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Original Article

Growth inhibitory effects of Chidamide on breast cancer cells

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Abstract: This study aimed to explore the effects of histone deacetylase inhibitor Chidamide on the proliferation of breast cancer cell lines MCF7 and MDA-MB453. The growth of MCF7 and MDA-MB453 cells were greatly inhibited by Chidamide in both time and dose dependent manners. Chidamide induced cell apoptosis and delayed G_2/M cell cycle progression. In addition, chidamide up-regulated the expression of Baxand p21 whereas down-regulated Bcl-2 and Cyclin B2 to promote the cleavage of PARP and caspase-3. In conclusion, Chidamide could induce cell apoptosis and cell cycle arrest to inhibit the growth of MCF7 and MDA-MB453 cells, possibly through regulating the expression of Bax, p21, Bcl-2 and Cyclin B2.

Keywords: MCF7, MDA-MB453, Chidamide, HDACi, apoptosis, cell cycle

Introduction

Histone acetylases (HAT) and histone deacetylases (HDAC) are a series of enzymes functioning to acetylate and deacetylate the amino-terminal lysine residues of histones, which result in remodeling of the chromatin structures and affect the accessibility of the chromatin to transcription factors in gene transcription. Imbalanced activities of HAT and HDAC promote cancer development by altering the regulation of gene transcription; this affects the expression of proteins involved in the tumor suppression, cell cycle, apoptosis [1]. HDAC inhibitors (HDACi) is a class of agents that have shown promising therapeutic effects on several types of cancer. HDACi can modulate gene expression and chromatin modification by inducing acetylation of histone proteins [2]. Hence, HAT and HDAC are considered as an important class of targets in cancer treatment strategies, and HDACi are becoming a new promising class of anticancer drugs [3].

Breast cancer is now the most common cancer among Chinese women; Cases in China account for 12.2% of all newly diagnosed breast cancers and 9.6% of all deaths from breast cancer worldwide [4]. Surgery, chemotherapy, hormonal therapy and molecular targeted therapy are important means of breast cancer treatment. Though there have been great advances in the treatment of breast cancer, the resistance to chemotherapy and endocrine therapy can occur at the inception of diseases or develop during treatment, which result in a high mortality rate of breast cancer. Thus, there is great need to develop new agents to improve treatment of breast cancer. Chidamide is a novel HDACi which specifically inhibits HDAC1, 2, 3, and 10 [5]. In this study, we showed that Chidamide possessed potent growth inhibition, cell cycle arrest and apoptosis in breast cancer cell lines.

Materials and methods

Reagents

Chidamide was supplied by Chipscreen Biosciences (Shenzhen, China) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 80 mM and then kept at -20°C until use.

Table 1. Primers used for quantification measurements of mRNA expression

Gene	Primers
Gapdh	F: 5'-AACGGATTTGGTCGTATTG-3'
	R: 5'-GGAAGATGGTGATGGGATT-3'
P21	F: 5'-GTGGCTCTGATTG GCTTTCTG-3'
	R: 5'-CTGAAAACAGGCAGCCCAAGG-3'
Bcl-2	F: 5'-AGCGTCAACCGGGAGATG-3'
	R: 5'-AGCCAGGAGAAATCAAACAGAG-3'
Bax	F: 5'-AGGCTGAGACGGGGTTATCT-3'
	R: 5'-GCGCAGAAGGAATTAGCAAG-3'

F, forward; R, reverse.

Cell culture

The human MCF7 and MDA-MB453 breast cancer cell lines were purchased from the cell bank of Shanghai, Chinese Academy of Sciences. They were cultured in RPMI 1640 or DMEM medium (Hangzhou bio Pharmaceutical Technology Co., Ltd, China) supplemented with 10% fetal bovine serum (Sijiqing biological engineering materials Co., Ltd, China) and incubated at 5% CO_2, 37°C and 95% humidity.

Cell growth assay

Cell viability was measured by using the tetrazolium salt-based 7Sea Cell Counting Kit (Seven Futai Biotechnology Co. Ltd, China). Cells were seeded in 96-well plate (5000 cells/well). After 24 hours, cells were treated with different doses of Chidamide (0, 20, 40, 60, 80, 100 μmol/L) for 24, 48 and 72 h. Then 20 μl of 7Sea Cell Counting Kit reagent was added to the wells and incubated for 2 h. And the OD was read at 450 nm within 15 min. The experiment was repeated 3 times and each sample had 4 duplications.

