Full Paper

Hirsutanol A Induces Apoptosis and Autophagy via Reactive Oxygen Species Accumulation in Breast Cancer MCF-7 Cells

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Abstract. Hirsutanol A is a novel sesquiterpene compound purified from the marine fungus *Chondrostereum* sp in the coral *Sarcophyton tortuosum*. Our previous studies had demonstrated that hirsutanol A exerted potent cytotoxic effect in many kinds of cancer cell lines. Here, the anti-cancer molecular mechanisms of hirsutanol A were investigated in breast cancer MCF-7 cells. The results showed that hirsutanol A could inhibit cell proliferation, elevate reactive oxygen species (ROS) level, and induce apoptosis and autophagy. Co-treatment with the potent antioxidant agent *N*-acetyl-L-cysteine could effectively reverse the effect of enhanced ROS production, which in turn, reduces growth inhibition, apoptosis, and autophagy mediated by hirsutanol A. In addition, blocking autophagy by bafilomycin A1 or Atg7-siRNA could synergistically enhance the antiproliferative effect and apoptosis induced by hirsutanol A. These data suggested that hirsutanol A could induce apoptosis and autophagy via accumulation of ROS and co-treatment with an autophagy inhibitor could sensitize MCF-7 cells to hirsutanol A.

Keywords: hirsutanol A, apoptosis, autophagy, reactive oxygen species (ROS), breast cancer

Introduction

Reactive oxygen species (ROS), a collective term for oxygen-derived species including superoxide anion radical $O_2^{\cdot-}$, hydrogen peroxide H_2O_2, hydroxyl radicals $\cdot OH$, and so on, are innitially considered as normal by-products of cellular metabolism (1, 2). Emerging evidences confirm that most cancer cells are associated with increased metabolic activity and production of ROS under oxidative stress, which renders cancer cells more vulnerable to damage by futher ROS insults since it is more difficult to scavenge the increased ROS induced by exogenous agents in cancer cells than in normal cells (3). Thus, an exogenous ROS inducer could selectively kill cancer cells by damaging the mitochondrial membrane and causing the activation of numerous pathways to trigger apoptosis (4). Moreover, ROS can function as signaling molecules to modulate autophagy (5).

Apoptosis (self-killing) is a highly regulated and organized cell death process controlling the development and homeostasis of multicellular organisms. It involves the demolition of cellular structures and organelles by the activation of catabolic enzymes including the decrease in cell volume, compaction of cytoplasmic organelles, condensation and fragmentation of nuclear chromatin, internucleosomal DNA cleavage, and membrane blebbing (6, 7). Induction of apoptosis by chemotherapeutic drugs is a key way to kill cancer cells.

Autophagy (self-eating) is a mechanism resulting in lysosomal degradation of cytoplasmic constituents. During autophagy, portions of the cytoplasm are first sequestered by double-membrane vesicles called autophagosomes and degraded after fusion with lysosomes for subsequent recycling (8). The role of autophagy is a double-edged sword. Proper autophagy provides a survival function but excessive autophagy might result in autophagic cell death (9, 10).

Accumulating evidences have described the complex interplay between apoptosis and autophagy. In some systems, the induction of autophagy can enhance apoptotic cell death. However, specific removal of the damaged mitochondria by autophagy can help to prevent the release of apoptotic factors such as cytochrome *c* from the mitochondria and activation of the apoptotic cascade, which will reduce the apoptotic response to stimulation (11, 12).

Hirsutanol A is a novel sesquiterpene compound purified from the marine fungus *Chondrostereum* sp in the coral *Sarcophyton tortuosum* (13). Our previous studies have shown that hirsutanol A exerted potent cytotoxic effect on many kinds of human cancer cell lines and induced autophagical cell death via increasing the ROS level (14, 15). However, in the present study, we examined the molecular mechanism of hirsutanol A implicated in anticancer activity in the MCF-7 cells and found that hirsutanol A could induce apoptosis and autophagy via accumulation of ROS and blockade of autophagy could contribute to hirsutanol A-induced apoptosis.

Materials and Methods

Drugs and reagents

Fetal bovine serum was purchased from Gibco® (New York, NY, USA). 3-(4,5-dime-thylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), CM-H2DCF-DA, DMSO, and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against LC3 were from Novus Biologicals, Inc. (Littleton, CO, USA). Chemiluminescence reagents were acquired from Cell Signaling Technology (Danvers, MA, USA). Antibodies against GAPDH and anti-mouse IgG-horseradish peroxidase and anti-rabbit IgG-horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell lysis buffer was from Upstate Biotech Co. (New York, NY, USA). Hirsutanol A, a sesquiterpene compound isolated from fungus *Chondrostereum* sp in *Sarcophyton tortuosum* in Dr. Hou-Jin Li's lab, was initially dissolved in 100% DMSO at 100 nmol/L and stored at −20°C. Its structure is shown in Fig. 1.

