1,25-Dihydroxyvitamin D_3 and cisplatin synergistically induce apoptosis and cell cycle arrest in gastric cancer cells

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Abstract. 1,25-Dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] plays an anticancer role in multiple types of cancer and potentiates the cytotoxic effects of several common chemotherapeutic agents. The hypercalcemia caused by 1,25(OH)$_2$D$_3$ alone or resistance to cisplatin weaken the anticancer effects of vitamin D. Thus, in this study, we aimed to investigate the synergistic effects of 1,25(OH)$_2$D$_3$ and cisplatin on the apoptosis and cell cycle progression of gastric cancer cells. BGC-823 human gastric cancer cells were treated with 1,25(OH)$_2$D$_3$ or cisplatin alone, or a combination of both agents. Cell apoptosis was assessed by TUNEL assay and flow cytometry. The expression of the apoptosis-related proteins, poly(ADP-ribose) polymerase (PARP), Bax, Bcl-2, caspase-3 and caspase-8, was examined using immunoblot analysis. ERK and AKT phosphorylation were examined by immunoblot analysis. The cell cycle distribution was determined by propidium iodide staining and flow cytometric analysis. p21 and p27 protein expression was also examined using immunoblot analysis. Our results revealed that co-treatment with 1,25(OH)$_2$D$_3$ enhanced cisplatin-induced apoptosis and upregulated the expression of Bax, and promoted the cleavage of PARP and caspase-3. The phosphorylation levels of ERK and AKT were reduced following combined treatment with 1,25(OH)$_2$D$_3$ and cisplatin. The percentage of cells in the G_0/G_1 phase was greater in the cells treated with the combined treatment than in those treated with either 1,25(OH)$_2$D$_3$ or cisplatin alone. p21 and p27 expression was upregulated following co-treatment with both agents. The results of this study suggest that 1,25(OH)$_2$D$_3$ potentiates cisplatin-mediated cell growth inhibition and cell apoptosis, which involves the upregulation of Bax, a decrease in ERK and AKT phosphorylation levels, and increased p21 and p27 levels.

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Introduction

It has been previously demonstrated that vitamin D_3 affects cell proliferation, differentiation and apoptosis (1). The anti-tumor activity of 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] is observed only when it is applied in supraphysiological doses, which may cause the side-effect of hypercalcemia (2). For this reason, the synthesis of analogs has been initiated in order to dissociate the calcemic effect from the anticancer activity of calcitriol. One strategy of improving the anticancer effects is to combine vitamin D with other agents in order to develop thera-peutic interventions that allow dose reduction, the alleviation of toxicity and the maintenance of the growth inhibitory potential. According to the World Health Organization International Agency for Research on Cancer (IARC), gastric cancer is the second leading cause of cancer-related mortality worldwide (3). Despite significant progress in the treatment of patients with gastric cancer in recent years, there is a constant need for new therapies (4). Due to the low responsiveness of some patients suffering from gastric cancer to cisplatin therapy, there is a need to explore new combined therapeutic methods.

The role of vitamin D in inhibiting cancer cell growth, inducing cell differentiation and promoting cell apoptosis has been a research hotspot for the prevention and therapy of certain types of cancer, including gastric cancer (5). Although vitamin D exerts a less progressive direct killing effect on cancer cells, it can enhance the cytotoxicity of certain anticancer drugs, such as paclitaxel and may synergistically suppress the prolifera-tion of cancer cells. The synergistic effects of vitamin D have been found in combination chemotherapy in various malignant somatic cells *in vitro* and *in vivo* (6-8).

Cisplatin is a major chemotherapeutic agent used in the treatment of gastric cancer. The National Comprehensive Cancer Network (NCCN) guideline suggested that cisplatin should be used as a first-line anticancer drug in the treatment of gastric cancer. Cisplatin exerts anticancer effects through various mechanisms; however, its most prominent mode of action is through the generation of DNA lesions followed by the activation of DNA damage response and the induction of cell apoptosis (9). Resistance to cisplatin arises through multiple mechanisms involving reduced drug uptake, increased drug inactivation, increased DNA damage repair, and the inhibition of transmission of DNA damage recognition signals to the

apoptotic pathway (10). However, some patients with gastric cancer are not sensitive to cisplatin treatment. In addition, high-dose chemotherapy often results in several side-effects; a high concentration of vitamin D also causes hypocalcemia; thus, this limits the single use of both drugs.

$1,25(OH)_2D_3$ has been shown to synergistically or additively enhance the antitumor activities of a number of chemotherapeutic agents, including carboplatin, cisplatin, docetaxel and paclitaxel in prostate cancer (11), breast cancer (12), bladder cancer (13) and murine models of squamous cell carcinoma (SCC) (14). In the present study, we investigated the synergistic effects of $1,25(OH)_2D_3$ and cisplatin on apoptosis and cell cycle distribution, as well as their mechanisms of action in BGC-823 gastric cancer cells *in vitro*. Our aim was to examine the biological effects of combined treatment with $1,25(OH)_2D_3$ and cisplatin against gastric cancer cells. We also wished to to evaluate the effects of co-treatment with $1,25(OH)_2D_3$ and cisplatin on the apoptosis and cell cycle distribution of gastric cancer cells, and to explore the possible mechanisms responsible for the the synergistic anticancer effects of $1,25(OH)_2D_3$ and cisplatin.

