Histone deacetylase inhibitor AR-42 inhibits breast cancer cell growth and demonstrates a synergistic effect in combination with 5-FU

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Abstract. AR-42 is a member of a novelly discovered class of phenylbutyrate-derived histone deacetylase inhibitors, and has a number of antitumor effects in a variety of tumor types; however, the role of AR-42 and its possible mechanisms have not been reported in the treatment of breast cancer. The aim of the present study was to investigate the antitumor effects of AR-42 and its associated mechanisms in breast cancer. MTT assays and colony formation assays were conducted to measure the proliferation of MCF-7 cells, and flow cytometry was used to analyze cell apoptosis. The results revealed that AR-42 induced cell apoptosis and suppressed cell growth in a dose- and time-dependent manner. Mechanistically, AR-42 treatment increased the acetylation of the p53 protein and prolonged the half-life of the p53 protein; furthermore, AR-42 treatment upregulated p21 and PUMA expression. Notably, AR-42 had a synergistic effect on MCF-7 cells in combination with fluorouracil, which is one of the most commonly used chemotherapeutic agents. In conclusion, the results indicated that AR-42 inhibits breast cancer cell proliferation and induces apoptosis, indicating that AR-42 is a potential therapeutic agent.

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Introduction

Breast cancer is one of the most common and malignant tumor types among females globally, which accounts for 30% all new cancer diagnoses in females (1). Additionally, with a global annual increase of ~200 million patients, the mortality rate is increasing each year (2). On average there is a female diagnosed with breast cancer every three minutes globally (3). In China, the annual incidence of female breast cancer has experienced a sharp increase from 3 to 4% of the female population, which is notably higher than the average global growth rate for the diagnosis of breast cancer (4). Chemotherapy remains an important breast cancer treatment; however, clinical practice has confirmed that 30-50% of patients with breast cancer are either not sensitive to the treatment or the treatment does not produce effective results (5). Rather, they demonstrate heart and kidney side effects, which frequently cause extensive physical and mental harm to patients (6). Thus, it is a common goal of doctors and patients to discover novel drugs that improve the efficacy and reduce the toxicity of cancer treatments.

An increasing number of studies have focused on histone deacetylation, which is an important epigenetic modification involved in the development of numerous malignant tumor types, including melanoma, leukemia, prostate cancer, lung cancer and colon cancer (7-10). In the case of breast cancer, histone deacetylation is closely associated with the apoptosis, differentiation and down-regulation of tumor suppressor gene expression and cell sensitivity to drugs (11,12). In the previous study, it was determined that the histone deacetylase (HDAC) regulator breast cancer metastasis-suppressor 1 like can regulate the activity of HDAC1/2 and inhibit the transcription of frizzled class receptor 10 and its downstream pathway, thus inhibiting the occurrence of epithelial-mesenchymal transition (EMT) in breast cancer (13). Inhibition of histone acetylase

activity can induce breast cancer cell apoptosis, promote cancer cell differentiation, reduce drug resistance and inhibit tumor cell proliferation and the occurrence of EMT in breast cancer cells (14); therefore, targeting the specific inhibition of protein acetylation of enzymes may present an alternative treatment strategy for breast cancer.

Apoptosis serves an important role in cancer treatment and is a popular target of numerous treatment strategies due to its disorder being closely associated with tumor development (15,16). In terms of cell growth arrest and apoptosis regulation, p53 serves an important role as a tumor suppressor (17,18). By inactivating p53, cancer cells can avoid arrest despite carrying genetic damage (18). Previous studies demonstrated that the apoptosis-stimulating proteins phorbol-12-myristate-13-acetate-induced protein 1, p21 and PUMA may affect the progression of breast cancer through mediating the p53 pathway (19-21); therefore, studying the p53 pathway may identify novel therapeutic methods for breast cancer.

