Send Orders for Reprints to reprints@benthamscience.net

Current Pharmaceutical Design, 2020, *26*, 1-15 1

RESEARCH ARTICLE

Autophagic Vacuole Secretion Triggered by Chidamide Participates in TRAIL Apoptosis Effect in Breast Cancer Cells

Weiqiang Zhou[2,#,*], Han Han[3,#], Junnan Xu[4], Tao Sun[4,*] and Xiuyan Feng[1,*]

[1]*The Second Affiliated Hospital of Shenyang Medical College, No.20 North 9th St, Heping Dis, Shenyang City, Liaoning Pro 110002, P. R. China;* [2]*Department of Pathogen Biology, Shenyang Medical College, No.146 North Huanghe St, Huanggu Dis, Shenyang City, Liaoning Pro 110034, P. R. China;* [3]*Department of Biochemistry and Molecular Biology, Shenyang Medical College, No.146 North Huanghe St, Huanggu Dis, Shenyang City, Liaoning Pro 110034, P. R. China;* [4]*Department of Medical Oncology, Cancer Hospital of China Medical University, Liaoning Cancer Hospital and & Institute, Key Laboratory of Liaoning Breast Cancer Research, No.44 Xiaoheyan Rd, Dadong Dis, Shenyang City, Liaoning Pro 110042, P.R. China*

ARTICLE HISTORY

Received: February 26, 2020
Accepted: July 31, 2020

DOI:
10.2174/1381612826666200811175513

Abstract: ***Background:*** Breast cancer is one of the most prevalent diseases threatening women's health today. In-depth research on breast cancer (BC) pathogenesis and prevention and treatment methods are gradually receiving attention. Chidamide is a novel histone deacetylase inhibitor (HDACi) that depresses the function of histone deacetylase, consequently affecting the growth of BC cells through epigenetic modification. However, preclinical and clinical studies show that chidamide is ineffective in long-term treatment. We demonstrated in previous experiments that TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in BC cells and is significantly less non-toxic to normal cells than chidamide. Therefore, in this study, we treated BC cells with chidamide and TRAIL to explore a novel option to reduce the clinical toxicity through augmenting the sensitivity for BC cells.

Methods and Results: Results from the MTT and cell viability assays indicated that the combination of chidamide and TRAIL in MCF-7 and MDA-MB-231 cells induced BC cell death, while maintaining a reduced concentration of chidamide. Autophagy assay and annexin V analysis showed that the autophagosome microtubule-associated protein1light chain3- II (LC3-II) was abnormally increased and much more early and late phase of apoptotic cells appeared during chidamide and TRAIL induction. Anti-tumor assays in a BC tumor xenograft model displayed that the mixture of chidamide and TRAIL exhibited stronger effects on inhibiting tumor growth. The data from real-time PCR and western blotting showed that the cytotoxic effect correlated with the expressions of related apoptosis and autophagy factors.

Conclusion: Our data are the first to demonstrate the synergistic effects of chidamide and TRAIL in BC cells, specifically, the pharmacological effects on cell death induction. These results lay a solid experimental and theoretical basis to solve the clinical resistance of chidamide.

Keywords: Chidamide, TRAIL, Apoptosis, drug resistance, Breast Cancer.

1. INTRODUCTION

The status report on the global burden of cancer across 20 world regions estimated 18.1 million new cases and 9.6 million cancer deaths in 2018. For both sexes combined, breast cancer (BC) is the second most commonly diagnosed cancer (11.6% of the total cases), with 2.1 million newly diagnosed female breast cancer cases being reported in 2018. This accounts for almost one in four cancer cases among women [1]. Approximately 60–70% of breast cancers are estrogen receptor (ER) or progesterone receptor (PR) positive, and 15–30% of cases have gene amplification and overexpression of the human epidermal growth factor receptor 2 (HER2) proteins. Additionally, 10–15% of breast cancers are called triple-negative (TNBC), defined by the lack of ER and PR expression and HER2 amplification [2]. While chemotherapy has proven to be more effective in the control of aggressive and metastatic BC in recent years, BC remains an incurable disease that has two obstacles to achieving successful treatment in a majority of patients: (i) adverse toxicities for healthy persons, and (ii) chemotherapy resistance and relapse [3-6].

Researchers have found that epigenetic changes have more significance in the occurrence and development of BC than was previously thought [7–9]. Histone acetylation, which regulates the homeostasis between histone acetyltransferase and histone deacetylase, is one of the most important epigenetic modifications participating in breast carcinogenesis [10-11].

Chidamide is a novel and potential HDACi against the major class I HDAC subtypes (HDAC1, 2, 3), which are closely related to tumorigenesis and disease progression. Chidamide has shown significant anti-tumor effects in the treatment of leukemia, pancreatic cancer, and colon cancer [12]. Also, chidamide was reported to be used for breast cancer. The study revealed that chidamide repressed the growth of triple-negative breast cancer cells, likely by the functioning of miR-33a-5p [13]. However, chidamide alone used clinically results in side effects such as anorexia, diarrhea, fatigue, nausea, thrombocytopenia, and vomiting. Moreover, like other antitumor drugs, chidamide also has a high cytotoxic effect and long-term drug resistance. Consequently, clinicians prefer to use HDAC inhibitors in combination with other drugs [14-16].

TRAIL (or Apo2L) can induce apoptosis in a variety of cells [17–20]. Interestingly, one of the crucial properties of TRAIL is its

*Address correspondence to these authors at the The Second Affiliated Hospital of Shenyang Medical College, No.20 North 9th St, Heping Dis, Shenyang City, Liaoning Pro 110002, P. R. China; Tel: 86-024-3123-6509; Fax. 86-024-6221-5656; Email: fengxy1972@hotmail.com; Department of Pathogen Biology, Shenyang Medical College, No.146 North Huanghe St, Huanggu Dis, Shenyang City, Liaoning Pro 110034, P. R. China; Tel: 86-024-6221-6825; Fax. 86-024-6221-5656; Email: zhouwq@hotmail.com; Department of Medical Oncology, Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, No.44 Xiaoheyan Rd, Dadong Dis, Shenyang City, Liaoning Pro 110042, P.R. China; Tel: 86-024-3191-6684; Fax. 86-024-6221-5656; E-mail: jianong@126.com
[#]*These authors contributed equally.*

1381-6128/20 $65.00+.00

selective cytotoxicity against cancer cells. Due to variations in drug resistance, overcoming drug resistance is only possible by co-administrating compounds with multiple functions that not only target the cancer cells with selective cytotoxicity but also protect healthy cells against chemo-toxicity. Therefore, in this study, we selected BC ER-positive MCF-7 cells and triple-negative MDA-MB-231 cells to investigate the pharmacological response of breast cancer cells initiated by chidamide and TRAIL.

2. MATERIALS AND METHODS

2.1. Cell Lines and Reagents

Human MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Leibovitz's L-15 medium, *RPMI-1640 medium,* Fetal Bovine Serum (FBS) and Penicillin-streptomycin Cocktails were purchased from Thermo Scientific (Rockford, IL). Chidamide was supplied by Chipscreen Biosciences Ltd. (Shenzhen, China). The CellTiter 96® AQueous One Solution Cell Proliferation assay was from Promega (Madison, MI). Muse Count & Viability kit and Muse Annexin V kit were from Millipore (Darmstadt, Germany). High Pure RNA Isolation kit and Transcriptor First Strand cDNA Synthesis kit were given from Roche Diagnostics GmbH (Mannheim, Germany). TRAIL powder, power SYBR Green PCR Master mix, RIPA Cell Lysis buffer and BCA Protein Assay kit were from Life Technologies (Austin, TX). Polyclonal anti-Bid antibody, polyclonal anti-ULK1 antibody, polyclonal anti-ATG4A antibody, polyclonal anti-ATG7 antibody, polyclonal anti-ATG9B antibody, polyclonal anti-caspase 3 antibody, polyclonal anti-caspase 8 antibody, polyclonal anti-CTSB antibody, polyclonal anti-LC3B antibody, polyclonal TRAIL DR5 antibody and polyclonal anti-β actin antibody were obtained from Abcam Inc (Cambridge, MA). Protease inhibitor and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

MCF-7 and MDA-MB-231 cells were grown in Leibovitz's L-15 medium or *RPMI-1640 medium,* respectively, with 15% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. All the cells were maintained at 37℃ with 5% CO_2 and 95% humidity. The cells were seeded at the density of 1.0×10^4 cells/ml in 96-well plate, 5.0×10^5 cells/ml in 6-well plate and 1.5×10^7 cells/ml in 100 mm dish. The cells were grown to 70-80% confluence and starved for 24 hours in basal medium (with DMSO) without FBS and treated with different compounds.

2.2.1. Chidamide Concentration-response Effects

MCF-7 and MDA-MB-231 cells were plated in 96-well plate (5.0×10^3 cells/ml). After 24 hours starvation, the cells were treated with different concentrations of chidamide (0, 0.5, 1, 2, 5, 10 and 20 µM) with or without TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) for 24 or 48 hours incubation. The same concentrations of DMSO were added as a control. The CellTiter 96® AQueous One Solution Cell Proliferation assay (MTT) was used and absorbance was measured at 490nm on a microplate reader.

2.3. Cell Viability and Apoptosis Assay

MCF-7 and MDA-MB-231 cells were cultured in 96-well plate and treated with different concentrations of chidamide with or without TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) as described previously. Muse Count & Viability reagent was used to assess cell viability. 2×10^5 of harvested cells (50 µl cell suspension) was added with 450 µl Count & Viability reagent. The results were obtained with Muse Count & Viability software module in Muse Cell Analyzer, and the statistics showed the concentrations and percentages of viable and dead cells.

For the apoptotic assay, 1×10^6 of cells were added with 100 µl of Muse Annexin V and Dead Cell reagent for 20 min at room temperature. Muse Cell Analyzer was determined the percentages of the cells represented by alive, apoptosis and dead population.

2.4. Autophagosome Associated LC3-II assay

MCF-7 and MDA-MB-231 cells were treated with chidamide and TRAIL at a 4×10^4/ml density in the 96-well plate for 48 hours, and then the cells were stained with anti-LC3/Alexa Fluro® 555 conjugated antibody for 4 hours. The autophagosome associated LC3-II (lipidated and sequestered in the autophagosomes) from cytosolic LC3-I was achieved by using autophagy enabling solution following the manufacturer's recommendations. Trapped LC3-II was measured using Muse Cell Analyzer, and statistical results were shown by the corresponding histogram plot.

2.5. Establishment of Breast Cancer Tumor Xenograft Models

For the establishment of breast cancer tumor xenograft models, the female nude mice were subcutaneously injected with 1×10^7 MDA-MB-231 cells suspended in 100 µL PBS. Length (L) and width (W) of the tumor was determined by a vernier caliper. The tumor volume (V) was calculated according to the equation: $V = L \times W^2/2$.