Materials and methods

Drugs. $1,25(OH)_2D_3$ was purchased from Sigma-Aldrich (St. Louis, MO, USA). $1,25(OH)_2D_3$ was dissolved in absolute ethanol (ETOH) to the concentration of 10^{-3} M and stored in solution at -20˚C. $1,25(OH)_2D_3$ was freshly diluted in culture medium to reach the required concentrations prior to each experiment. The ethanol concentration in each test condition never exceeded 0.1%.

Cisplatin was purchased from Shanghai Haoran Bio-Technology Co., Ltd. (Shanghai, China). Cisplatin was dissolved in sterile 0.9% NaCl to the concentration of 50 μg/ml and stored in solution at 4˚C. Cisplatin was freshly diluted in culture medium to reach the required concentrations prior to each experiment.

Cell culture. The BGC-823 gastric cancer cell line was purchased from the Central Laboratory of Xiangya Medical College of Central South University, Changsha, China. The cells were cultured according to standard conditions. In brief, the BGC-823 gastric cancer cells were grown in RPMI-1640, 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humid environment with 5% CO_2. Cell media and reagents were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). The culture media were changed every 48 h, and the cells were passaged every 2-3 days to produce new generations. The cells were plated in 25-cm² flasks (Costar Life Sciences, Tewksbury MA, USA), and split every 48 h by washing with D-Hank's solution and detached using 0.05% trypsin-EDTA. Half of the cells were plated in new flasks with fresh culture medium and the remaining cells were used for the experiments. The cells which had undergone 3 passages were selected for the experiments.

Drug treatment. The cells in the $1,25(OH)_2D_3$ treatment group were cultured in RPMI-1640 culture medium with 10 nM $1,25(OH)_2D_3$ for 72 h. The cells in the cisplatin treatment group were treated with 0.2 μg/ml cisplatin solution for 2 h following normal culture for 24 h; the cells were then cultured with fresh medium, followed by washing twice with D-Hank's solution.

The cells in the group co-treated with $1,25(OH)_2D_3$ and cisplatin were treated with 0.2 μg/ml cisplatin for 2 h following culture for 24 h with $1,25(OH)_2D_3$ alone, and were then cultured with fresh culture medium with 10 nM $1,25(OH)_2D_3$. The control group was treated with ETOH in RPMI-1640 culture medium. The total culture time was 72 h for each group.

Preparation of cell extracts. The BGC-823 cells were seeded in 6-well plates ($5x10^4$ cells/well) and left overnight to attach. The cells were then treated with $1,25(OH)_2D_3$ or cisplatin alone, or a combination of both. The cells treated with an equivalent amount of ETOH were used as the vehicle control. The cells were washed with D-Hank's solution and replenished with fresh medium every 24 h. Following treatment for 72 h, the cells were harvested for immunoblot analysis using RIPA buffer (Thermo Scientific, Waltham, MA, USA) supplemented with protease inhibitors.

Immunoblot analysis. The cell protein concentration was measured using a BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions. An immunoblot analysis was performed using a Bio-Rad wet electroblotting system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The results from immunoblot analysis were quantified by measuring the optical density of the immunoreactive bands using ImageJ software.

Mouse and rabbit antibodies against Bax, Bcl-2, caspase-3, caspase-8, poly(ADP-ribose) polymerase (PARP), cleaved PARP, phosphorylated (p-)ERK1/2, ERK1/2, p-AKT, AKT, p21, p27 and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin was used as the loading control to ensure equal protein loading among all wells in immunoblot analysis.

