Original Article

Molecular, biological characterization and drug sensitivity of chidamide-resistant MCF7 cells

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Background: Chidamide (CHI) is a subtype-selective histone deacetylase inhibitor (HDACI) developed in China and approved as a second-line treatment combined with the aromatase inhibitor for hormone receptor-positive (HR^+)/human epidermal growth factor receptor 2-negative ($HER2^-$) advanced breast cancer. However, drug resistance is commonly occurred after a long period of medication. This study aimed to investigate the characterization of induced resistance to CHI and explore the potential cross-resistance to chemotherapeutic agents.

Methods: CHI with gradually increasing concentrations was added to breast cancer MCF7 cells to establish a CHI-resistant MCF7 (MCF7-CHI-R) cell line. Cell counting kit-8 (CCK-8) assays were performed to detect half-maximal inhibitory concentration (IC_{50}) of CHI. Colony formation was used to determine the proliferation inhibition rate. Western blot analysis was conducted to detect expressions of protein related with cell cycle, apoptosis, ferroptosis, and histone deacetylase (HDAC). Flow cytometry was used to analyze apoptosis and cell cycle.

Results: The IC_{50} value of CHI of MCF7-CHI-R cells was increased in comparison with MCF7 cells. And CHI led to cell cycle arrest and ferroptosis, which were not exhibited in MCF7-CHI-R cells. Moreover, HDAC activity decreased in MCF7-CHI-R cells in comparison with MCF7 cells, and HDAC1 and HDAC10 might be involved in the resistance to CHI. In addition, MCF7-CHI-R cells were resistant to gemcitabine (GEM), doxorubicin (ADM), docetaxel (DXT), albumin-bound paclitaxel (nab-PTX) and paclitaxel (PTX).

Conclusions: The MCF7-CHI-R was established and the anti-ferroptosis pathway activation was involved in the resistance of MCF-CHI-R cells. Also, MCF7-CHI-R cells were resistant to GEM, ADM, DXT, nab-PTX and PTX.

Keywords: Chidamide (CHI); acquired resistance; histone deacetylase (HDAC); MCF7 cell; ferroptosis

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Introduction

Breast cancer is the leading cause of cancer death among women worldwide (1). Breast cancer accounts for 30% of newly diagnosed malignancies in women and causes 15% of women's cancer deaths, according to data released in 2019 (2). The incidence of breast cancer increases slightly by 0.3% per year, mainly due to the increased incidence of local stage and hormone receptor-positive (HR^+) disease (3). HR^+ breast cancers are those that express estrogen receptor (ER) or progesterone receptor (PR) or both and account for 70–80% of all breast cancers (4). Previous study has indicated that the treatment of HR^+ advanced/metastatic breast cancers has historically been endocrine therapies, including aromatase inhibitors, selective ER modulators, and selective ER down-regulators (5). Pharmacotherapy is one of the most important treatments, but resistance to cancer medication is a huge burden on clinical treatment after a long period of medication (6).

Histone deacetylases (HDACs) are responsible for regulating chromatin remodeling. Chromatin is composed of DNA and histones, and HDACs lead to chromatin compaction and transcriptional suppression by the deacetylation of lysine residues of histone H2A, H2B, H3, and H4 (7). The 18 human HDACs were divided into four groups based on their homology with yeast (8). In the past few decades, accumulating evidence has shown that epigenetic dysregulation is closely related to the development and progression of many diseases, mainly including various cancers (9). HDAC inhibitors (HDACIs) have been used to treat various types of hematologic diseases and solid tumors for several years. So far, five HDACIs, including vorinostat (SAHA, 1), romidepsin (FK228, 2), belinostat (PXD101, 3), panobinostat (LBH589, 4), and chidamide (CHI; CS055, 5) have been approved by the US Food and Drug Administration (FDA) or the China FDA (CFDA) for the treatment of various hematological malignancies (10).

CHI is a novel benzamide HDACI developed in China and is also the first oral subtype selective HDACI targeting HDAC1/2/3/10 in the world (11,12). It has been approved in China for relapsed or refractory peripheral T-cell lymphoma and HR^+/human epidermal growth factor receptor 2-negative ($HER2^-$) advanced breast cancer (13,14). In $HR^+/HER2^-$ advanced breast cancer, it is used as a second-line treatment that complies with aromatase inhibitor. Previous study has shown that CHI induces cell proliferation inhibition and apoptosis in hematologic malignancies (15). But it has been shown in clinical studies that the clinical response to treatment with CHI in relapsed/refractory diffuse large B-cell lymphoma is poor because the majority of patients exhibit drug resistance and cell cycle mechanism played an important role in CHI resistance (16).

Although a few studies have reported CHI resistance in breast cancer, one shortcoming of antitumor drugs is the emergence of drug resistance (17). In the present study, the CHI-resistant MCF7 (MCF7-CHI-R) cell line was established to investigate the characterization of CHI resistance in detail. In addition, possible cross-resistance to chemotherapeutic drugs was investigated, and the results showed that MCF7-CHI-R cells were resistant to gemcitabine (GEM), doxorubicin (ADM), docetaxel (DXT), albumin-bound paclitaxel (nab-PTX), and paclitaxel (PTX). We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2169/rc).

Highlight box

Key findings

- The chidamide (CHI)-resistant MCF7 (MCF7-CHI-R) cell line was established and the anti-ferroptosis pathway activation was involved in the resistance of MCF7-CHI-R cells. Also, MCF7-CHI-R cells were resistant to gemcitabine (GEM), doxorubicin (ADM), docetaxel (DXT), albumin-bound paclitaxel (nab-PTX) and paclitaxel (PTX).

What is known and what is new?

- CHI is a subtype-selective histone deacetylase (HDAC) inhibitor developed in China and approved as a second-line treatment combined with the aromatase inhibitor for hormone receptor-positive/human epidermal growth factor receptor 2-negative advanced breast cancer. However, drug resistance is commonly occurred after a long period of medication. But there are few studies on the drug resistance of CHI in breast cancer.
- CHI with gradually increasing concentrations was added to breast cancer MCF7-CHI-R. We found that the half-maximal inhibitory concentration value of CHI of MCF7-CHI-R cells was increased in comparison with MCF7 cells. And CHI led to cell cycle arrest and ferroptosis, which were not exhibited in MCF7-CHI-R cells. Moreover, HDAC activity decreased in MCF7-CHI-R cells in comparison with MCF7 cells, and HDAC1 and HDAC10 might be involved in the resistance to CHI. MCF7-CHI-R cells were resistant to GEM, ADM, DXT, nab-PTX, and PTX.

What is the implication, and what should change now?

- The results revealed that the induction of ferroptosis might alleviate the resistance to CHI in MCF7-CHI-R cells. And they indicated that GEM, ADM, DXT, nab-PTX, and PTX may not be good options after resistance to CHI for breast cancer patients.

Methods

Chemicals and reagents

CHI was obtained from Shenzhen ChipScreen Biosciences, Ltd. (Shenzhen, China) and was dissolved in dimethyl sulfoxide (DMSO). Fetal bovine serum (FBS) and minimum essential medium (MEM) medium were purchased from Procell (Wuhan, China). Cell counting kit-8 (CCK-8) was obtained from Proteintech (Wuhan, China). The annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit and cell cycle detection kit were supplied by KeyGEN BioTECH (Nanjing, China). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime (Shanghai, China). Bicinchoninic acid assay (BCA) was obtained from Biosharp (Hefei, China). Polyvinylidene difluoride (PVDF) membrane was supplied by Merck Millipore (Burlington, MA, USA). The chemiluminescence detection kit was purchased from Meilunbio (Dalian, China). The detailed information of antibodies and chemotherapy drugs is shown in Table S1.

Cell culture and establishment of CHI-resistant cell lines

MCF7 cells were purchased from Procell and were cultured in MEM medium supplemented with 10% FBS and 0.01 mg/mL insulin in a humidified incubator at 37 ℃ with 5% CO_2. MCF7 cells were treated with CHI at increasing concentrations of 1, 2, 4, 8, and 16 μM for about 6 months to establish a CHI-resistant breast cancer cell line named MCF7-CHI-R. MCF7 cells were authenticated prior to their use and tested for mycoplasma contamination. MCF7-CHI-R cells were authenticated using STR analysis and the results showed that it retained the identity as MCF7 cells. The detailed report of STR identification of MCF7-CHI-R cells is shown in appendix available at https://cdn.amegroups.cn/static/public/tcr-23-2169-1.pdf.