Flow cytometry analysis

The proportion of apoptotic cells was quantified by FITC Annexin V Apoptosis Detection Kit I (Becton, Dickinson and Company, USA). Following drug treatment, cells were harvested and washed with phosphate-buffered saline (PBS), and re-suspended in 100 μl of binding buffer. Then cells were incubated with 5 μl Annexin V and 5 μl PI for 15 minutes in the dark at room temperature, before adding 400 μL of binding buffer. The stained cells were analyzed by flow cytometry in an hour.

The cell cycle distribution was quantified by Cell Cycle Staining Kit (MultiSciences, Lianke Biotechnology Co., Ltd. China). Following drug treatment, cells were harvested and washed with phosphate-buffered saline (PBS), and then re-suspended in 1 ml DNA Staining solution. Cells were then incubated with 10 μl Permeabilization solution for 30 minutes before analyzing the stained cells by flow cytometry as soon as possible. Both apoptosis and cell cycle distribution were analyzed using Beckman Coulter Flow Cytometer.

Western blotting

Western blotting was applied to evaluate the content of pro-Caspase-3, pro-PARP, cleaved-PARP and Cyclin B2 in cell extracts after treatment of Chidamide with various concentrations. Cells were lysed with a lysis buffer, and the protein concentrations were determined with BCA Protein Assay Kit (ComWin Biotech Co., Ltd. China). Proteins (30 μg) were separated using 12% SDS-poly-acrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the SDS-PAGE was transferred electronically to polyvinylidenedifluoride (PVDF) membrane. PVDF membranes were blocked with a solution containing 5% skim milk and then incubated overnight at 4°C with the following antibodies: β-actin (Santa Cruz Biotechnology, USA), caspase 3, PARP and Cyclin B2 (Cell Signaling Technology, USA). After washed with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated for 2 h at room temperature with anti-rabbit IgG sheep antibody (Jackson ImmunoResearch, USA) or anti-mouse IgG sheep antibody (Jackson ImmunoResearch, USA) coupled to horseradish peroxidase. Reactive proteins were visualized using the EZ-ECL kit (Biological Industries, Israel).

Quantitative real time polymerase chain reaction (RT-qPCR)

Total RNA of the breast cancer cell lineswere extracted using TRIzol reagent. Total mRNA was reverse transcribed using the PrimeScript™ RT reagent Kit (TaKaRa, Japan). Primers (Shanghai Jierui Biotechnology Co., Ltd. China) used for quantification measurements are shown in **Table 1**. Total cDNA was used as a template for RT-qPCR with the standard protocol using 2 μl cDNA template, 12.5 μl SYBR Premix Ex Taq™ (TaKaRa, Japan), 1 μl forward primer, 1 μl

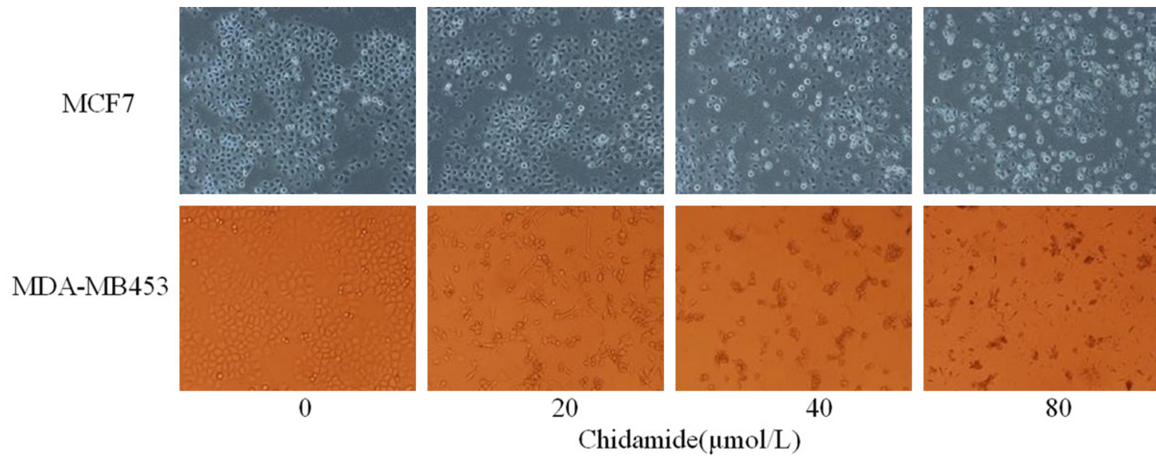

Figure 1. MCF7 and MDA-MB453 were treated with Chidamide for 48 h.