TUNEL assay. The In Situ Cell Death Detection Fluorescein kit (Roche Applied Science, Indianapolis, IN, USA) was used to detect cell apoptosis. Cell apoptosis was analyzed by TUNEL assay. In brief, the procedure was as follows: BGC-823 gastric cancer cell suspension was prepared following scheduled experiment treatment, the test sample was washed 3 times in phosphate-baffered saline (PBS) and adjusted to $2x10^7$ cells/ml. Subsequently, 100 μl/tube suspension were transferred into a V-bottomed EP tube, and then 100 μl/tube of a freshly prepared fixation solution were added (4% paraformaldehyde in PBS, pH 7.4) to the cell suspension. The cells were then resuspended and incubated for 60 min at 22˚C. The EP tubes were centrifuged at 300 x g for 10 min and the fixative was removed by flicking off or suction. The cells were then washed once with PBS, the EP tubes were centrifuged at 300 x g for 10 min again, and finally, the cells were resuspended in 100 μl/tube permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice. The TUNEL reaction mixture was prepared immediately according to the kit's instructions prior to use in the experiments and kept on ice until use. The cells were washed twice with PBS, then resuspended in 50 μl/tube TUNEL reaction mixture and incubated for 60 min at 37˚C in a humidified atmosphere in the dark. The cells were then transferred to a tube to a final volume of 500 μl in PBS. The well-prepared samples were tested in a Beckman flow cytometry (Beckman Coulter, Miami, FL, USA) for the analysis of cell apoptosis.
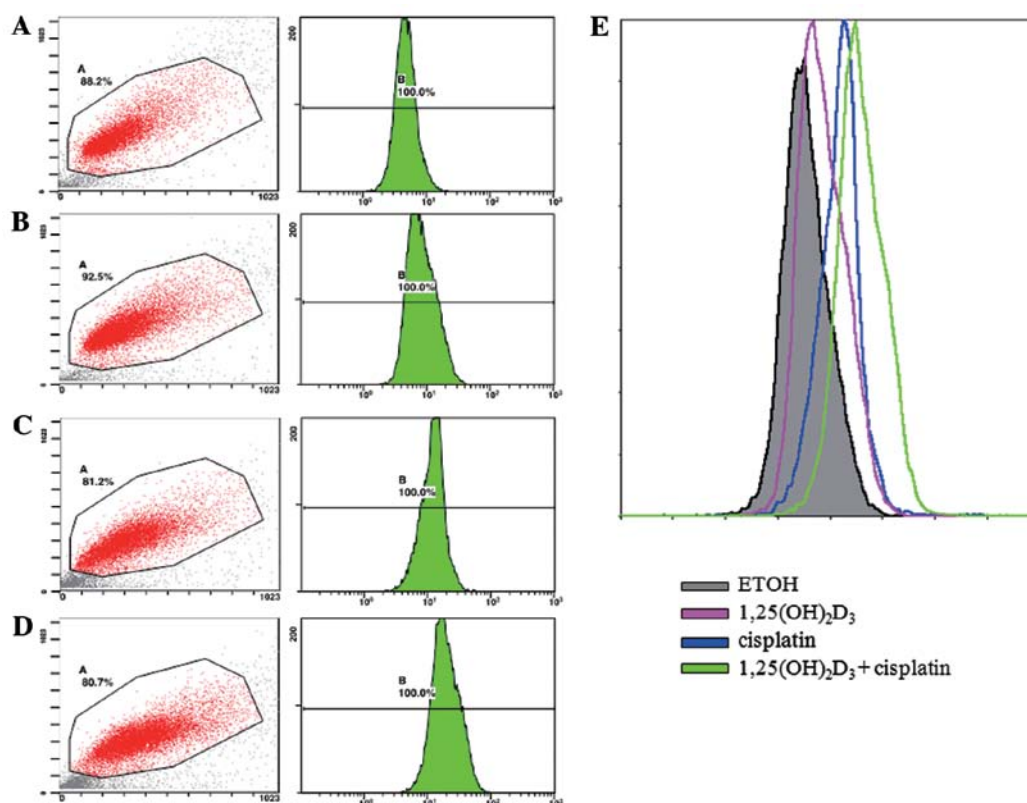
Figure 1. Apoptosis of BGC-823 cells determined by TUNEL assay following co-treatment with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and cisplatin. Apoptotic intensity of BGC-823 cells was determined by flow cytometry after TUNEL assay. The effects of treatment with (A) vehicle-treated control (ETOH), (B) 1,25(OH)₂D₃, (C) cisplatin, and (D) co-treatment with 1,25(OH)₂D₃ and cisplatin are indicated. (E) The overlapped peak demonstrates the effects as a whole. The experiments were repeated 3 times.

Cell cycle analysis. The BGC-823 gastric cancer cells were treated with the vehicle control (ETOH), 1,25(OH)₂D₃ alone, cisplatin alone, or combined treatment with 1,25(OH)₂D₃ and cisplatin for 24 h. Following treatment, the BGC-823 cells (1x10⁶/sample) were collected by trypsin digestion, then washed twice in cold PBS and fixed for 24 h in 70% ETOH at -20˚C. The cells were then washed twice in PBS and incubated with RNAse (8 µg/ml; Fermentas, St. Leon-Rot, Germany) at 37˚C for 1 h. The cells were stained with propidium iodide (0.5 mg/ml; Sigma-Aldrich) for 30 min at 37˚C in the dark. The cellular DNA content was determined using a Beckman flow cytometry (Beckman Coulter) and ModFit LT 3.0 software (Verity Software House Inc., Topsham, ME, USA). The experiment was repeated 3 times.

Data analysis. Statistical analysis was performed by employing GraphPad Prism 5.0 software (GraphPad Software, CA, USA). All data are presented as the means ± standard error of the mean (SEM). Each experiment was repeated 3 times. The difference between the mean values of 2 groups was evaluated using the Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

1,25(OH)₂D₃ enhances the anticancer and apoptotic effects of cisplatin and in BGC-823 gastric cancer cells. The apoptosis of BGC-823 gastric cancer cells was evaluated by TUNEL assay. The apoptosis of the treated cancer cells was expressed using a fluorescent signal determined by flow cytometry. The density plots obtained by flow cytometry are shown as Fig. 1. The peaks shown in different colors represent the intensity of cell apoptosis following treatment with ETOH (vehicle control), 1,25(OH)₂D₃ or cisplatin alone, as well as co-treatment with 1,25(OH)₂D₃ and cisplatin.

Treatment with 10 nM 1,25(OH)₂D₃ or 0.2 µg/ml cisplatin alone significantly enhanced cell apoptosis compared with the control, as indicated by the increased fluorescence intensity of the DNA fragments in apoptotic cells ($P<0.05$). Co-treatment with 10 nM 1,25(OH)₂D₃ and 0.2 µg/ml cisplatin led to a significantly ($P<0.05$) greater number of apoptotic BGC-823 cells compared to treatment with cisplatin or 1,25(OH)₂D₃ alone (Table I).