Cell viability analysis

Cell viability was evaluated by CCK-8 assay. The cells were seeded into a 96-well plate at a density of 5×10^3 cells/well. To assess cell viability, 100 μL medium containing serial dilutions of CHI, PTX, DXT, ADM, nab-PTX, 5-fluorouracil (5-FU), GEM, eribulin, or cyclophosphamide (CTX) were added. After 72 hours treatment, the cells were incubated in 100 μL MEM containing 10 μL CCK-8 at 37 ℃ for 3 hours. The absorbance detection was measured at 450 nm using a microplate reader. On the basis of the results, the half-maximal inhibitory concentration (IC_{50}) was calculated.

Clone formation assay

A total of 800 cells were plated on six-well plates, and were treated with 5 μM CHI for 10 days. Cells were washed with phosphate-buffered saline (PBS), fixed in 100% methanol for about 5 min at room temperature, and stained with 0.005% crystal violet (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature.

Cell cycle arrest

The cells were treated with 5 μM CHI for 72 hours. Then, the cells were fixed in 70% ethanol at 4 ℃ overnight, treated with 450 μL Rnase A, and stained with 50 μL propidium iodide for 30 min at room temperature away from light. Then, the cell cycle was analyzed using a Beckman flow cytometer.

Apoptosis assay

The cells were treated with 5 μM CHI for 72 hours. Subsequently, the cells were collected, and stained with 5 μL annexin V and 5 μL propidium iodide for 15 min at room temperature in the dark, and then analyzed by flow cytometry.

Western blot analysis

Western blot analysis was performed using standard procedures. Briefly, cells were lysed with RIPA lysis buffer, and then total protein was extracted and isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. To block non-specifically bound, the membrane was incubated with 5% skim milk powder for 1 hour at room temperature. Membranes were then incubated with primary antibody overnight at 4 ℃ followed by horseradish peroxidase (HRP)-labeled secondary antibody, and then detected by chemiluminescence. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands were used as control for normalization. The density of the target bands was analyzed using ImageJ software.

Statistical analysis

Data were presented as mean ± standard error. Statistical comparisons were analyzed using two-way analysis of variance (ANOVA) and Student's *t*-test using Graph Prism 8 (Graph Pad, La Jolla, CA, USA). P<0.05 was considered statistically significant.

Results

A CHI-resistant breast cancer cell line was established

Resistance to antitumor drugs is commonly occurred. CHI can improve progression-free survival in patients with HR^+/$HER2^-$ advanced breast cancer (13), but few studies have reported CHI resistance in breast cancer. To investigate the acquired resistance to CHI in MCF7 cells, MCF7-CHI-R cells were established after CHI treatment for about 6 months. The IC_{50} values of CHI of MCF7 and MCF7-CHI-R cells after 72 hours were 7.8 and 220.2 μM, respectively (*Figure 1A*). The IC_{50} value of MCF7-CHI-R cells was 28-fold compared with that of MCF7 cells. The morphology of MCF7-CHI-R cells was bigger than MCF7 cells (*Figure 1B*). MCF7 cells exhibited markedly decreased colony formation rate (88%) after CHI treatment for 10 days in comparison with untreated controls (*Figure 1C*, P<0.001), whereas the change in the MCF7-CHI-R cells was not so evident. The colony formation numbers of MCF7, MCF7/CHI 5 μM, MCF7-CHI-R, and MCF7-CHI-R/CHI 5 μM cells were 1,208, 147, 968, and 541, respectively (*Figure 1D*). The growth rate of MCF7-CHI-R cells was lower than that of MCF7 cells (*Figure 1E*). And the growth of MCF7 cells is dependent on estrogen but MCF7-CHI-R cells are less dependent on estrogen (*Figure 1F*). The results showed that MCF7-CHI-R cells decreased the colony formation rates, but their sensitivity to CHI was decreased. These results indicated that the MCF7-CHI-R cell line was established successfully.

Cell cycle arrest induced by CHI was not induced in MCF7-CHI-R cells

To determine the possible intrinsic mechanism of CHI-induced MCF7 cell proliferation inhibition, the cell cycle was detected by flow cytometry after CHI treatment. Cell cycle arrest was induced by CHI during G0/G1 phase in MCF7 cells (*Figure 2A*, P<0.001) but unchanged in MCF7-CHI-R cells.

The expressions of cell cycle-related protein were detected by Western blot analysis. The results showed that CHI significantly increased p21 expression in MCF7 cells (*Figure 2B*, P=0.009), but the effect of CHI on MCF7-CHI-R cells was abolished. The p16 was reduced in MCF7 cells after CHI treatment (*Figure 2C*, P=0.04), but it had no significant change in MCF7-CHI-R cells after CHI treatment. These results indicated that CHI did not induce cell cycle arrest in MCF7-CHI-R cells, and p21 might be involved in CHI resistance in MCF7-CHI-R cells.

Cell apoptosis did not play a key role after CHI treatment in MCF7 and MCF7-CHI-R cells

Apoptosis is one of the main ways of cell death, and to investigate whether CHI induces apoptosis, cell apoptosis was detected by flow cytometry. Results showed that CHI did not induce apoptosis in MCF7 cells and MCF7-CHI-R cells (*Figure 3A*).

The expressions of apoptosis-related protein caspase-3, PARP, and p53 were further detected by Western blot analysis (*Figure 3B-3D*). The results showed that CHI treatment had no effect on caspase-3 and p53 but decreased cleaved caspase-3 (P=0.009) and cleaved PARP (P<0.001) in the MCF7 cells. However, all of them were not changed after CHI treatment in the MCF7-CHI-R cells. The expressions of bcl family members were detected, and the results exhibited that the ratio of bax to bcl2 was upregulated after CHI treatment in MCF7 cells (*Figure 3E*, P=0.03), but it was unchanged after CHI treatment in MCF7-CHI-R cells. The expression of DNA damage repair-related protein γH2AX was detected, and the results showed that CHI decreased the expression of γH2AX in the MCF7 cells (*Figure 3F*, P<0.001), but it had no effect on γH2AX in the MCF7-CHI-R cells. These results suggested that apoptosis did not account for the cell death induced by CHI in MCF7 cells.

Ferroptosis was induced by CHI in MCF7 but not in MCF7-CHI-R cells

Ferroptosis is an iron- and reactive oxygen species (ROS)-dependent programmed cell death that is distinct from apoptosis and has been paid much attention in recent years (18). Extensive study suggests that ferroptosis plays a pivotal role in tumor suppression and is correlated with cancer therapy resistance (19-21). To investigate the effects of CHI on MCF7 cells, the expressions of the ferroptosis-related protein xCT and GPX4 were detected by Western