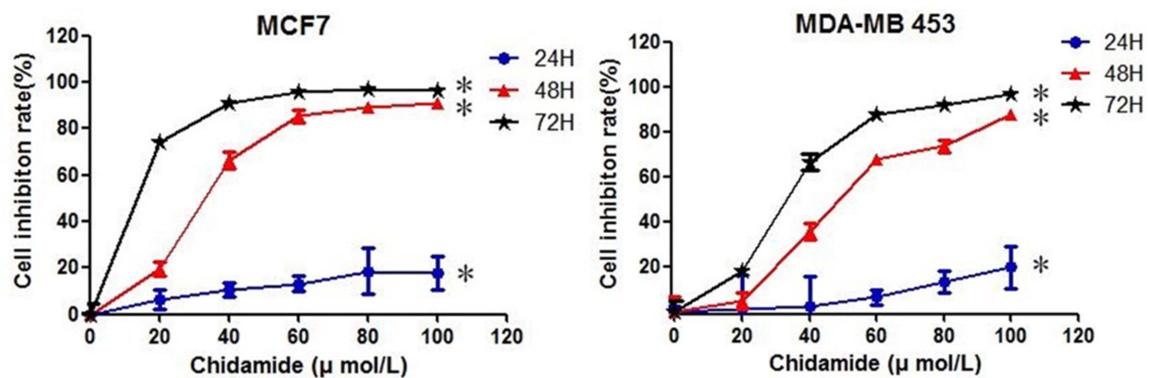

Figure 2. Time and dose-dependent effect of Chidamide on the inhibition of cell growth in MCF7 and MDA-MB453 cells lines. MCF7 and MDA-MB453 cells were treated with different concentrations of Chidamide for 24, 48 and 72 h, and then cell viability was determined by 7Sea-CCK assay.

reverse primer and 8.5 μl dH_2O. The thermal cycling conditions for RT-qPCR included 40 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The results were analyzed using $2^{-\Delta\Delta Ct}$, in which ΔCt = Ct (target gene) - Ct (internal reference), ΔΔCt = ΔCt (sample) - ΔCt (control).

Statistical analysis

Student's t test were used to compare the difference in 7Sea-CCK, RT-qPCR and flowcytometry analysis.

Results

Chidamide inhibits cell proliferation

To determine whether Chidamide had an inhibitory effect on breast cancer cell growth, cell proliferation was determined by 7Sea-CCK. MCF7 and MDA-MB453 cells were treated with 0, 20, 40, 80, 100 μM Chidamide for 24, 48 or 72 h. Treated with Chidamide, the cells began to crease, then turned into the round shape and shed off (**Figure 1**). Moreover, Chidamide suppressed breast cancer cell lines proliferation in a concentration and time-dependent manner, with an stronger effect on inhibiting cell proliferation in the 72 h group (**Figure 2**).

Chidamide induces apoptosis and cell-cycle arrest

To further investigate the mechanism underlying the growth inhibitory effect of Chidamide, apoptosis and cell cycle distribution were analyzed by flow cytometry. After the treatment of Chidamide with different concentrations such as 20, 40, 80 μM, the percentages of apoptosis detected by Annexin V and PI staining were sig-