Effects of co-treatment with 1,25(OH)₂D₃ and cisplatin on the expression of apoptosis-related proteins in BGC-823 gastric cancer cells. Following treatment with 1,25(OH)₂D₃ alone or in combination with cisplatin for 72 h, the cells were harvested for immunoblot analysis. The expression of a series of apoptosis-related proteins was then determined. The cleavage of PARP was significantly higher in the group co-treated with 1,25(OH)₂D₃ and cisplatin ($P<0.01$) compared with the group treated with cisplatin or 1,25(OH)₂D₃ alone (Fig. 2). In addition, the expression of caspase-3, a key member of the caspase family, was significantly reduced in the cells co-treated with 1,25(OH)₂D₃ and cisplatin ($P<0.01$) compared with the cells treated with cisplatin or 1,25(OH)₂D₃ alone. However, no signifi-
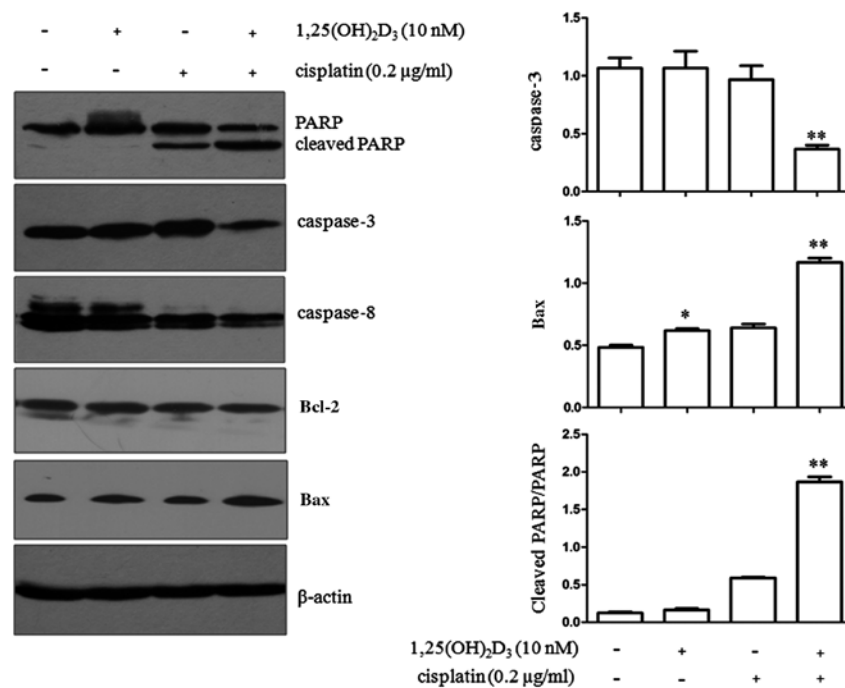
Figure 2. Effects of co-treatment with 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] and cisplatin on the expression of apoptosis-related proteins in BGC-823 cells. BGC-823 gastric cancer cells were treated with 1,25(OH)$_2$D$_3$, or cisplatin alone or a combination of both agents. Each experiment was repeated independently 3 times, and representative blots are shown. The expression of poly(ADP-ribose) polymerase (PARP), cleaved PARP, caspase-3, caspase-8, Bcl-2 and Bax is demonstrated. β-actin was used as a loading control for total cellular proteins. Values represent the means ± standard error of the mean (SEM) of triplicate assays. *P<0.05 compared with ETOH treatment alone; **P<0.01 compared with ETOH, 1,25(OH)$_2$D$_3$, or cisplatin treatment alone.

Table I. Effects of treatment with 1,25(OH)$_2$D$_3$ alone or in combination with cisplatin on the apoptosis of BGC-823 cells.

Group	Fluorescence intensity (means ± SEM)
Control (ETOH)	4.73±0.55
1,25(OH)$_2$D$_3$	9.2±1.14[a]
Cisplatin	14.17±4.01[a]
1,25(OH)$_2$D$_3$ + cisplatin	23.07±3.00[a,b]

[a]P<0.05 compared to the control; [b]P<0.05 compared to treatment with cisplatin or 1,25(OH)$_2$D$_3$ alone. 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; SEM, standard error of the mean.

cant change was observed in caspase-8 expression in all the treatment groups [1,25(OH)$_2$D$_3$ or cisplatin treatment alone or combined treatment (P>0.05)]. Bax expression was significantly upregulated by 1,25(OH)$_2$D$_3$ treatment alone (P<0.05) or the combined treatment with 1,25(OH)$_2$D$_3$ and cisplatin (P<0.01).

Regulation of AKT and ERK1/2 phosphorylation by co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin. Cell apoptosis is regulated by multiple pathways. AKT and ERK1/2 are two important kinases involved in cell proliferation and apoptosis in gastric cancer (15,16). In the present study, we wished to explore the effects of 1,25(OH)$_2$D$_3$ or cisplatin treatment alone, as well as the effects of co-treatment with both agents on the phosphorylation levels of AKT and ERK1/2. As illustrated in Fig. 3, treatment with 1,25(OH)$_2$D$_3$ (P<0.05) or cisplatin

(P<0.01) alone, as well as the combined treatment (P<0.01) significantly reduced the phosphorylation level of AKT. Furthermore, co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin further reduced the phosphorylation level of AKT compared to treatment with cisplatin alone (P<0.05).