Recent advances in HDAC inhibitors have been encouraging. This is a class of compounds that target HDAC and focus on the malignant proliferation of cells through selective inhibition of growth and induction of apoptosis (14). Additionally, a recent study also determined that inhibitors may reverse multidrug resistance of tumors, and significantly reverse cisplatin resistance in ovarian cancer and colorectal cancer cells (22,23). This demonstrates the potential research and developmental value of multidrug resistance drug reversal agents.

AR-42 is a novelly discovered class of phenylbutyrate protein deacetylase inhibitors that display localized enrichment in tumor tissues (24). AR-42 was initially determined to be effective in various blood tumor types, including leukemia, lymphoma and other blood tumor types, and it serves a role in the inhibition of tumor growth (25,26). Previous studies demonstrated AR-42 to have antitumor effects in solid tumor types, including hepatocellular carcinoma, ovarian cancer and pancreatic cancer (27-29). In addition, AR-42 was determined to have a synergistic effect with cisplatin (30), indicating a potential antitumor effect of AR-42. The role of AR-42 in breast cancer remains unclear; therefore, the aim of the present study was to investigate the antitumor effects of AR-42 and its associated mechanisms in breast cancer.

Materials and methods

Cell culture. The human breast cancer MCF-7 cell line was provided by the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and incubated in a 5% CO_2 atmosphere at 37˚C.

Antibodies and chemicals. AR-42 (Arno Therapeutics, Flemington, NJ, USA) and cycloheximide (CHX) were prepared in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The CHX chase assay identified the p53 degradation half-life. Anti-β-actin (cat. no. sc-47778; 1:1,000), rabbit anti-p53 (cat. no. sc-6243; 1:1,000) and anti-Ac-lysine (cat. no. sc-81623; 1:1,000) antibodies were purchased from Santa Cruz Biotechnology (New York, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse (cat. no. 7076; 1:2,000) and anti-rabbit (cat. no. 7074; 1:2,000) IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell viability assay. Cells were plated in 96-well plates at density of 5,000 cells/well and were treated with AR-42 (0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 μmol/l; cat. no. S2244; Selleck Chemicals, Houston, TX, USA) and/or 5-FU (0, 0.25, 0.5, 0.1 and 0.2 μmol/l; cat. no. F6627; Sigma Aldrich; Merck KGaA). After 72 h of drug exposure at 37˚C, cells were treated with MTT solution (5 mg/ml), to dissolve the purple formazan, for an additional 4 h at 37˚C, and the optical density (OD)490 value was detected using enzyme labeling apparatus (ELx800 Strip Reader; BioTek Instruments, Inc., Winooski, VT, USA), reflecting the number of viable cells. Cytotoxicity (%)=(1-OD490 of experimental well)/OD490 of control well.

Colony formation assays. The cells were digested with 0.25% trypsin solution at 37˚C for 2 mins and prepared for single cell suspension. They were seeded into 6-well plates (Wuxi Nest Biotechnology, Co. Ltd., Jiangsu, China) with 1,000 cells/per well cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), then gently shook to evenly disperse in the cells normal culture conditions. Following incubation at 37˚C for two weeks, the cells were carefully washed twice with PBS, fixed for 15 min with 100% methanol and stained for 15 min with 0.1% crystal violet dye at room temperature. Water was then used to slowly wash away the dyeing liquid, and the cells were left to dry naturally in the air. The 0 μM AR-42 was the negative control group. Each experiment was repeated at least three times.

Apoptosis assay. MCF-7 cells were treated with 0, 0.025, 0.05, 0.1 or 0.2 μM AR-42 at 37˚C for 48 h in culture conditions, and then harvested and washed with PBS twice. The cells were collected and stained with an Annexin V/PI double flow cytometry kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China), according to the manufacturer's protocol, to detect the cell apoptosis rate. The 0 μM AR-42 were the negative control. Each experiment was repeated at least three times.

Analysis of in vitro drug interaction. Analysis of the drug's synergistic inhibitory effect was determined using the coefficient of drug interaction (CDI) metric. CDI was calculated as follows: CDI=AB/(A*B). A or B is the ratio of the single drug group to the control group in OD490 and AB represents the ratio of the two-drug combination group to the control group in OD490. CDI>1 signifies antagonism, CDI=1 indicates additivity, and CDI<1 indicates synergism. A CDI<0.7 was considered to indicate a statistically significant synergistic effect.

