-qPCR, with *β-actin* as the control. The histogram shows the relative gene expression levels of the RT-qPCR results. Mean ± SD, n = 3, $*p < 0.05$ and $**p < 0.01$. (C) MDA-MB-231 was treated with Akt and ERK inhibitors for 24 h, and luciferase activity was measured using the luciferase reporter assay. The histogram shows the relative luciferase activity of each group. Mean ± SD, n = 3, $**p < 0.01$. (D) MDA-MB-231 was treated with Akt and ERK inhibitors for 24 h, and the mRNA levels of *IL-6*, *IL-8*, *IL-1β*, *MMP1* and *MMP3* were detected by RT-qPCR, with *β-actin* as the control. The histogram shows the relative gene expression levels of the RT-qPCR results. Mean ± SD, n = 3, $*p < 0.05$ and $**p < 0.01$.

that the number of stained cells was significantly decreased when p21 was knocked down (Fig. 5C). Thus, the inhibition of pAkt and pERK1/2 signaling nearly imitates NCTD-induced cell cycle arrest and cell senescence, and NCTD induces cell senescence via p21 activation, at least partly.

3.5. NCTD suppresses cell proliferation in TNBC cells in vivo

To further study the function of NCTD *in vivo*, we constructed a xenograft model using MDA-MB-231 cells in nude mice, which were divided into the NCTD group and the control group. After 4 weeks, tumor size in the NCTD-treated group was significantly smaller than that in the control group (Fig. 6A and B), and the body weight of mice in the two groups was not significantly different (data not shown). To test the consistency between the *in vitro* assay and the *in vivo* assay, the protein levels of pAkt, pERK1/2 and p21 were measured by immunohistochemistry. The results showed that the expression of pAkt and pERK1/2 was decreased, while the level of p21 was increased in the NCTD-treated group compared with the control group. Moreover, NCTD effectively inhibited tumor growth *in vivo* by downregulating pAkt and pERK1/2 and upregulating p21.

3.6. NCTD inhibits NF-κB signaling but upregulates markers of the SASP

Recent evidence has shown that in addition to its tumor-suppressive function, cellular senescence also has the potential to promote tumor progression by inducing a senescence-associated secretory phenotype (SASP) [36,37]. NCTD was shown to promote cellular senescence by inhibiting pAkt and pERK1/2 signaling pathways. In order to test our hypothesis that NCTD will induce the SASP, we firstly evaluated the effect of NCTD on NF-κB, a crucial transcription factor which mediates the expression of SASP-related genes [38], using the NF-κB reporter gene assay. We found that luciferase activity was significantly decreased in the NCTD group compared with the control group (Fig. 7A). Next, we conducted RT-qPCR to detect major components (soluble signaling factors) of SASP. The result showed that the mRNA levels of interleukins *IL-6*, *IL-8* and *IL-1β*, and extracellular proteases *MMP-1* and *MMP-3* were significantly upregulated in the NCTD group compared with the control group (Fig. 7B). Similarly, Akt and ERK inhibitors suppressed luciferase activity, but decreased the majority of SASP-related genes. Thus, these data indicate that NCTD suppresses NF-κB signaling but increases the mRNA levels of SASP markers, which may not depend on Akt and ERK signaling.

4. Discussion

It is well known that Akt and ERK signaling are crucial regulators in cell biological processes such as the cell cycle, proliferation, survival, cell senescence and metabolism, and are mutated, self-activated and interact with each other in breast cancer, especially in triple-negative breast cancer (TNBC) [4–7]. Due to the complexity of signaling pathways, inhibition of one signaling pathway may cause compensatory activation of other signaling pathways [14]. As a result, how to simultaneously suppress Akt and ERK signaling is unclear. In this study, the results showed that norcantharidin (NCTD) suppressed cell proliferation *in vitro* and *in vivo*, and induced cell apoptosis, cell cycle arrest and cell senescence mainly by simultaneously suppressing pAkt and pERK1/2 signaling and activating p21 and p16. Following the confirmation of potential crosstalk of the pAkt and pERK1/2 signaling pathways, the combination of LY294002 and U0126 was assessed and was shown to induce a similar effect to that of NCTD. These findings indicate that NCTD induces cell apoptosis, cell cycle arrest and cell senescence mainly via dual inhibition of pAkt and pERK1/2 signaling in TNBC.