ERK1/2 phosphorylation was also observed following treatment with 1,25(OH)$_2$D$_3$ or cisplatin alone or the combined treatment. Both agents decreased the phosphorylation levels of ERK1/2 (P<0.01). Similarly, co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin significantly reduced the phosphorylation levels of ERK1/2 compared to treatment with cisplatin alone (P<0.05). These results indicated that co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin further enhanced the anti-proliferative effects of cisplatin on BGC-823 gastric cancer cells.

Effects of co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin on cell cycle distribution of BGC-823 gastric cancer cells. The evaluation of the cell cycle distribution was carried out following treatment with 1,25(OH)$_2$D$_3$ or cisplatin alone or the comined treatment. Co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin significantly increased the percentage of cells in the G$_0$/G$_1$ phase when compared to the group treated with ETOH (vehicle control), 1,25(OH)$_2$D$_3$ or cisplatin alone (P<0.05) (Fig. 4). We observed that the cells treated with both 1,25(OH)$_2$D$_3$ and cisplatin had accumulated in the G$_0$/G$_1$ phase and the number of cells was decreased in the G$_2$/M phase, when compared to the cells treated with cisplatin or 1,25(OH)$_2$D$_3$ alone. However, the percentage of cells in the G$_2$/M phase was not significantly affected by treatment with 1,25(OH)$_2$D$_3$ alone. The percentage of cells in the different cell cycle phases is shown in Table II. 1,25(OH)$_2$D$_3$, in combination with cisplatin, significantly increased the
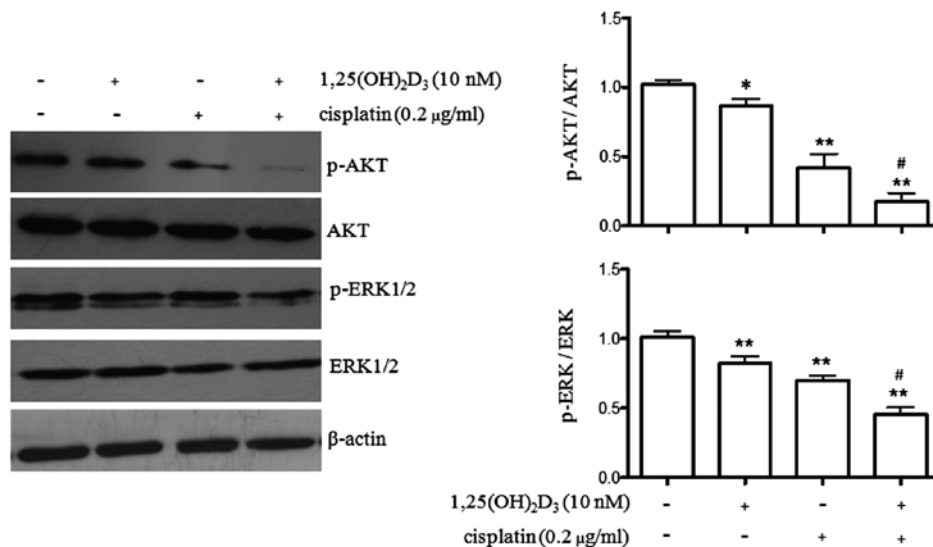
Figure 3. Regulation of AKT and ERK1/2 phosphorylation by co-treatment with 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] and cisplatin. BGC-823 gastric cancer cells were treated with 1,25(OH)$_2$D$_3$, cisplatin alone or a combination of both agents. Each experiment was repeated independently 3 times, and representative blots are shown. The expression of AKT, pAKT, ERK1/2, pERK1/2 is demonstrated. β-actin was used as a loading control for total cellular proteins. Values represent the means ± standard error of the mean (SEM) of triplicate assays. *P<0.05 compared with ETOH; **P<0.01 compared with ETOH; #P<0.05 compared with cisplatin treatment alone.
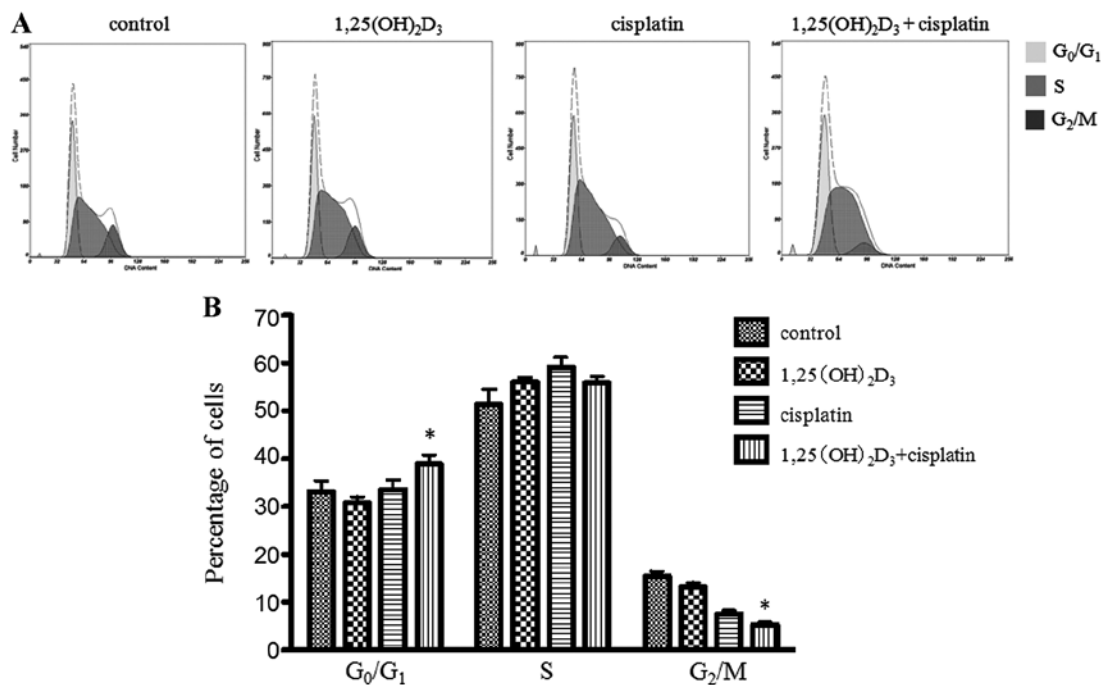