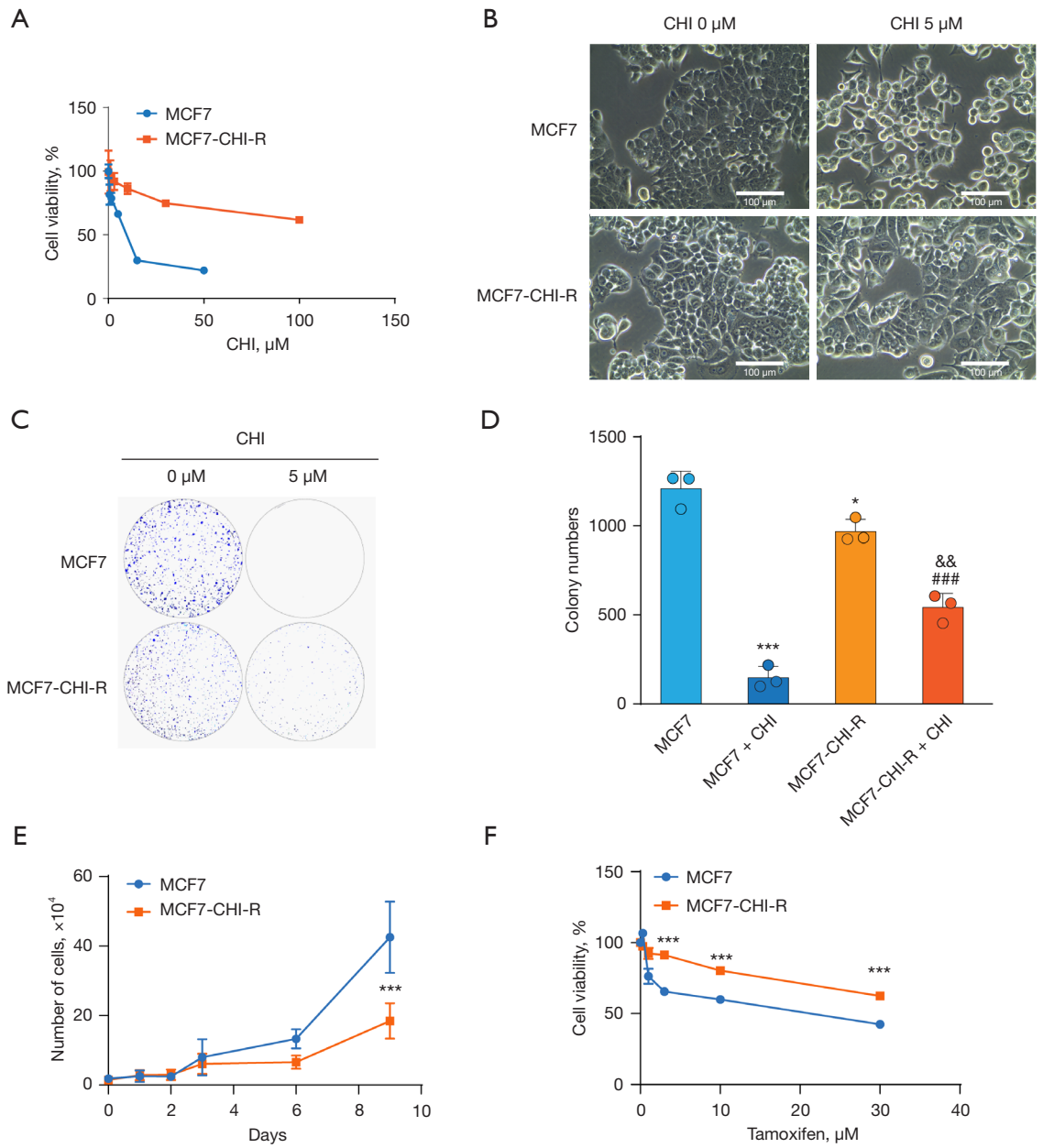


Figure 1 Establishment of CHI-resistant cell line. (A) Survival curves of MCF7 and MCF7-CHI-R cells after CHI treatment for 72 hours. (B) Representative bright-field images of MCF7 and MCF7-CHI-R cells. (C) Colony formation of MCF7 and MCF7-CHI-R cells after stained with 0.005% crystal violet. (D) Quantitative determination of colony formation in MCF7 and MCF7-CHI-R cells. (E) The numbers of MCF7 and MCF7-CHI-R cells. (F) Survival curves of MCF7 and MCF7-CHI-R cells after tamoxifen treatment for 72 hours. *, P≤0.05, ***, P≤0.001, *vs.* MCF7 group; ###, P≤0.001, *vs.* MCF7-CHI-R group; &&, P≤0.01, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. CHI, chidamide.

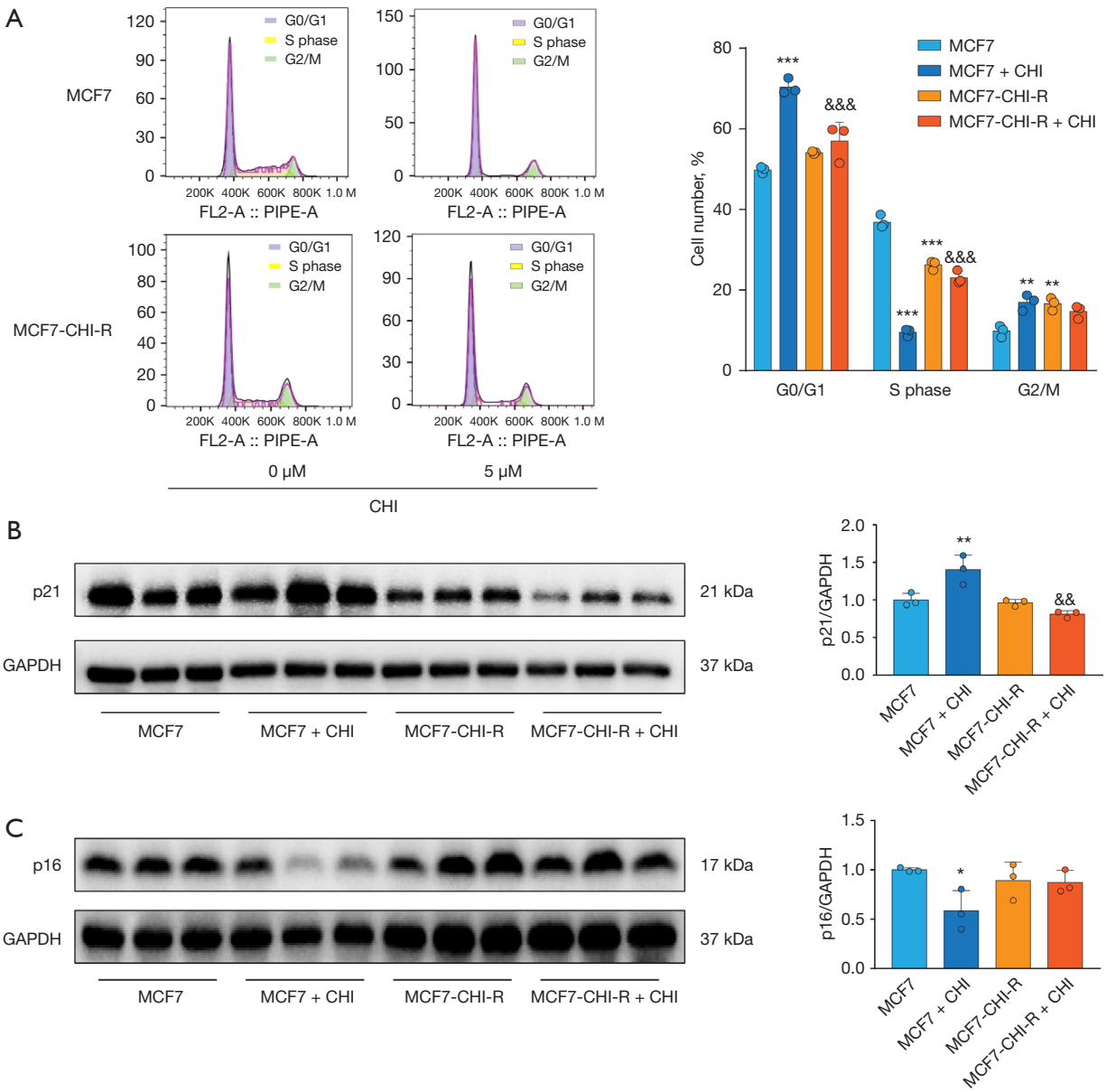
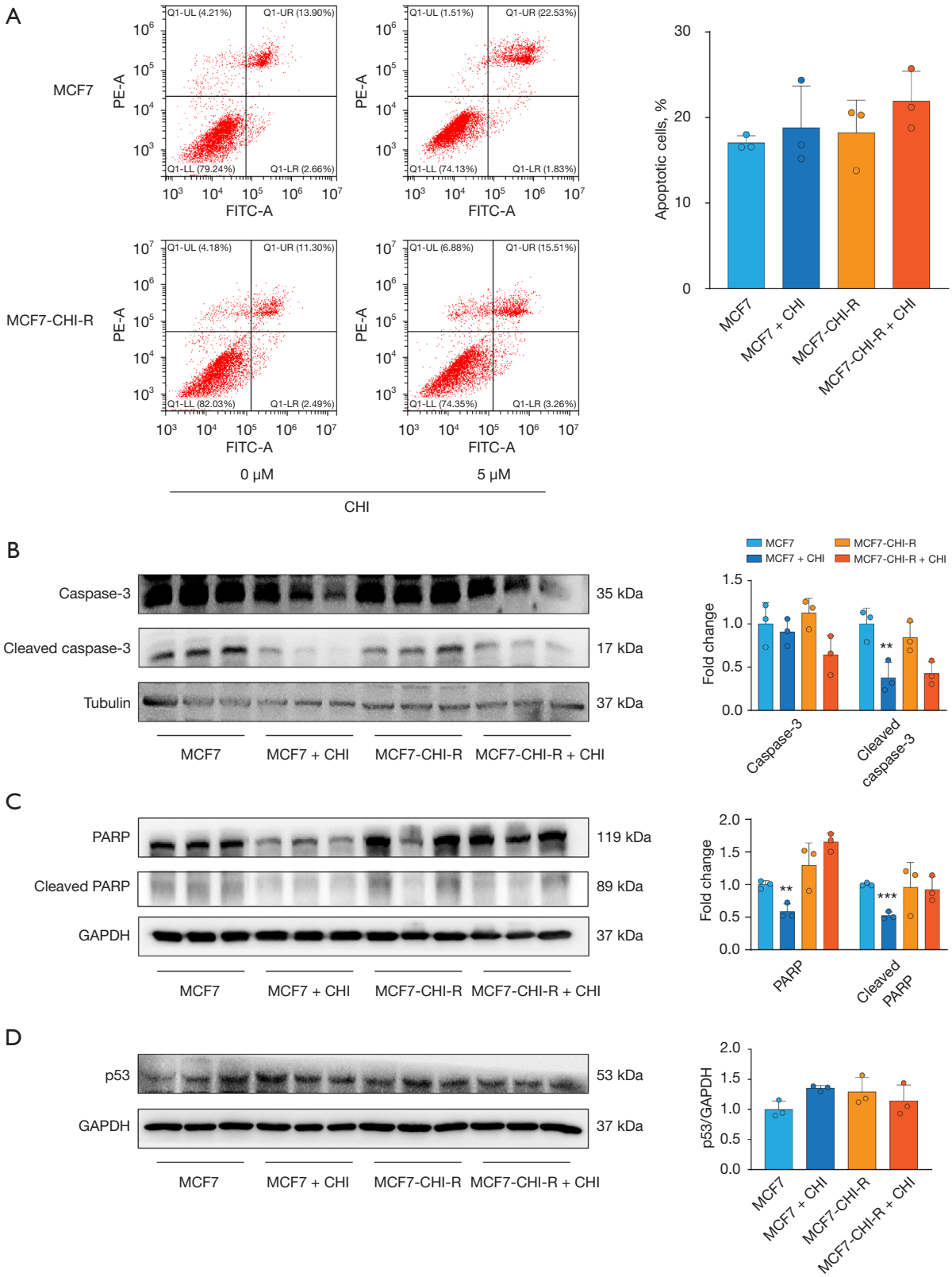


