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# Chidamide, a novel histone deacetylase inhibitor, synergistically enhances gemcitabine cytotoxicity in pancreatic cancer cells

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ABSTRACT

Pancreatic cancer is a lethal human malignancy with an extremely poor prognosis and urgently requires new therapies. Histone deacetylase inhibitors (HDACIs) represent a new class of anticancer agents and have shown promising antitumor activities in preclinical models of pancreatic cancer. In this study, we sought to determine the antitumor effects of a novel HDACI, chidamide (CS055), in pancreatic cancer cells alone or in combination with gemcitabine. Treatments of BxPC-3 or PANC-1 pancreatic cancer cell lines with chidamide resulted in dose- and time-dependent growth arrest, accompanied by induction of p21 expression. When combined in a sequential schedule, chidamide synergistically enhanced gemcitabine-induced cell growth arrest and apoptosis, accompanied by cooperative downregulation of Mcl-1 and loss of mitochondrial membrane potential ($\Delta\Psi_m$). Chidamide enhanced gemcitabine-induced DNA double-strand breaks and S phase arrest, and abrogated the G2/M cell cycle checkpoint, potentially through suppression of CHK1 expression. Our results suggest that chidamide has a therapeutic potential for treating pancreatic cancer, especially in combination with gemcitabine.



## 1. Introduction

Pancreatic cancer is a common human gastrointestinal malignancy worldwide and is the fourth leading cause of death from cancer in the US. It is estimated that 43,920 people will be newly diagnosed with pancreatic cancer and 37,390 pancreatic cancer patients will die in the US in 2012 [1]. Despite the extremely poor prognosis with a 5-year survival rate less than 5% [2,3], little improvement has been made in prognosis in the past 20 years.

Histone deacetylase inhibitors (HDACIs) are promising new agents for the treatment of cancer and have demonstrated anticancer efficacy across a range of cancers, most impressively in hematological malignancies [4]. HDACIs have been shown to induce cell-cycle arrest, differentiation and apoptosis in tumor cells, but less so in normal cells [5–8]. Further, some HDACIs including Vorinostat (SAHA), MS-275, Trichostatin A (TSA), and valproic acid (VPA) have been shown to synergistically enhance anticancer activities of conventional chemotherapeutic drugs [8–10]. In pancreatic cancer cells, synergistic antitumor interactions between HDACIs, such as TSA, Vorinostat, or MGCD0103, and gemcitabine have been previously reported [11–14].

Chidamide (CS055/HBI-8000), a novel HDACI of the benzamide class, has been in phase I clinical trial in the US and phase II/III trials for cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) in China [15]. Chidamide has shown anticancer activity in colon, lung, breast, and liver solid tumor cells and in myeloid leukemia cells [15–17]. In this study, we hypothesize that chidamide synergistically augments the anticancer activity of gemcitabine in pancreatic cancer cells by inducing apoptosis. We demonstrate that chidamide induces growth arrest of pancreatic cancer cells, accompanied by cell cycle arrest and up-regulation of $p21^{CIP1/WAF1}$. Further, we show that chidamide cooperatively augments gemcitabine-induced apoptosis in pancreatic cancer cells by enhancing gemcitabine-induced DNA double-strand breaks (DSBs) and S phase arrest and abrogating the G2/M cell cycle checkpoint. These results indicate that chidamide may have a therapeutic potential in treating pancreatic cancer, especially in combination with gemcitabine.

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## 2. Materials and methods

### 2.1. Chemicals

Chidamide, N-(2-amino-5-fluorine benzyl)-4-[N-(pyridine-3-acrylyl) ammonia methyl] benzamide (patent No. PCT/IB04/000401) was designed and synthesized by Shenzhen Chipscreen Biosciences Ltd. (Shenzhen, China). Gemcitabine (GEM; Gemzar) was purchased from Eli Lilly (Fegersheim, France). All other chemicals were of analytical grade and obtained commercially.

### 2.2. Cell culture

Human pancreatic cancer cell lines BxPC-3 and PANC-1 were obtained from the Cell Biology Research Institute of Shanghai, Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin/streptomycin (100 U/ml each), $NaHCO_3$ (2 g/L), and Hepes (2.4 g/L) in an incubator at 37 C with 95% air and 5% $CO_2$.