Figure 4. Effects of co-treatment with 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] and cisplatin on the cell cycle distribution in BGC-823 gastric cancer cells. BGC-823 gastric cancer cells were treated with 1,25(OH)$_2$D$_3$, or cisplatin alone or a combination of both agents. (A) Flow cytometric analysis of BGC-823 cells with propidium iodide staining. (B) Cell cycle distribution of BGC-823 cells following treatment. The percentages of cells in different cell cycle phases are expressed as the means ± standard error of the mean (SEM). The experiment was repeated independently 3 times. *P<0.05 compared with ETOH, 1,25(OH)$_2$D$_3$, or cisplatin treatment alone.

percentage of cells in the G_0/G_1 phase and decreased the percentage of cells in the G_2/M phase, when compared to treatment with cisplatin or 1,25(OH)$_2$D$_3$ alone (P<0.05).

Effects of co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin on p21 and p27 protein expression in BGC-823 gastric cancer cells. As the anti-proliferative effects of 1,25(OH)$_2$D$_3$ commonly involve the upregulation of p21 and/or p27 (17,18), we wished

to determine the effects of 1,25(OH)$_2$D$_3$ and cisplatin on the protein expression of p21 and p27 in BGC-823 cells. p21 and p27 are important cell cycle regulators (19) and they were also examined in our study. As shown in Fig. 5, co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin significantly increased the expression of p21 and p27 (P<0.01) compared to treatment with ETOH, 1,25(OH)$_2$D$_3$ or cisplatin alone. The effects on p21 and p27 expression induced by treatment with 1,25(OH)$_2$D$_3$ or cisplatin

Table II. Effects of treatment with 1,25(OH)$_2$D$_3$ alone or in combination with cisplatin on cell cycle distribution of BGC-823 cells.

	Percentage of cells in each cell cycle phase (means ± SEM)		
	G$_0$/G$_1$	S	G$_2$/M
Control (ETOH)	33.17±1.27	51.37±1.82	14.27±0.73
1,25(OH)$_2$D$_3$	30.77±0.73	56.03±0.58	13.17±0.59
Cisplatin	33.57±1.17	59.14±1.21	7.60±0.44
1,25(OH)$_2$D$_3$ + cisplatin	38.87±1.14[a]	55.83±0.79	5.28±0.35[a]

[a]$P<0.05$ compared to control, or treatment with 1,25(OH)$_2$D$_3$ or cisplatin alone. 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D3; SEM, standard error of the mean.
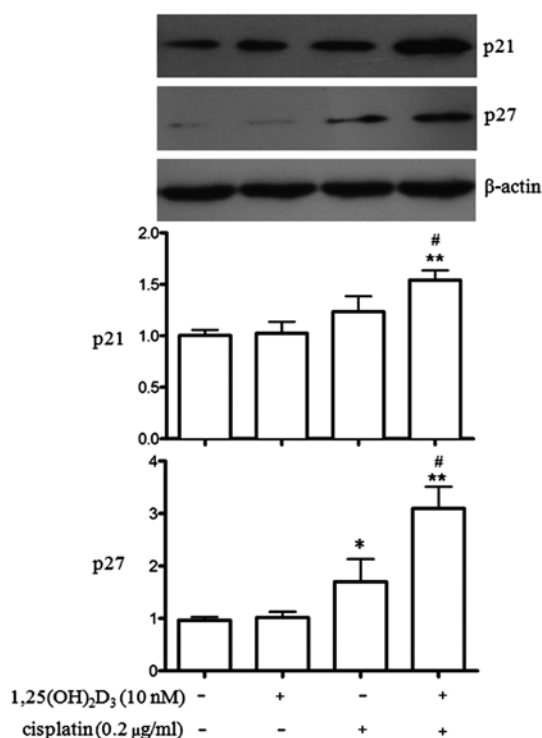

