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Induction of apoptosis of human ovarian cancer cells by SGI-1776 combination with DDP in sub-toxic concentration in vitro*

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Abstract *Objective:* The aim of the study was to investigate the effect of SGI-1776 combination with DDP in sub-toxic concentration on induction of apoptosis of human ovarian cancer HO-8910 cells *in vitro* and to unravel the associated mechanisms. *Methods:* Human ovarian cancer HO-8910 cells were cultured *in vitro*. The inhibitory effect of SGI-1776 combination with DDP in sub-toxic concentration on induction on viability of human ovarian cancer HO-8910 cells was evaluated by the MTT assay. Cell apoptosis rate was analyzed by flow cytometry. The proteins expression level related to apoptosis were analyzed by Western blot. *Results:* SGI-1776 combination with DDP in sub-toxic concentration significantly inhibited the proliferation of human ovarian cancer HO-8910 cells, and proliferation inhibition rate was increased drastically compared with normal saline (NS) group or DDP group in sub-toxic concentration or SGI-1776 group in sub-toxic concentration ($P < 0.01$). Apoptosis rate markedly increased after the treatment of SGI-1776 combination with DDP in sub-toxic concentration for 48 h. Western blot showed that the expression of bcl-2 protein was down-regulated and protein level of Bax and Cyto-c were depressed by SGI-1776 combination with DDP in sub-toxic concentration. *Conclusion:* SGI-1776 combination with DDP in sub-toxic concentration could inhibit the cell proliferation and lead to cell apoptosis inhuman ovarian cancer HO-8910 cells, and its mechanism may be related to through mitochondrial apoptotic pathway.

Key words ovarian cancer; SGI-1776; DDP; apoptosis

Cis-diammin-odichloroplatinum II dichloridle (DDP) was one of chemicals whose center poses one complex that heavy metal divalent platinum integrating with two chorine atoms and two ammonia molecules, and it poses broad anticancer spectrum, strong anticancer effect, synergistic action with variety of antineoplastic drug, and no cross-drug-resistance characteristics. At present, DDP and its derivatives has come to become one of most common drugs in carcinoma chemotherapy, especically in the treatments in ovarian cancer [1]. SGI-1776, an imidazo pyridazine compound initially found through virtual screening, which has been shown to have inhibitory activity against Pim-1, Pim-2, Pim-3 kinase, and induced tumor cells apoptosis and inhibited cancer cell proliferation, has already entered phase I of clinical trials [2], but the effects and mechanism of SGI-1776 combination with DDP on antitumor effect has not been reported in public. Thus, in the present study, experiments were designed to investigate the effects of SGI-1776 combination with DDP in their sub-toxic concentrations on cell induction of apoptosis in HO-8910 cell.

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Materials and methods

Experimental materials

The human ovarian cancer HO-8910 cells were pur-

chased from China Centre for Type Culture Collection (China) and were maintained in Dulbecco's minimum essential medium (DMEM) medium (Invitrogen Corp., USA) supplemented with 10% fetal bovine serum (Boster Wuhan Biological Technology Ltd., China), 4 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37 ˚C in a humidified atmosphere of 5% CO_2. SGI-1776 was kindly provided by Super-Gen Inc (Dublin, USA). Primary antibodies for Bcl-2, Bax, Cyto-c, GAPDH, and horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody were purchased from Beyotime Company (China). 3-(4, 5-dimethylthi-azol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), SGI-1776 was dissolved to make a 1 μmol/L stock solution and was added directly to the media at different concentrations.

Experimental methods

Cell Culture

The human ovarian cancer HO-8910 cells were maintained in DMEM medium (Invitrogen Corp., USA) supplemented with 10% fetal bovine serum, 4 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37 ˚C in a humidified atmosphere of 5% CO_2. Exponentially growing cells were used in experiments [3].

Cell proliferation inhibition analysis

The effect of SGI-1776 combination with DDP on the proliferation ability of HO-8910 cells was determined by using MTT assy. 2.5×10^4 cells/mL exponentially growing cells were plated into 96-well flat-bottom microplates, and treated with DDP 0.2 μmol/L, SGI-1776 0.2 μmol/L, DDP 0.2μmol/L + SGI-1776 0.2 μmol/L, normal saline (NS). The total incubational volume of each well was 200 μL. After incubation for 24, 48, 72 h, 20 μL MTT was added to each well and incubated for additional 6 h, and then the supernatants were removed and 100 μL. Dimethyl sulfoxide was added to dissolve the fromazan crystals. The viable cell number was directly proportional to the production of formazan [4]. The plate was then read in a micro-plate reader (ELX-800) at 570 nm. We counted the relative viable cells inhibit rate (IR) = $(1 - A_{\text{average value of experimental group}} / A_{\text{average value of NS}}) \times 100\%$. The experiment was repeated three times.