### 2.3. In vitro cytotoxicity assays

*In vitro* cytotoxicities of chidamide or gemcitabine alone or in combination in the pancreatic cancer cell lines were determined by using MTT reagent [10]. Briefly, BxPC-3 or PANC-1 cells were plated in 96-well plates ($3 \times 10^3$ cells/well) and were treated with chidamide or gemcitabine at the indicated concentrations, alone or sequentially combined (gemcitabine treatment for 48 h followed by chidamide treatment for 72 h), for up to 120 h. MTT was then added to a final concentration of 1 mM. After 4 h, formazan crystals were dissolved by the addition of 100 μl of 10% SDS in 10 mM HCl. Optical densities were measured with a visible microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. The extent and direction of antitumor interactions between the two agents were determined by calculating combination index (CI) using the CalcuSyn software (Biosoft, Cambridge, UK) [12]. CI < 1, CI = 1, or CI > 1 indicates synergistic, additive, or antagonistic effects, respectively.

### 2.4. Cell cycle analysis

The effects of chidamide or gemcitabine, alone or in combination, on cell cycle distribution in the pancreatic cancer cell clines were analyzed by using propidium iodide (PI) staining and flow cytometry analysis with a Beckman Colter flow cytometer (Brea, CA). Cell cycle analysis was performed with the ModFit LT™3.0 DNA analysis software (Becton Dickinson, Franklin Lakes, NJ).

### 2.5. Assessment of baseline and drug-induced apoptosis

The effects of chidamide or gemcitabine, alone or in combination, on apoptosis in BxPC-3 or PANC-1 cell clines were analyzed by flow cytometry analysis, as previously described [10,18,19]. The treated cells were harvested and apoptosis was determined using an Apoptosis Annexin V-FITC kit (Keygen Biotechnology Co. Ltd., Nanjing, China) and flow cytometry analysis. Results are presented as percent of Annexin $V^+$ cells (mean ± standard errors).

### 2.6. Western blots

BxPC-3 or PANC-1 cells treated with chidaminde or gemcitabine alone or combined were washed and lysed in a lysis buffer [20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM phenylmethylsulfonyl and protease inhibitor cocktail]. The protein lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4 °C and subjected to SDS–PAGE and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). The membrane was immunoblotted with anti-acetylated histone H3 (ac-H3), -p21, -Bax, -Bcl-2, -Bim, -caspase 3, -caspase 9, -phospho-H2AX (γH2AX), -GAPDH (Cell Signaling Technology, Danvers, MA, USA), -Mcl-1, or -CHK1 (Santa Cruz Biotechnology Inc., CA, USA) antibodies. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA).

### 2.7. Analysis of mitochondrial membrane potential (MMP, $\Delta\Psi_m$)

MMP was assessed by the retention of Rhodamine 123 [20,21]. BxPC-3 or PANC-1 cells were treated with chidamide or gemcitabine alone or combined, washed with PBS, and incubated with serum free DMEM containing Rhodamine 123 (1 μM) at 37 °C for 30 min in the dark. The cells were then washed and the fluorescence was measured using a fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 507 nm and an emission wavelength of 529 nm.

### 2.8. Statistical analysis

The differences between two experimental groups were analyzed by Student's *t* test (GrpahPad Prism version 5.0). A *p* value less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Chidamide caused growth arrest in pancreatic cancer cell lines

To test whether chidamide possesses anti-proliferation activity in pancreatic cancer cells, BxPC-3 or PANC-1 cells were treated with variable concentrations of chidamide (0–50 μM) for up to 96 h and cell viability was assessed by MTT assays. Chidamide treatments caused growth arrest of the pancreatic cancer cell lines in a dose- and time-dependent manner (Fig. 1A and B), accompanied by dose-dependent hyperacetylation of histone H3. In contrast, chidamide treatments had no effects on protein levels for total histone H3 (Fig. 1C and D). Chidamide treatments caused G2/M cell cycle arrest ($p < 0.05$), along with induction of p21 expression in both cell lines (Fig. 1E–H). These results indicate that chidamide exerts its anti-proliferation effects by causing G2/M cell cycle arrest through induction of p21.