Figure 5. Effects of co-treatment with 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] and cisplatin on p21 and p27 protein expression in BGC-823 gastric cancer cells. BGC-823 gastric cancer cells were treated with 1,25(OH)$_2$D$_3$, or cisplatin alone or a combination of both agents. Each experiment was repeated independently 3 times, and representative blots are shown. β-actin was used as a loading control for total cellular proteins. Values represent the means ± standard error of the mean (SEM) of triplicate assays. *$P<0.05$ compared with ETOH; **$P<0.01$ compared with ETOH or 1,25(OH)$_2$D$_3$ alone; #$P<0.05$ compared with cisplatin treatment alone.

alone differed. Treatment with cisplatin alone significantly increased p27 expression ($P<0.05$), whereas 1,25(OH)$_2$D$_3$ had no such effect. Treatment with 1,25(OH)$_2$D$_3$ or cisplatin alone did not upregulate p21 expression. Co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin further increased the expression of p21 and p27 compared to treatment with cisplatin alone ($P<0.05$).

Discussion

Gastric cancer remains the second most common cause of cancer-related mortality worldwide (20). Surgery remains as the most common curative treatment. However, the majority of patients with gastric cancer develop local or distant recurrence (21). Meta-analyses have indicated that certain patients treated with chemotherapy following surgery benefit from this treatment strategy, while other patients have undergone expensive and potentially toxic therapy without any beneficial effects (22). Cisplatin is widely used and has been demonstrated to be effective in the palliative treatment of gastric cancer (23). Oxaliplatin, the third generation platinum compound, plus 5-fluorouracil modulated with leucovorin (FOLFOX) has been widely used as the first-line treatment in advanced gastric cancer (24,25). However, resistance to oxaliplatin and cisplatin remains a major obstacle to further increasing the treatment response rate. In the present study, to the best of our knowledge, we demonstrate for the first time the biological effects of combined treatment with 1,25(OH)$_2$D$_3$ and cisplatin against gastric cancer cell growth. We observed that 1,25(OH)$_2$D$_3$ induced the apoptosis of BGC-823 cells as shown by TUNEL assay. However, when used in combination with cisplatin, the apoptotic signal significantly increased compared to treatment with cisplatin alone. Previous studies have indicated that the anticancer effects of vitamin D are limited in certain types of cancer (8). Preclinical experiments have suggested that vitamin D exerts minor effects on the prevention or therapy of cancer *in vivo*, although it inhibits cell proliferation and induces cell apoptosis *in vitro* (26,27). However, vitamin D and its analogues are still being focused on, as they exert synergistic anticancer effects when used in combination with chemotherapeutic drugs, such as platinum (28). The mechanisms of cancer progression have been clarified and a number of target proteins have been identifed to be important in the treatment of cancer (29).

In the present study, we demonstrated the differential effects of treatment with vitamin D$_3$ alone or in combination with cisplatin on the apoptosis of BGC-823 gastric cancer cells. Treatment with 1,25(OH)$_2$D$_3$ and cisplatin alone induced the apoptosis of BGC-823 cells, as shown by TUNEL assay. Furthermore, enhanced apoptosis was observed following co-treatment with both agents, and the fluorescence intensity of apoptotic cells markedly increased by 5-fold of the control, and by 1.6-fold that of cisplatin (Table I). We also observed the changes in protein expression following co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin. The caspase pathway is involved in vitamin D-induced cell apoptosis (18,30). In the present study, caspase-3 expression was reduced following co-treatment with 1,25(OH)$_2$D$_3$ and cisplatin, which indicated a greater apoptotic status in the BGC-823 cells. In addition, the significantly increased cleavage of PARP and the

expression of pro-apoptotic Bax were also observed following co-treatment with $1,25(OH)_2D_3$ and cisplatin when compared to treatment with cisplatin alone.

Having demonstrated the synergism between $1,25(OH)_2D_3$ and cisplatin, we sought to explore the underlying mechanisms. Caspases play a crucial role in apoptotic cell death induced by vitamin D_3 (30). The apoptosis of gastric cancer cells can be triggered by the extrinsic pathway activated by death receptor and the intrinsic pathway regulated by Bcl-2 family members and caspase cascades in the mitochondrion (31). Previous studies have indicated that $1,25(OH)_2D_3$-mediated apoptosis is caspase-dependent and appears to act through the mitochondrial pathway of cytochrome c release, caspase-9 activation, and subsequent caspase-3 activation, finally the processing of PARP (32). In our study, we found that caspase-3 expression was significantly decreased following treatment with $1,25(OH)_2D_3$ and cisplatin, while caspase-8 expression remained unaltered following the combined treatment [$1,25(OH)_2D_3$ and cisplatin] or treatment with $1,25(OH)_2D_3$ and cisplatin alone. This suggests that the mitochondrial pathway is involved in vitamin D-mediated apoptosis, although the involvement of other pathways cannot be ruled out.