Cell apoptosis analysis

Exponentially growing cells were simultaneously dealt in DMEM medium supplemented with 1% fetal calf serum for 24 h, and then the cells were exposed in different experimental drug groups and continued to incubate in DMEM medium supplemented with 10% fetal calf serum for 48 h, then the cells were harvested and washed by cold phosphate sulfoxide (PBS) twice and fixed in ethanol 70% (4 ℃), then stained in Annexin V-Propidium Iodide(AV-PI) in darkness. Apoptotic rate of cells was analyzed by flow cytometry. The experiment was repeated three times [5].

Western blot

HO-8910 cells were exposed to NS, DDP 0.2 μmol/L, SGI-1776 0.2 μmol/L, and DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L for 48 h, and then washed 3 times in cold PBS, then harvested and treated with cell lysate [3]. The protein amount was detected with BCA kit, samples containing 30 μg of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresisgel electrophoresis, and then electro-transferred to the polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline Tween containing 5% non-flat dry milk and incubated with the indicated primary antibodies overnight at 4 ℃, and then membranes were incubated with Horseradish Peroxidase-conjugated second antibody. Protein-antibodies complexes were detected by enhanced chemiluminescene according to the manufacturer's recommendations. Band densities in western blot measured using Imaging J for windows software (NIH) [6]. The experiment was repeated three times.

Statistical analysis

All the experimental data were expressed in $\bar{\chi} \pm S$, and statistic analyses were performed using SPSS 13.0 software. Differences between groups were examined with One-Way ANOVA, and a probability level of 0.05 was chosen for statistical significance.

Results

SGI-1776 combination with DDP inhibits the proliferation of HO-8910 cells

MTT assay showed that the proliferation of HO-8910 cells was inhibited when cells exposed to DDP 0.2 μmol/L or SGI-1776 0.2μmol/L for 24, 48, 72 h compared with NS group ($P < 0.05$), but the inhibition rate was less than 15%, which suggested DDP 0.2 μmol/L and SGI-1776 0.2 μmol/L was sub-toxic drug concentration. While the proliferation of HO-8910 cells was significantly inhibited when cells exposed to DDP 0.2 μmol/L + proliferation 0.2 μmol/L for 24, 48, 72 h compared with NS group, DDP 0.2 μmol/L group, and SGI-1776 0.2 μmol/L group ($P < 0.05$). And the inhibition rate of DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L group more than the addition between the DDP 0.2 μmol/L group and SGI-1776 0.2 μmol/L group, Which indicated there was synergistic effect between SGI-1776 and DDP in sub-toxic drug concentration (Fig. 1).

Effect of SGI-1776 combination with DDP on the apoptotic rate of HO-8910 cells

HO-8910 cells indicated apoptosis when they were ex-

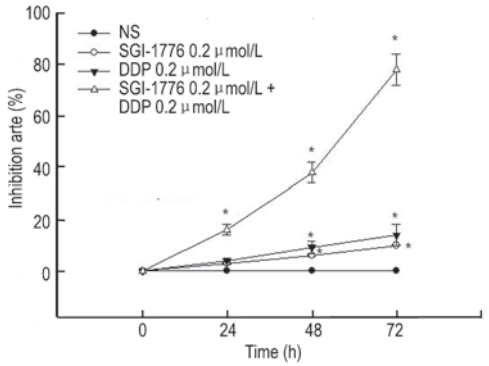

Fig. 1 Effects of different experimental factors on proliferation of HO-8910 cells in different times (* $P < 0.05$ vs NS group)

posed to NS group, DDP 0.2 μmol/L group, SGI-1776 0.2 μmol/L group, and DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L for 48 h. and the apoptosis rate respectively 1.10%, 12.3%, 12.3% and 520.7%, and there were significant difference between DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L and other experimental groups ($P < 0.1$). And the apoptosis rate of DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L was more than the addition between DDP 0.2 μmol/L group and SGI-1776 0.2 μmol/L group, which showed there was synergistic effect between SGI-1776 and DDP ($P < 0.5$; Fig. 2).