Figure 2 Effects of CHI on the cell cycle of MCF7 and MCF7-CHI-R cells. (A) Cell cycle distribution was examined by propidium iodide staining and was analyzed by two-way ANOVA. (B,C) The expression of p21 and p16 after the treatment of CHI was determined by Western blot analysis, and the quantification was analyzed. *, P≤0.05, **, P≤0.01, ***, P≤0.001, *vs.* MCF7 group; $^{\&\&}$, P≤0.01, $^{\&\&\&}$, P≤0.001, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. CHI, chidamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance.

blot analysis. The results showed that the expressions of xCT (P<0.001) and GPX4 (P=0.04) were downregulated in the MCF7 cells after CHI treatment, but they were not significantly changed in the MCF7-CHI-R cells after CHI treatment (*Figure 4A,4B*). These results indicated that ferroptosis might account for the cell death induced by CHI in MCF7 cells, whereas anti-ferroptosis pathway activation resulted in CHI resistance in MCF7-CHI-R cells.



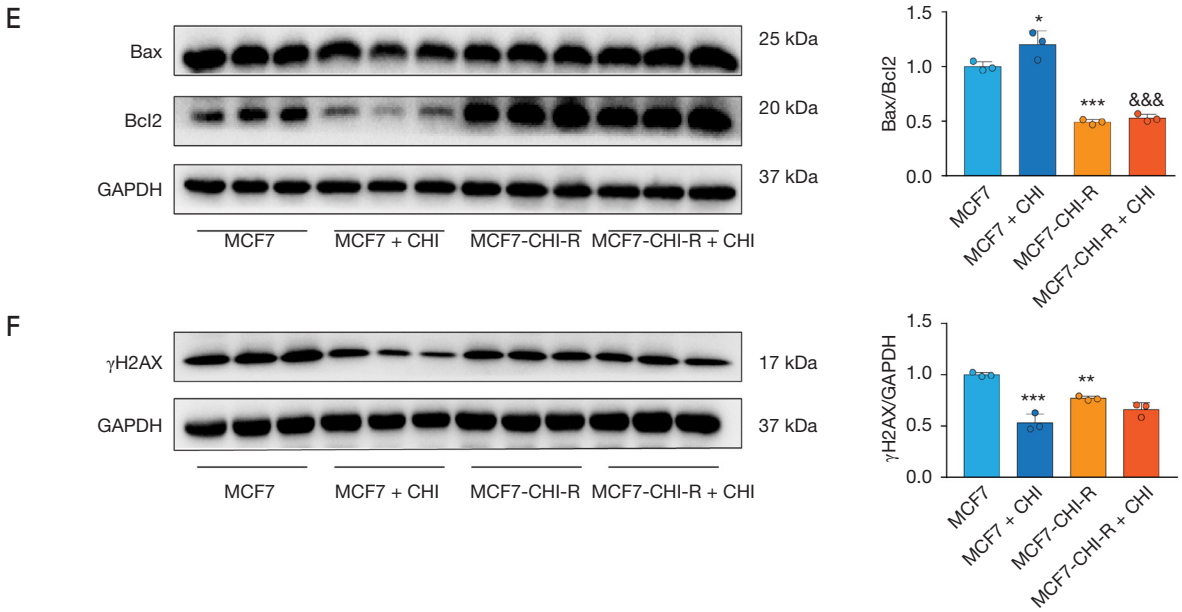


Figure 3 Effects of CHI on the apoptosis of MCF7 and MCF7-CHI-R cells. (A) Apoptosis was measured by flow cytometry analysis and apoptotic cells rate was analyzed by two-way ANOVA. (B) The expressions of caspase-3 and cleaved caspase-3 after the treatment of CHI were determined by Western blot analysis, and the quantification was analyzed. (C-F) The expression of PARP, cleaved PARP, p53, bax, bcl2, and γH2AX were determined by Western blot analysis, and the quantification was analyzed. *, P≤0.05, **, P≤0.01, ***, P≤0.001, *vs.* MCF7 group; $^{\&\&\&}$, P≤0.001, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. PE, phycoerythrin; FITC, fluorescein isothiocyanate; CHI, chidamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance.

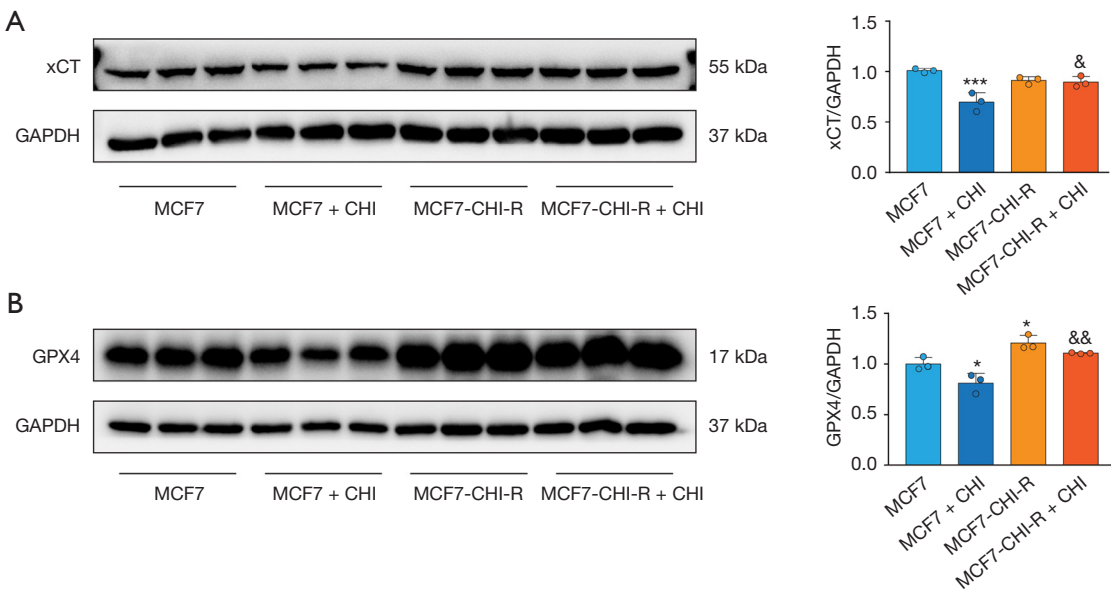


Figure 4 Effects of CHI on the ferroptosis of MCF7 and MCF7-CHI-R cells. (A,B) The expression of xCT and GPX4 after the treatment of CHI was determined by Western blot analysis, and the quantification was analyzed. *, P≤0.05, ***, P≤0.001, *vs.* MCF7 group; $^{\&}$, P≤0.05, $^{\&\&}$, P≤0.01, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHI, chidamide.