### 3.2. Synergistic antitumor interactions between chidamide and gemcitabine in pancreatic cancer cells

Despite the well characterized mechanisms of action of HDACIs, they show modest clinical activities. Therefore, rational combinations of HDACIs with conventional chemotherapy drugs seem a promising approach to enhance the clinical effects of these agents. Previous studies have demonstrated *in vitro* synergisms between HDACIs (e.g., MGCD0103 and TSA) and gemcitabine in pancreatic cancer cells [11,12,14]. It is conceivable that chidamide may also exert the same effects on gemcitabine in pancreatic cancer cells. Treatments of BxPC-3 or PANC-1 cells with variable concentrations of gemcitabine for 120 h resulted in dose-dependent growth arrest in both cell lines with $IC_{50}$s of 9.62 nM and 26.97 nM, respectively (Fig. 2A and B). To determine the effects of chidamide on gemcitabine cytotoxicity, three drug administration schedules were tested. Interestingly, pretreatment of the pancreatic cancer cell lines with gemcitabine for 48 h followed by chidamide treatment for 72 h was found to be the optimal schedule for drug administration (data not shown). As shown in Fig. 2C and D, chidamide significantly enhanced gemcitabine-induced growth arrest determined by MTT

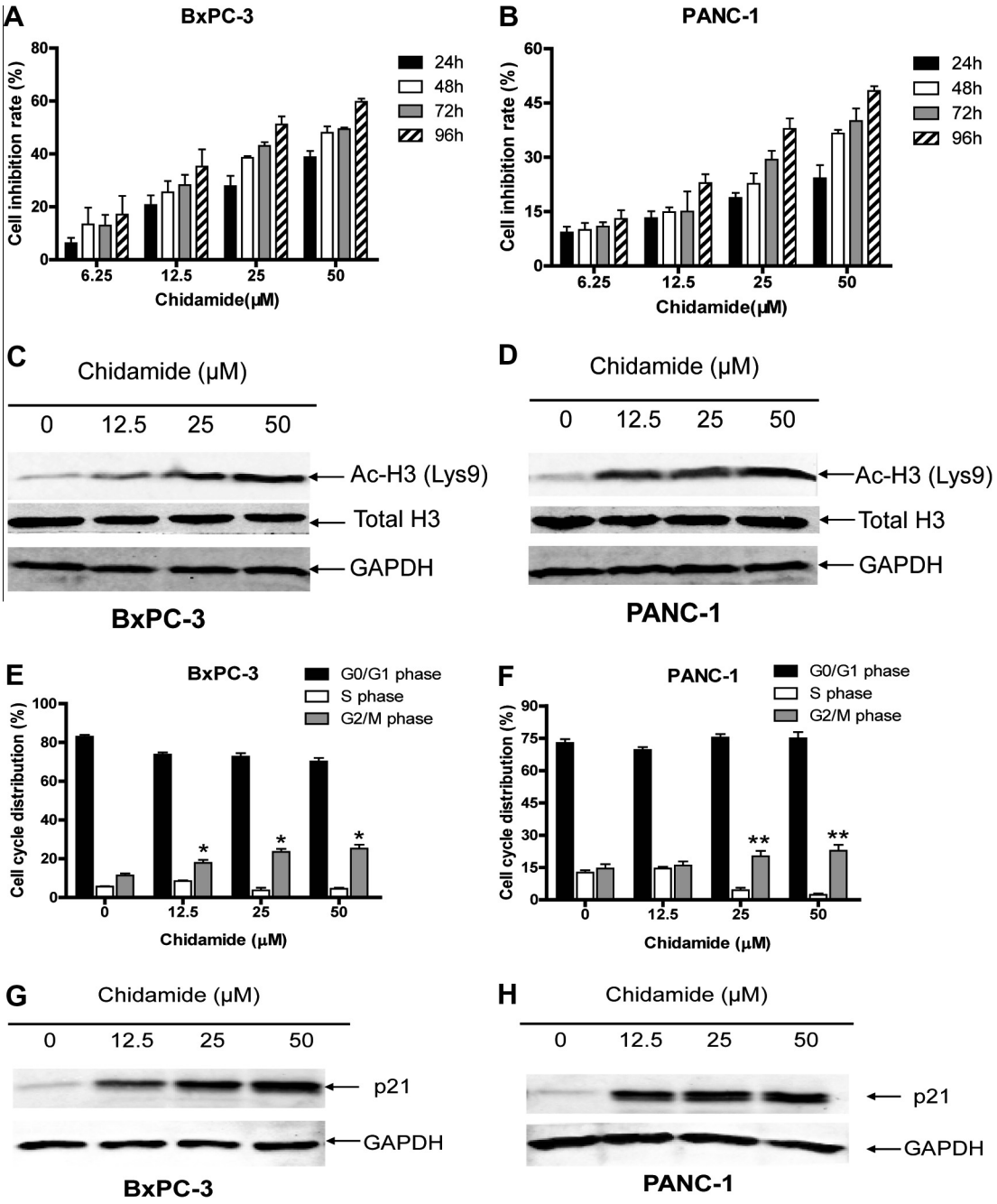


**Fig. 1.** Effects of chidamide on cell proliferation, histone H3 acetylation, and cell cycle distribution in pancreatic cancer cells. (A and B) BxPC-3 or PANC-1 cells were treated with vehicle control or chidamide at the indicated doses for up to 96 h. Cell viability was determined by MTT assays. The data are presented as mean inhibition rates ± SE from at least three independent experiments. (C and D) BxPC-3 or PANC-1 cells were harvested and lysed after incubation with variable concentrations of chidamide (0–50 μM) for 72 h. Soluble proteins were