Once the tumor reached 80 mm^3, the tumor-bearing mice were randomized into treatment groups. The mice were intravenously administrated with chidamide or chidamide + TRAIL (12 mg/kg chidamide and 0.2 mg/kg TRAIL) and saline as a negative control for every two days (totally 12 injections). The tumor size and bodyweight of the mice were monitored every 2 days. On day 23, mice were sacrificed. The tumors were harvested and weighted.

2.6. cDNA Synthesis and Real-time PCR Analysis

Total RNA was extracted from MCF-7 and MDA-MB-231 cells using the High Pure RNA Isolation kit according to the manufacturer's instructions. RNA quantitation was performed via real-time PCR. The total RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis kit and amplified by Power SYBR Green PCR Master mix in an Applied Biosystems 7500 real-time PCR system. The primers were designed by Primer 3 suit and libraries. The sequences of primers were in Table **1**. Data normalization was based on correcting all C_t values for the average C_t values of the GAPDH gene present on the array. Three independent biological replicates were performed.

2.7. Western Blot Analysis

The MCF-7 and MDA-MB-231 cells pellet collected from 6-well plate were incubated in RIPA buffer containing 0.1 mg/ml protease inhibitor, 1mM PMSF. The cellular lysate was rotated for 2 hours at 4°C followed by centrifugation for 10 minutes at 14,000 g at 4°C. Proteins were quantified using the BCA protein assay kit. For immunoblotting, 20µg proteins were separated by SDS-polyacrylamide gels electrophoresis and transferred to PVDF membranes. Western blot analyses were performed using the antibodies described above. The level of β-actin was used as loading controls. Protein bands were detected using ECL Western blot substrate and exposed on DNR MF-Chemi Bio-Imaging Systems.

2.8. Data analysis

All data in the text and figures are provided as means±S.D. The results were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey Post hoc comparisons. All of the analyses were performed using the Statistical Package for Social Science (SPSS) software v22.0 (IBM, Armonk, NY). $p < 0.05$ was considered significant.

3. RESULTS

3.1. Only a High Dose of Chidamide can Show the Cytotoxic Effect on Breast Cancer Cells

Varied types of breast cancer cells had different drug sensitivities to chidamide treatment, MCF-7 cells were mildly sensitive to chidamide treatment. After 24 hours of incubation, high concentrations of chidamide (10–20 µM) inhibited the growth of MCF-7 cells (Fig. **1A**), and the half-maximal inhibitory *concentration* (IC50) of chidamide after 48 hours was 20 µM (Fig. **1C**). MDA-MB-231 cells were more sensitive to chidamide, with 5 µM chidamide visibly inhibiting cell growth (Fig. **1B**). However, the IC50 effect was also 20 µM chidamide after 48 hours of treatment (Fig. **1D**).

To verify the cytotoxicity of chidamide in breast cancer cells, we quantified cell viability with the Muse Count & viability reagent on a Muse Cell Analyzer. After 24 hours, treatment with 0.5–5 µM chidamide had little effect on MCF-7 and MDA-MB-231 cells. Cell mortality increased from 3.8% to 5.8% in MCF-7 cells and from 4.5% to 7.2% in MDA-MB-231 cells compared to the controls. Only high concentrations of chidamide (10 µM or 20 µM) exhibited a pharmacological effect, as cell mortality rose more than 10% in this group compared to controls (Fig. **2A** and 3A). However, within 48 hours of treatment, the cytotoxic effects of the varying chidamide concentrations in breast cancer cells were significantly different. A chidamide concentration of 20 µM exhibited the highest effect on cell death in both cell lines (Fig. **2B**, **3B**).

Table 1. The primers used for detecting factors are summarized in the table.

Gene Name	Primer Orientation	Sequence
ULK1	Forward	5’-GTAAACTGGGGTCGCATTGT-3’
	Reverse	5’-TGGATCCAAGGCTCTAGGTG-3’
ATG3	Forward	5’-TTTGGCTATGATGAGCAACG-3’
	Reverse	5’-GTGGCAGATGAGGGTGATTT-3’
ATG4A	Forward	5’-AAAAATGTGCCGTGTCCTTC-3’
	Reverse	5’-GCAGAGGTGCCCTTACTCTG-3’
ATG5	Forward	5’-GCAAGCCAGACAGGAAAAAG-3’
	Reverse	5’-GACCTTCAGTGGTCCGGTAA-3’
ATG7	Forward	5’-GAACATGGTGCTGGTTTCCT-3’
	Reverse	5’-CATCCAGGGTACTGGGCTAA-3’
ATG9B	Forward	5’-CCTTGGGCAGTTCTTCTTTG-3’
	Reverse	5’-CTTCCTGGTGCCTGGTACAT-3’
ATG10	Forward	5’-GCATTGTAGGGCCAGTTGTT-3’
	Reverse	5’-GCTGGCCAGGTAAACTCTTG-3’
ATG12	Forward	5’-AGGTCTGTAGTCGCGGAGAA-3’
	Reverse	5’-GTTCCCGGCTAGTCATTCAA-3’
ATG16L2	Forward	5’-CCAGATCATCCCTGTGTGTG-3’
	Reverse	5’-CGAACAGCATTGACCTCAGA-3’
Beclin 1	Forward	5’- AGGTTGAGAAAGGCGAGACA-3’
	Reverse	5’- GCTTTTGTCCACTGCTCCTC-3’
LC3B	Forward	5’-AACGGGCTGTGTGAGAAAAC-3’
	Reverse	5’-AGTGAGGACTTTGGGTGTGG-3’
Bax	Forward	5’-GACGGCCTCCTCTCCTACTT-3’
	Reverse	5’-CCTCCCAGAAAAATGCCATA-3’
Bcl-2	Forward	5’-GGATGCCTTTGTGGAACTGT-3’
	Reverse	5’-AGCCTGCAGCTTTGTTTCAT-3’
Bid	Forward	5’-GTGCAACACTGGTCTGCTGT-3’

(Table 1) Contd....

Gene Name	Primer Orientation	Sequence
	Reverse	5’-CCTCATGTTGTGGTCACAGG-3’
Caspase 2	Forward	5’-ATTGGATCCCTTGGGCACCT-3’
	Reverse	5’-GGCAGGCATAGCCGCATATC-3’
Caspase 3	Forward	5’-GGTTCATCCAGTCGCTTTGT-3’
	Reverse	5’-CGGTTAACCCGGGTAAGAAT-3’
Caspase 8	Forward	5’-CACGTATGGTGGCTCATGTC-3’
	Reverse	5’-ACGGGGTCTTGTTCTGTCAC-3’
CTSB	Forward	5’- AGCAGGCCCTCTTTCCATCC -3’
	Reverse	5’- GCAGGCAGCTTCAGGTCCTC -3’
TRAIL DR5	Forward	5’-ATTTCAGCCTCTTTCCAGCA-3’
	Reverse	5’-CGGAACAAAACACACAATGC-3’
Nf-κB1 p65	Forward	5’- TCTGCTTCCAGGTGACAGTG -3’
	Reverse	5’- ATCTTGAGCTCGGCAGTGTT -3’
GAPDH	Forward	5’-GAGTCAACGGATTTGGTCGT-3’
	Reverse	5’-GACAAGCTTCCCGTTCTCAG-3’

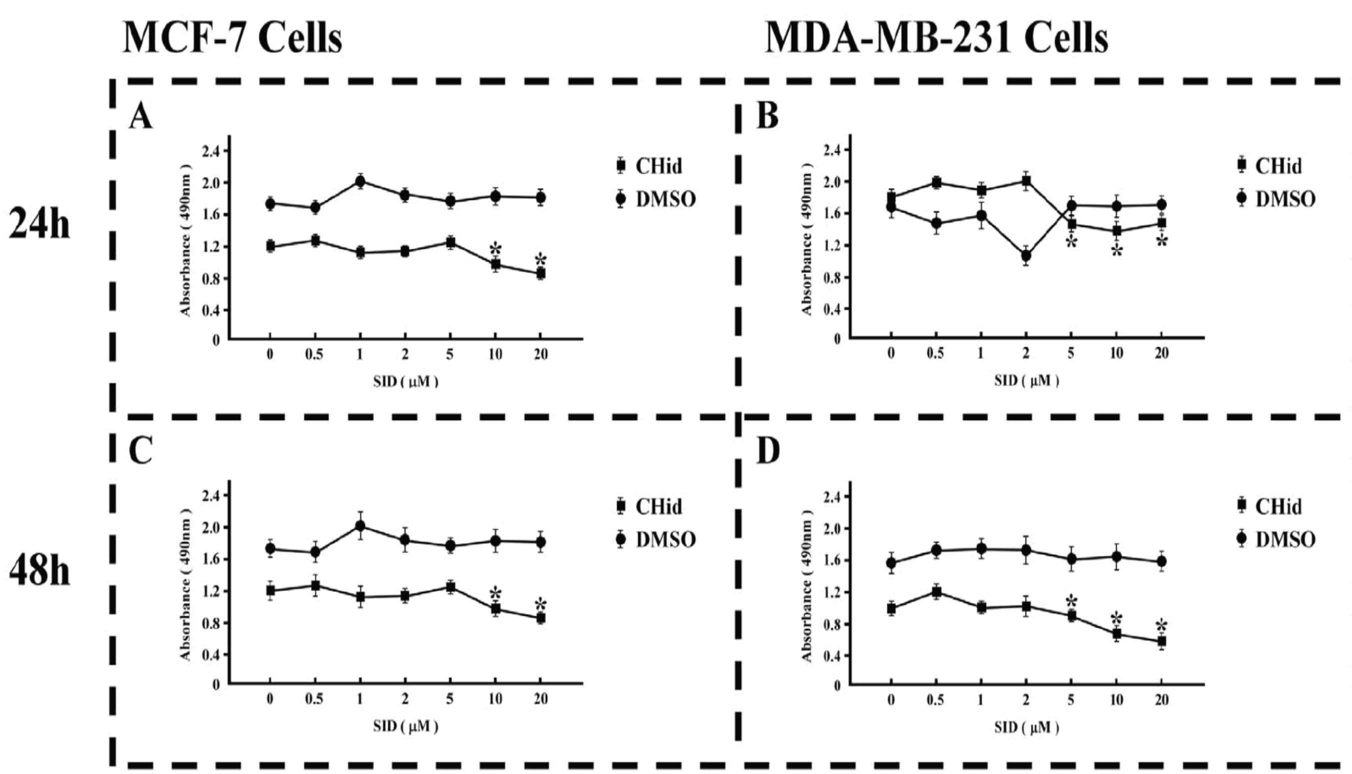

Fig. (1). The assay for chidamide concentration-response effects by MTT. MCF-7 or MDA-MB-231 cells were cultured for 24 or 48 hours in the presence of chidamide at varying concentrations. The same concentrations of DMSO were used as control. The cell proliferation effect was measured by MTT assay. A. MCF-7 cells treated with chidamide (0–20 μM) for 24 hours. B. MDA-MB-231 cells treated with chidamide (0–20 μM) for 24 hours. C. MCF-7 cells treated with chidamide (0–20 μM) for 48 hours. D. MDA-MB-231 cells treated with chidamide (0–20 μM) for 48 hours. means±S.D. for three independent experiments and analyzed using SPSS software, chidamide versus Basal at $p<0.05$.