Cantharidin is a natural element extracted from blister beetles, and has been proven to be effective in cancer [16]. NCTD, the demethylated analog of cantharidin, has been synthesized and been shown to have an equal anti-cancer effect with fewer side effects than cantharidin [39]. It was confirmed that NCTD suppresses cell proliferation, cell migration and invasion, and induces cell apoptosis, cell cycle arrest and cell senescence in several cancers, including breast cancer [40–43]. Previous data have shown that NCTD suppresses the growth of MDA-MB-231 (ER-/PR-/HER2-), a TNBC cell line [28]. Due to the lack of a therapeutic target, TNBC is more aggressive and has a worse prognosis than other subtypes of breast cancer [3]. In this study, the results showed that NCTD more effectively suppressed the proliferation of TNBC cells compared with non-TNBC cells, indicating that NCTD may act as a potential adjuvant treatment in TNBC. However, the underlying mechanism is unclear.

It has been confirmed that the inhibition of Akt or ERK signaling is closely related to cell proliferation, cell cycle distribution, invasion and metastasis in several cancers, including breast cancer [8–11,13]. In recent years, small molecule inhibitors targeting the pAkt or pERK1/2 signaling pathways have been shown to effectively attenuate tumor cell proliferation and invasion, and induce cell cycle arrest, cell apoptosis and even cell senescence [12,44]. However, the effect, especially that of ERK inhibitors in TNBC, is limited. This may be related to the relatively low level of phosphorylated ERK in TNBC [45]. Akt inhibitors have a better effect, which is probably due to the level of phosphorylated Akt being significantly higher in TNBC than in non-TNBC [46]. Furthermore, NCTD suppressed cell proliferation, angiogenesis, epithelial-mesenchymal transition and induced cell apoptosis by blocking ERK signaling in colon cancer and hepatoma [20,47,48]. NCTD also inhibited cell proliferation, angiogenesis, metastasis and overcame drug-resistance by targeting Akt signaling in several cancers [19,28]. Moreover, it has been widely reported that crosstalk exists between the Akt and ERK signaling pathways, and inhibition of one pathway leads to enhanced activation of the other, which may weaken their anti-tumor effect and result in drug resistance [49–51]. Therefore, dual inhibition of Akt and ERK signaling might result in better tumor inhibition in TNBC [52]. However, little is known about the effect of NCTD on both Akt and ERK signaling in cancers, especially in TNBC. In this study, NCTD simultaneously inhibited Akt and ERK signaling both *in vitro* and *in vivo*. The potential crosstalk between these two signaling pathways was observed when either LY294002 or U0126 was used, which partly explains the ineffectiveness or drug resistance of single inhibition in some studies [49–51]. It is worth mentioning that further studies are required to determine the detailed mechanism between Akt and ERK signaling in NCTD-induced tumor suppression.

Cell senescence is the biological response to abnormal extracellular or intracellular stress. Its tumor-suppressive function has been confirmed, since it blocks tumor cells at a certain phase of cell cycle, thereby decreasing cell proliferation [36]. It has been widely confirmed that Akt or ERK signaling is closely related to cell senescence in several cancers, including breast cancer [11–13]. However, no study has shown that dual inhibition of Akt and ERK signaling significantly induces cell senescence in cancer. Also there are no data to show that NCTD induces cell senescence in breast cancer. In this study, the results showed that NCTD induced cell senescence in TNBC by mainly targeting Akt and ERK signaling.