SGI-1776 combination with DDP represses the expression of bcl-2 protein and up-regulates the expression Bax protein and Cyto-c protein in HO-8910 cells

The expression of Bcl-2 protein was showed to be repressed by different experimental factors in HO-8910 cells ($P_{\text{NS vs SGI-1776 0.2 μmol/L}} = 0.017$, $P_{\text{NS vs DDP 0.2 μmol/L}} = 0.028$, $P_{\text{NS vs DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L}} = 0.006$). Expression of Bax protein was activated by different experimental factors represses in HO-8910 cells ($P_{\text{NS vs SGI-1776 0.2 μmol/L}} = 0.018$, $P_{\text{NS vs DDP 0.2 μmol/L}} = 0.022$, $P_{\text{NS vs DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L}} = 0.004$). Expression of Cyto-c protein was up-regulated by different experimental factors represses in HO-8910 cells ($P_{\text{NS vs SGI-1776 0.2 μmol/L}} = 0.015$, $P_{\text{NS vs DDP 0.2 μmol/L}} = 0.017$, $P_{\text{NS vs DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L}} = 0.005$). Those results indicated that the SGI-1776 combination with DDP induced cell cycle apoptosis might be through mitochondrial apoptotic pathway (Fig. 3).

Discussion

DDP pose broad anticancer spectrum, strong anticancer effect, synergistic with variety of antineoplastic, and no cross-drug-resistance characteristics [1]. At present, DDP and its derivatives is one of most commonly used drugs in carcinoma chemotherapy, especically in the treatments in ovarian cancer, but the side effect of DDP limit it's clinical application, such as bone marrow suppression, kidney toxicity, neurotoxicity, allergic reaction and so on. SGI-1776, an imidazo pyridazine compound, which has been shown to have inhibitory activity against tumor cells proliferation through induction apoptosis, Xie and so on discovered SGI-1776 affected the ovarian cancer cell cycle by regulating the expression levels of CDK2, CDK4, CDK6, and CDK inhibitors p27 and p21 through down-regulation of Pim-1 expression [2].

Human ovarian cancer HO-8910 cells were used in

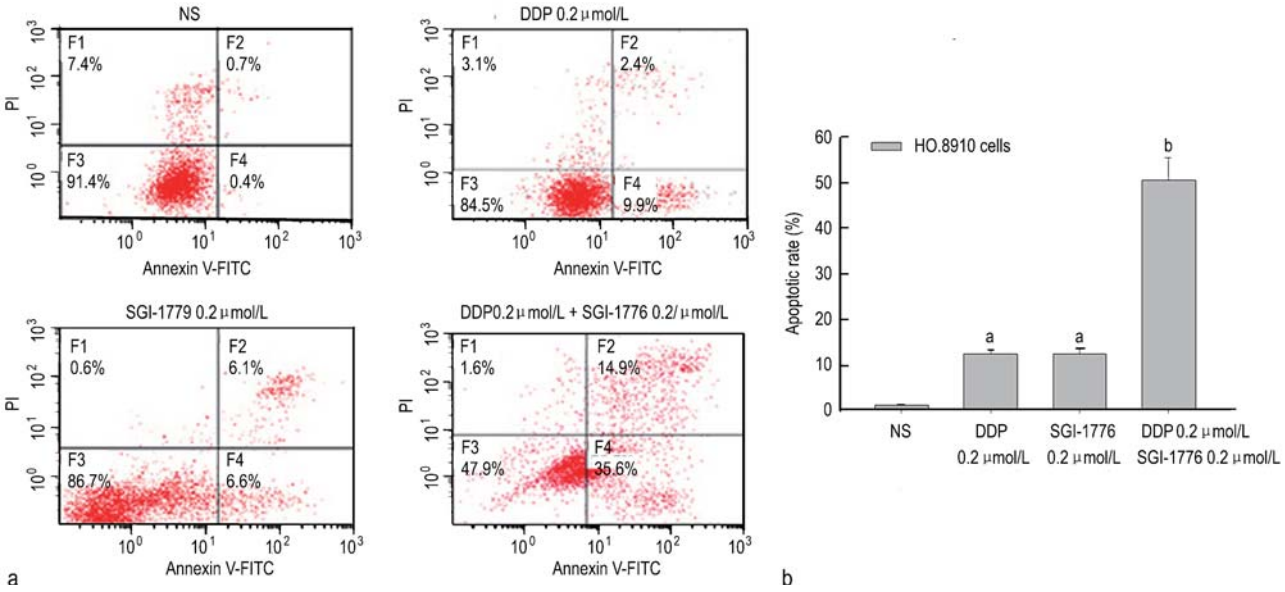

Fig. 2 Effects of different experimental factors on apoptosis rate of HO-8910 cells. (a) the apoptotic rate of HO-8910 cells of AV-PI staining; (b) compare of apoptotic rate of HO-8910 cells treated by experimental factors. [a] $P < 0.05$ vs NS group, [b] $P < 0.05$ vs NS group