analyzed on Western blots probed by anti-acetylated (ac)-H3, -H3 or -GAPDH antibody. (E–H) BxPC-3 or PANC-1 cells were treated with vehicle control or variable concentrations of chidamide for 72 h. The cells were harvested, fixed, stained with propidium iodide, and subjected to flow cytometry analysis to determine cell cycle distribution. Results are presented as means of triplicates from one representative experiment. The same experiment was repeated three times. Soluble proteins were analyzed on Western blots probed by anti-p21 or -GAPDH antibody. * indicates $p < 0.05$, while ** indicates $p < 0.01$, relative to vehicle control.

assays (2.6- to 9.2-fold in BxPC-3 cells and 1.8- to 7.7-fold in PANC-1 cells, $p < 0.05$). The combined effects of chidamide and gemcitabine were clearly synergistic as determined by calculating combination index (CI) values. CI < 1, indicative of synergism, was calculated for each of the drug combinations (Fig. 2E and F).

### 3.3. Chidamide and gemcitabine cooperatively induced apoptosis and MMP loss in pancreatic cancer cells

Efforts were then undertaken to determine if the synergistic antitumor effects of combined gemcitabine and chidamide were due to induction of apoptosis. Although chidamide alone induced modest apoptosis, it potently enhanced gemcitabine-induced apoptosis in BxPC-3 cells sequentially treated with 40 nM gemcitabine for 24 h followed by 25 μM chidamide for 48 h (>2-fold, $p < 0.05$). This was accompanied by cooperative cleavage/activation of caspases 9 and 3 (Fig. 3A). Similar results were also obtained in PANC-1 cells, though to a lesser extent (Fig. 3B).

Treatment with gemcitabine or chidamide alone caused MMP loss in both cell lines (Fig. 3C and D). Consistent with the results shown in Fig. 3A and B, combined treatments of the cell lines with the two agents resulted in further MMP loss compared to