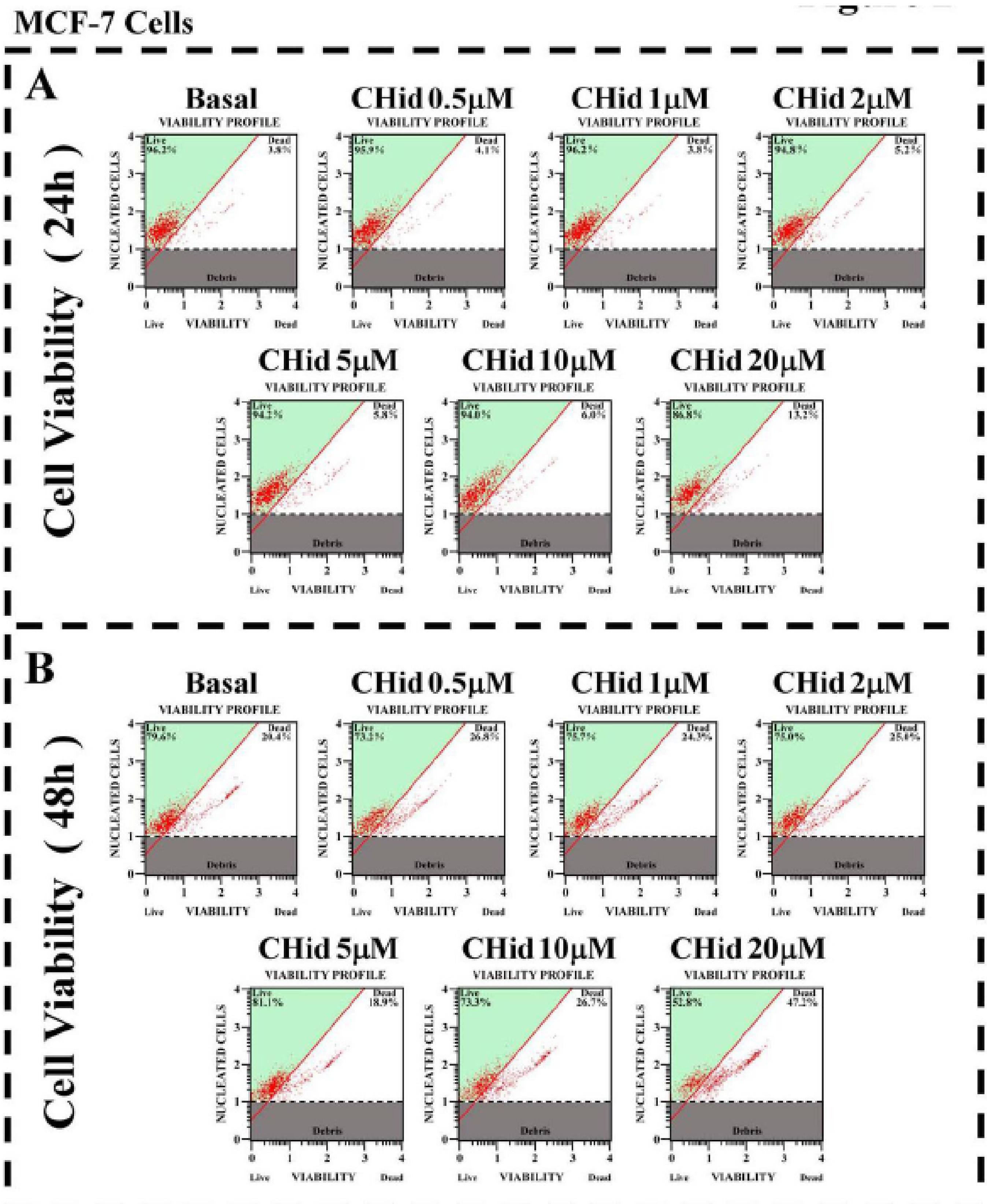

Fig. (2). Cell Viability assay by Muse Cell Analyzer in MCF-7 cells. MCF-7 cells were treated with different concentrations of chidamide (0–20 μM), as described previously for 24 or 48 hours. Muse Count & Viability reagent was used to assess cell viability. A. Cells treated for 24 hours. B. Cells treated for 48 hours. The statistics showed the concentrations and percentages of viable and dead cells.

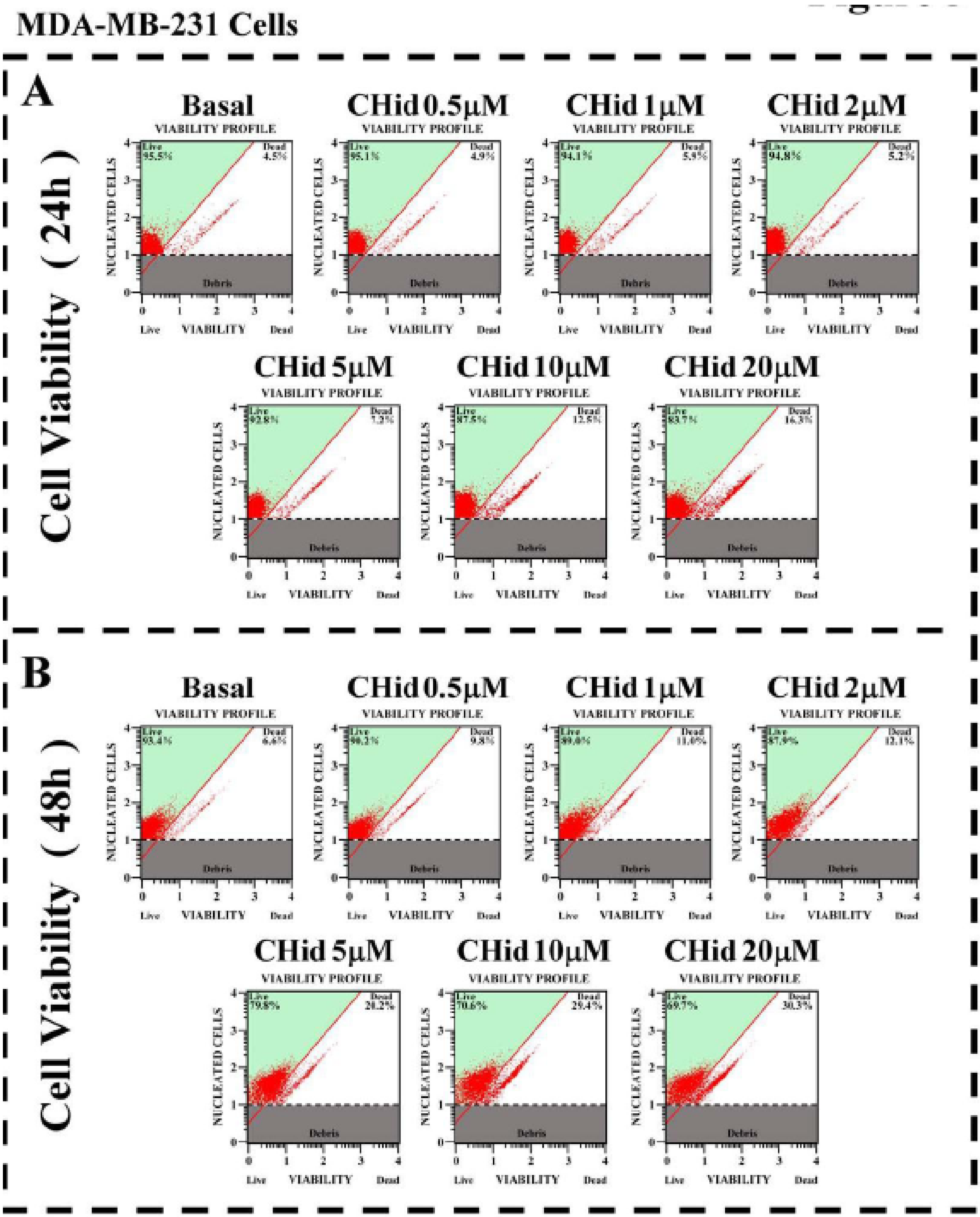

Fig. (3). Cell Viability assay by Muse Cell Analyzer in MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of chidamide (0–20 μM), as described previously for 24 or 48 hours. Muse Count & Viability reagent was used to assess cell viability. A. Cells treated for 24 hours. B. Cells treated for 48 hours. The statistics showed the concentrations and percentages of viable and dead cells.