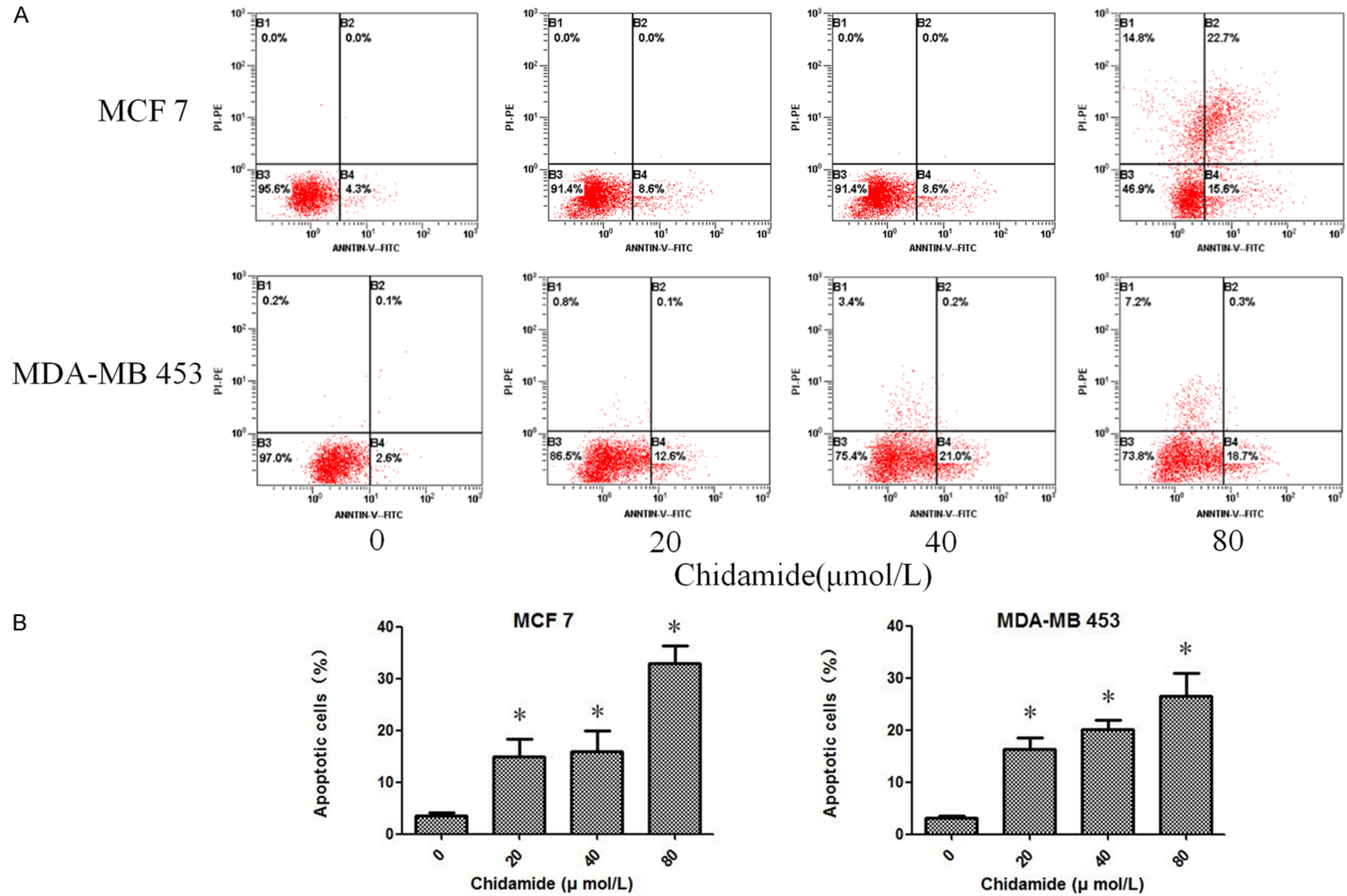

Figure 3. A. MCF7 and MDA-MB453 were treated with Chidamide for 48 h, and then apoptosis were determined by flow cytometry. B. The percentages of apoptosis was quantified in Chidamide treated and untreated cells.