Cell lines and cell culture

Human breast cancer cell line MCF-7 prepared by our lab was cultured in DMEM (Gibco®) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37°C in 5% CO_2. All experiments were carried out with cells in the logarithmic growth phase.

MTT assay

MCF-7 cells were seeded in 96-well plates at the density of 8,000 cells per well in 195 μL DMEM and cultivated at 37°C in 5% CO_2 overnight. Cells were either pre-incubated with 1 mmol/L antioxidant NAC or 1 nmol/L autophagic inhibitor bafilomycin A1 for 1 h and then cultivated for 72 h at 37°C or they were treated with 5 μL different concentrations of hirsutanol A for indicated times. A 10-μL aliquot of 5 mg/mL MTT was then added into each well and the plates were incubated at 37°C in 5% CO_2 for 4 h. After complete removal of the medium, 100 μL of DMSO was added into each well to dissolve the insoluble purple formazan product. Absorbance values of the resultant purple solution were obtained with a test wavelength of 570 nm and a reference wavelength of 650 nm. The rates of cell growth inhibition were calculated. The 50% inhibitory rates (%) were calculated by the Bliss method. Inhibitory rate = (1 − the average OD value of the treatment group / the average OD value of the control group) × 100% (16).

Annexin V / propidium iodide double-staining assay

Annexin V – fluorescein isothiocyanate / propidium iodide staining was performed using the Annexin V – fluorescein isothiocyanate apoptosis detection kit. Cells (3.0×10^5 per mL) were seeded into six-well plates with 2 mL in each well and then treated with different concentrations of hirsutanol A for 72 h or pre-treated with 1 mmol/L NAC followed by treatment with 30 μmol/L hirsutanol A for 72 h. Both floated and attached cells were collected, washed with ice-cold PBS twice, and then incubated at room temperature in the presence of media binding reagent and Annexin V-FITC (Invitrogen, New York, NY, USA) for 15 min in the dark. After washing with PBS, the cells were re-suspended in ice-cold 1 × binding buffer and treated with 10 μL propidium iodide (30 μg/mL) on ice in the dark. Apoptosis was quantified by flow cytometry (Becton Dickinson, Oxford, UK) at the wavelength of 488 nm immediately and analyzed by the Cell-Quest software (17).

Flow cytometry assay

Cells were diluted to 3.0×10^5 per mL and seeded into six-well plates with 2 mL in each well. Cells were treated either with hirsutanol A for the indicated times or with various concentrations of hirsutanol A for 24 h or they were pre-incubated with 1 mmol/L NAC followed by
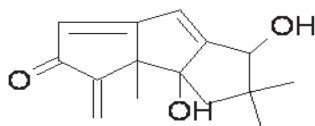

Fig. 1. The chemical structure of hirsutanol A.

treatment with 30 μmol/L hirsutanol A for 24 h and then incubated with 1 μmol/L (final concentration) CM-H2DCF-DA or DHE fluorescent dye in the dark for 1 h at 37°C. After washing twice with PBS at 4°C, cells were centrifuged and re-suspended in PBS. The level of intracellular ROS was detected by flow cytometry with the FACS Calibur system and CellQuestPro analysis software [18].

Immunoblotting analysis

Cells were seeded into six-well plates, treated with hirsutanol A for the indicated times, or pre-incubated with NAC for 1 h followed by treatment with hirsutanol A for 24 h. Cells were harvested and washed twice with PBS and lysed in lysis buffer. The cell lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C and the concentrations of protein were determined with the Bio-Rad protein assay (Hercules, CA, USA). SDS-PAGE sample buffer was added to the cell lysates. Then the cell lysates were heated at 100°C for 5 min, and cell lysate containing 20 – 40 μg protein was loaded in each well of 8% and 15% SDS-PAGE gels. Resolved proteins were electrophoretically transferred to a PVDF membrane and incubated sequentially with primary antibodies and horseradish peroxidase–conjugated secondary antibodies. After washing, the protein complex was detected using an ECL chemiluminescence reagent and XAR film (Kodak, New York, NY, USA) as described by the manufacturer [19, 20].

siRNA transfection

The target sequence for Atg7-specific siRNA was 5′-CAGUGGAUCUAAAU CUCAAACUGAUTT-3′ and control siRNA (no silencing) was synthesized by GenChem Co. (Yeong Kang City, Taiwan) [21]. One day before transfection, cells were plated in six-well plates with antibiotic-free growth medium at a density of 1.5×10^5 cells per well. When cells grew to a confluency of 30% – 50% on the second day, transfection was performed by using Opti-MEM media (Gibco®), lipofectamine 2000 (Invitrogen), and Atg7 siRNA according to manufacturer's recommendations. After 6 h, the media was replaced with antibiotic-free growth medium and cells were treated with 20 μmol/L hirsutanol A for 3 h. Cells transfected with lipofectamine were used as the control [22].