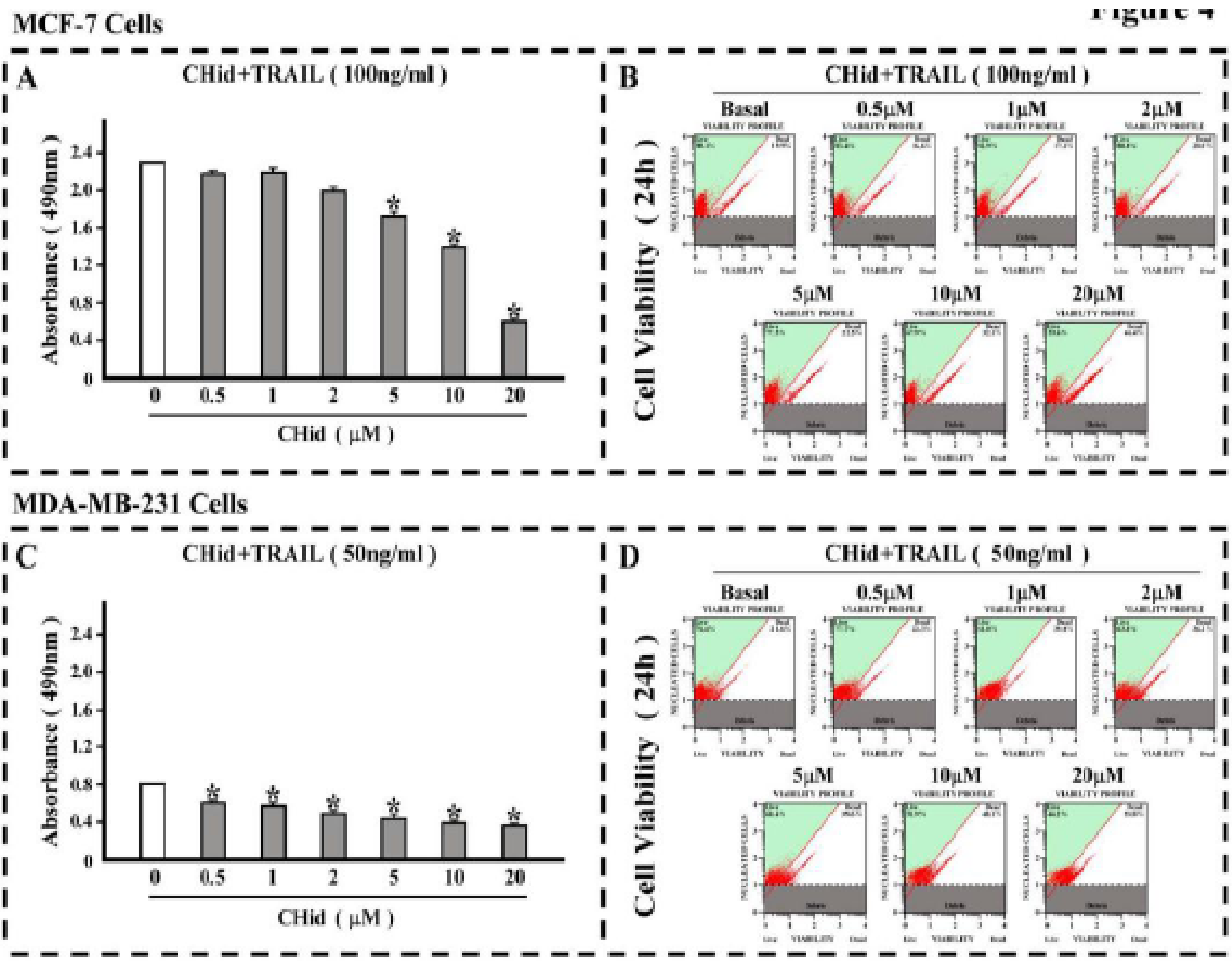

Fig. (4). The effect of chidamide and TRAIL combination on cell viability and cell proliferation of breast cancer cells. Breast cells were plated in 96-well plate and different concentrations of chidamide (0–20 μM) were treated with TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) for 48 hours. The cell proliferation effects of chidamide and TRAIL treatment were measured by MTT and Muse cell viability assay. A. MTT assay for chidamide (0–20 μM) with TRAIL (100 ng/ml) in MCF-7 cells. B. Muse cell viability assay for chidamide (0–20 μM) with TRAIL (100 ng/ml) in MCF-7 cells. C. MTT assay for chidamide (0–20 μM) with TRAIL (50 ng/ml) in MDA-MB-231 cells. D. Muse cell viability assay for chidamide (0–20 μM) with TRAIL (50 ng/ml) in MDA-MB-231 cells. mean±S.D. for three independent experiments, chidamide and TRAIL versus TRAIL at $p<0.05$.

Combining the above results, we verified that 20 μM chidamide after 48 hours of incubation significantly inhibited growth and induced cell death in MCF-7 and MDA-MB-231 breast cancer cells.

3.2. Combination of Chidamide and TRAIL Significantly Induced Breast Cancer Cell Death

Our previous experimental results showed that breast cancer cell lines treated with TRAIL show varying sensitivity [21]. Therefore, the breast cancer cells were incubated with two different concentrations of TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) with increasing concentrations of chidamide (10–20 μM) for 48 hours. We then used the MTT and *Muse Cell* Analyzer to analyze breast cancer cell viability. The data in Fig. (**4**) indicate that the addition of TRAIL significantly reduced the effective dose of chidamide, with 10 μM of chidamide markedly displaying cytotoxic effects on MCF-7 and MDA-MB-231 cells co-treated with TRAIL.

3.3. Anti-tumor Effects in a Xenograft Breast Cancer Tumor Model

The therapeutic potential of chidamide and TRAIL was evaluated in MDA-MB-231 tumor-bearing nude mice. Compared with the saline negative control, tumor growth was significantly inhibited after successive treatment with TRAIL alone or with chidamide. The mixture of chidamide and TRAIL exhibited stronger effects on tumor growth inhibition compared to chidamide alone, potentially owing to a synergistic anti-tumor effect (Fig. **5A** and **5B**).

3.4. Combination of Chidamide and TRAIL Generated a Large Number of Autophagosome in BC Cells

We incubated chidamide and TRAIL with breast cancer cells for 48 hours and used the Muse Autophagy Assay to measure the mean autophagy intensity value, which was represented the producing degree of the autophagosome. As shown in Fig. (**6A** and **6B**), the effect of chidamide was similar in both MDA-MB-231 and MCF-7 cells; the amount of trapped autophagosome increased with chidamide induction. However, the pharmacological effects of TRAIL were more significant in MDA-MB-231 cells than in MCF-7 cells. TRAIL alone did not affect the autophagosome formation in MCF-7 cells, but in MDA-MB-231 cells, the mean autophagy intensity value more than doubled compared with the control. The co-treatment of chidamide and TRAIL significantly increased the amount of autophagosome in both of the cells.

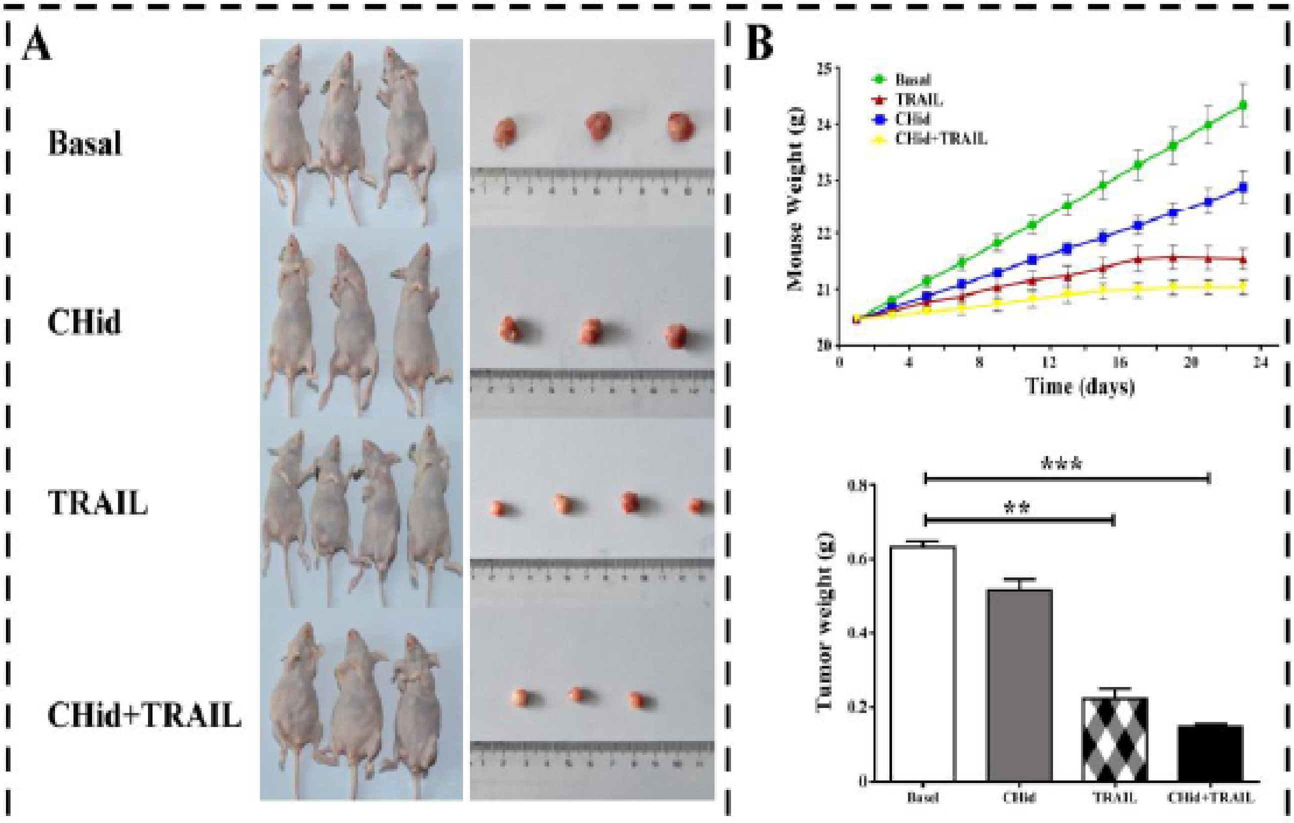

Fig. (5). Anti-tumor efficacy on breast cancer tumor xenograft model. (**A**) The representative images of the breast cancer xenograft tumors collected from the mice after treatment with different formulations on day 23. (**B**) Changes in the body weights of MDA-MB-231 nude mice receiving intravenous injection of different formulations. ** TRAIL versus saline group (n=3) at p<0.05. *** chidamide and TRAIL combination versus saline group (n=3).

Next, we estimated the apoptosis induction of chidamide and TRAIL in breast cancer cells. Results showed that the apoptotic effect was significantly brought out by TRAIL alone in both of the cells. The function of chidamide was dependent on cell selective. It had a slight effect on MDA-MB-231 cells; however, chidamide obviously enhanced the late apoptosis rate and the number of necrotic cells in MCF-7 cells. Co-treatment undoubtedly activated the early and late apoptosis rate and necrosis rate in MCF-7 and MDA-MB-231 cells (Fig. **6C** and **6D**).

3.5. The Pharmacological Effects of Chidamide and TRAIL were Associated with High Transcriptional Activity of Genes Related to Apoptosis and Autophagy

We used qPCR to screen the changes in mRNA levels of various genes that participated in apoptosis and autophagy as implementers or regulators. As seen in Fig. (**7**), change in ATGs mRNA expression following chidamide and TRAIL treatment varied, and the amounts of ULK1 (ATG1A), ATG4A and ATG9B were significantly increased in both cell lines (Fig. **7A**, **B**, **H**, **K**, **L**, **R**). ATG5 and ATG7 were expressed only in MCF-7 cells (Fig. **7G**, **C**) and no significant changes were observed in MDA-MB-231 cells (Fig. **7Q**, **M**). Also, other ATGs such as ATG3, ATG10, ATG12, and ATG16L2 did not distinctly change with the treatment of chidamide and TRAIL (Fig. **7D**, **E**, **F**, **I**, **N**, **O**, **P**, **S**). Only Beclin 1, an important factor in autophagy, was induced in MDA-MB-231 cells following treatment with TRAIL alone; it was not stimulated with any compounds in MCF-7 cells (Fig. **7J**, **T**).

For apoptosis assay, the main functions for drug treatment were reflected in the mRNA changes of Bid, caspase 3 and caspase 8. The effect of TRAIL on both breast cancer cell lines was more obvious than that of chidamide. Chidamide has little stimulation on Bid, caspase 3 and caspase 8, but TRAIL could activate the expressions of related genes at an unusual level either alone or in combination (Fig. **8B**, **C**, **H**, **L**, **M**, **R**). In addition, we also observed that the changes of CTSB and TRAIL DR5 were also apparent with drug treatment (Fig. **8D**, **I**, **N**, **S**).