Western blotting and immunoprecipitation. Procedures were conducted as previously described (31). Following extraction of total cellular protein, the protein concentration was determined by the BCA method. The sample was mixed

with loading buffer (cat. no. P0015; Beyotime Institute of Biotechnology, Shanghai, China) and placed in a boiling water bath for 10 min, and following 10% SDS-PAGE electrophoresis (30 μg/lane) was transferred to a polyvinylidene fluoride membrane. The membranes were incubated in 5% skim milk at room temperature for 2 h. The membranes were incubated with antibodies against human p53 or β-actin overnight at 4˚C and incubated at room temperature for 2 h with anti-mouse and anti-rabbit IgG HRP-conjugated antibodies. The chemiluminescence system was exposed to enhanced chemiluminescent (Advansta, Inc., Menlo Park, CA, USA; R-03025-D25), and the experiment was repeated three times. ImageJ 1.50f software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis of the experimental data.

To investigate the interaction between AR-42 and Ack-p53 at the endogenous level, the clarified supernatants were first incubated with anti-p53 or anti-Ac-lysine for 2 h at 4˚C. Protein A/G-agarose was then added and incubated for 2 h to overnight. Precipitates were washed four times with RIPA buffer (Beyotime Institute of Biotechnology) and analyzed by western blotting as aforementioned. ImageJ 1.50f software was used for densitometric analysis of the experimental data.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. cDNA was synthesized with the MLv transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.). The quantitative analysis of p21 and puma expression was assayed using a SYBR® green kit (Takara Bio, Inc., Otsu, Japan) with gene-specific primers. Primer sequences for p21, puma, and β-actin were as follows: p21, forward,. 5'-GCTCGGCTCTTCACCAAG-3', and reverse, 5'-GTCACTGTCTTGTACCCTTGTG-3'; puma, forward, 5'-CGACCTCAACGCACAGTACGA-3', and reverse, 5'-AGGCACCTAATTGGGCTCCAT-3'; β-Actin, forward, 5'-GGTGGCTTTTAGGATGGCAAG-3', and reverse, 5'-ACTGGAACGGTGAAGGTGACAG-3'. β-Actin was used as a normalization control. The standard PCR conditions were: 95˚C for 15 mins, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, and 72˚C for 40 sec. The fold changes were calculated through relative quantification with $2^{-\Delta\Delta Cq}$ (32). All of the reactions were performed in a 20 μl reaction volume in triplicate. These experiments were repeated at least three times independently.

Statistical analysis. SPSS 16 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the experimental data. Values are expressed in triplicate and presented as the mean ± standard deviation. Comparisons were conducted using One-way analysis of variance followed by Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AR-42 inhibits the growth of the MCF-7 cell line in a dose- and time-dependent manner. Previous studies have demonstrated the antitumor effects of AR-42 in blood tumor and solid tumor types (25-29). To investigate the antitumor effects of AR-42 in breast cancer, the MCF-7 cell line was treated with AR-42 at concentrations of 0-0.8 μM for different periods of time. The MTT assay was used to evaluate cell viability. As depicted in Fig. 1A and B, AR-42 inhibits MCF-7 cell growth in a dose- and time- dependent manner. Furthermore, the colony formation assays (Fig. 1C) indicated that AR-42 inhibited colony formation in a dose-dependent manner.

AR-42 inhibits the growth of MCF-7 cells by promoting apoptosis. Inhibition of cell growth may be the result of apoptosis, induction of necrosis or cell cycle arrest (15). Apoptosis is a programmed cell death process regulated by multiple genes, and its disorder is closely associated with the development of tumors (16). To detect whether AR-42 affects the apoptotic rate of MCF-7 cells, flow cytometry was performed on the cells. As depicted in Fig. 2, following treatment with 0-0.2 μM AR-42 for 48 h, the survival ratios of MCF-7 cells were decreased from 86.05% to 81.77, 74.18, 63.51 and 60.86% as the AR-42 concentration increased from 0.0 to 0.2 μm (Fig. 2. $P<0.01$ vs. control group). These results indicated that AR-42 inhibits the growth of MCF-7 cells by promoting cell apoptosis.