Oxidative stress was induced by CHI in MCF7 but not in MCF7-CHI-R cells

As an iron-dependent mode of cell death, ferroptosis is characterized by the accumulation of lipid ROS (22). To investigate the underlying mechanism of CHI-induced ferroptosis, the expressions of the oxidative stress-related protein were detected by Western blot analysis. The results showed that CHI markedly decreased the expression of Keap1 (P<0.001) and increased the expression of Nrf2 (P=0.002) in the MCF7 cells, but the influence of CHI on MCF7-CHI-R cells was not evident (*Figure 5A*,*5B*). Moreover, the expression of HO-1 was increased in MCF7 cells after CHI treatment (P<0.001), but it had no significant change in MCF7-CHI-R cells after CHI treatment (*Figure 5C*). As the oxidative stress-related protein, SOD1 was downregulated in the MCF7 cells after treatment with CHI (P=0.003), but it did not change significantly in the MCF7-CHI-R cells after treatment with CHI (*Figure 5D*). MCF7 cells after CHI treatment contained significantly higher concentrations of ROS than the parental cells (P=0.007). But the baseline of ROS levels in MCF7-CHI-R was upregulated compared to MCF cells (P=0.02) and CHI treatment had no effect on ROS levels in MCF7-CHI-R cells (*Figure 5E*). In conclusion, these results might indicate that MCF7-CHI-R cells acquired resistance to CHI via the Keap1-Nrf2/HO-1 pathway, and antioxidative stress pathway activation might account for the reduced stimulation of CHI on MCF7-CHI-R cells.

HDAC1 and HDAC10 were decreased in MCF7-CHI-R cells

As inhibitors of HDAC, CHI is responsible for inhibiting HDAC1/2/3/10, and their expressions were detected by Western blot analysis (*Figure 6A-6C*). Results showed that the expressions of HDAC1 (P=0.008), HDAC2 (P<0.001), and HDAC10 (P=0.003) were downregulated in the MCF7-CHI-R cells in comparison with MCF7 cells. Moreover, CHI reduced the expressions of HDAC1 (P=0.008), HDAC3 (P=0.04), and HDAC10 (P=0.04) in MCF7 cells, but the effect of CHI was disappeared in the MCF7-CHI-R cells. The expressions of HDAC2 were decreased by 40% in the MCF7 cells after CHI treatment (P<0.001), but they were decreased by 33% in the MCF7-CHI-R cells after CHI treatment (P=0.005). In addition, the acetylation of histones H3 (P<0.001) and H4 (P=0.01) was markedly increased in MCF7 cells after CHI treatment, but the acetylation was not remarkedly changed in the MCF7-CHI-R cells after CHI treatment (*Figure 6D*,*6E*). These results further indicated that MCF7-CHI-R cells were established successfully.

Cross-resistance of the CHI-resistant breast cancer cell line

To investigate whether the MCF7-CHI-R was cross-resistant to other antitumor drugs, CCK-8 assays were performed. As shown in *Figure 7A-7H*, MCF7-CHI-R cells were still sensitive to 5-FU, eribulin, and CTX. Compared with the MCF7 cells, the resistance of MCF7-CHI-R cells to GEM, ADM, DXT, nab-PTX, and PTX increased by 4.22, 6.52, 2.88, 6.76, and >100 times, respectively. The results showed that MCF7-CHI-R cells were also resistant to GEM, ADM, DXT, nab-PTX, and PTX, which might remind the choice of chemotherapy drugs for the patients with CHI resistance in the future.

Discussion

As the inhibitor of class I HDACs, CHI has been reported to have antitumor effects in pancreatic cancer, non-small cell lung cancer, colon cancer, breast cancer, and NK/T lymphoma cells (23). Moreover, CHI has been approved as a second-line treatment that complies with aromatase inhibitors for $HR^+/HER2^-$ advanced breast cancer. The long-term treatment of CHI currently used in the clinic is likely to lead to the emergence of CHI-resistant human breast cancer cells, which might undermine the long-term efficacy of CHI in the treatment of human breast cancer. In the present study, a MCF7-CHI-R cell line was established successfully after CHI treatment for 6 months. These results suggested that ferroptosis might be involved in CHI resistance in MCF7-CHI-R cells, HDAC1 and HDAC10 play a pivotal role in CHI resistance, and MCF7-CHI-R cells were found to be resistant to GEM, ADM, DXT, nab-PTX, and PTX.

Several HDACI-resistant cancer cell lines have been reported (24,25). HDACIs are supposed to inhibit cell proliferation and mediate cell death through several pathways. It has been determined that HDACIs can induce G1 cell cycle arrest by inducing the cyclin-dependent kinase inhibitor p21 (26). In the present study, results showed that the cell cycle was arrested in MCF7 cells after CHI treatment, but it was not influenced in MCF7-CHI-R cells after CHI treatment. In addition, previous