Finally, the protein analysis confirmed that the expression of ATG4A and LC3B in MCF-7 and MDA-MB-231 cells was also highly enhanced with the induction of chidamide and TRAIL, accompanied by a significant increase in Bid, caspase 3 and caspase 8. Furthermore, CTSB and TRAIL DR5, which were important targets of chidamide and TRAIL, were also highly expressed (Fig. **9**).

4. DISCUSSION

The problem of drug resistance of clinical anti-cancer drugs has been accompanied by the whole process of treatment [22, 23]. As a new generation of HDACi drugs, chidamide has the greatest advantage that it has better low-toxicity than the lower generation of HDACi drugs such as trichostatin A (TSA) and vorinostat (SAHA) [24-25]. For example, a clinical study was performed to evaluate the safety, pharmacokinetics, and preliminary efficacy of chidamide in combination with exemestane in postmenopausal women with

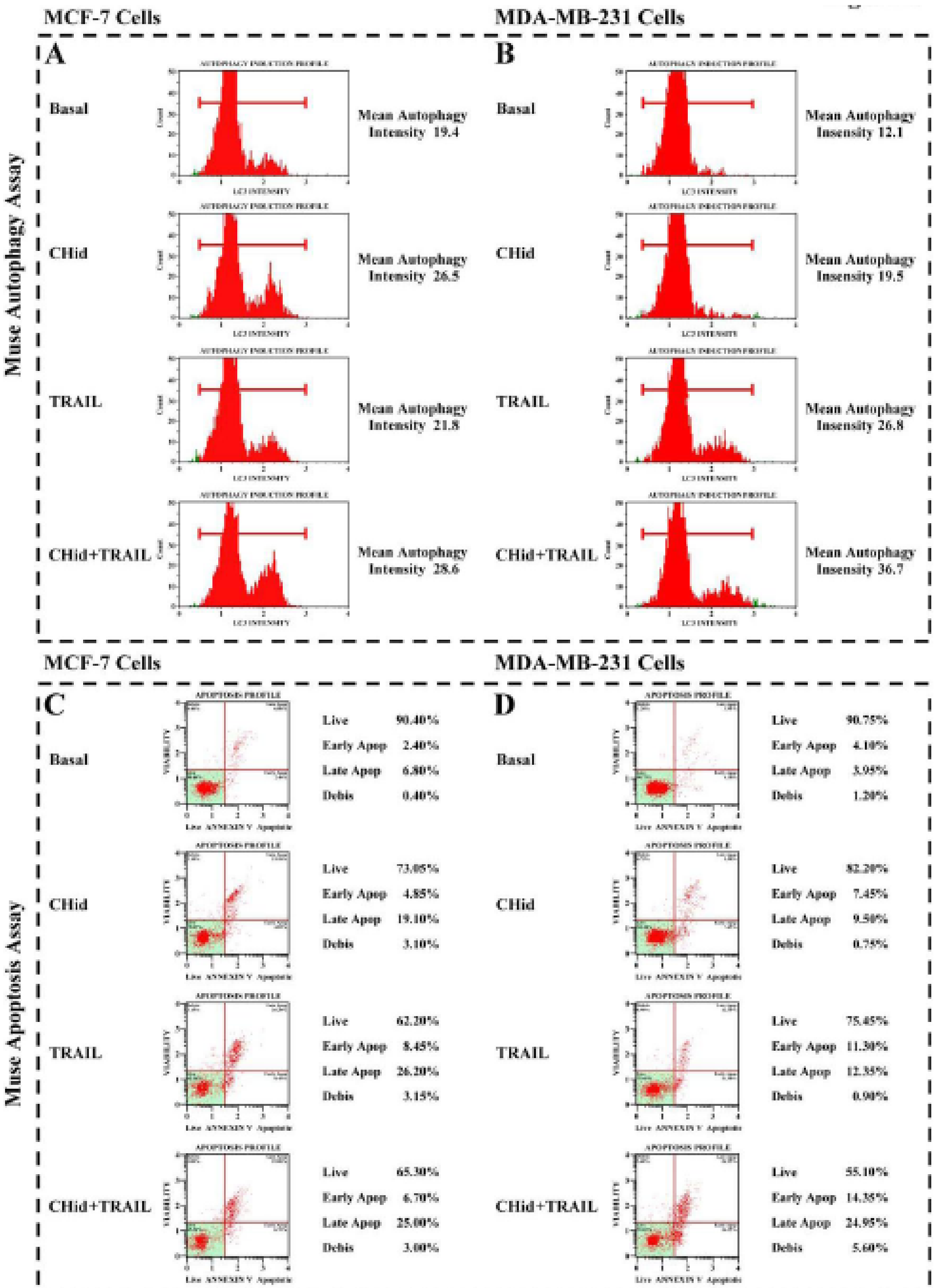

Fig. (6). The autophagy and apoptosis assay induction by chidamide and TRAIL. MCF-7 or MDA-MB-231 cells were incubated with chidamide and TRAIL in 96-well plate for 48 hours described as before. Autophagy assay was used to measure the amount of autophagosome-associated LC3-II with compounds. The values of trapped LC3-II were shown by the corresponding histogram plot. The apoptotic assay was used to determine the percentages of the cells represented by alive, apoptosis and dead population. A. Autophagy assay for MCF-7 cells. B. Autophagy assay for MDA-MB-231 cells. C. Apoptosis assay for MCF-7 cells. D. Apoptosis assay for MDA-MB-231 cells.

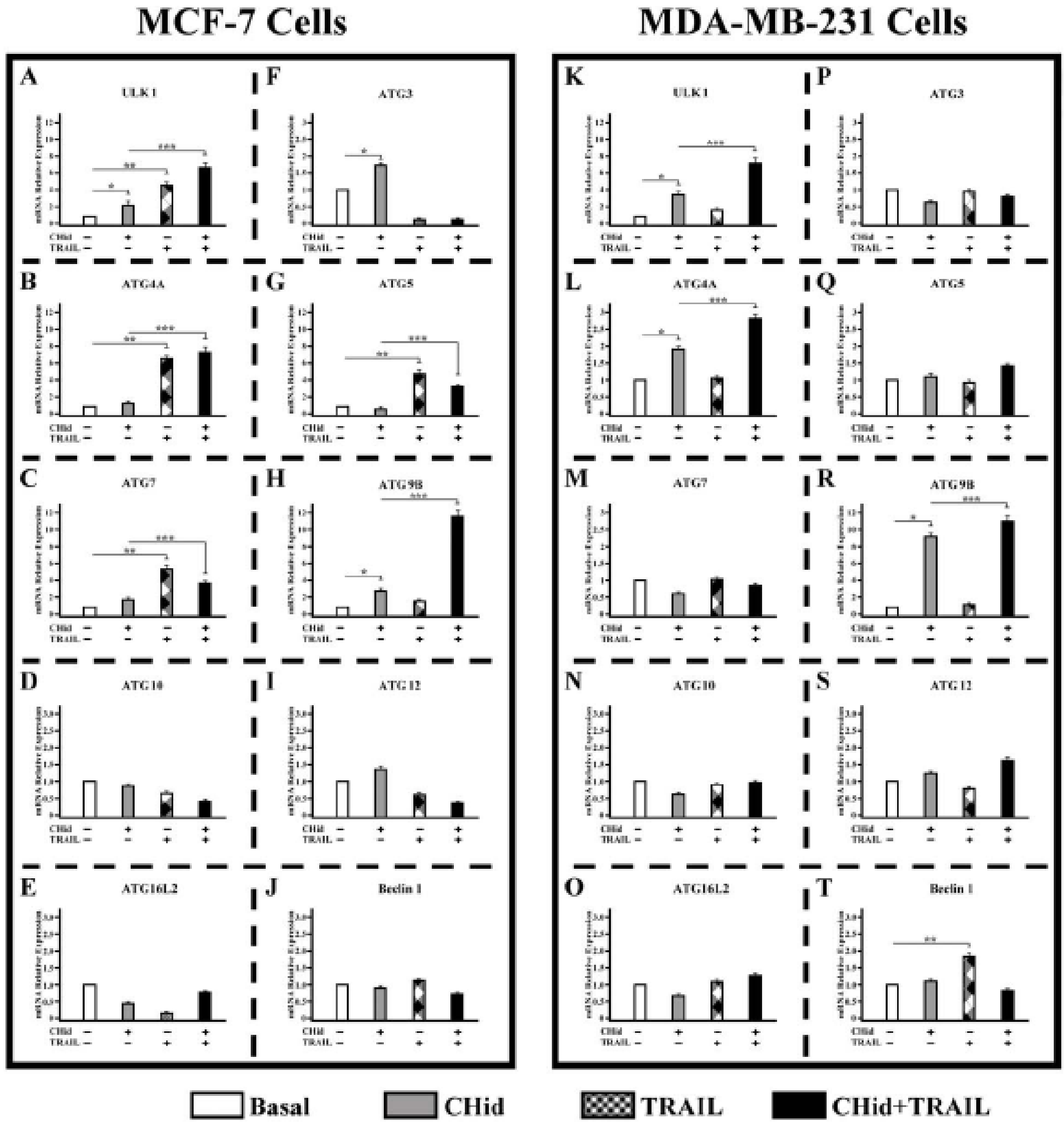

Fig. (7). The effects of chidamide and TRAIL combination on the mRNA expressions of autophagy-related ATGs in breast cancer cells. Breast cells were plated in 96-well plate and 10 µM chidamide was treated with TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) for 48 hours. QPCR was used to determine the changes in mRNA levels of various ATGs induction by chidamide and TRAIL treatment in MCF-7 (Fig. 7A-7J) and MDA-MB-231 (Fig. 7K-7T) cells. means±S.D. for three independent experiments and analyzed using SPSS software, * chidamide versus Basal at $p<0.05$. ** TRAIL versus Basal at $p<0.05$. *** chidamide and TRAIL combination versus chidamide at $p<0.05$.