AR-42 induces MCF-7 cell apoptosis by increasing the acetylation level of p53. Acetylation has been reported to increase the activity and stability of p53 (33,34). To understand the mechanisms underlying AR-42 mediated apoptosis in MCF-7 cells, whether AR-42 affects p53 acetylation level was investigated. As depicted in Fig. 3A and B, the expression of acetylated p53 was elevated following treatment with AR-42. Furthermore, the CHX chase assay results indicated that AR-42 prolonged the half-life of the p53 protein, indicating that AR-42 increased the stability of the p53 protein. p21 and PUMA are important downstream transcriptional targets of p53 (20); their mRNA levels following AR-42 treatment in MCF-7 cells was examined. As depicted in Fig. 3C, AR-42 treatment induced p21 and PUMA expression in MCF-7 cells in a dose-dependent manner.

AR-42 combined with 5-FU has a synergistic inhibitory effect on MCF-7 cells. Chemotherapy remains an important breast cancer treatment. As a conventional chemotherapy drug, 5-FU can effectively improve the survival rate of clinical patients (35). Previous study demonstrated that 5-FU has serious side effects, such as cytotoxicity, leading to a narrow therapeutic effect (35); therefore, it's necessary to investigate possible synergistic effects of combining 5-FU and AR-42 in MCF-7 cells. As depicted in Fig. 4A, the results revealed that AR-42 increased the cytotoxicity of 5-FU towards MCF-7 cells. The synergistic effect is indicated as a CDI value. As presented in Fig. 4B, 0.25 μM 5-FU combined with 0.2 μM AR-42 had the most significant synergistic effect (CDI<0.7).

Discussion

Breast cancer is known to be one of the most widespread and prevalent tumor types globally (1). Breast cancer cases in China account for 12% of the global total novel diagnosed cases of breast cancer each year (4); therefore, it is necessary to investigate novel strategies for breast cancer treatment.