Statistical analysis

Data were analyzed by Student's t-test with SPSS 11.0 analysis software, and results were considered to be significant at $P < 0.05$. Results are presented as the mean and standard deviation ($\pm$ S.D.).

Results

Hirsutanol A exhibited potent cytotoxicity against MCF-7 cells

The MTT assay measures the activity of mitochondrial dehydrogenase enzymes based on its ability to cleave the terazolium ring to produce formazan [23]. The cytotoxic effect of hirsutanol A on MCF-7 cells was evaluated by MTT assay. Hirsutanol A inhibited cell proliferation in a dose- and time-dependent manner. The half-maximal inhibitory concentrations (IC$_{50}$) were 10.69, 18.36, and 33.51 μmol/L for MCF-7 cells after treatment with hirsutanol A for 72, 48, and 24 h, respectively (Fig. 2).

Hirsutanol A increased the production of intrinsic ROS

ROS mainly include superoxide anion radical $O_2{\cdot}^-$ and hydrogen peroxide H_2O_2. Our previous studies had confirmed that hirsutanol A could induce autophagical cell death by causing an accumulation of ROS in human hepatocellular carcinoma cells. DHE and CM-H2DCF-DA could be used as fluorescent probes to detect the level of $O_2{\cdot}^-$ and H_2O_2. In the present study, with flow cytometry analysis, we measured the effects of hirsutanol A on cellular superoxide and hydrogen peroxide level in human breast cancer MCF-7 cells stained with DHE and CM-H2DCF-DA [24, 25]. The results showed that there was no significant change in DHE fluorescence after treatment with hirsutanol A for 3h but markedly increased in CM-H2DCF-DA fluorescence in a dose-dependent fashion(Fig. 3: A, B), which suggested that the ROS induced by hirsutanol A were mainly hydrogen peroxide but not superoxide.

Hirsutanol A induced apoptosis and autophagy in MCF-7 cells

Translocation of phosphatidyl serine to the cell surface is an indicator of early cell apoptosis [26]. Annexin V –
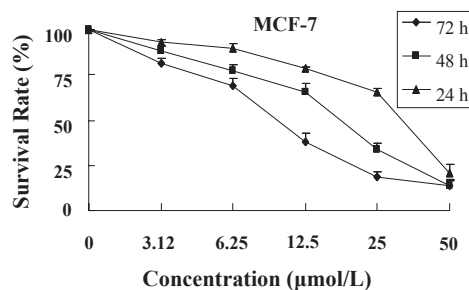
Fig. 2. Anti-proliferative effect of hirsutanol A in breast cancer MCF-7 cells. Cells were treated with different concentrations of hirsutanol A for 72, 48, and 24 h. Inhibitory rates were determined by MTT assay. Mean and error bars for triplicate determinations are shown.

fluorescein isothiocyanate / propidium idodide staining assay was employed to monitor the apoptotic cells. The percentages of Annexin V-positive MCF-7 cells were 2.3%, 10.0%, 20.8%, and 31.3% after treatment with 0, 7.5, 15, and 30 μmol/L of hirsutanol A for 72 h, respectively. These data indicated that hirsutanol A could induce apoptosis in a dose-dependent manner (Fig. 4A).

The amount of PE-conjugated form of LC3 (LC3-II) correlates with the number of autophagosomes and the conversion of LC3-I to LC3-II can be used as a autophagy marker (27, 28). Thereby, the conversion of LC3-I to LC3-II was measured by western blot analysis. LC3-I to

LC3-II conversion was markedly increased in a dose- and time- dependent manner after treatment with hirsutanol A, indicating induction of autophagy (Fig. 4B).