However, recent studies have revealed the two sidedness of cell senescence [37]. SASP cells secrete a series of soluble signaling factors, such as interleukins, inflammatory cytokines and growth factors, which affect the surrounding environment and consequently change cell behavior. Numerous studies have reported that SASP promotes cell proliferation and cell motility in several cancers, including breast cancer [53–55]. Our data indicate that NCTD induces TNBC cell cycle arrest and cell senescence, while also upregulating the level of soluble signaling factors of SASP in a NF-κB-independent manner. However, luciferase activity was significantly decreased under Akt and ERK inhibitors treatment, accompanied by a decrease of the majority of these SASP-related genes. Whether this will weaken or even reverse the anti-cancer effect of NCTD requires further research.

p21 and p16, which are downstream of pAkt and pERK1/2, are tumor suppressors which induce cell senescence and cell cycle arrest [56–58]. Inhibitors of pAkt or pERK1/2 can activate p21 and p16 and promote cell senescence and cell cycle arrest [11,12,59–61]. It is well known that p21 is closely related to both G0/1 and G2/M phase arrest [62,63]. However, in our study, NCTD induced G2/M phase arrest and signaling inhibitors induced G0/1 arrest in TNBC. This inconsistency may be explained by NCTD, but not the inhibitors, causing DNA damage [18]. This might be the underlying mechanism of the different phase of cell cycle arrest caused by NCTD.

It has been reported that NCTD can suppress the pAkt or pERK1/2 signaling pathway [19,47]. However, we did not find research describing suppression of both these pathways by NCTD. In the present study, we found that NCTD simultaneously inhibited pAkt and pERK1/2 in TNBC cells. As pAkt and pERK1/2 were both inhibited by NCTD, their potential crosstalk was also blocked, which resulted in effective tumor repression *in vitro* and *in vivo*. Thus, this study provides a theoretical basis for the application of NCTD in the treatment of TNBC.

Collectively, the present findings prove that NCTD exerts an anticancer effect *in vitro* and *in vivo* by simultaneously suppressing Akt and ERK signaling. We also show that NCTD upregulates the level of soluble signaling factors of SASP in a NF-κB-independent manner, which provides a novel explanation of the molecular mechanism of NCTD.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This research was approved by the Institutional Ethics Committees of The First Affiliated Hospital of Chongqing Medical University and conformed to the principles of the Declaration of Helsinki.

Acknowledgements

The authors thank Prof. Qian Tao (the Chinese University of Hong Kong, Hong Kong, China) for generously providing cell lines. This study was supported by the National Natural Science Foundation of China (NO. 81572769, NO.81372238), the Natural Science Foundation of Chongqing (2016ZDXM006) and the Scientific Research Foundation of Chongqing Medical University (NO. 201408).

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, Ca - Cancer J. Clin. 68 (2018) 7–30.
[2] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[3] W.D. Foulkes, I.E. Smith, J.S. Reis-Filho, Triple-negative breast cancer, N. Engl. J. Med. 363 (2010) 1938–1948.
[4] E. Lopez-Knowles, S.A. O'Toole, C.M. McNeil, E.K. Millar, M.R. Qiu, P. Crea, R.J. Daly, E.A. Musgrove, R.L. Sutherland, PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality, Int. J. Cancer 126 (2010) 1121–1131.
[5] A. Adeyinka, Y. Nui, T. Cherlet, L. Snell, P.H. Watson, L.C. Murphy, Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression, Clin. Cancer Res.: Offic. J. Am. Assoc. Canc. Res. 8 (2002) 1747–1753.
[6] O.K. Mirzoeva, D. Das, L.M. Heiser, S. Bhattacharya, D. Siwak, R. Gendelman, N. Bayani, N.J. Wang, R.M. Neve, Y. Guan, Z. Hu, Z. Knight, H.S. Feiler, P. Gascard, B. Parvin, P.T. Spellman, K.M. Shokat, A.J. Wyrobek, M.J. Bissell, F. McCormick, W.L. Kuo, G.B. Mills, J.W. Gray, W.M. Korn, Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition, Cancer Res. 69 (2009) 565–572.
[7] Comprehensive molecular portraits of human breast tumours, Nature 490 (2012) 61–70.
[8] K. Balakrishnan, M. Peluso, M. Fu, N.Y. Rosin, J.A. Burger, W.G. Wierda,