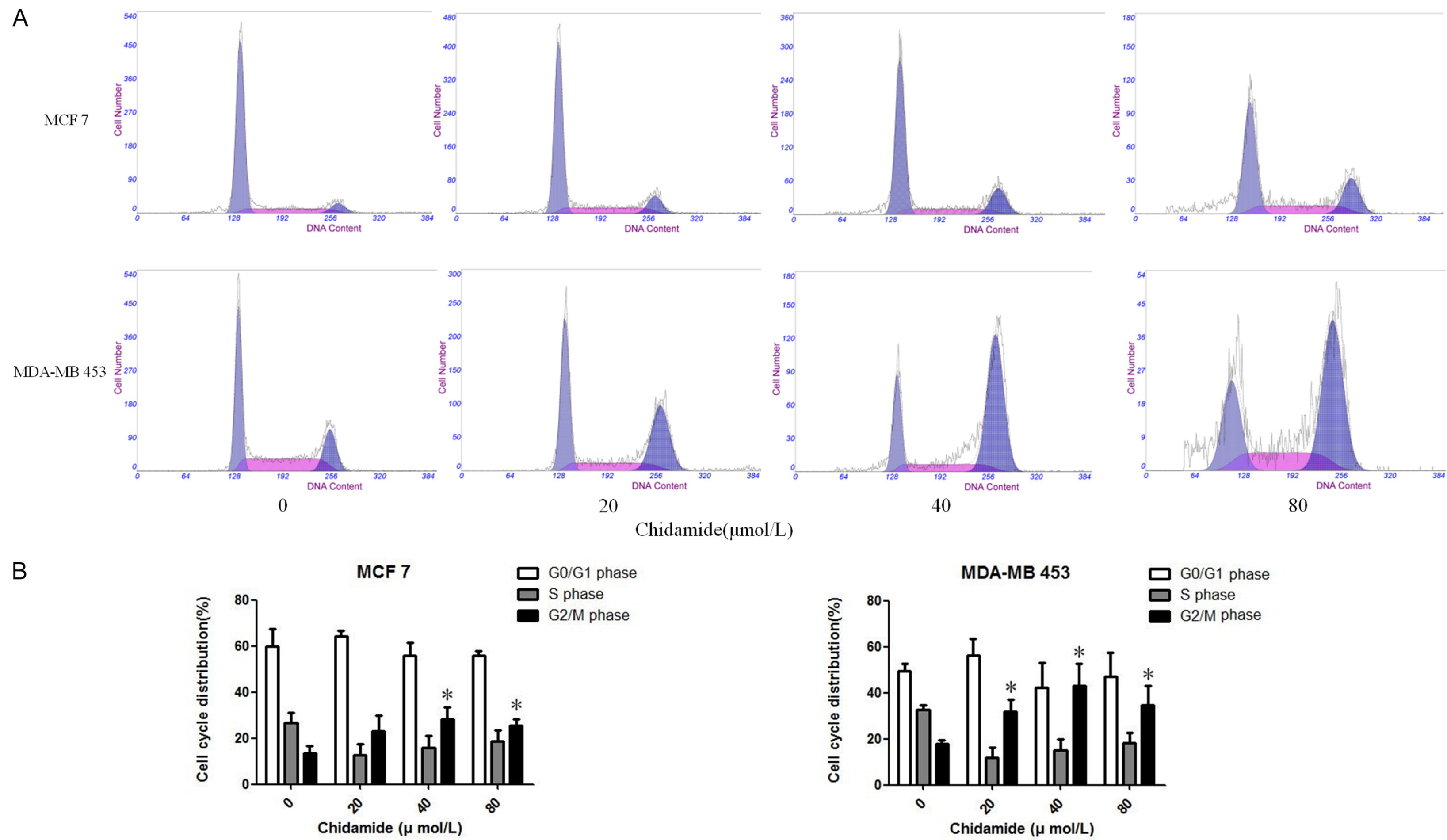

Figure 4. A. MCF7 and MDA-MB453 were treated with Chidamide for 48 h, and then cell cycles were determined by flow cytometry. B. The cell cycle distribution was quantified in Chidamide treated and untreated cells.