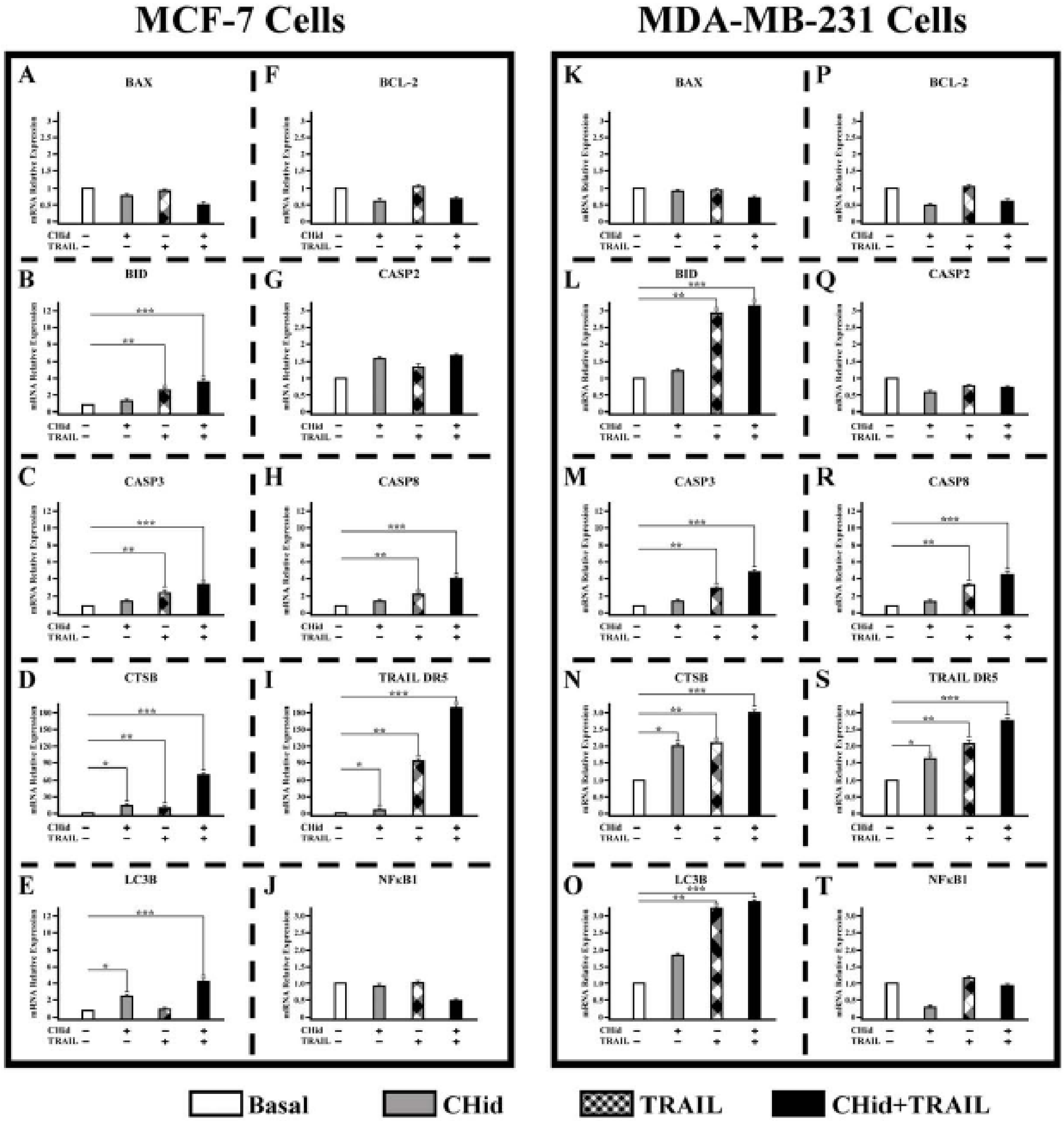

Fig. (8). The effects of chidamide and TRAIL combination on the mRNA expressions of apoptosis-related ATGs in breast cancer cells. Breast cells were treated with chidamide and TRAIL described as before. QPCR was used to determine the changes in mRNA levels of various factors in MCF-7 (Fig. 8A-8J) and MDA-MB-231 (Fig. 8K-8T) cells. means±S.D. for three independent experiments and analyzed using SPSS software, * chidamide versus Basal at $p<0.05$. ** TRAIL versus Basal at $p<0.05$. *** chidamide and TRAIL combination versus chidamide at $p<0.05$.

HR+ and HER2-negative advanced breast cancer (ABC) that recurrent or progressed to at least one endocrine therapy [26]. But it also has the shortcomings and deficiencies of high dosage and potential drug resistance [27]. This was also verified in our experiment. The cytotoxic effect of a low dose of chidamide on breast cancer cells was not very significant. It needed to increase the dose up to 20 µm for 48 hours duration to perform the full cytotoxic effect. Therefore, it is essential to search for compatibility with other drugs with the aim of deep optimizing the efficacy of chidamide. Based on this idea, we found that TRAIL may be a more suitable candidate. Recently, it was reported that a promoter SNP of TRAIL functionally modulates the gene expression, and they advise considering the -716 TRAIL SNP status in patients undergoing TRAIL therapy. High TRAIL expressing individuals with -716 CC genotype are at a

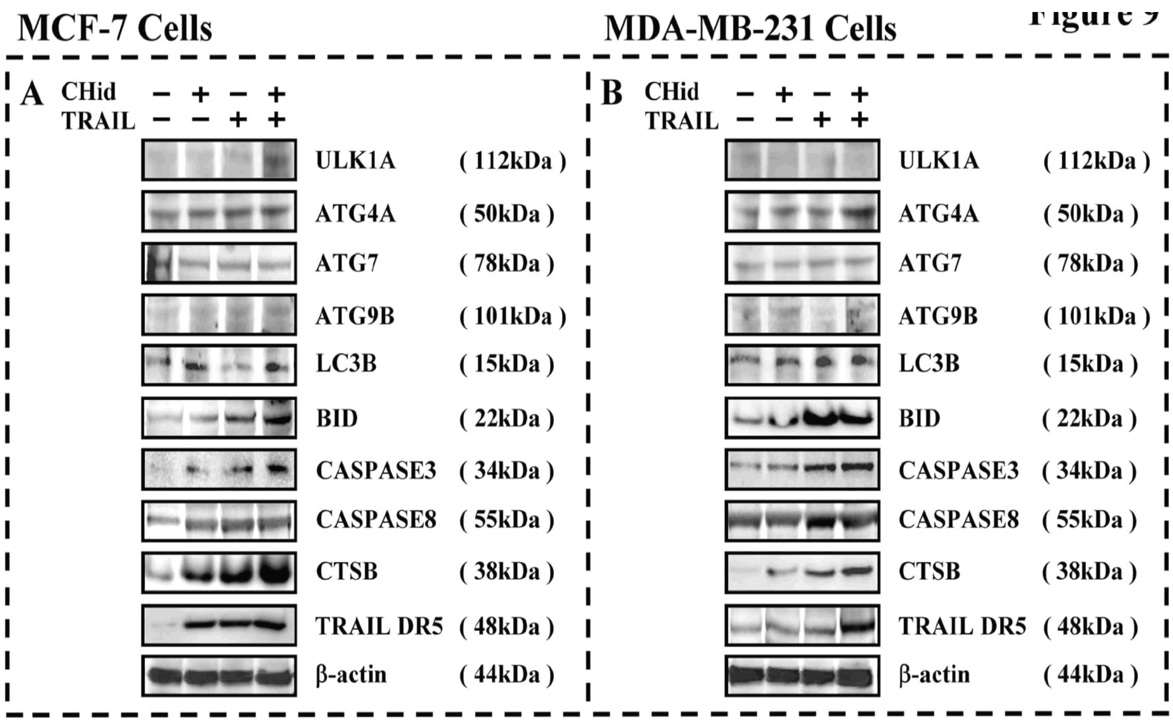

Fig. (9). The effects of chidamide and TRAIL combination on the protein expression of related factors. Breast cells were plated in 96-well plate and 10 μM chidamide was treated with TRAIL (100 ng/ml for MCF-7 and 50 ng/ml for MDA-MB-231) for 48 hours. Western blot analysis was used to determine the changes in protein levels of various factors. **A**. MCF-7 cells. **B**. MDA-MB-231 cells.

greater risk of developing tumor. The findings indicated that genetic variants of TRAIL at position 1595 in exon 5 might be associated with the progression of breast cancer [28]. Combined with our previous experiments, it showed that TRAIL, as a strong inducer, can induce breast cancer apoptosis, and it had a selective cytotoxic effect that was no obvious toxic reaction to normal cells [29]. In this experiment, when TRAIL and chidamide were combined to treat breast cancer cells, we found that their effect on cells was obviously better than that of chidamide alone, and the dosage of chidamide reaching or close to IC50 was also reduced from 20 μM to 10 μM.

At present, it is generally accepted that apoptosis and autophagy function on cell survival in two different aspects. Autophagy is a physiological reaction for cells under normal conditions, which is mainly characterized by autophagic lysosomes. Its contribution to cells is more for efficient recovery and utilization of all necessary components [30-32]. But apoptosis is a kind of active cell death process which is characterized by chromatin condensation of the nucleus [33, 34]. Both of them can induce cell death, but autophagy induced cell death is more a mechanism of cell self-protection, and cell apoptosis is the result of cell response to external stimulation such as chemical drugs [35, 36]. When we analyzed the apoptosis and autophagy effects of chidamide and TRAIL on breast cancer cells, we found that different types of cells had different responses to the drugs. For ER+ breast cancer MCF-7 cells, chidamide could strongly activate the apoptotic and autophagic responses, in which the ratio of living cells was greatly reduced, the ratios of early, late apoptosis and necrosis cells were markedly increased. For MDA-MB-231 cells, the role of TRAIL seemed to be more powerful than that of chidamide. Chidamide only induces a certain degree of autophagy and had a general effect on apoptosis induction.

When we determined the changes of some factors associated with apoptosis and autophagy, we found that the expression levels of ATG4A and ATG9B were raised following treatment with TRAIL or chidamide. The ATG4 family (ATG4A, 4B, 4C, 4D) is critical to the biological formation of autophagosomes [37, 38]. The ATG4-catalyzed delipidation reaction is also necessary for the fusion of autophagosomes and lysosomes to form autophagosomes [39–41]. Moreover, the essential role of ATG9B is also required for the formation of autophagosomes in mammalian cells [42]. Therefore, we implied that with drug treatment, a series of autophagy-related protein (ATG) molecules are involved in the formation of autophagosomes and autophagic lysosomes.

In addition, we found that the expressions of Bid, caspase-3 and caspase-8, which are closely related to apoptosis [43-45], were active in the breast cancer cells treated with chidamide and TRAIL. In MCF-7 and MDA-MB-231 cells, the expression of Bid was significantly increased both in TRAIL alone and in combination with chidamide, while the mRNA expressions of caspase-3 and caspase-8, which play important roles in apoptosis signal transduction, were enlarged in co-administration. However, the expressions of Bax and Bcl-2, which are the classical apoptosis markers [46, 47], did not change strongly.

It should be noted that chidamide alone had no effect on the expressions of caspases. Chidamide mainly initiated autophagic effect by activating autophagy-related factors such as ATG4A, ATG9B and LC3B, to promote the formation and maturation of autophagy vesicles, and finally to wrap cathepsin B (CTSB) for intracellular material transport. TRAIL, on the other hand, can activate apoptosis through its specific receptor, TRAIL DR5 (TRAIL Death Receptor 5), and induce breast cancer cell death through caspase 3.