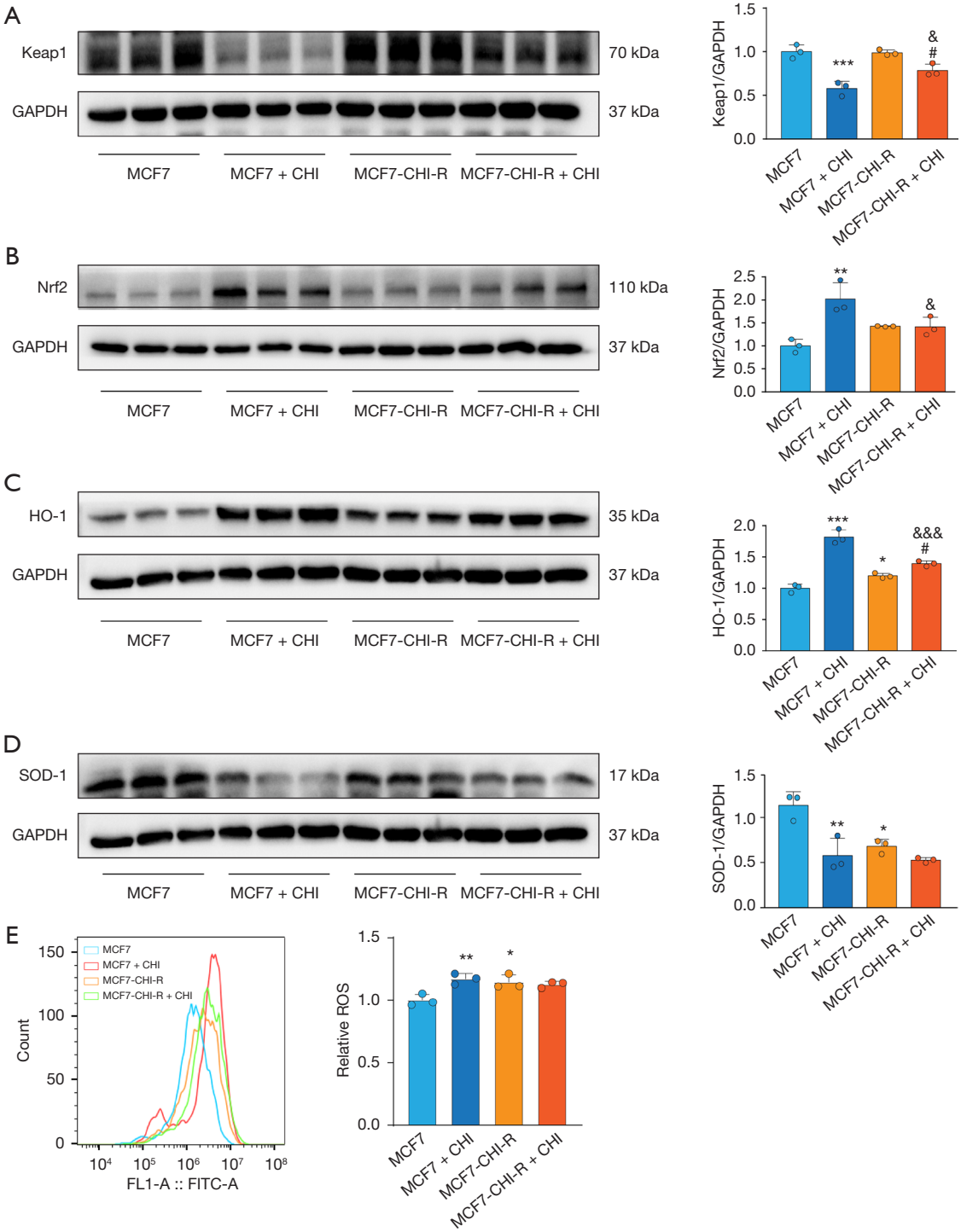


Figure 5 Effects of CHI on the oxidative stress of MCF7 and MCF7-CHI-R cells. (A-D) The expression of Keap1, Nrf2, HO-1, and SOD-1 after the treatment of CHI was determined by Western blot analysis, and the quantification was analyzed. (E) Intracellular ROS concentrations were measured in MCF7 and MCF7-CHI-R cells after CHI treatment. *, P≤0.05, **, P≤0.01, ***, P≤0.001, *vs.* MCF7 group; #, P≤0.05, *vs.* MCF7-CHI-R group; &, P≤0.05, &&&, P≤0.001, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHI, chidamide; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species.

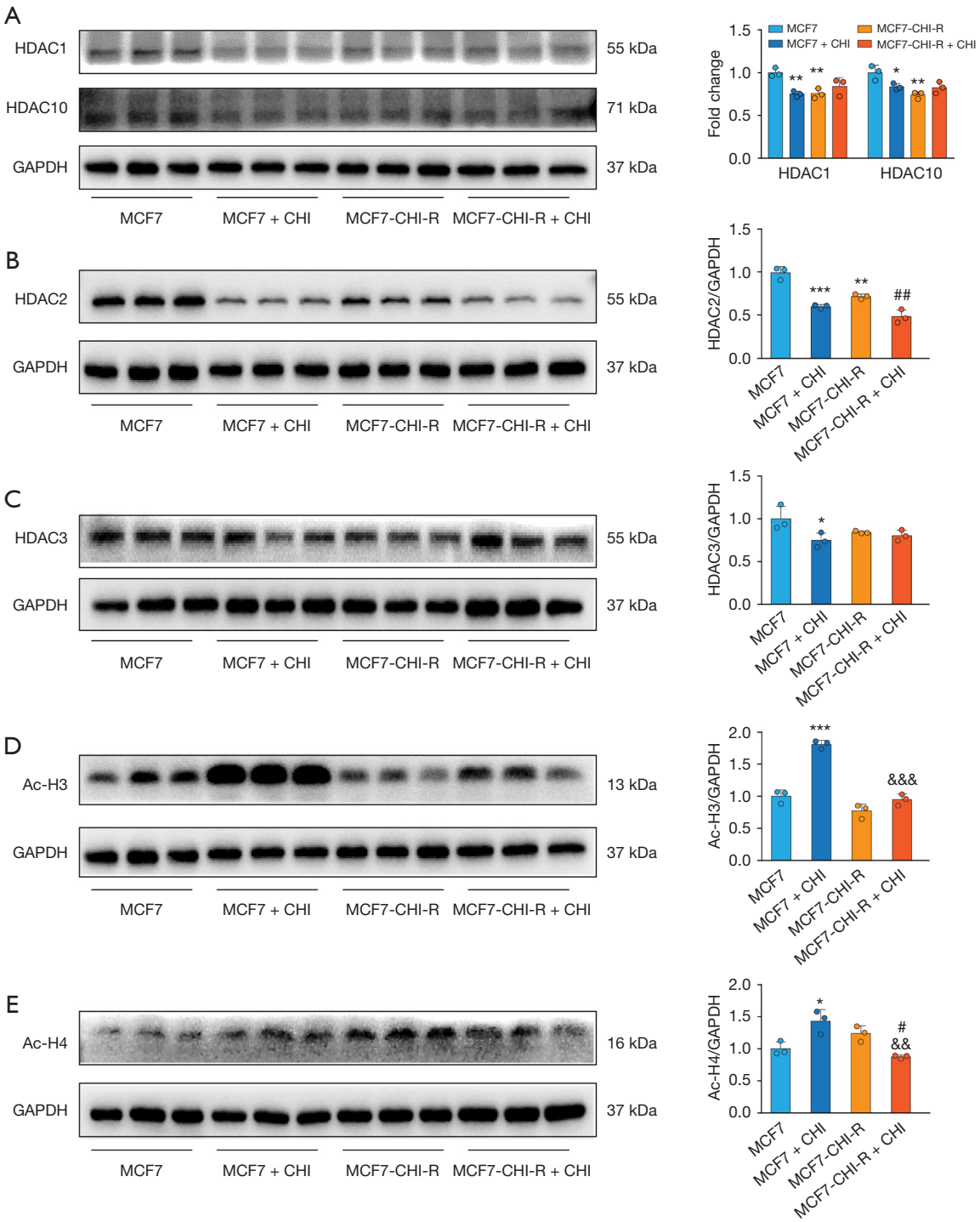


Figure 6 Effects of CHI on the HDAC activity and acetylation of histones H3 and H4 of MCF7 and MCF7-CHI-R cells. (A) The expression of HDAC1 and HDAC10 after the treatment of CHI was determined by Western blot analysis, and the quantification was analyzed. (B-E) The expression of HDAC2, HDAC3, Ac-H3, and Ac-H4 after the treatment of CHI was determined and the quantification was analyzed. *, P≤0.05, **, P≤0.01, ***, P≤0.001, *vs.* MCF7 group; $^{\#}$, P≤0.05, $^{\#\#}$, P≤0.01, *vs.* MCF7-CHI-R group; $^{\&\&}$, P≤0.01, $^{\&\&\&}$, P≤0.001, *vs.* MCF7 + CHI group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7; MCF7 + CHI, MCF7 cells after treatment with CHI; MCF7-CHI-R + CHI, CHI-resistant MCF7 cells after treatment with CHI. HDAC, histone deacetylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHI, chidamide.