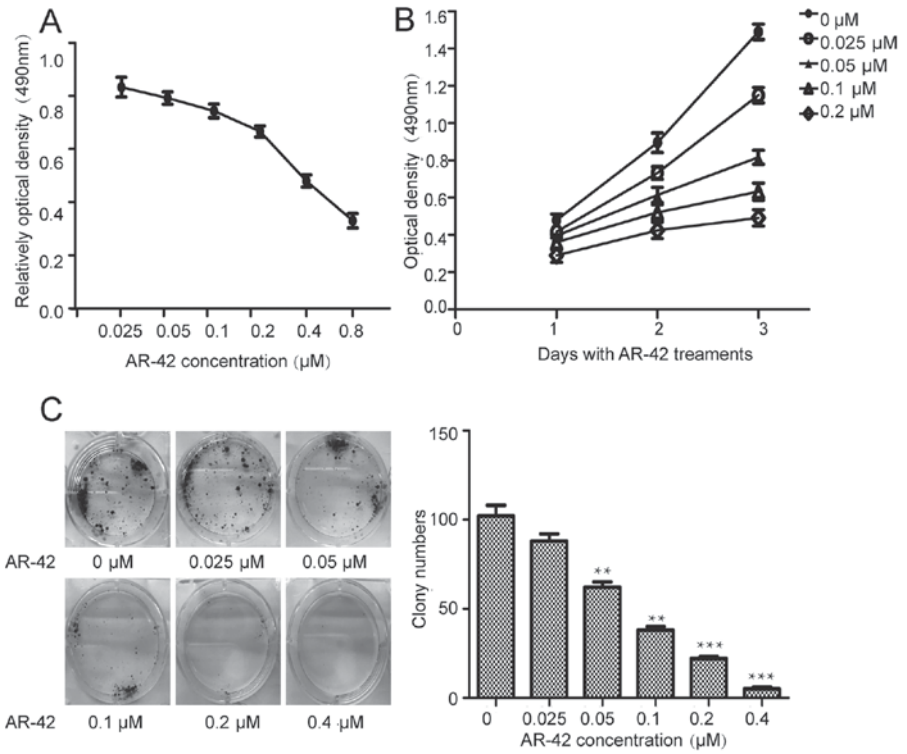

Figure 1. AR-42 inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. (A) MCF-7 cells underwent treatment with various concentrations of AR-42 (0.0-0.8 μM) for three days. The average of three independent experiments is displayed. (B) A total of 0.0-0.2 μM AR-42 was administered to MCF-7 cells for three days, and the absorbance was quantified daily. The average of three independent experiments is displayed. (C) MCF-7 cells were plated into 6-well plates (1,000 cells/well). The medium with 0.0-0.4 μM AR-42 was changed every four days, and for colony assays, the cells were stained with crystal violet two weeks following each treatment. **P<0.01; ***P<0.001 vs. control group. OD, optical density.

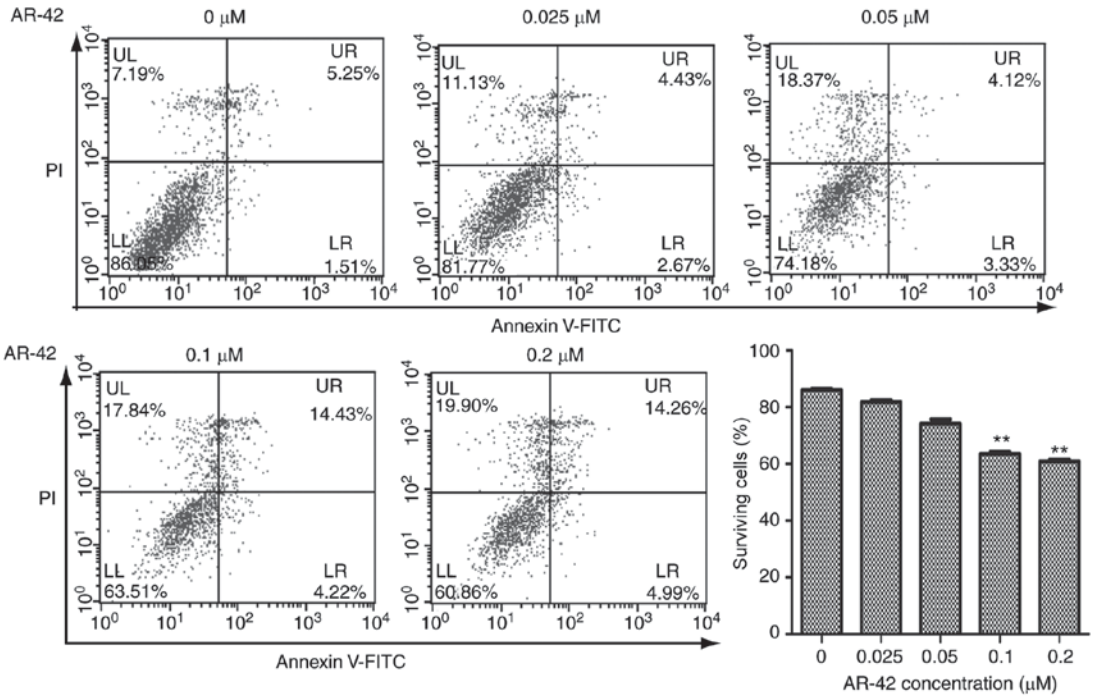

Figure 2. AR-42 inhibited the growth of MCF-7 cells by promoting apoptosis. Cell apoptosis levels were evaluated by flow cytometry. Apoptosis analysis was conducted on cells treated with (0.0-0.2 μM) AR-42 for 48 h. n=3. Surviving cells were determined to be the total Annexin V/FITC-negative cells. Data are presented as the mean ± standard deviation, n=3. **P<0.01 vs. control group. PI, propidium iodide; FITC, fluorescein isothiocyanate.