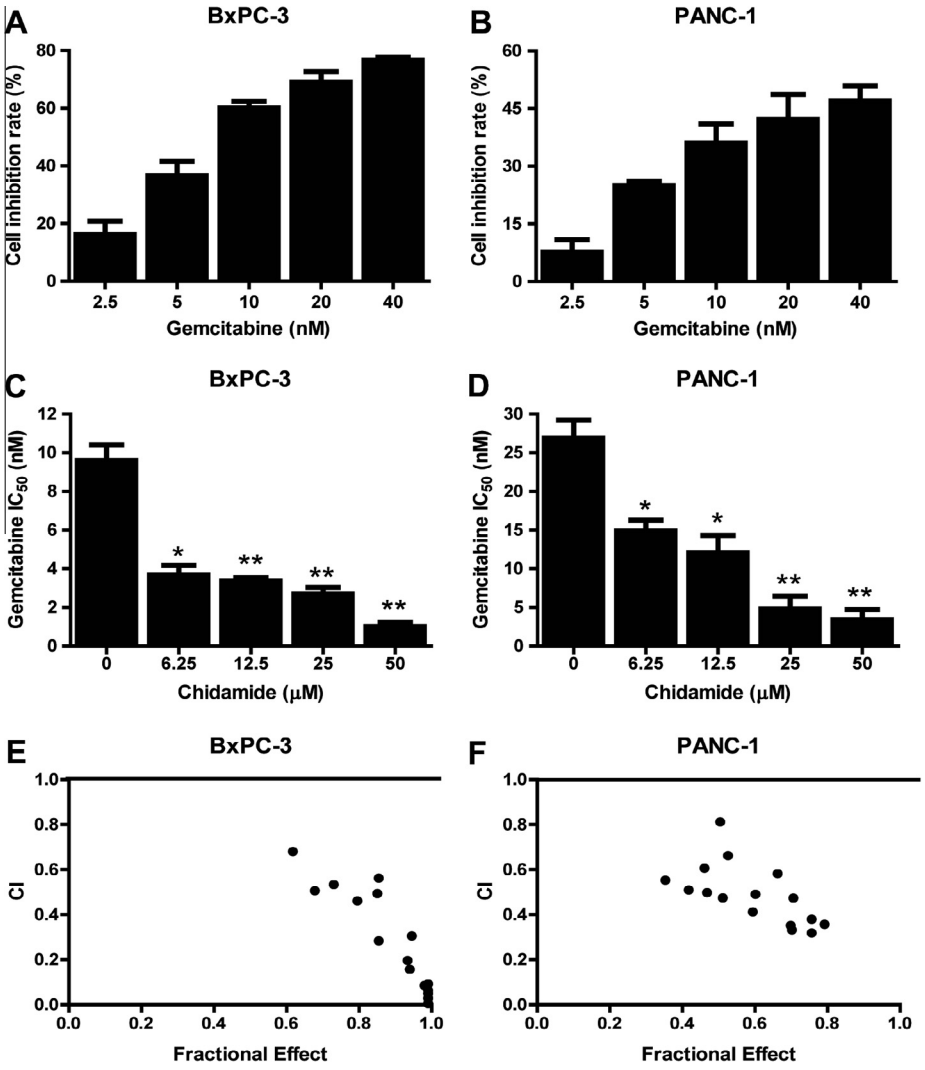


**Fig. 2.** Synergistic antitumor interactions between chidamide and gemcitabine in pancreatic cancer cells. (A and B) BxPC-3 and PANC-1 cells were treated with a range of concentrations of gemcitabine for 120 h. Cell viability was determined by MTT assays. The $IC_{50}$ values were calculated as the concentrations of gemcitabine necessary to inhibit 50% proliferation compared to control cells cultured in the absence of gemcitabine. The data are presented as mean values ± standard errors from at least 3 independent experiments. (C and D) Gemcitabine $IC_{50}$ of BxPC-3 or PANC-1 cells were determined in the absence or presence of chidamide treated sequentially (gemcitabine followed by chidamide). * indicates $p < 0.05$, while ** indicates $p < 0.01$, relative to vehicle control. (E and F) BxPC-3 or PANC-1 cells were treated with gemcitabine (4 concentrations) or chidamide (4 concentrations) alone or combined sequentially (16 combined groups). Combination index values were calculated with the CalcuSyn software.

treatments with gemcitabine or chidamide alone ($p < 0.05$, Fig. 3C and D). This was accompanied by cooperative downregulation of Mcl-1 (Fig. 3E and F). In contrast, no obvious changes on protein levels for Bax, Bcl2, and Bim were observed (data not shown). These results suggest that gemcitabine and chidamide cooperate in downregulation of Mcl-1 expression, leading to MMP loss and subsequent apoptosis in pancreatic cancer cells.

### *3.4. Chidamide cooperatively enhanced gemcitabine-induced cell cycle arrest and DNA DSBs by suppressing CHK1 expression in pancreatic cancer cells*

Besides apoptosis, cell cycle arrest may also contribute to the synergistic antitumor effects of chidamide and gemcitabine in pancreatic cancer cells. Treatments of BxPC-3 or PANC-1 cells with gemcitabine resulted in S and G2/M arrest, while chidamide alone caused only G2/M arrest ($p < 0.05$). Interestingly, combined treatments of the cell lines with the two agents resulted in cooperative induction of S arrest ($p < 0.01$). The combined treatment also caused abrogation of the G2/M cell cycle checkpoint in BxPC-3 cells but not in PANC-1 cells (Fig. 4A and B).