M.J. Keating, K. Faia, S. O'Brien, J.L. Kutok, V. Gandhi, The phosphoinositide-3-kinase (PI3K)-delta and gamma inhibitor, IPI-145 (Duvelisib), overcomes signals from the PI3K/AKT/S6 pathway and promotes apoptosis in CLL, Leukemia 29 (2015) 1811–1822.
[9] Y. Gao, X. Xiao, C. Zhang, W. Yu, W. Guo, Z. Zhang, Z. Li, X. Feng, J. Hao, K. Zhang, B. Xiao, M. Chen, W. Huang, S. Xiong, X. Wu, W. Deng, Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF-kappaB/iNOS signaling pathways, J. Pineal Res. (2017) 62.
[10] P. de la Puente, B. Muz, A. Jin, F. Azab, M. Luderer, N.N. Salama, A.K. Azab, MEK inhibitor, TAK-733 reduces proliferation, affects cell cycle and apoptosis, and synergizes with other targeted therapies in multiple myeloma, Blood Canc. J. 6 (2016) e399.
[11] X. Xu, Z. Lu, W. Qiang, V. Vidimar, B. Kong, J.J. Kim, J.J. Wei, Inactivation of AKT induces cellular senescence in uterine leiomyoma, Endocrinology 155 (2014) 1510–1519.
[12] T.K. Hayes, N.F. Neel, C. Hu, P. Gautam, M. Chenard, B. Long, M. Aziz, M. Kassner, K.L. Bryant, M. Pierobon, R. Marayati, S. Kher, S.D. George, M. Xu, A. Wang-Gillam, A.A. Samatar, A. Maitra, K. Wennerberg, E.F. Petricoin 3rd, H.H. Yin, B. Nelkin, A.D. Cox, J.J. Yeh, C.J. Der, Long-term ERK inhibition in KRAS-mutant pancreatic cancer is associated with MYC degradation and senescence-like growth suppression, Cancer Cell 29 (2016) 75–89.
[13] C.D. Britten, PI3K and MEK inhibitor combinations: examining the evidence in selected tumor types, Cancer Chemother. Pharmacol. 71 (2013) 1395–1409.
[14] M.C. Mendoza, E.E. Er, J. Blenis, The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation, Trends Biochem. Sci. 36 (2011) 320–328.
[15] E.E. Talbert, J. Yang, T.A. Mace, M.R. Farren, A.B. Farris, G.S. Young, O. Elnaggar, Z. Che, C.D. Timmers, P. Rajasekera, J.M. Maskarinec, M. Bloomston, T. Bekaii-Saab, D.C. Guttridge, G.B. Lesinski, Dual inhibition of MEK and PI3K/Akt rescues cancer cachexia through both tumor-extrinsic and -intrinsic activities, Mol. Canc. Therapeut. 16 (2017) 344–356.
[16] G.S. Wang, Medical uses of mylabris in ancient China and recent studies, J. Ethnopharmacol. 26 (1989) 147–162.
[17] L. Mo, X. Zhang, X. Shi, L. Wei, D. Zheng, H. Li, J. Gao, J. Li, Z. Hu, Norcantharidin enhances antitumor immunity of GM-CSF prostate cancer cells vaccine by inducing apoptosis of regulatory T cells, Cancer Sci. 109 (2018) 2109–2118.
[18] C.C. Yu, F.Y. Ko, C.S. Yu, C.C. Lin, Y.P. Huang, J.S. Yang, J.P. Lin, J.G. Chung, Norcantharidin triggers cell death and DNA damage through S-phase arrest and ROS-modulated apoptotic pathways in TSGH 8301 human urinary bladder carcinoma cells, Int. J. Oncol. 41 (2012) 1050–1060.
[19] F. Chen, S. Wang, Y. Wei, J. Wu, G. Huang, J. Chen, J. Shi, J. Xia, Norcantharidin modulates the miR-30a/Metadherin/AKT signaling axis to suppress proliferation and metastasis of stromal tumor cells in giant cell tumor of bone, Biomed. Pharmacother. = Biomedecine and pharmacotherapie 103 (2018) 1092–1100.
[20] L. Zhang, Q. Ji, X. Liu, X. Chen, Z. Chen, Y. Qiu, J. Sun, J. Cai, H. Zhu, Q. Li, Norcantharidin inhibits tumor angiogenesis via blocking VEGFR2/MEK/ERK signaling pathways, Cancer Sci. 104 (2013) 604–610.