Of course, we also noticed that the transport of CTSB by vesicles was also enormously risen. CTSB is a corporate effector of apoptosis and autophagy [48, 49]. It is an important component of lysosomes and the main enveloping protease of autophagic lysosomes. Moreover, it is still an important regulatory factor in the apoptosis pathway. TRAIL can transmit signals to CTSB through TRAIL DR5. It has been reported that CTSB can activate the expression of Bid. Bid is activated by the cleavage of the carboxyl

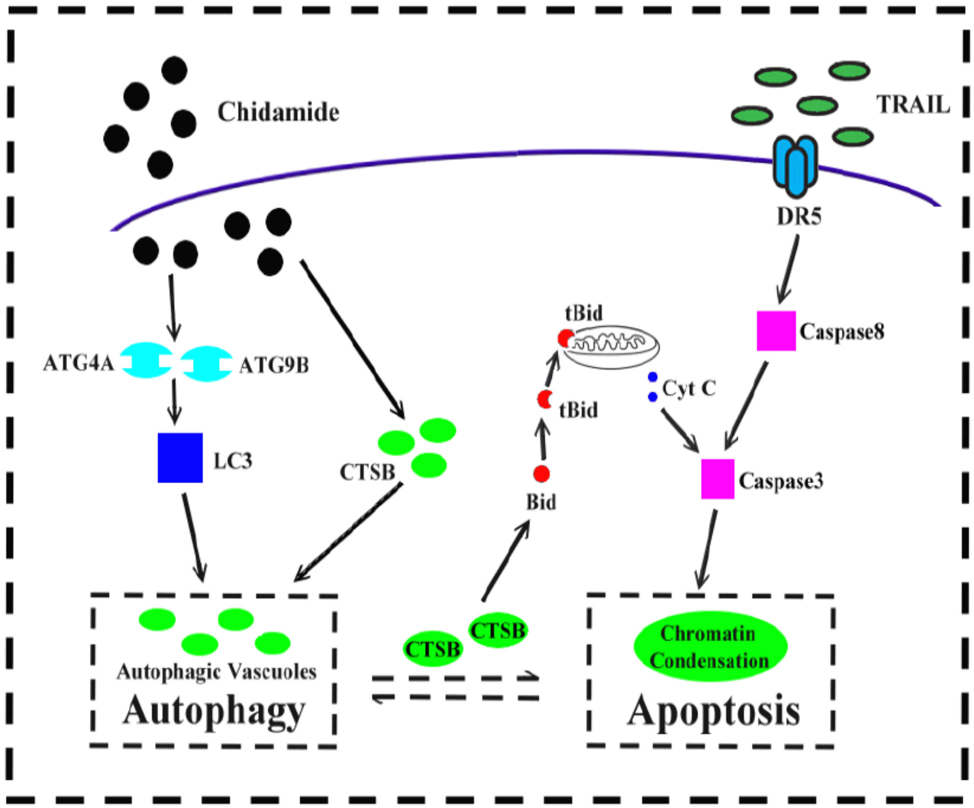

Fig. (10). Schematic representation of the signaling transduction pathway induced by chidamide and TRAIL combination in MCF-7 and MDA-MB-231 breast cancer cells. The autophagic vesicle secretion induced by chidamide-LC3-CTSB axis coordinates with TRAIL-caspase 3 pathway initiated apoptosis of breast cancer cells.

domain under caspase 8 to form tBID. The transfer of tBid to mitochondria leads to the release of cytochrome C to induce apoptosis [50]. Therefore, there may be a transduction chain of TRAIL DR5-CTSB-Bid in the pharmacological action of chidamide and TRAIL. The chain initiates the occurrence and intensity of apoptosis through the interaction with caspase 3 and caspase 8 (Fig. **10**).

Interestingly, combined with the experiment of breast cancer tumor xenograft (Fig. **5**), we also suggested that TRAIL should be paid more attention to its anti-cancer effect, which has potential clinical application value for triple-negative breast cancer, a malignant type with high recurrence, death and metastasis [51, 52].

CONCLUSION

In summary, we report an original study revealing the pharmacological effects of chidamide and TRAIL in BC cells. This chidamide and TRAIL combination may be used as a novel drug strategy in the future.

LIST OF ABBREVIATIONS

CHiD	=	Chidamide
SAHA	=	Vorinostat
TSA	=	Richostatin A
TRAIL	=	TNF-related Apoptosis Inducing Ligand
TRAIL DR5	=	TRAIL Death Receptor 5
HDACi	=	Histone Deacetylase Inhibitor
Apo2L	=	Recombinant Human TNF-Related Apoptosis Inducing Ligand
CTSB	=	Cathepsin B
ATG	=	Autophagy-related Protein
LC3-II	=	Light chain3- II
BC	=	Breast Cancer
ER	=	Estrogen Receptor
PR	=	Progesterone Receptor
HER2	=	Human Epidermal Growth Factor Receptor 2
TNBC	=	Triple-negative Breast Cancer
ABC	=	Advanced Breast Cancer
PK	=	Pharmacokinetics
FBS	=	Fetal Bovine Serum
BCA	=	Bicinchoninic Acid
MTT	=	The CellTiter 96® AQueous One Solution Cell Proliferation Assay
PCR	=	Polymerase Chain Reaction
ANOVA	=	A One-way Analysis of Variance
IC50	=	Half Maximal Inhibitory Concentration
ATCC	=	American Type Culture Collection
DMSO	=	Dimethylsulfoxide

AUTHORS'CONTRIBUTIONS

WZ, XF and TS participated in the design of the study; WZ, JX, HH and XF performed experiments; WZ, HH, XF analyzed data; WZ wrote the manuscript; All authors reviewed and participated in the revision of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The contents of animal experiments were approved by the ethics committee of Shenyang Medical College.

HUMAN AND ANIMAL RIGHTS

The contents of animal experiments in this manuscript were in accordance with the relevant regulations of the ethics committee of Shenyang Medical College.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

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

FUNDING

The work is supported by the Natural Science Foundation of China (81172509, 81502188), the Innovative Talents Support Foundation for Liaoning Universities of Department of Education of Liaoning Province (LR2017040), the Research Foundation of Science and Technology Department of Liaoning Province (2018010621-301, 2018225016, 2017012046-301, 2015020251), and the Research Foundation of Shenyang Science and Technology Bureau (18-013-0-88).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68(6): 394-424. http://dx.doi.org/10.3322/caac.21492 PMID: 30207593

[2] Greer YE, Gilbert SF, Gril B, *et al.* MEDI3039, a novel highly potent tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor 2 agonist, causes regression of orthotopic tumors and inhibits outgrowth of metastatic triple-negative breast cancer. Breast Cancer Res 2019; 21(1): 27. http://dx.doi.org/10.1186/s13058-019-1116-1 PMID: 30777098

[3] Gao A, Sun T, Ma G, *et al.* LEM4 confers tamoxifen resistance to breast cancer cells by activating cyclin D-CDK4/6-Rb and ERα pathway. Nat Commun 2018; 9(1): 4180. http://dx.doi.org/10.1038/s41467-018-06309-8 PMID: 30301939

[4] Coté D, Eustace A, Toomey S, *et al.* Germline single nucleotide polymorphisms in ERBB3 and BARD1 genes result in a worse relapse free survival response for HER2-positive breast cancer patients treated with adjuvant based docetaxel, carboplatin and trastuzumab (TCH). PLoS One 2018; 13(8)e0200996 http://dx.doi.org/10.1371/journal.pone.0200996 PMID: 30071039

[5] Elias D, Vever H, Lænkholm AV, *et al.* Correction: Gene expression profiling identifies FYN as an important molecule in tamoxifen resistance and a predictor of early recurrence in patients treated with endocrine therapy. Oncogene 2018; 37(41): 5585-6. http://dx.doi.org/10.1038/s41388-018-0495-6 PMID: 30242243

[6] Palomeras S, Ruiz-Martínez S, Puig T. Targeting breast cancer stem cells to overcome treatment resistance. Molecules 2018; 23(9)E2193 http://dx.doi.org/10.3390/molecules23092193 PMID: 30200262

[7] Kim HS, Kacew S, Lee BM. Genetic and epigenetic cancer chemoprevention on molecular targets during multistage carcinogenesis. Arch Toxicol 2016; 90(10): 2389-404. http://dx.doi.org/10.1007/s00204-016-1813-9 PMID: 27538406

[8] Kazanets A, Shorstova T, Hilmi K, Marques M, Witcher M. Epigenetic silencing of tumor suppressor genes: Paradigms, puzzles, and potential. Biochim Biophys Acta 2016; 1865(2): 275-88. PMID: 27085853

[9] Feinberg AP, Koldobskiy MA, Göndör A. Epigenetic modulators, modifiers and mediators in cancer aetiology and progression. Nat Rev Genet 2016; 17(5): 284-99. http://dx.doi.org/10.1038/nrg.2016.13 PMID: 26972587

[10] Rifaï K, Idrissou M, Penault-Llorca F, Bignon YJ, Bernard-Gallon D. Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer. Cancers (Basel) 2018; 10(11)E409 http://dx.doi.org/10.3390/cancers10110409 PMID: 30380732

[11] Han YJ, Boatman SM, Zhang J, *et al.* LncRNA BLAT1 is upregulated in basal-like breast cancer through epigenetic modifications. Sci Rep 2018; 8(1): 15572. http://dx.doi.org/10.1038/s41598-018-33629-y PMID: 30349062

[12] Mao J, Li S, Zhao H, *et al.* Effects of chidamide and its combination with decitabine on proliferation and apoptosis of leukemia cell lines. Am J Transl Res 2018; 10(8): 2567-78. PMID: 30210693

[13] Bai X, Jiang H, Han G, He Q. Chidamide suppresses the glycolysis of triple negative breast cancer cells partially by targeting the miR-33a-5p-LDHA axis. Mol Med Rep 2019; 20(2): 1857-65. http://dx.doi.org/10.3892/mmr.2019.10425 PMID: 31257519

[14] Liu Z, Chen J, Wang H, *et al.* Chidamide shows synergistic cytotoxicity with cytarabine via inducing G0/G1 arrest and apoptosis in myelodysplastic syndromes. Am J Transl Res 2017; 9(12): 5631-42. PMID: 29312515

[15] Zhang H, Li L, Li M, *et al.* Combination of betulinic acid and chidamide inhibits acute myeloid leukemia by suppression of the HIF1α pathway and generation of reactive oxygen species. Oncotarget 2017; 8(55): 94743-58. http://dx.doi.org/10.18632/oncotarget.21889 PMID: 29212263

[16] Li Y, Wang Y, Zhou Y, *et al.* Cooperative effect of chidamide and chemotherapeutic drugs induce apoptosis by DNA damage accumulation and repair defects in acute myeloid leukemia stem and progenitor cells. Clin Epigenetics 2017; 9: 83. http://dx.doi.org/10.1186/s13148-017-0377-8 PMID: 28814980

[17] Shankar S, Davis R, Singh KP, Kurzrock R, Ross DD, Srivastava RK. Suberoylanilide hydroxamic acid (Zolinza/vorinostat) sensitizes TRAIL-resistant breast cancer cells orthotopically implanted in BALB/c nude mice. Mol Cancer Ther 2009; 8(6): 1596-605. http://dx.doi.org/10.1158/1535-7163.MCT-08-1004 PMID: 19509267