Although induction of p21 by chidamide (Fig. 4C and D) could contribute to growth inhibition and cell cycle arrest, it seemed not responsible for the cooperative S phase arrest and abrogation of the G2/M cell cycle checkpoint induced by the two agents. Gemcitabine exerts it antitumor activity by inhibiting DNA synthesis and inducing DNA damage. The cooperative induction of S phase arrest and abrogation of the G2/M checkpoint indicated that chidamide may enhance gemcitabine-induced DNA damage. Indeed, gemcitabine and chidamide cooperated (if not synergized) in inducing DNA double-strand breaks (DSBs) as reflected by the induction of γH2AX. Interestingly, gemcitabine treatment resulted in induction of CHK1 (checkpoint kinase 1, a critical protein in cell cycle checkpoint pathways and DNA DSB repair [22]) which was abolished by chidamide (Fig. 4C and D). These results suggest that chidamide cooperated with gemcitabine in inducing DNA DSBs by suppressing the expression of CHK1 in pancreatic cancer cells.

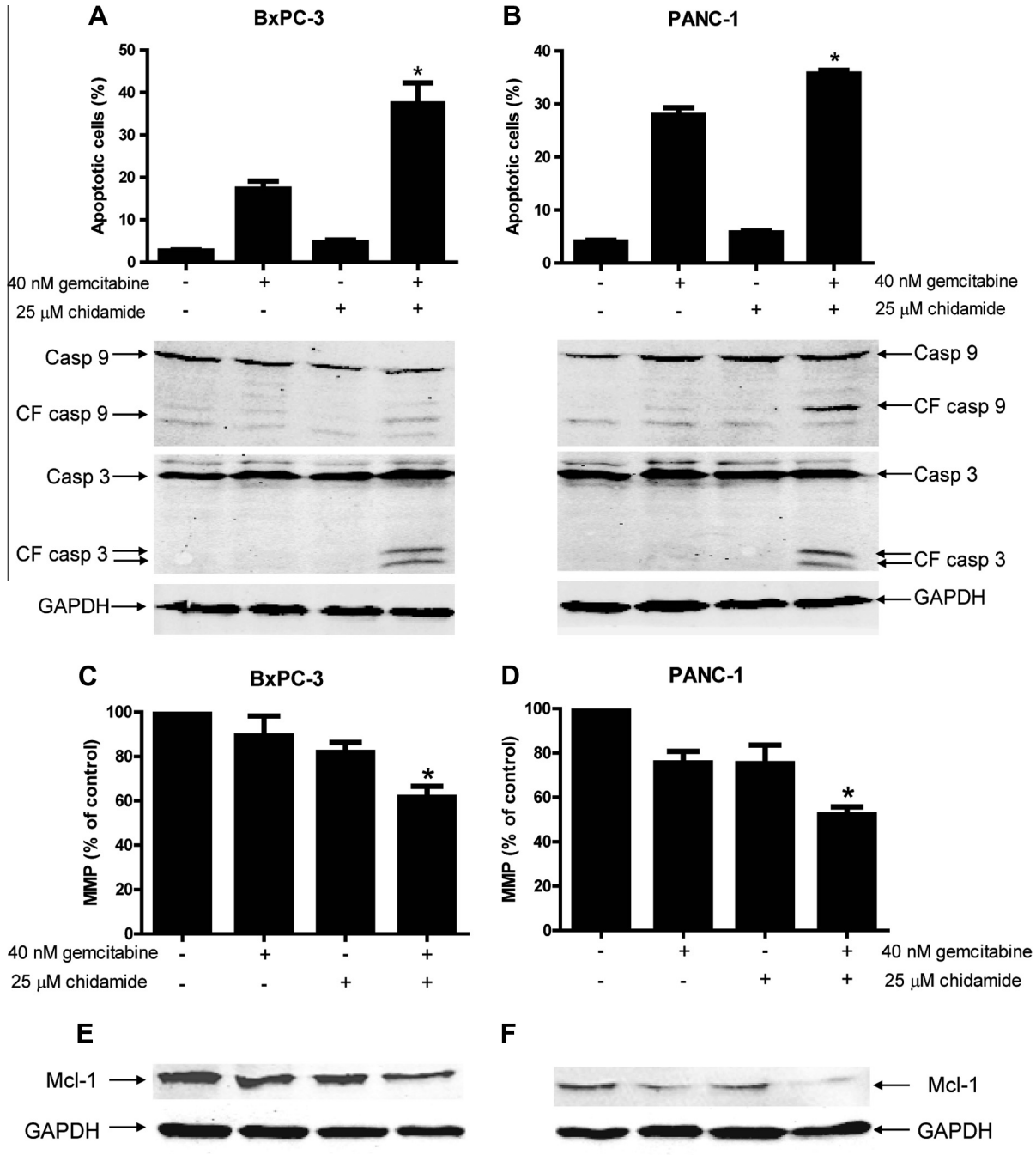


**Fig. 3.** Effects of gemcitabine or chidamide alone or sequentially combined on apoptosis and mitochondrial membrane potential (MMP) in pancreatic cancer cells. (A and B) BxPC-3 or PANC-1 cells were treated first with gemcitabine for 24 h then treated with chidamide for 48 h without washing out gemcitabine. Apoptosis analysis was performed as described in Section 2. Results are shown as mean percent of