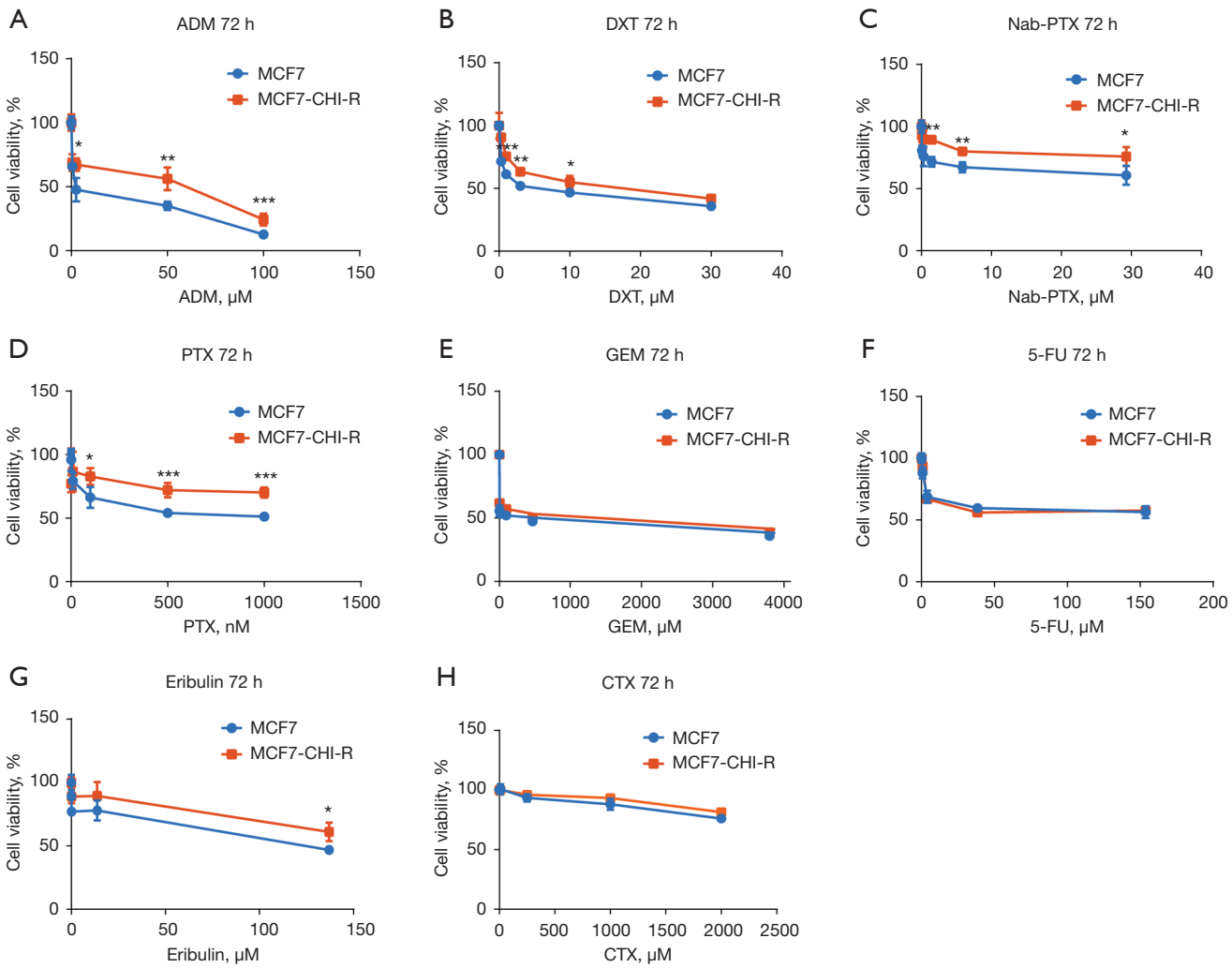


Figure 7 Effects of other chemotherapy drugs on the MCF7 and MCF7-CHI-R cells. (A-H) IC_{50} values of ADM, DXT, nab-PTX, PTX, GEM, 5-FU, eribulin, and CTX of MCF7 and MCF7-CHI-R cells. *, P≤0.05, **, P≤0.01, ***, P≤0.001, *vs.* MCF7 group. n=3 in each group of each figure. MCF7-CHI-R, CHI-resistant MCF7. CHI, chidamide; ADM, doxorubicin; DXT, docetaxel; nab-PTX, albumin-bound paclitaxel; PTX, paclitaxel; GEM, gemcitabine; 5-FU, 5-fluorouracil; CTX, cyclophosphamide; IC_{50}, half-maximal inhibitory concentration.

study has identified that HDACIs could induce apoptosis through many ways, including selective upregulation of death receptors and ligands in tumor cells, downregulation of pro-survival proteins, and upregulation of pro-apoptotic proteins (27). However, in the present study, the effect on apoptosis in MCF7 cells after CHI treatment was not prominent, which was reflected through the cell apoptosis rate and the expressions of protein related with apoptosis.

Ferroptosis is an iron-dependent form of non-apoptotic cell death; previous study identified that HDACIs suppressed tumor by ferroptosis induction (28). Similar to the reported research, the results in the present study showed that the expressions of xCT and GPX4 were decreased in MCF7 cells after treatment with CHI, but they were not changed in the MCF7-CHI-R cells after treatment with CHI. Numerous previous studies have indicated that the regulation of ferroptosis could influence the efficacy of cancer treatment and even reverse cancer therapy resistance (21,29,30). Similar to previous research, the results in the present study revealed that the induction of ferroptosis might alleviate the resistance to CHI in MCF7-CHI-R cells. However, subsequent experiments are still needed

to demonstrate induction of ferroptosis to overcome drug resistance.

Previous research has shown that the activation of the Keap-Nrf2 pathway can inhibit ferroptosis, and the Nrf2 signaling pathway is responsible for antioxidation (31). Nrf2 localizes in the cytoplasm under normal conditions where it interacts with Keap1. The oxidative modification of Keap1 causes it to dissociate from Nrf2, allowing Nrf2 translocation to the nucleus where it binds to antioxidant responsive elements, such as HO-1 and SOD (32). We postulated that CHI-induced oxidative stress may cause Nrf2-mediated upregulation of antioxidants. However, the effect of CHI was abolished in the MCF7-CHI-R cells, which indicated that the stimulation of CHI to MCF7-CHI-R cells decreased and a CHI-resistant cell line was established.

CHI inhibits HDAC1, 2, 3, and 10 at low nanomolar concentrations (33) and HDAC1 is most closely associated with the malignant phenotype (34). In our studies, HDAC1, HDAC3, and HDAC10 were significantly downregulated in MCF7 cells after CHI treatment, but they were not changed in MCF7-CHI-R cells after CHI treatment. It has been exhibited that HL-60/LR cells possess significantly higher expressions of HDAC1, HDAC2, and HDAC4 than HL-60 cells, but HL-60/LR cells lack HDAC6 (24). In the present study, MCF7-CHI-R cells manifested lower levels of HDAC1, HDAC2, and HDAC10 than MCF7 cells, which might explain the resistance of MCF7-CHI-R cells.

An important finding from this study is that the CHI-resistant cells showed cross-resistance to other chemotherapy drugs for breast cancer. For instance, vorinostat-resistant cells showed cross-resistance to other first-generation and second-generation HDACIs (35). In addition, the A549-CHI-R cell line was cross-resistant to other chemotherapeutic drugs, including GEM, vinorelbine, and PTX (34). In the present study, MCF7-CHI-R cells with decreased HDAC activity showed cross-resistance to GEM, ADM, DXT, nab-PTX, and PTX. For $HR^+/HER2^-$ advanced breast cancer, if the disease progresses on second-line or third-line endocrine therapy, chemotherapy will be given. Thus, our results indicated that GEM, ADM, DXT, nab-PTX, and PTX may not be good options after resistance to CHI. By contrast, 5-FU, eribulin, and CTX may be used.

There are some limitations in our current study. First, we did not conduct animal experiments to demonstrate CHI resistance of MCF7-CHI-R cells. Second, we only indicate that ferroptosis might play an important role in the CHI resistance, but data must be supplemented to elucidate the mechanism of resistance and overcome CHI resistance. Despite these shortcomings, our study revealed that a CHI-resistant cell line was established.

Conclusions

In conclusion, a CHI-resistant cell line was established, and it was proposed that cell cycle, ferroptosis, and HDAC downregulation may contribute to CHI resistance. The results also revealed that the induction of ferroptosis might alleviate the resistance to CHI in MCF7-CHI-R cells. In addition, the CHI-resistant cell line remained sensitive to 5-FU, eribulin, and CTX but cross-resistant to GEM, ADM, DXT, nab-PTX, and PTX.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2169/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2169/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2169/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