[18] Zhou W, Feng X, Han Han , Guo S, Wang G. Synergistic effects of combined treatment with histone deacetylase inhibitor suberoylanilide hydroxamic acid and TRAIL on human breast cancer cells. Sci Rep 2016; 6: 28004. http://dx.doi.org/10.1038/srep28004 PMID: 27292433

[19] Chen Y, Wei L, Zhang X, *et al.* 3-Bromopyruvate sensitizes human breast cancer cells to TRAIL-induced apoptosis via the phosphorylated AMPK-mediated upregulation of DR5. Oncol Rep 2018; 40(5): 2435-44. http://dx.doi.org/10.3892/or.2018.6644 PMID: 30132536

[20] Cano-González A, López-Rivas A. Opposing roles of TGF-β and EGF in the regulation of TRAIL-induced apoptosis in human breast epithelial cells. Biochim Biophys Acta 2016; 1863(8): 2104-14. http://dx.doi.org/10.1016/j.bbamcr.2016.05.011 PMID: 27208428

[21] Han H, Zhou H, Li J, Feng X, Zou D, Zhou W. TRAIL DR5-CTSB crosstalk participates in breast cancer autophagy initiated by SAHA. Cell Death Discov 2017; 3: 17052. http://dx.doi.org/10.1038/cddiscovery.2017.52 PMID: 29018571

[22] Ekinci E, Rohondia S, Khan R, Dou QP. Repurposing disulfiram as an anti-cancer agent: updated review on literature and patents. Recent Patents Anticancer Drug Discov 2019; 14(2): 113-32. http://dx.doi.org/10.2174/1574892814666190514104035 PMID: 31084595

[23] Namee NM, O'Driscoll L. Extracellular vesicles and anti-cancer drug resistance. Biochim Biophys Acta Rev Cancer 2018; 1870(2): 123-36. http://dx.doi.org/10.1016/j.bbcan.2018.07.003 PMID: 30003999

[24] Nagini S. Breast cancer: current molecular therapeutic targets and new players. Anticancer Agents Med Chem 2017; 17(2): 152-63.

http://dx.doi.org/10.2174/1871520616666160502122724 PMID: 27137076

[25] Muraoka M, Akagi T, Ueda A, *et al.* C/EBPε ΔRS derived from a neutrophil-specific granule deficiency patient interacts with HDAC1 and its dysfunction is restored by trichostatin A. Biochem Biophys Res Commun 2019; 516(1): 293-9. http://dx.doi.org/10.1016/j.bbrc.2019.06.130 PMID: 31256937

[26] Zhang Q, Wang T, Geng C, *et al.* Exploratory clinical study of chidamide, an oral subtype-selective histone deacetylase inhibitor, in combination with exemestane in hormone receptor-positive advanced breast cancer. Chin J Cancer Res 2018; 30(6): 605-12. http://dx.doi.org/10.21147/j.issn.1000-9604.2018.06.05 PMID: 30700929

[27] Derakhshani A, Rezaei Z, Safarpour H, *et al.* Overcoming trastuzumab resistance in HER2-positive breast cancer using combination therapy. J Cell Physiol 2020; 235(4): 3142-56. http://dx.doi.org/10.1002/jcp.29216 PMID: 31566722

[28] Yildiz Y, Yaylim-Eraltan I, Arikan S, Ergen HA, Küçücük S, Isbir T. Is there any correlation between TNF-related apoptosis-inducing ligand (TRAIL) genetic variants and breast cancer? Arch Med Sci 2010; 6(6): 932-6. http://dx.doi.org/10.5114/aoms.2010.19304 PMID: 22427769

[29] Trivedi R, Mishra DP. Trailing TRAIL Resistance: Novel targets for TRAIL sensitization in cancer cells. Front Oncol 2015; 5: 69. http://dx.doi.org/10.3389/fonc.2015.00069 PMID: 25883904

[30] Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. Oncogene 2017; 36(12): 1619-30. http://dx.doi.org/10.1038/onc.2016.333 PMID: 27593926

[31] Munson MJ, Ganley IG. MTOR, PIK3C3, and autophagy: Signaling the beginning from the end. Autophagy 2015; 11(12): 2375-6. http://dx.doi.org/10.1080/15548627.2015.1106668 PMID: 26565689

[32] Herrero-Martín G, Høyer-Hansen M, García-García C, *et al.* TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. EMBO J 2009; 28(6): 677-85. http://dx.doi.org/10.1038/emboj.2009.8 PMID: 19197243

[33] Tabatabayi ZS, Homayouni-Tabrizi M, Neamati A, Beyramabadi SA. Mn(II) complex of a vitamin B6 Schiff base as an exclusive apoptosis inducer in human MCF7 and HepG2 cancer cells: Synthesis, characterization, and biological studies. J Cell Biochem 2020; 121(3): 2677-89. http://dx.doi.org/10.1002/jcb.29488 PMID: 31680295

[34] Jamali T, Kavoosi G, Ardestani SK. In-vitro and in-vivo anti-breast cancer activity of OEO (Oliveria decumbens vent essential oil) through promoting the apoptosis and immunomodulatory effects. J Ethnopharmacol 2020; 248112313 http://dx.doi.org/10.1016/j.jep.2019.112313 PMID: 31655147

[35] Rai S, Arasteh M, Jefferson M, *et al.* The ATG5-binding and coiled coil domains of ATG16L1 maintain autophagy and tissue homeostasis in mice independently of the WD domain required for LC3-associated phagocytosis. Autophagy 2019; 15(4): 599-612. http://dx.doi.org/10.1080/15548627.2018.1534507 PMID: 30403914

[36] Cui Y, Wang J, Liu S, *et al.* miR-216a promotes breast cancer cell apoptosis by targeting PKCα. Fundam Clin Pharmacol 2019; 33(4): 397-404. http://dx.doi.org/10.1111/fcp.12481 PMID: 31119784

[37] Wolf J, Dewi DL, Fredebohm J, *et al.* A mammosphere formation RNAi screen reveals that ATG4A promotes a breast cancer stem-like phenotype. Breast Cancer Res 2013; 15(6): R109. http://dx.doi.org/10.1186/bcr3576 PMID: 24229464

[38] Bortnik S, Choutka C, Horlings HM, *et al.* Identification of breast cancer cell subtypes sensitive to ATG4B inhibition. Oncotarget 2016; 7(41): 66970-88. http://dx.doi.org/10.18632/oncotarget.11408 PMID: 27556700

[39] Zhang X, Li C, Wang D, Chen Q, Li CL, Li HJ. Aberrant methylation of ATG2B, ATG4D, ATG9A and ATG9B CpG island promoter is associated with decreased mRNA expression in sporadic breast carcinoma. Gene 2016; 590(2): 285-92. http://dx.doi.org/10.1016/j.gene.2016.05.036 PMID: 27265029

[40] Antonelli M, Strappazzon F, Arisi I, *et al.* ATM kinase sustains breast cancer stem-like cells by promoting ATG4C expression and autophagy. Oncotarget 2017; 8(13): 21692-709. http://dx.doi.org/10.18632/oncotarget.15537 PMID: 28423511

[41] Zhang L, Guo M, Li J, *et al.* Systems biology-based discovery of a potential Atg4B agonist (Flubendazole) that induces autophagy in breast cancer. Mol Biosyst 2015; 11(11): 2860-6. http://dx.doi.org/10.1039/C5MB00466G PMID: 26299935

[42] Claude-Taupin A, Fonderflick L, Gauthier T, *et al.* *ATG9A* Is Overexpressed in Triple Negative Breast Cancer and Its In Vitro Extinction Leads to the Inhibition of Pro-Cancer Phenotypes. Cells 2018; 7(12)E248 http://dx.doi.org/10.3390/cells7120248 PMID: 30563263

[43] Wang S, Xu Y, Li C, *et al.* Gambogic acid sensitizes breast cancer cells to TRAIL-induced apoptosis by promoting the crosstalk of extrinsic and intrinsic apoptotic signalings. Food Chem Toxicol 2018; 119: 334-41. http://dx.doi.org/10.1016/j.fct.2018.02.037 PMID: 29458160

[44] Bagherabad MB, Afzaljavan F, Vahednia E, *et al.* Association of caspase 8 promoter variants and haplotypes with the risk of breast cancer and its molecular profile in an Iranian population: A case-control study. J Cell Biochem 2019; 120(10): 16435-44. http://dx.doi.org/10.1002/jcb.28781 PMID: 31257627

[45] Jiao C, Chen W, Tan X, *et al.* Ganoderma lucidum spore oil induces apoptosis of breast cancer cells in vitro and in vivo by activating caspase-3 and caspase-9. J Ethnopharmacol 2020; 247112256 http://dx.doi.org/10.1016/j.jep.2019.112256 PMID: 31586690

[46] Ren L, Li Y, Zhao Q, *et al.* miR-519 regulates the proliferation of breast cancer cells via targeting human antigen R. Oncol Lett 2020; 19(2): 1567-76. PMID: 31966082

[47] Zoeller JJ, Vagodny A, Taneja K, *et al.* Neutralization of BCL-2/XL enhances the cytotoxicity of T-DM1 in vivo. Mol Cancer Ther 2019; 18(6): 1115-26. http://dx.doi.org/10.1158/1535-7163.MCT-18-0743 PMID: 30962322

[48] Toupin NP, Arora K, Shrestha P, *et al.* BODIPY-caged photoactivated inhibitors of cathepsin B flip the light switch on cancer cell apoptosis. ACS Chem Biol 2019; 14(12): 2833-40. http://dx.doi.org/10.1021/acschembio.9b00711 PMID: 31750642

[49] Wang Y, Niu H, Hu Z, *et al.* Targeting the lysosome by an aminomethylated riccardin D triggers DNA damage through cathepsin B-mediated degradation of BRCA1. J Cell Mol Med 2019; 23(3): 1798-812. http://dx.doi.org/10.1111/jcmm.14077 PMID: 30565390

[50] Hodgkinson VC, ELFadl D, Agarwal V, *et al.* Proteomic identification of predictive biomarkers of resistance to neoadjuvant chemotherapy in luminal breast cancer: a possible role for 14-3-3 theta/tau and tBID? J Proteomics 2012; 75(4): 1276-83. http://dx.doi.org/10.1016/j.jprot.2011.11.005 PMID: 22115752

[51] Castelli V, Piroli A, Marinangeli F, *et al.* Local anesthetics counteract cell proliferation and migration of human triple-negative breast cancer and melanoma cells. J Cell Physiol 2020; 235(4): 3474-84. http://dx.doi.org/10.1002/jcp.29236 PMID: 31541469

[52] Menbari MN, Rahimi K, Ahmadi A, *et al.* miR-483-3p suppresses the proliferation and progression of human triple negative breast cancer cells by targeting the HDAC8>oncogene. J Cell Physiol 2020; 235(3): 2631-42. http://dx.doi.org/10.1002/jcp.29167 PMID: 31508813