annexin V+ cells of triplicates from one representative experiment. Cleavage of caspases 9 and 3 was detected by immunoblotting analysis. Gem, gemcitabine; Chi, chidamide. (C and D) BxPC-3 or PANC-1 cells used in A&B were subjected to MMP analysis by Rhodamine 123 staining. * indicates $p < 0.05$, relative to vehicle or individual drug treatments. (E and F) Whole cell lysates from BxPC-3 or PANC-1 cells used in A-D were subjected to immunoblotting to determine protein levels for Bcl2 family members, Bax, Bcl2, Bim, and Mcl-1. Cooperative suppression of Mcl-1 by the two agents in both cell lines was detected.

## 4. Discussion

In this study, we demonstrate that chidamide induces growth inhibition and cell cycle arrest of BxPC-3 and PANC-1 pancreatic cancer cell lines, and it synergistically augments gemcitabine-induced apoptosis. This was accompanied by cooperative induction of DNA DSBs by the two agents and suppression of CHK1 by chidamide. This may represent a common mechanism underlying the synergistic antitumor interactions between HDACIs and DNA damaging agents in cancer cells.

MTT assays revealed that chidamide causes growth arrest in a dose- and time-dependent manner in the pancreatic cancer cell lines, accompanied by G2/M arrest. Consistent with previous reports that induction of p21 is a crucial mechanism for growth arrest in some pancreatic cancer cells induced by HDACIs [13], chidamide also potently induces the expression of p21. When