We also found that the pro-apoptotic protein, Bax, was upregulated following co-treatment with $1,25(OH)_2D_3$ and cisplatin, while treatment with cisplatin alone did not increase the expression of Bax. The translocation of Bax to the mitochondria has been shown to be of particular importance for the induction of vitamin D-mediated apoptosis in certain cell types. The treatment of MCF-7 breast cancer cells with $1,25(OH)_2D_3$ has been shown to result in the redistribution of Bax from the cytosol to the mitochondria (33,34). Changes in the expression or cellular distribution of Bcl-2 anti-apoptotic proteins are a possible mechanism of $1,25(OH)_2D_3$-mediated apoptosis (35). However, Bcl-2 expression did not show a downregulation in the BGC-823 gastric cancer cells treated with $1,25(OH)_2D_3$ or cisplatin. Cisplatin cytotoxicity results from the formation of bifunctional, intrastrand DNA adducts (36). Cisplatin activates p53 and then results in the increased transcription of p53 target genes, including Bax and p21, as well as in cell cycle arrest and apoptosis (37). In our study, the combined use of cisplatin and $1,25(OH)_2D_3$ enhanced the apoptosis of BGC-823 cells.

In our previous study, we reported that $1,25(OH)_2D_3$-mediated apoptosis is associated with the downregulation of the AKT and ERK survival signaling pathways (18). Activated AKT phosphorylates a host of proteins that affect cell growth, cell cycle entry and cell survival. The decreased phosphorylation of AKT may contribute to the anti-proliferative effects of $1,25(OH)_2D_3$. siRNA-AKT has been shown to promote $1,25(OH)_2D_3$-induced apoptosis in SCC cells through the caspase-10-caspase-3 pathway, whereas caspase-8 and caspase-9 are not involved (38). Akt may regulate apoptosis through a number of different mechanisms depending on the apoptotic stimuli and cell types, which involve the regulation of phosphorylation and protein expression (39,40). In our study, as compared to treatment with cisplatin alone, AKT phosphorylation was further decreased in the cells that became apoptotic following combined treatment with $1,25(OH)_2D_3$ and cisplatin. These data indicate that $1,25(OH)_2D_3$ further enhances the cisplatin-induced loss of survival signaling, and thus further inhibits the proliferation of gastric cancer cells. The AKT

pathway presents an attractive target for anticancer therapies, which may be applied in future anticancer chemotherapy.

It has been demonstrated that the MAPK-ERK pathway is one of the most significant signal transduction pathways (41), and several key growth factors and genes promote tumor growth by activating this signaling cascade. The down-regulation of ERK phosphorylation is a contributing factor to cellular apoptosis in gastric cancer (42). The vitamin D analog, Gemini, has been shown to suppress ErbB2-positive mammary tumor growth through the inhibition of ErbB2/AKT/ERK signaling (43), and the knockdown of ZFX has been shown to inhibit gastric cancer cell growth *in vitro* and *in vivo* by downregulating the MAPK-ERK pathway (44). In our previous study, we observed that the pERK/ERK ratio was decreased in $1,25(OH)_2D_3$-treated BGC-823 cells (18), and we found a further reduction in pERK/ERK in BGC-823 cells co-treated with $1,25(OH)_2D_3$ and cisplatin. Therefore, the promotion of gastric cancer cell apoptosis or inhibition of cell growth due to the combined effects of vitamin D and cisplatin may be explained, at least in part, by the inhibition of the ERK and AKT pathway. However, the direct link between vitamin D and the ERK or AKT pathway requires further investigation.

The anti-proliferative effects of $1,25(OH)_2D_3$ commonly involve the cell cycle arrest of different cancer cells. $1,25(OH)_2D_3$ inhibits cell proliferation, induces cell cycle arrest and promotes the accumulation of cells in the G_0/G_1 phase in multipotent mesenchymal cells (MMCs) (45). The vitamin D analogue, EB1089, has been shown to significantly reduce cell growth in human hepatoma cells (Hep-G2) and block Hep-G2 cell-associated tumor formation in nude mice through cell cycle G_1 phase arrest by the accumulation of p27 (46). In this study, we demonstrated that $1,25(OH)_2D_3$ alone did not induce cell cycle G_0/G_1 arrest or G_2/M cell cycle change. However, $1,25(OH)_2D_3$, in conjunction with cisplatin, induced G_0/G_1 cell cycle arrest or a decrease in the number of cells in the G_2/M phase in BGC-823 cells, and we observed that the effects of this combined treatment were more potent compared to the effects induced by cisplatin alone.

Although a number of $1,25(OH)_2D_3$ responsive genes are known, the exact mechanisms of growth regulation by $1,25(OH)_2D_3$ have not been completely defined. However, an increase in p21 and/or p27 expression is an almost universal feature (47). In our study, p21 protein expression increased significantly following co-treatment with $1,25(OH)_2D_3$ and cisplatin, and p27 was upregulated to a much higher degree following the combined treatment compared to treatment with cisplatin alone. This indicates that $1,25(OH)_2D_3$ promotes the effects of cisplatin, inducing cell cycle arrest in the G_0/G_1 phase.

In conclusion, to the best of our knowledge, the present study demonstrates for the first time that $1,25(OH)_2D_3$ plays a synergistic role in cisplatin-mediated growth inhibition and apoptosis in gastric cancer cells. The combined use of $1,25(OH)_2D_3$ and cisplatin may be used as a strategy to overcome resistance to cisplatin and dose limitations, and to improve the anticancer effects of chemotherapy.

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