HDAC inhibitors represent a novel anticancer therapeutic strategy (36). A significant number of HDAC inhibitors have been developed in the past decade (3). Previous studies have demonstrated that AR-42, a member of a novelly discovered

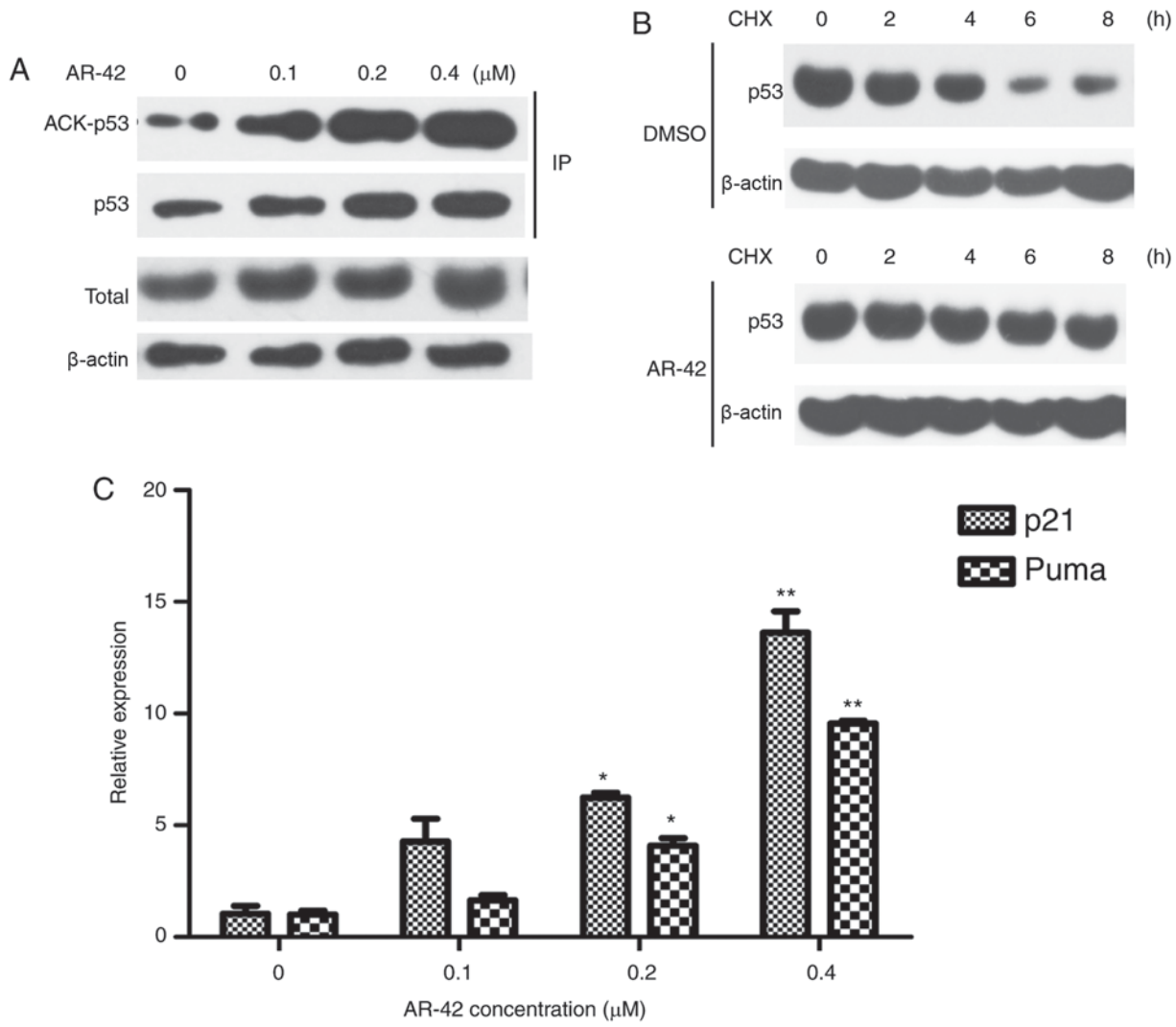

Figure 3. AR-42 induced MCF-7 cells apoptosis by increasing p53, its acetylation and expression of p21 and PUMA. (A) AR-42 treatment upregulated p53 and its acetylation. Western blotting and immunoprecipitation procedures were conducted on MCF-7 cells treated with 0.0-0.4 μM AR-42 for 48 h. (B) AR-42 prolonged the half-life of the p53 protein, as measured by the CHX chase assay, which identified the p53 degradation half-life. (C) p21 and PUMA were expressed in a dose-dependent manner in MCF-7 cells in response to AR-42 treatment. Using reverse transcription-quantitative polymerase chain reaction, the gene expression of p21 and PUMA was determined in cells treated with 0-0.4 μM AR-42 for 48 h. Data are the mean of at least three independent experiments. *P<0.05; **P<0.01 vs. control group. CHX, cycloheximide; DMSO, dimethyl sulfoxide; IP, immunoprecipitation.

class of phenylbutyrate-derived HDAC inhibitors, has been demonstrated to have antitumor effects in blood tumor and solid tumor types (24-26). Apoptosis disorders are closely associated with the development of tumors (15); therefore, the induction of tumor cell apoptosis may be an efficient strategy to prevent tumor progression. In the present study, it was demonstrated that AR-42 inhibited the proliferation of MCF-7 breast cancer cells by indirectly regulating acetylation of the p53 protein, which also indicated a synergistic effect when combined with 5-FU. The results indicated that the proliferation of breast cancer cells may be suppressed by AR-42 treatment, which may prove to be an alternative therapeutic approach for the future treatment of breast cancer.