1. Zeng C, Zhang J. A narrative review of five multigenetic assays in breast cancer. Transl Cancer Res 2022;11:897-907.
2. Wang X, Wang C, Guan J, et al. Progress of Breast Cancer basic research in China. Int J Biol Sci 2021;17:2069-79.
3. DeSantis CE, Ma J, Gaudet MM, et al. Breast cancer statistics, 2019. CA Cancer J Clin 2019;69:438-51.
4. Kay C, Martínez-Pérez C, Meehan J, et al. Current trends in the treatment of HR+/HER2+ breast cancer. Future Oncol 2021;17:1665-81.
5. Rascon K, Flajc G, De Angelis C, et al. Ribociclib in HR+/HER2- Advanced or Metastatic Breast Cancer Patients. Ann Pharmacother 2019;53:501-9.
6. Dan VM, Raveendran RS, Baby S. Resistance to Intervention: Paclitaxel in Breast Cancer. Mini Rev Med Chem 2021;21:1237-68.
7. Ramaiah MJ, Tangutur AD, Manyam RR. Epigenetic modulation and understanding of HDAC inhibitors in cancer therapy. Life Sci 2021;277:119504.
8. Li Y, Seto E. HDACs and HDAC Inhibitors in Cancer Development and Therapy. Cold Spring Harb Perspect Med 2016;6:a026831.
9. Sang Cho K, Song B, Lee IS. Neurodevelopmental disorders with epigenetic dysregulation and Drosophila. Front Biosci (Landmark Ed) 2019;24:1330-49.
10. Zhao C, Dong H, Xu Q, et al. Histone deacetylase (HDAC) inhibitors in cancer: a patent review (2017-present). Expert Opin Ther Pat 2020;30:263-74.
11. Huang H, Wenbing Y, Dong A, et al. Chidamide Enhances the Cytotoxicity of Cytarabine and Sorafenib in Acute Myeloid Leukemia Cells by Modulating H3K9me3 and Autophagy Levels. Front Oncol 2019;9:1276.
12. Ding N, You A, Tian W, et al. Chidamide increases the sensitivity of Non-small Cell Lung Cancer to Crizotinib by decreasing c-MET mRNA methylation. Int J Biol Sci 2020;16:2595-611.
13. Jiang Z, Li W, Hu X, et al. Tucidinostat plus exemestane for postmenopausal patients with advanced, hormone receptor-positive breast cancer (ACE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2019;20:806-15.
14. Shi Y, Dong M, Hong X, et al. Results from a multicenter, open-label, pivotal phase II study of chidamide in relapsed or refractory peripheral T-cell lymphoma. Ann Oncol 2015;26:1766-71.
15. Yuan XG, Huang YR, Yu T, et al. Chidamide, a histone deacetylase inhibitor, induces growth arrest and apoptosis in multiple myeloma cells in a caspase-dependent manner. Oncol Lett 2019;18:411-9.
16. Sun Y, Gao Y, Chen J, et al. CREBBP cooperates with the cell cycle machinery to attenuate chidamide sensitivity in relapsed/refractory diffuse large B-cell lymphoma. Cancer Lett 2021. [Epub ahead of print]. doi: 10.1016/j.canlet.2021.09.002.
17. Xu J, Ni L, Zhao F, et al. Overexpression of hsa_circ_0002874 promotes resistance of non-small cell lung cancer to paclitaxel by modulating miR-1273f/MDM2/p53 pathway. Aging (Albany NY) 2021;13:5986-6009.
18. Wang H, Cheng Y, Mao C, et al. Emerging mechanisms and targeted therapy of ferroptosis in cancer. Mol Ther 2021;29:2185-208.
19. Zhang C, Liu X, Jin S, et al. Ferroptosis in cancer therapy: a novel approach to reversing drug resistance. Mol Cancer 2022;21:47.
20. Viswanathan VS, Ryan MJ, Dhruv HD, et al. Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. Nature 2017;547:453-7.
21. Friedmann Angeli JP, Krysko DV, Conrad M. Ferroptosis at the crossroads of cancer-acquired drug resistance and immune evasion. Nat Rev Cancer 2019;19:405-14.
22. Li J, Cao F, Yin HL, et al. Ferroptosis: past, present and future. Cell Death Dis 2020;11:88.
23. Bai X, Jiang H, Han G, et al. Chidamide suppresses the glycolysis of triple negative breast cancer cells partially by targeting the miR-33a-5p-LDHA axis. Mol Med Rep 2019;20:1857-65.
24. Fiskus W, Rao R, Fernandez P, et al. Molecular and biologic characterization and drug sensitivity of pan-histone deacetylase inhibitor-resistant acute myeloid leukemia cells. Blood 2008;112:2896-905.
25. Zhu Y, Das K, Wu J, et al. RNH1 regulation of reactive oxygen species contributes to histone deacetylase

inhibitor resistance in gastric cancer cells. Oncogene 2014;33:1527-37.
26. Marks PA. The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. Expert Opin Investig Drugs 2010;19:1049-66.
27. Dickinson M, Johnstone RW, Prince HM. Histone deacetylase inhibitors: potential targets responsible for their anti-cancer effect. Invest New Drugs 2010;28 Suppl 1:S3-20.
28. Zille M, Kumar A, Kundu N, et al. Ferroptosis in Neurons and Cancer Cells Is Similar But Differentially Regulated by Histone Deacetylase Inhibitors. eNeuro 2019;6:ENEURO.0263-18.2019.
29. Li B, Yang L, Peng X, et al. Emerging mechanisms and applications of ferroptosis in the treatment of resistant cancers. Biomed Pharmacother 2020;130:110710.
30. Wu Y, Yu C, Luo M, et al. Ferroptosis in Cancer Treatment: Another Way to Rome. Front Oncol 2020;10:571127.
31. Li X, Chen J, Yuan S, et al. Activation of the P62-Keap1-NRF2 Pathway Protects against Ferroptosis in Radiation-Induced Lung Injury. Oxid Med Cell Longev 2022;2022:8973509.
32. Hu Y, Lu W, Chen G, et al. Overcoming resistance to histone deacetylase inhibitors in human leukemia with the redox modulating compound β-phenylethyl isothiocyanate. Blood 2010;116:2732-41.
33. Ning ZQ, Li ZB, Newman MJ, et al. Chidamide (CS055/HBI-8000): a new histone deacetylase inhibitor of the benzamide class with antitumor activity and the ability to enhance immune cell-mediated tumor cell cytotoxicity. Cancer Chemother Pharmacol 2012;69:901-9.
34. Luo S, Ma K, Zhu H, et al. Molecular, biological characterization and drug sensitivity of chidamide-resistant non-small cell lung cancer cells. Oncol Lett 2017;14:6869-75.
35. Dedes KJ, Dedes I, Imesch P, et al. Acquired vorinostat resistance shows partial cross-resistance to 'second-generation' HDAC inhibitors and correlates with loss of histone acetylation and apoptosis but not with altered HDAC and HAT activities. Anticancer Drugs 2009;20:321-33.

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Supplementary

Table S1 The detailed information of antibodies and chemotherapy drugs

Reagent	Company	Cat. No.	Dilution
PTX	Aladdin	33069-62-4	–
DXT	Jiangsu Hengrui Medicine Co., Ltd.	H20020543	–
CTX	Baxter Oncology GmbH	HJ20160467	–
Adriamycin	Rhawn	R034347	–
GEM	Lilly France S.A.S.	No.7501	–
Nab-PTX	Qilu Pharmaceutical Co., Ltd.	H20193309	–
Eribulin	Eisai Pharmaceutical Co., Ltd.	02220803	–
Fluorouracil	Shanxi Pude Pharmaceutical Co., Ltd.	H20051113	–
Bax	Beyotime	AF5120	1:1,000
Bcl2	Proteintech	12789-1-AP	1:1,000
p21	Beyotime	AF5252	1:1,000
p16	Bimake	A5163	1:1,000
Caspase 3	Beyotime	AF5132	1:1,000
PARP	CST	9532	1:1,000
HDAC1	Proteintech	10197-1-AP	1:1,000
HDAC2	Beyotime	AF1555	1:1,000
HDAC3	Proteintech	10255-1-AP	1:1,000
HDAC10	Proteintech	67646-1-Ig	1:5,000
Ac-H3	Beyotime	AF5611	1:2,000
Ac-H4	Beyotime	AF5629	1:1,000
xCT	Bimake	A5912	1:1,000
GPX4	Bimake	A5569	1:1,000
Keap1	Proteintech	60027-1-1G	1:1,000
Nrf2	Proteintech	16396-1-AP	1:1,000
HO-1	Proteintech	10701-1-AP	1:1,000
SOD-1	Affinity	AF5198	1:1,000
GAPDH	Proteintech	60004-1-Ig	1:50,000
p53	Beyotime	AF0255	1:1,000
γH2AX	Abcam	Ab22551	1:1,000

PTX, paclitaxel; DXT, docetaxel; CTX, cyclophosphamide; GEM, gemcitabine; nab-PTX, albumin-bound paclitaxel; HDAC, histone deacetylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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