Hirsutanol A induced apoptosis and autophagy via accumulation of ROS

Accumulating evidences demonstrated that ROS serving as a second messenger can modulate the associated signaling pathways to induce apoptosis and autophagy (29, 30). In the present study, we found that hirsutanol A could elevate ROS level and induce apoptosis and autophagy. Therefore, we further elucidated whether the
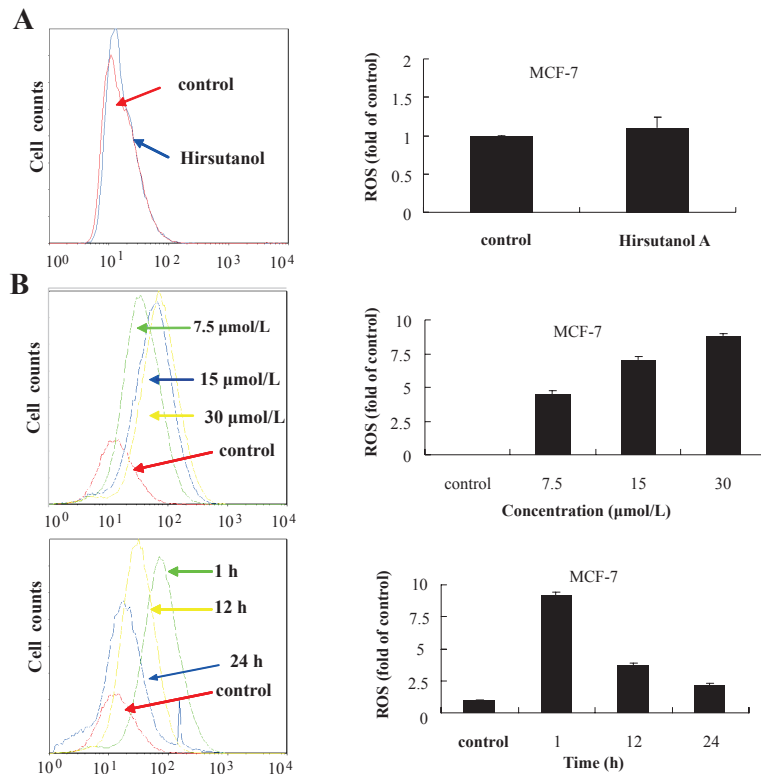

Fig. 3. Hirsutanol A-induced accumulation of intrinsic reactive oxygen species (ROS). MCF-7 cells were stained with DHE for 30 min after exposure to 30 μmol/L hirsutanol A for 3 h. The cellular O_2 level was monitored by flow cytometry (A). MCF-7 cells were first treated with various concentrations of hirsutanol A for 3 h or with 30 μmol/L hirsutanol A for the indicated times and then stained with CM-H2DCF-DA. The cellular H_2O_2 level was monitored by flow cytometry (B). Mean and error bars for triplicate determinations are shown.
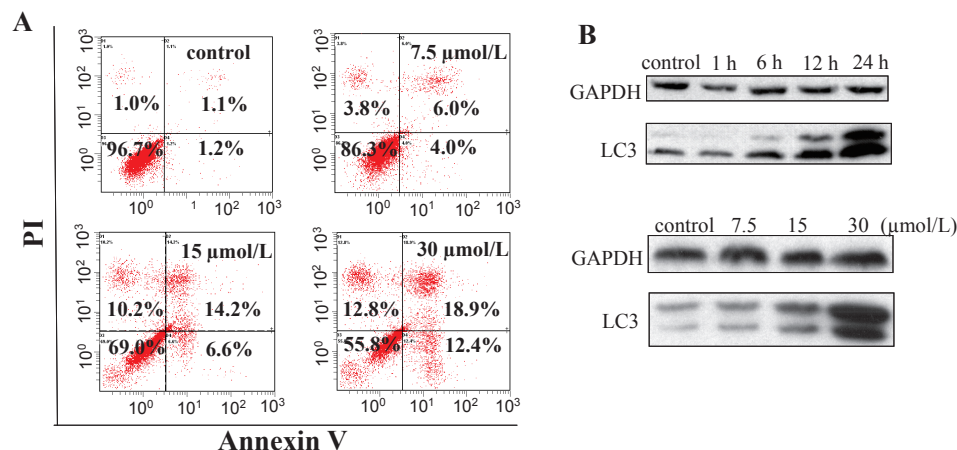

Fig. 4. Induction of apoptosis by hirsutanol A in breast cancer MCF-7 cells. Cells were treated with various concentrations of hirsutanol A for 72 h. Annexin V/PI analysis was used to detect the apoptosis. The cells in the lower right quadrant (Annexin V$^+$/PI$^-$) and in the upper right quadrant (Annexin V$^+$/PI$^+$) were apoptotic cells (A). Proteins were harvested from the MCF-7 cells after treatment with 30 μmol/L hirsutanol A for the indicated times or treatment with various concentrations of hirsutanol A for 24 h. Immunoblotting assay was used to measure the conversion of LC3-I to LC3-II (B).