Apoptosis is a programmed cell death process regulated by multiple genes, and its disorder is closely associated with the development of tumors (15). In terms of cell growth arrest and apoptosis regulation, p53 serves an important role as a tumor suppressor by inducing extrinsic and intrinsic apoptotic pathways to ensure efficient death responses (17,37). Acetylation is essential for p53 activity, and its function is important in transcriptional activation and senescence (33,34). Acetylation of p53 affects cell activity and function, such as Lys-373 and Lys-382 acetylation, which inhibits p53 degradation, thereby mediating apoptosis and enhancing cell drug sensitivity (34). It has been demonstrated that treatment of colon cancer cells with SIRTI siRNA or the HDACI MS275 can increase p53 acetylation levels and enhance paclitaxel-induced apoptosis (38). The SIRT1 small molecule inhibitor Tenovin-1 can inhibit increased p53 expression and activity levels and inhibit melanoma cell proliferation (38,39). As depicted in Fig. 3, the results indicated that AR-42 treatment increased the level of p53 protein and its acetylation formation. In addition, AR-42 treatment significantly prolonged the half-life of the p53 protein. It is notable that p53 acetylation itself is effective in stabilizing and increasing the level of total p53 (40).

As a result, the accumulation of the p53 protein and its acetylation may be the consequence of AR-42-mediated apoptosis in MCF-7 cells. The tumor suppressor protein p53 affects