[21] J. Guo, Y. Wu, L. Yang, J. Du, K. Gong, W. Chen, J. Dai, X. Li, S. Xi, Repression of YAP by NCTD disrupts NSCLC progression, Oncotarget 8 (2017) 2307–2319.
[22] C. Muller, S. Monferran, A.C. Gamp, P. Calsou, B. Salles, Inhibition of Ku heterodimer DNA end binding activity during granulocytic differentiation of human promyelocytic cell lines, Oncogene 20 (2001) 4373–4382.
[23] F. Shao, M. Lv, Y. Zheng, J. Jiang, Y. Wang, L. Lv, J. Wang, The anti-tumour activity of rLj-RGD4, an RGD toxin protein from Lampetra japonica, on human laryngeal squamous carcinoma Hep-2 cells in nude mice, Biochimie 119 (2015) 183–191.
[24] G. Semenova, D.S. Stepanova, C. Dubyk, E. Handorf, S.M. Deyev, A.J. Lazar, J. Chernoff, Targeting group I p21-activated kinases to control malignant peripheral nerve sheath tumor growth and metastasis, Oncogene 36 (2017) 5421–5431.
[25] E. Velez, S.N. Goldberg, G. Kumar, Y. Wang, S. Gourevitch, J. Sosna, T. Moon, C.L. Brace, M. Ahmed, Hepatic thermal ablation: effect of device and heating parameters on local tissue reactions and distant tumor growth, Radiology 281 (2016) 782–792.
[26] S. Naito, A.C. von Eschenbach, R. Giavazzi, I.J. Fidler, Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice, Cancer Res. 46 (1986) 4109–4115.
[27] L. Ye, T. Xiang, Y. Fan, D. Zhang, L. Li, C. Zhang, X. He, Q. Xiang, Q. Tao, G. Ren, The 19q13 KRAB Zinc-finger protein ZFP82 suppresses the growth and invasion of esophageal carcinoma cells through inhibiting NF-kappaB transcription and inducing apoptosis, Epigenomics 11 (2019) 65–80.
[28] Y. Huang, Q. Liu, K. Liu, K. Yagasaki, G. Zhang, Suppression of growth of highly-metastatic human breast cancer cells by norcantharidin and its mechanisms of action, Cytotechnology 59 (2009) 201–208.
[29] M. Ingham, G.K. Schwartz, Cell-cycle therapeutics come of age, J. Clin. Oncol.: Offic. J. Am. Soc. Clin. Oncol. 35 (2017) 2949–2959.
[30] F. Braso-Maristany, S. Filosto, S. Catchpole, R. Marlow, J. Quist, E. Francesch-Domenech, D.A. Plumb, L. Zakka, P. Gazinska, G. Liccardi, P. Meier, A. Gris-Oliver, M.C. Cheang, A. Perdrix-Rosell, M. Shafat, E. Noel, N. Patel, K. McEachern, M. Scaltriti, P. Castel, F. Noor, R. Buus, S. Mathew, J. Watkins, V. Serra, P. Marra, A. Grigoriadis, A.N. Tutt, PIM1 kinase regulates cell death, tumor growth and chemotherapy response in triple-negative breast cancer, Nat. Med. 22 (2016) 1303–1313.
[31] X. Yang, K. Qu, J. Tao, G. Yin, S. Han, Q. Liu, H. Sun, Inhibition of CIP2A attenuates tumor progression by inducing cell cycle arrest and promoting cellular senescence in hepatocellular carcinoma, Biochem. Biophys. Res. Commun. 495 (2018) 1807–1814.
[32] M.V. Astle, K.M. Hannan, P.Y. Ng, R.S. Lee, A.J. George, A.K. Hsu, Y. Haupt, R.D. Hannan, R.B. Pearson, AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy, Oncogene 31 (2012) 1949–1962.
[33] A.L. Kennedy, J.P. Morton, I. Manoharan, D.M. Nelson, N.B. Jamieson, J.S. Pawlikowski, T. McBryan, B. Doyle, C. McKay, K.A. Oien, G.H. Enders, R. Zhang, O.J. Sansom, P.D. Adams, Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis, Mol. Cell 42 (2011) 36–49.