induction of apoptosis and autophagy was triggered by excessive ROS. NAC, a potent antioxidant agent, was used to reverse the effect of enhanced ROS production induced by hirsutanol A (31) (Fig. 5A). The hirsutanol A-induced ROS accumulation was prevented by pre-incubation with NAC for 1 h. Hirsutanol A-induced cell growth inhibition and apoptosis were significantly inhibited in the presence of NAC (Fig. 5: B, C). In addition, inhibition of hirsutanol A-induced ROS accumulation could prevent the conversion of LC3-I to LC3-II (Fig. 5D). These data indicated that hirsutanol A-induced apoptosis and autophagy was triggered by accumulation of ROS.

Blockade of autophagy promoted cell growth inhibition and apoptosis induced by hirsutanol A

Hirsutanol A could induce apoptosis and autophagy, which prompted us to further dissect the contribution of
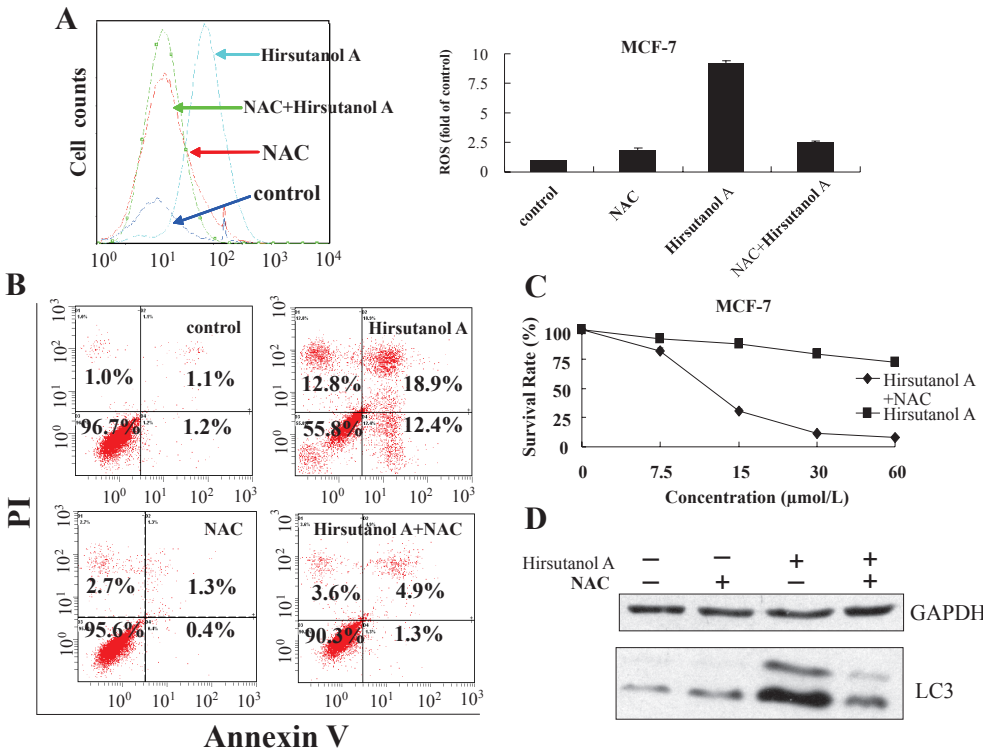