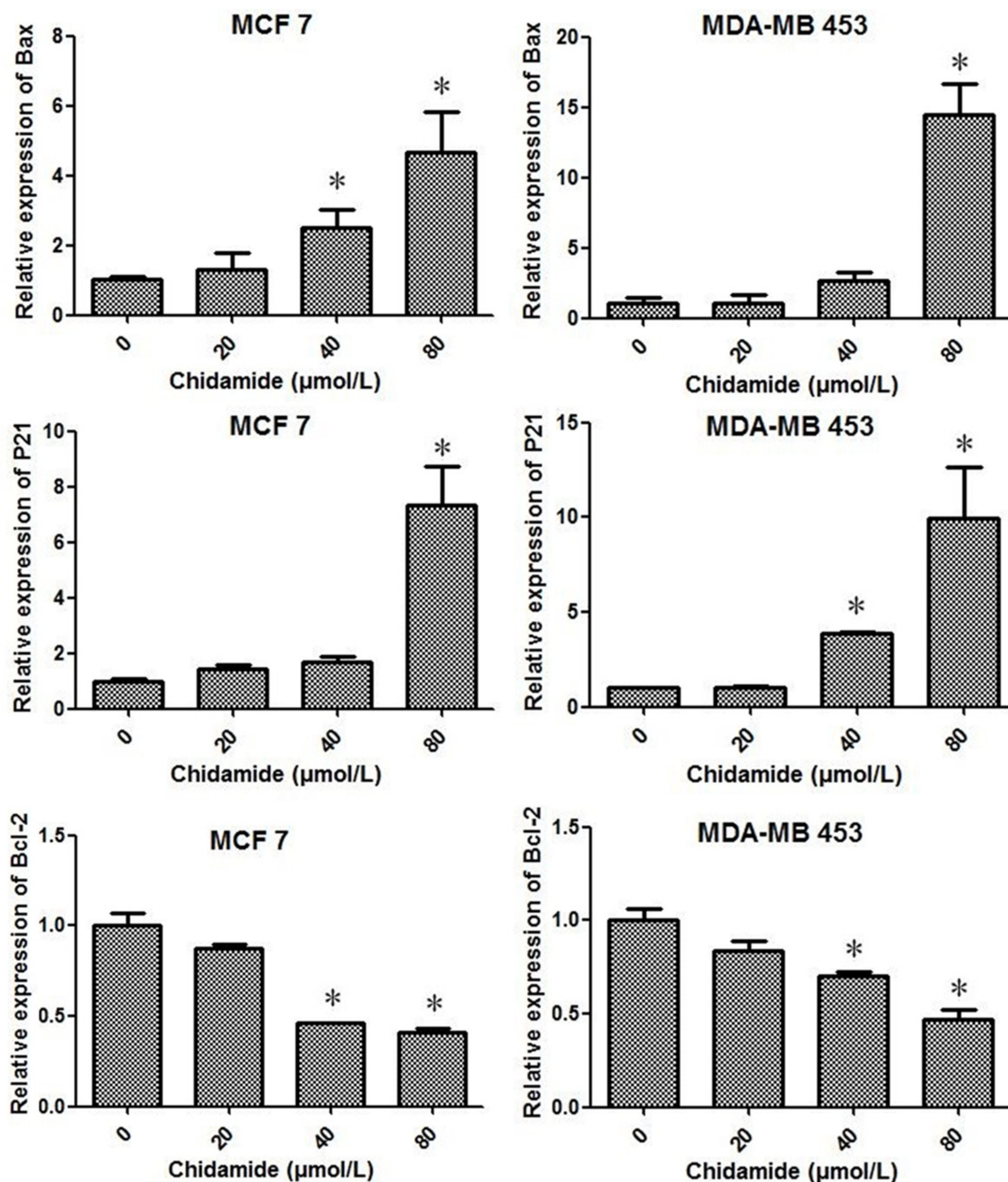

Figure 5. Effect of Chidamide on the mRNA expression of Bax, Bcl-2 and p21. MCF7 and MDA-MB453 cells were treated with Chidamide for 48 h, and then harvested for mRNA expression analysis using RT-qPCR.

nificantly increased (**Figure 3**). In addition, Chidamide treatment also notably altered the cell cycle distributions in both breast cancer cell lines. Cells arrested in G_2/M phase were greatly increased upon Chidamide treatments (**Figure 4**). Consistently, western blotting and RT-qPCR analysis further indicated that pro-Caspase-3, pro-PARP, Cyclin B2 and Bcl-2 expression were down-regulated (**Figure 5**). Whereas cleaved-PARP, Bax and p21 were up-regulated after Chidamide treatment (**Figure 6**). It is therefore likely that the decrease of pro-Caspase-3, pro-PARP, Cyclin B2 and Bcl-2 levels, increase of pro-PARP, Bax and P21 levels by Chidamide induce apoptosis and cell-cycle arrest at the G_2/M phase.

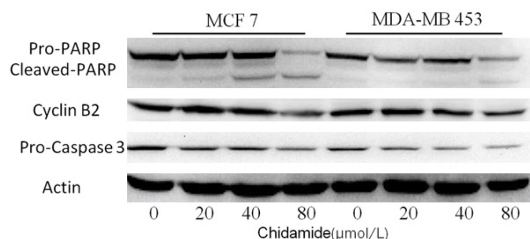

Figure 6. Effect of Chidamide on the protein expression of Pro-PARP, Pro-Caspase3, Cleaved-PARP, Cyclin B2 and Actin. Cells were treated with Chidamide for 48 h, and then harvested for western blot analysis.