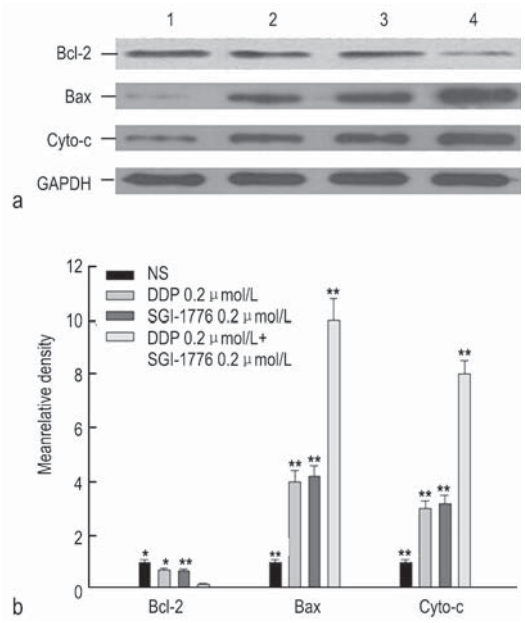

Fig. 3 The expression of proteins of HO-8910 cells treated with different experimental factors for 48 h were analyzed by Western blot. * P < 0.05 vs NS group, ** P < 0.05 vs NS group. 1: NS ; 2: DDP 0.2 μmol/L; 3: SGI-1776 0.2 μmol/L; 4: DDP 0.2 μmol/L + SGI-1776 0.2 μmol/L

our study, the inhibitory effect of SGI-1776 combination with DDP on the proliferation ability of human ovarian cancer HO-8910 cells and on apoptotic were evaluated. Bcl-2 protein is one kind of inhibiting components on cell apoptosis, it is able to inhibit the permeability of mitochondrial adventitia and prevent Cyto-c protein release from mitochondrion through integrating Bax protein and forming hemerodimer and regulate intrinsic apoptotic pathway. Bax protein is one of promoting components on cell apoptosis, which form channels to promote Cyto-c protein release from mitochondrion, and the change of the permeability of mitochondrial adventitia is the key factors [7]. The homodimer of Bax protein compose the releasing channel of Cyto-c protein, and it is necessary factor to form mitochondrial apoptotic pathway. Only Bcl-2 protein integrating Bax protein and forming hemerodimer, Bcl-2 protein educe its anti-apoptotsis effects, thus the relative expression of Bcl-2 protein and Bax protein is the key factor to regulate apoptotic progress [8]. Cyto-c protein transfer from cellular mucleus to cytoplasm and integrate with Apaf-1 and ATP/dATP to form complex of apoptosis which pose ability to activate caspase cascade proteins, and trigger cellular constructive protein and functional protein degradation and cause cell apoptosis [7, 8].

Our data showed that the expression protein level of Bcl-2 was decreased accompanied with HO-8910 cells proliferating inhibition and apoptosis when cells exposed to SGI-1776 combination with DDP in sub-toxic concentration *in vitro*, which mean the factor of inhibiting apoptosis declined and cells would tend to apoptosis, which theory was proved by our experimental again. Bax proteins were activated accompanied with HO-8910 cells proliferation inhibition and apoptosis when cells exposed to SGI-1776 combination with DDP in sub-toxic concentration *in vitro*, which implied that more Cyto-c proteins will be released from from cellular mucleus to cytoplasm and trigger caspase cascade proteins and induce cell apoptosis, and our experimental results indicated the proteins expression of Bax and Cyto-c came to enhance when HO-8910 cells were exposed to SGI-1776 combination with DDP in sub-toxic concentration accompanied with HO-8910 cells proliferation inhibition and apoptosis, and our experimental results were consistent with the upper theory, and all our experimental results were similar with the research made by Xie and so on [2].

In summary, SGI-1776 combination with DDP in sub-toxic could significantly inhibit proliferation and induce apoptosis of human ovarian cancer HO-8910 cells, and the induction of apoptosis through mitochondrial apoptotic pathway was one of possible mechanisms. Our study indicated that SGI-1776 combination with DDP not only could inhibit significantly human ovarian cancer proliferation and induce apoptosis, but also reduced the concentration of drug and toxic side effects, SGI-1776 combination with DDP might become a good candidate for an new combination therapy in anti-tumor aspect, but the present study is limited in the experiment *in vivo*, and further studies *in vivo* are required to confirm the validation.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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