Fig. 5. Hirsutanol A-induced cell growth inhibition, apoptosis, and autophagy via accumulation of ROS. Cells were pre-incubated with NAC for 1 h and then exposed to hirsutanol A for 3 h. The cellular H_2O_2 level was monitored by flow cytometry (A). Cells were pre-incubated with 1 mmol/L NAC for 1 h and then treated with various concentrations of hirsutanol A for 72 h. MTT assay was used to detect the cell growth inhibition. Apoptosis was evaluated by Annexin V/PI analysis (B, C). Cells were pre-incubated with 1 mmol/L NAC for 1 h and then treated with 30 μmol/L hirsutanol A for 24 h. The protein expression of LC3 was evaluated by immunoblotting assay (D). Mean and error bars for triplicate determinations are shown.
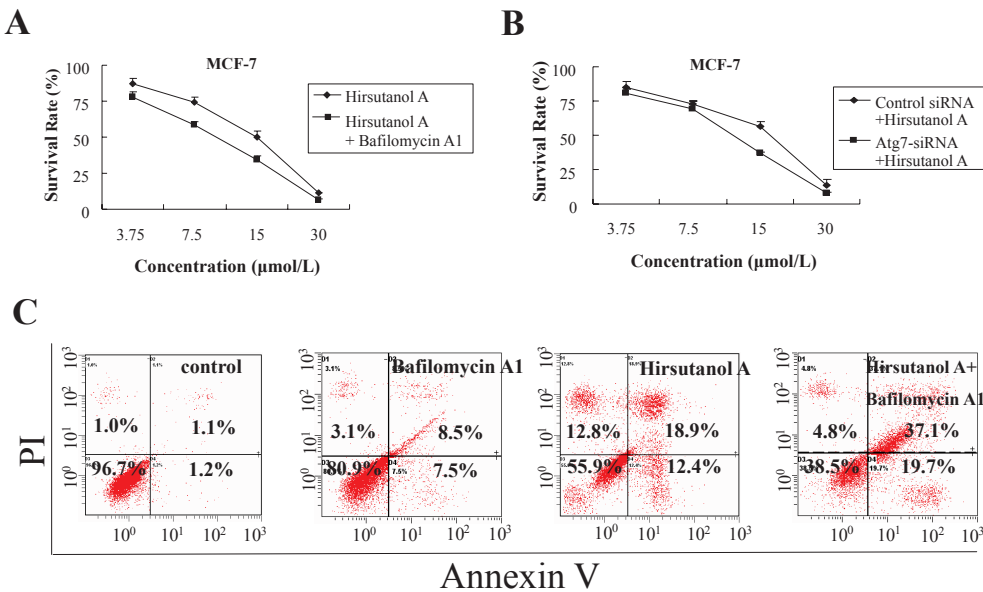

Fig. 6. Blockade of autophagy enhanced growth inhibition and apoptosis. MCF-7 cells were first pre-incubated with 1 nmol/L bafilomycin A1 for 1 h (A) or transformed with scrambled Atg7-siRNA (B) and then detected by MTT assay or by flow cytometry (C) after treatment with hirsutanol A for 72 h. The cells in the lower right quadrant (Annexin V^+/PI^-) and in the upper right quadrant (Annexin V^+/PI^+) were apoptotic cells. Mean and error bars for triplicate determinations are shown.

autophagy to hirsutanol A-induced apoptosis. Bafilomy-cin A1 or Atg7-siRNA was used to block autophagy induced by hirsutanol A in MCF-7 cells. Then the cell growth inhibition and apoptosis induced by hirsutanol A were detected (32, 33). The results showed that blocking autophagy remarkably increased the cell growth inhibition and apoptosis induced by hirsutanol A (Fig. 6).

Discussion

Therapeutic selectivity is an important issue in cancer treatment. An ideal anticancer agent should be toxic to malignant cells with minimum toxicity in normal cells (33). However, multiple genetic and epigenetic alterations would cause drug resistance in cancer cells, which is a serious challenge for gene-targeted drugs (34). Thus, searching for an attractive target for novel selective anticancer drugs is an important and challenging task. Recently, many researchers have focused on the biological differences between normal and cancer cells and they believed that manipulating these differences is essential in designing novel selective anticancer drugs (35). Numerous evidences suggested that most cancer cells were under oxidative stress due to the increased production of intrinsic ROS (36). High ROS level and duration of ROS stress made cancer cells up-regulate redox enzyme to adapt this abnormal situation. So, the redox system was viewed as an attractive target for selective cancer therapy and a ROS inducer has become a potential anticancer drug for targeting primarily cancer cells (37).

Our previous studies showed that hirsutanol A exerted potent cytotoxic effects on many kinds of human cancer cells. In addition, we also confirmed that hirsutanol A could induce autophagical cell death by causing accumulation of ROS in human hepatocellular carcinoma cells. Some evidences supported that as a potent oxidant agent, ROS could damage mitochondrial membrane, which results in mitochondrial membrane potential disorder and release of cytochrome *c* from mitochondria that can activate caspase-3-triggering mitochondria / cytochrome *c*-mediated apoptosis (38, 39). Thus, we suggested that hirsutanol A-induced apoptosis was associated with the increased level of ROS. The results showed that apoptotic cells were up to 31.3% after treatment with hirsutanol A for 72 h, but only 6.2% when MCF-7 cells were pre-incubated with NAC for 1 h to reverse the effect of enhanced ROS production induced by hirsutanol A, suggesting that hirsutanol A could serve as a ROS inducer to trigger apoptosis via accumulation of ROS.