Discussion

Formed by DNA and an octamer of histones consisting of H3, H4, H2A and H2B, the nucleosomes are fundamental for building block of chromatin [6]. The structure and function of nucleosome is regulated predominantly by post-translational modifications (PTMs) in these core histones. An astonishing number of PTMs, including acetylation, methylation, citrullination, ubiquitination, sumoylation, ribosylation, isomerization and phosphorylation occur on histones [7, 8]. Such PTMs are dynamically regulated, making them potential targets for chemotherapeutic intervention in the treatment of cancer. Among them, histone acetylation is the major epigenetic events that are validated targets for the treatment of cancer. For example, vorinostat and romidepsin are HDAC inhibitors approved for the treatment of cutaneous T-cell lymphoma.

Chidamide is a novel Class I selective HDACi. Increasing findings from in vitro and in vivo studies supported that Chidamide is a potential therapeutic drug in the treatment of a variety of cancers such as hepatocellular carcinoma [9], leukaemia [10, 11], lung cancer [12], colon cancer [13], pancreatic cancer [14], lymphoma cancer [5]. In this study, we attempted to clarify the effect and possible mechanism of Chidamide in breast cancer cell. For this point, we studied the changes in cell proliferation, cell cycle distribution and apoptosis after Chidamide treatment. We found Chidamide inhibited proliferation, induced apoptosis and arrested at G_2/M phase in the two independent breast cancer cell lines.

There are two major apoptosis pathways including intrinsic mitochondrial apoptosis and extrinsic death receptor pathways or endoplasmicreticulum stress pathways [15]. The mitochondrial apoptosis pathway is the most important pathway by regulating the ratio of Bcl-2 and Bax. Specifically, Bcl-2 suppresses apoptosis partly by blocking efflux of cytochrome which activates downstream caspase signals, while Bax hasan apoptosis promoting effect by antagonizing Bcl-2 [16]. Therefore, the ratio of Bcl-2/Bax expression determines whether the apoptotic process occurs. An increased Bax/Bcl-2 ratio can activate pro-Caspase-3 and result in cell death [17-19]. Caspase-3 plays a central role in the execution of cancer cell apoptosis and also responsible for the cleavage of PARP during cell death. PARP, which is generally recognized as a substrate of caspases-3 with a strong affinity, could be a very useful marker of apoptosis. In this study, the effect of Chidamide on apoptosis was investigated. We found that in MCF7 and MDA-MB453 cells, Chidamide treatment promoted the expression of Bax and cleaved-PARP, but inhibited the expression of Bcl-2, pro-Caspase-3 and pro-PARP in a dose-dependent manner. The results suggested that application of Chidamide significantly enhanced the apoptotic process probably by activating mitochondrial apoptotic pathway.

It is well known that cyclin family proteins regulate cell cycle progression through the association with cyclin-dependent kinases (CDK). Cell cycle progression follows periodic alternations in the levels of D-, E-, A-, and B-type cyclins. In general, inhibition of proliferation and cycle arrest in the G_2/M phase could be due to the reduction of cyclin B. Cyclin B2 is a member of the cyclin B, which plays a key role in mediating entry into G_2/M phase. P21 is an universal inhibitor of CDKs and it is required to arrest cells at the G_1 and G_2 checkpoints, which plays a critical role in the suppression of tumor cell proliferation [20]. Our data showed that Chidamide significantly increased the expression of p21 and decreased the level of Cyclin B2, suggesting that cell cycle arrest at G_2/M phrase induced by Chidamide may be attributed to its promotion of p21 and suppression of Cyclin B2.

In summary, we have shown that Chidamide possesses potent HDACi property and exhibits efficient anti-proliferative activity on breast cancer cells, accompanied by cell apoptosis

and cell cyclearrest at G_2/M phase. Chidamide is a potential candidate agent in the treatment of breast cancer. Certainly, further studies including in vivo studies and the combined effects of Chidamide with other chemotherapeuticagents are needed before clinical applications.

Acknowledgements

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Disclosure of conflict of interest

None.

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