[34] Q. Liu, X. Xu, M. Zhao, Z. Wei, X. Li, X. Zhang, Z. Liu, Y. Gong, C. Shao, Berberine induces senescence of human glioblastoma cells by downregulating the EGFR-MEK-ERK signaling pathway, Mol. Canc. Therapeut. 14 (2015) 355–363.
[35] O. Clark, S. Daga, A.W. Stoker, Tyrosine phosphatase inhibitors combined with retinoic acid can enhance differentiation of neuroblastoma cells and trigger ERK- and AKT-dependent, p53-independent senescence, Cancer Lett. 328 (2013) 44–54.
[36] F. Rodier, J. Campisi, Four faces of cellular senescence, J. Cell Biol. 192 (2011) 547–556.
[37] J.P. Coppe, P.Y. Desprez, A. Krtolica, J. Campisi, The senescence-associated secretory phenotype: the dark side of tumor suppression, Ann. Rev. Pathol. 5 (2010) 99–118.
[38] W. Xu, Y. Li, W.W. Yuan, Y. Yin, W.W. Song, Y. Wang, Q.Q. Huang, W.H. Zhao, J.Q. Wu, Membrane-bound CD40L promotes senescence and initiates senescence-associated secretory phenotype via NF-kappaB activation in lung adenocarcinoma, Cell. Physiol. Biochem.: Int. J. Exp. Cell Physiol. Biochem., Pharmacol. 48 (2018) 1793–1803.
[39] S.H. Kok, S.J. Cheng, C.Y. Hong, J.J. Lee, S.K. Lin, Y.S. Kuo, C.P. Chiang, M.Y. Kuo, Norcantharidin-induced apoptosis in oral cancer cells is associated with an increase of proapoptotic to antiapoptotic protein ratio, Cancer Lett. 217 (2005) 43–52.
[40] Y.N. Chen, J.C. Chen, S.C. Yin, G.S. Wang, W. Tsauer, S.F. Hsu, S.L. Hsu, Effector mechanisms of norcantharidin-induced mitotic arrest and apoptosis in human hepatoma cells, Int. J. Cancer 100 (2002) 158–165.
[41] P. Qiu, S. Wang, M. Liu, H. Ma, X. Zeng, M. Zhang, L. Xu, Y. Cui, H. Xu, Y. Tang, Y. He, L. Zhang, Norcantharidin Inhibits cell growth by suppressing the expression and phosphorylation of both EGFR and c-Met in human colon cancer cells, BMC Canc. 17 (2017) 55.
[42] D. Jin, Y. Wu, C. Shao, Y. Gao, D. Wang, J. Guo, Norcantharidin reverses cisplatin resistance and inhibits the epithelial mesenchymal transition of human nonsmall lung cancer cells by regulating the YAP pathway, Oncol. Rep. 40 (2018) 609–620.
[43] D. Liu, P. Shi, X. Yin, Z. Chen, X. Zhang, Effect of norcantharidin on the human breast cancer Bcap-37 cells, Connect. Tissue Res. 53 (2012) 508–512.
[44] E. Jokinen, J.P. Koivunen, MEK and PI3K inhibition in solid tumors: rationale and evidence to date, Therapeut. Adv. Med. Oncol. 7 (2015) 170–180.
[45] J. Huang, Q. Luo, Y. Xiao, H. Li, L. Kong, G. Ren, The implication from RAS/RAF/ERK signaling pathway increased activation in epirubicin treated triple negative breast cancer, Oncotarget 8 (2017) 108249–108260.
[46] S. Umemura, S. Yoshida, Y. Ohta, K. Naito, R.Y. Osamura, Y. Tokuda, Increased phosphorylation of Akt in triple-negative breast cancers, Cancer Sci. 98 (2007) 1889–1892.
[47] C. Peng, Z. Li, Z. Niu, W. Niu, Z. Xu, H. Gao, W. Niu, J. Wang, Z. He, C. Gao, P. Lin, M. Agrez, Z. Zhang, J. Niu, Norcantharidin suppresses colon cancer cell epithelial-mesenchymal transition by inhibiting the alphavbeta6-ERK-ets1 signaling pathway, Sci. Rep. 6 (2016) 20500.