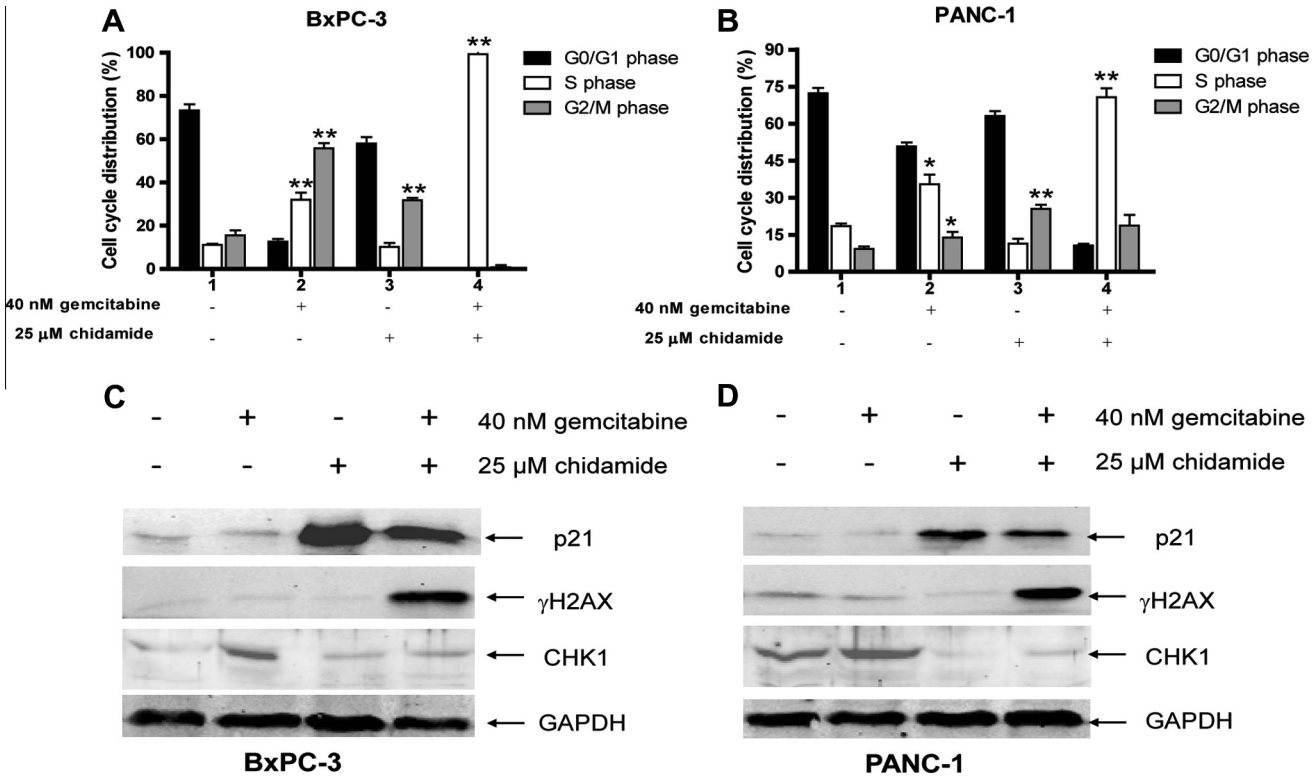


**Fig. 4.** Chidamide synergistically enhanced gemcitabine-induced cell cycle arrest and DNA DSBs by suppressing CHK1 expression in pancreatic cancer cells. (A and B) BxPC-3 or PANC-1 were treated first with gemcitabine for 24 h, then treated with chidamide for 48 h without washing out gemcitabine. Cell cycle distribution was determined by PI staining and flow cytometry analysis. Results are presented as mean values of triplicates from one representative experiment. * indicates $p < 0.05$, while ** indicates $p < 0.01$, relative to vehicle or individual drug treatments. (C and D) BxPC-3 or PANC-1 were treated first with gemcitabine for 24 h then treated with chidamide for 48 h without washing out gemcitabine. Cells were harvested and whole cell lysates were prepared and subjected to immunoblotting analysis of γH2AX, p21, CHK1, and GAPDH.

combined in a sequential fashion (gemcitabine first followed by chidamide), chidamide potently and synergistically enhanced the cytotoxicity of gemcitabine in both BxPC-3 and PANC-1 cell lines. This synergistic cytotoxicity is clearly due to cell death since cooperative induction of apoptosis by the two agents was detected. Interestingly, combined treatments of the pancreatic cancer cell lines with chidamide and gemcitabine resulted in cooperative MMP loss along with Mcl-1 downregulation. These results strongly suggest that gemcitabine and chidamide cooperate in inducing Mcl-1 downregulation which leads to MMP loss and apoptosis.

The effects of gemcitabine and chidamide on S phase arrest and the G2/M checkpoint suggest that chidamide may enhance DNA damage and/or abrogate the G2/M cell cycle checkpoint induced by gemcitabine. Interestingly, gemcitabine and chidamide cooperatively (if not synergistically) induced γH2AX, a biomarker of DNA DSBs, in both cell lines, which may explain the cooperative induction of S phase arrest. Based on these observations and previous studies that have demonstrated that high level of DNA damage causes Mcl-1 degradation in cancer cells [23], it is conceivable that chidamide enhances gemcitabine-induced DNA DSBs, potentially leading to S phase arrest, Mcl-1 degradation, and subsequent MMP loss and apoptosis.

The HDACI VPA has been shown to suppress DNA repair genes (*RAD51*, *BRCA1*, and *CHK1*) to increase sensitivity to radiation in prostate cancer cells [24]. CHK1 plays critical roles in the repair of DNA DSBs and all the cell cycle checkpoint pathways [22]. It is conceivable that chidamide may downregulate CHK1 in pancreatic cancer cells since it enhanced gemcitabine-induced DNA DSBs and abrogated the G2/M checkpoint. Western blots of CHK1 in both BxPC-3 and PANC-1 cells strongly support this hypothesis. It is important to note that downregulation of CHK1 by chidamide did not result in abrogation of the G2/M checkpoint in PANC-1 cells. This may explain the lesser enhancement by chidamide on gemcitabine-induced apoptosis.

In conclusion, the present study demonstrates that chidamide causes growth arrest of pancreatic cancer cells by inducing p21 expression and cell cycle arrest. Furthermore, chidamide augments gemcitabine-induced DNA DSBs and apoptosis, and abrogates the G2/M checkpoint. These results suggest that the novel HDACI chidamide has an antitumor potential against pancreatic cancer cells, especially in combination with gemcitabine.

## Acknowledgments

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