ROS can also function as a molecular signal to mediate the initiation of autophagy. Thereby, we detected the autophagy and further explored the role of hirsutanol A-induced excessive ROS in induction of autophagy. The western blot analysis for measuring the conversion of LC3-I to LC3-II, which is an autophagy marker, indicated that hirsutanol A could significantly induce the conversion of LC3-I to LC3-II, while pre-treatment with NAC could reverse this conversion induced by hirsutanol A. The interaction between autophagy and apoptosis still remained to be elucidated. It has been reported that damage to the lysosomal compartment could activate apoptosis, while others observed that sequestration of mitochondria in autophagic vacuoles could protect cells against apoptosis (40, 41). Here, we explored the function of autophagy on the hirsutanol A-induced apoptosis. The results showed that blocking autophagy by bafilo-mycin A1 or Atg7-siRNA significantly enhanced the apoptosis induced by hirsutanol A.

In summary, hirsutanol A is a ROS inducer that exhibits anticancer effects and induces apoptosis and autophagy via accumulation of ROS. Hirsutanol A-induced autophagy provides a survival function to protect MCF-7 cancer cells from apoptosis. These evidences imply that treatment with hirsutanol A in combination with inhibitors of autophagy may exhibit a synergistic effect. In addition, the characteristics of the specific antitumor mechanism and natural occurrence of hirsutanol A provides some advantages as an anticancer agent. Our results also revealed that hirsutanol A may be a promising lead compound and deserves further investigation as a potential anticancer agent.

References

1 Chen YQ, Gibson SB. Is mitochondrial generation of reactive oxygen species a trigger for autophagy? Autophagy. 2008; 4:246–248.

2 Nogueira V, Park Y, Chen CC, Xu PZ, Chen ML, Tonic I, et al. Akt determines replicative senescence and oxidative or onco-genic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell. 2008;14:458–470.

3 Rachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009;8:579–591.

4 Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, et al. Selective killing of oncogenically transformed cells through a ROS mediated mechanism by *β*-phenylethyl isothio-cyanate. Cancer Cell. 2006;10:241–252.

5 Bensaad K, Cheung EC, Vousden KH. Modulation of intracellular ROS levels by TIGAR controls autophagy. EMBO J. 2009; 28:3015–3026.

6 Ko H, Kim YJ, Park JS, Park JH, Yang HO. Autophagy inhibition enhances apoptosis induced by ginsenoside Rk1 in hepatocellular carcinoma cells. Biosci Biotechnol Biochem. 2009;73:2183–2189.

7 Lockshin RA, Zahra Z. Apoptosis, autophagy, and more. Int J Biochem Cell Biol. 2004;36:2405–2419.

8 Li DD, Guo JF, Huang JJ, Wang LL, Deng R, Liu JN, et al. Rhabdastrellic acid-A induced autophagy-associated cell death

through blocking Akt pathway in human cancer cells. PLoS One. 2010;5:1–10.

9   Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451: 1069–1075.

10 Bursch W, Karwan A, Mayer M, Dornetshuber J, Fröhwein U, Schulte-Hermann R, et al. Cell death and autophagy: cytokines, drugs, and nutritional factors. Toxicology. 2008;254:147–157.

11 Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med. 2005;11:725–730.

12 Kim R, Emi M, Tanabe K, Murakami S, Uchida Y, Arihiro K. Regulation and interplay of apoptotic and non-apoptotic cell death. J Pathol. 2006;208:319–326.

13 Wang GYS, Abrell LM, Avelar A, Borgeson BM, Crews P. New hirsutane based sesquiterpenes from salt water cultures of a marine sponge-derived fungus and the terrestrial fungus coriolus consors. Tetrahedron. 1998;54:7335–7342.

14 Li HJ, Lan WJ, Lam CK, Yang F, Zhu XF. Hirsutane sesquiterpenoids from marine-derived fungus chondrostereum sp. Chem Biodivers. 2011;8:317–324.

15 Yang F, Gao YH, Wu KW, Deng R, Li DD, Wei ZX, et al. A novel sesquiterpene Hirsutanol A induces autophagical cell death in human hepatocellular carcinoma cells by increasing reactive oxygen species. Chin J Caner. 2010;29:655–660.

16 Chen LM, Wu XP, Ruan JW, Liang YJ, Ding Y, Shi Z, et al. Screening novel, potent multidrug- resistant modulators from imidazole derivatives. Oncol Res. 2004;14:355–362.