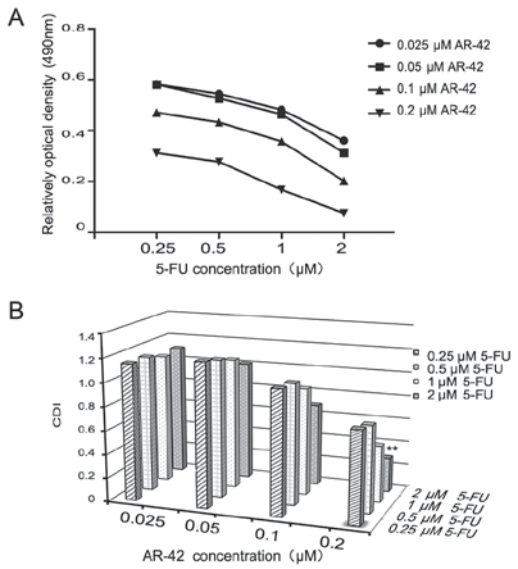

Figure 4. Synergistic inhibitory effect of AR-42 combined with 5-FU in MCF-7 cells. (A) MCF-7 cells were either treated exclusively with 0.25-2.0 μM 5-FU or combined with 0.025, 0.05, 0.1 or 0.2 μM AR-42. Cell viability was examined using an MTT assay three days following treatment. (B) Analysis of the synergistic inhibitory effect of different concentrations of AR-42 and 5-FU was revealed using CDI. n=3. **P<0.01 vs. 0.25 μM 5-FU and 0.025 μM AR-42. CDI, coefficient of drug interaction; OD, optical density; 5-FU, fluorouracil.

a vast number of downstream targets and performs a braking function by blocking injured cells from entering the cell cycle and promoting apoptosis (18). Among these downstream targets are p21 and PUMA, which are major mediators of the function of p53 (41). Previous studies performed on other cancer cell lines have demonstrated that HDAC inhibition results in the upregulation of p21 and PUMA expression through increasing the acetylation of p53 (20,21,42). In the present study, AR-42 increased p21 and PUMA expression, confirming p53 activation; therefore, AR-42 treatment may successfully induce molecular mediators of cell cycle arrest and apoptosis.

Currently, chemotherapy remains an important breast cancer treatment. As a conventional chemotherapy drug, 5-FU can effectively improve the survival rate of clinical patients. However, it has serious limitations, such as cytotoxicity, which leads to its narrow therapeutic effect (35,43). Thus, combining 5-FU with other pharmaceutical agents will provide more effective ways to sensitize cancer cells to chemotherapy, while reducing toxicity to normal cells (35). Combination therapy may achieve fewer side effects and greater therapeutic efficacy. The results indicated that AR-42 increased the cytotoxicity of 5-FU and indicated a significant synergistic effect when combined with 5-FU, which indicates that the combined treatment of AR-42 and 5-FU may be an effective strategy for treating breast cancer.

5-FU serves an important role in early breast cancer treatment following adjuvant therapy, breast cancer recurrence and metastasis following palliative care (43,44). It is an anti-metabolic chemotherapeutic agent, acting mainly through the irreversible inhibition of thymidylate synthase (TS), which results in a lack of thymine and the synthesis of non-functional DNA (35). A previous study demonstrated that p53 is important for drug sensitivity to TS inhibitors, such as 5-FU (45). It has been previously reported that acetylation of p53 is essential for preventing degradation and opening a conformation that allows binding to DNA (46). 5-FU mediated DNA damage activates numerous signaling pathways, such as p53-mediated apoptosis. The results indicated that AR-42 treatment caused MCF-7 cell apoptosis. It was demonstrated that AR-42 and 5-FU have the ability to activate p53-mediated apoptosis, which may clarify how AR-42 synergistically combines with 5-FU to restrain the growth of MCF-7 cells; however, the specific mechanisms underlying the synergistic effect of AR-42 and 5-FU require further study.

In conclusion, the present study investigated the anti-tumorigenic role of AR-42 in breast cancer cells. The results demonstrated that AR-42 inhibits the proliferation of MCF-7 breast cancer cells via the induction of cell apoptosis. Notably, combination assays demonstrated that joint AR-42 and 5-FU treatment have a significant synergistic effect on MCF-7 breast cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RZ and JW designed the research, analyzed data, and wrote the manuscript. XT, XW, CJ, FZ performed experiments and prepared the figures. JS, DS and ZZ were involved in drafting the manuscript, revising it critically for important intellectual content, designed the study and acquired the data. XBL and QL participated in the design of the study and helped finalize the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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