[48] Y.N. Chen, C.C. Cheng, J.C. Chen, W. Tsauer, S.L. Hsu, Norcantharidin-induced apoptosis is via the extracellular signal-regulated kinase and c-Jun-NH2-terminal kinase signaling pathways in human hepatoma HepG2 cells, Br. J. Pharmacol. 140 (2003) 461–470.
[49] A.B. Turke, Y. Song, C. Costa, R. Cook, C.L. Arteaga, J.M. Asara, J.A. Engelman, MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors, Cancer Res. 72 (2012) 3228–3237.
[50] V. Serra, M. Scaltriti, L. Prudkin, P.J. Eichhorn, Y.H. Ibrahim, S. Chandarlapaty, B. Markman, O. Rodriguez, M. Guzman, S. Rodriguez, M. Gili, M. Russillo, J.L. Parra, S. Singh, J. Arribas, N. Rosen, J. Baselga, PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer, Oncogene 30 (2011) 2547–2557.
[51] E. Jokinen, N. Laurila, J.P. Koivunen, Alternative dosing of dual PI3K and MEK inhibition in cancer therapy, BMC Canc. 12 (2012) 612.
[52] Y.H. Ibrahim, V. Serra, M. Scaltriti, L. Prudkin, P. Anton, J. Perez, J. Balmaña, J. Baselga, Combined PI3K and MEK Inhibition in Patient-Derived Xenografts Generated from Recurrent Triple Negative Breast Cancer, AACR, 2011.
[53] D. Liu, P.J. Hornsby, Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion, Cancer Res. 67 (2007) 3117–3126.
[54] P. Sun, N. Yoshizuka, L. New, B.A. Moser, Y. Li, R. Liao, C. Xie, J. Chen, Q. Deng, M. Yamout, M.Q. Dong, C.G. Frangou, J.R. Yates 3rd, P.E. Wright, J. Han, PRAK is essential for ras-induced senescence and tumor suppression, Cell 128 (2007) 295–308.
[55] J.P. Coppe, M. Boysen, C.H. Sun, B.J. Wong, M.K. Kang, N.H. Park, P.Y. Desprez, J. Campisi, A. Krtolica, A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion, Mol. Canc. Res.: MCR 6 (2008) 1085–1098.
[56] T. Abbas, A. Dutta, p21 in cancer: intricate networks and multiple activities, Nature reviews, Cancer 9 (2009) 400–414.
[57] H. Rayess, M.B. Wang, E.S. Srivatsan, Cellular senescence and tumor suppressor gene p16, Int. J. Cancer 130 (2012) 1715–1725.
[58] T. Bai, F. Liu, F. Zou, G. Zhao, Y. Jiang, L. Liu, J. Shi, D. Hao, Q. Zhang, T. Zheng, Y. Zhang, M. Liu, S. Li, L. Qi, J.Y. Liu, Epidermal growth factor induces proliferation of hair follicle-derived mesenchymal stem cells through epidermal growth factor receptor-mediated activation of ERK and AKT signaling pathways associated with

upregulation of cyclin D1 and downregulation of p16, Stem Cell. Dev. 26 (2017) 113–122.
[59] S.K. Roy, R.K. Srivastava, S. Shankar, Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer, J. Mol. Signal. 5 (2010) 10.
[60] S.B. Seo, J.J. Lee, H.H. Yun, C.N. Im, Y.S. Kim, J.H. Ko, J.H. Lee, 14-3-3beta depletion drives a senescence program in glioblastoma cells through the ERK/SKP2/p27 pathway, Mol. Neurobiol. 55 (2018) 1259–1270.
[61] H. Hu, Z. Li, J. Chen, D. Wang, J. Ma, W. Wang, J. Li, H. Wu, L. Li, M. Wu, Q. Qian, J. Chen, C. Su, P16 reactivation induces anoikis and exhibits antitumour potency by downregulating Akt/survivin signalling in hepatocellular carcinoma cells, Gut 60 (2011) 710–721.
[62] T. Waldman, K.W. Kinzler, B. Vogelstein, p21 is necessary for the p53-mediated G1 arrest in human cancer cells, Cancer Res. 55 (1995) 5187–5190.
[63] C. Cayrol, M. Knibiehler, B. Ducommun, p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells, Oncogene 16 (1998) 311–320.