17 Sewell JM, Mayer I, Langdon SP, Smyth JF, Jodrell DI, Guichard SM. The mechanism of action of Kahalalide F: variable cell permeability in human hepatoma cell lines. Eur J Cancer. 2005; 41:1637–1644.

18 Zhang JY, Wu HY, Xia XK, Liang YJ, Yan YY, She ZG, et al. Anthracenedione derivative 1403P-3 induces apoptosis in KB and KBv200 cells via reactive oxygen species-independent mitochondrial pathway and death receptor pathway. Cancer Biol Ther. 2007;6:1413–1421.

19 Zhou JM, Zhu XF, Lu YJ, Deng R, Huang ZS, Mei YP, et al. Senescence and telomere shortening induced by novel potent G-quadruplex interactive agents, quindoline derivatives, in human cancer cell lines. Oncogene. 2006;25:503–511.

20 Liu JN, Deng R, Guo JF, Zhou JM, Feng GK, Huang ZS, et al. Inhibition of myc promoter and telomerase activity and induction of delayed apoptosis by SYUIQ-5, a novel G-quadruplex interactive agent in leukemia cells. Leukemia. 2007;21:1300–1302.

21 Chen Y, Mcmillan WE, Kong J, Israels SJ, Gibson SB. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ. 2008;15: 171–182.

22 Deng R, Li W, Guan Z, Zhou JM, Wang Y, Mei YP, et al. Acetyl-cholinesterase expression mediated by c-Jun-NH2-terminal kinase pathway during anticancer drug-induced apoptosis. Oncogene. 2006;25:7070–7077.

23 Yan YY, Su XD, Liang YJ, Zhang J, Shi C, Lu Y, et al. Emodin azide methyl anthraquinone derivative triggers mitochondrial-dependent cell apoptosis involving in caspase-8-mediated Bid cleavage. Mol Cancer Ther. 2008;7:1688–1697.

24 Oyama Y, Hayashi A, Ueha T, Maekawa K. Characterization of 2′,7′-dichlorofluorescin fluorescence in dissociated mammalian brain neurons: estimation on intracellular content of hydrogen peroxide. Brain Res. 1994;635:113–117.

25 Bindokas VP, Jordan J, Lee CC, Miller RJ. Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J Neurosci. 1996;16:1324–1336.

26 Deng R, Tang J, Xia LP, Li DD, Zhou WJ, Wang LL, et al. ExcisaninA, a diterpenoid compound purified from Isodon MacrocalyxinD, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of its signal pathway. Mol Cancer Ther. 2009;8: 873–882.

27 Nakamura Y, Yogosawa S, Izutani Y, Watanabe H, Otsuji E, Sakai T. A combination of indol-3-carbinol and genistein synergistically induces apoptosis in human colon cancer HT-29 cells by inhibiting Akt phosphorylation and progression of autophagy. Mol Cancer. 2009;12:1–15.

28 Tanida L, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2004;36: 2503–2518.

29 Kanzawa T, Germano TM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ. 2004;11:448–457.

30 Insil K, Sara RE, John JL. Minireview: selective degradation of mitochondria by mitophagy. Arch Biochem Biophys. 2007; 462:245–253.

31 Han YH, Park WH. The effects of N-acetyl cysteine, buthionine sulfoximine, diethyldithiocarbamate or 3-amino-1,2,4-triazole on antimycin A-treated Calu-6 lung cells in relation to cell growth, reactive oxygen species and glutathione. Oncol Rep. 2009;22:385–391.

32 Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct. 1998;23:33–42.

33 Wu Z, Chang PC, Yang JC, Chu CY, Wang LY, Chen NT, et al. Autophagy blockade sensitizes prostate cancer cells towards src family kinase inhibitors. Genes Cancer. 2010;1:40–49.

34 Fang J, Seki T, Maeda H. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. Adv Drug Deliv Rev. 2009;61:290–302.

35 Zhang H, Trachootham D, Lu W, Carew J, Giles FJ, Keating MJ, et al. Effective killing of Gleevec-resistant CML cells with T3151 mutation by a natural compound PEITC through redox- mediated mechanism. Leukemia. 2008;22:1191–1199.

36 Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implication. Drug Resist Updat. 2004;7:97–110.

37 Schumacker PT. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell. 2006;10:175–176.

38 Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature. 2000;407:390–395.

39 Benhar M, Engelberg D, Levitzki A. ROS stress-activated kinases and stress signaling in cancer. EMBO. 2002;3:420–425.

40 Cheng JP, Lane JD. Organelle dynamics and membrane trafficking in apoptosis and autophagy. Histol Histopathol. 2010;25: 1457–1472.

41 Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010;221:3–12.