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Gemcitabine and cisplatin for treatment of lung cancer *in vitro* and *vivo*

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Abstract. – OBJECTIVE: To evaluate the antitumor activity of gemcitabine (GEM), cisplatin (DDP) as well as the combination of these two agents in lung cancer cells and mice.

MATERIALS AND METHODS: The cell viability was evaluated by the CCK-8 assay. Cell apoptosis was measured by flow cytometry assay and Hoechst staining. The protein expression of VEGF, VEGFR2, Ang II, AT1R, and ACE2 was examined by Western blotting. The effect of GEM and DDP on tumor growth and survival time was also measured in lung cancer mice *in vivo*.

RESULTS: The results revealed that alone or combined administration of GEM and DDP could inhibit the growth, induce apoptosis and apoptotic body formation of A549 cells compared with control cells, with the most significance detected in a combination of GEM and DDP administration. It is indicated that combined administration of GEM and DDP could delay the progress of tumor formation in nude mice. The cell apoptosis- and angiogenesis-related proteins expressions were decreased both in A549 cells and lung cancer mice.

CONCLUSIONS: GEM plus DDP can be an option for patients with lung cancer treatment. However, further prospective evaluation and randomized trials are to provide more accurate information through clinical trials.

Key Words:
lung cancer, Gemcitabine, Cisplatin, Cell apoptosis, Angiogenesis.

Introduction

As one of the most common cancer in the world, lung cancer can be seen about 1.5 million newly diagnosed cases and 1 million deaths every year[1]. Most non-small cell lung cancer (NSCLC) patients are diagnosed early[2]. Over the past decade, chemotherapy remains a major method of standard therapy and radiotherapy.

Gemcitabine (2'2'-difluorodeoxycytidine, GEM) is a chemotherapeutic agent, acting as a pyrimidine nucleoside antimetabolite which has a comparatively low toxicity. It can achieve 14-37% of response rates (RR) in first-line and about 25% RR in salvage therapy[3-6]. These characteristics indicate that GEM may be a good candidate for combination with other cytotoxic drugs, especially those who cause DNA damage. In some phase II trials, GEM combinations have enhanced objective remission rated (ORR) and overall median survival (OS). Then, many prospective randomized phase III clinical trials were compared. However, these tests had different results, and the number of admissions is very small.

Cisplatin (cis-diamminedichloroplatinum, DDP) as a DNA cross-linking agent presents distinguished activity in many solid tumors. The biochemical mechanism of DDP cytotoxicity involves the binding of drugs to DNA and non-DNA targets, and subsequent cell death through apoptosis, necrosis, or both[7]. DDP is very valid in the treatment of testes and human cancers, including ovarian, bladder, neck, head and neck, esophagus, and small cell lung cancer[8]. Some tumors, however, like colon and non-small cell lung cancer, have intrinsic resistance to DDP while other tumors like ovarian and small cell lung cancer produce acquired resistance after initial treatment[9]. More than 3 decades of in-depth studies starting with the discovery of anti-tumor activity of DDP reported that no more than 30 compounds showed sufficient pharmacological superiority compared to DDP tested in clinical trials[10].

What's more, experiments show that GEM-DDP combination, in close sequence or simultaneous exposure, should be in synergies *in vitro* and *in vivo*[11-13]. The combination of GEM and DDP showed a very high activity in phase II trials with stage IV NSCLC, with an RR of 54% and OS of 14 months[14]. Given these findings and the different mechanisms of action of GEM and DDP, we investigated the therapy's efficacy of a combination of two agents in lung cancer *in vitro* and *in*

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vivo. Combination of these two agents was found to achieve higher antitumor effects than either GEM or DDP alone.

Materials and Methods

Cell Culture

Lung cancer cell line A549 (Academia Sinica Cell Bank, Shanghai, China) were cultured in F-12K culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assay

The cell viability was assessed by Cell Counting Kit 8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD, USA). A549 cells were plated in the 96-well plates (5×10^3 cells/well) and incubated for 24 h. GEM, DDP, and GEM plus DDP were introduced into the medium of the cells. Cells cultured in the medium without the addition of the reagents were applied as controls. After 6, 12, 24, 48, and 72 h incubation, the cells were washed with D-Hanks buffer. 200 μl of CCK-8 solution was put to each well and incubated at 37°C for another 3 h. The optical density (OD) at each hole at 450 nm was recorded on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Apoptosis Assay

Apoptosis kit (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, San Jose, CA, USA) was applied to detect apoptotic cells. Then, A549 cells were plated in the 6-well plates (1×10^5 cells/well) and incubated for 24 h. GEM, DDP, and GEM plus DDP were introduced to the cells and incubated for 24 h. A549 cells were collected, washed twice with cold D-Hank's buffer and re-suspended in binding buffer (1×10^6 cells/mL). After 100 μl of A549 cells transferred to a tube, 5 μl of fluorescein isothiocyanate (FITC)-conjugated Annexin V (Annexin V-FITC) and 5 μl of propidium iodide (PI) were put followed by incubation for 15 min at inside temperature in the dark. The stained A549 cells were diluted by the binding buffer and analyzed by flow cytometry's (EPICS XL MCL, Beckman Coulter, Brea, CA, USA) Cell Quest software.

Hoechst Staining

A549 cells were centrifuged (800×*g* for 5 min) and fixed in 4% formalin for 10 min. After washing twice with PBS, the cells were stained with Hoechst 33258 in phosphate-buffered saline (PBS) containing 80% (v/v) glycerol in PBS according to the manufacturer's specifications, mounted on a slide, and observed with a fluorescence microscope (Nikon, Tokyo, Japan).

Western Blot

50 micrograms of protein from lung cancer A549 cells were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) through a Bio-Rad microplate gel device (Hercules, CA, USA) and electrophoresed to nitrocellulose tablets. The antibody was incubated with the blot overnight at 4°C. The film was washed and incubated with the corresponding secondary antibody and displayed by enhanced chemiluminescence (Millipore, Beijing, China) as the manufacturer instructs. Antibodies for VEGF, VEGFR2, ATIR, and ACE2 were gained from Abcam (Cambridge, MA, USA) and Ang II from Santa (Shanghai, China). Antibody for GAPDH was bought from Cell Signaling Technology (Danvers, MA, USA).

Cells Growth and Tumor Size Determination in Nude Mice Without Thymus

A549 cells were digested with trypsin, washed and re-suspended in F-12K without FBS. Cell concentration and viability were decided by trypan blue. Eight male non-thymus nude mice were divided into 2 groups (4 mice/group) at random, and subcutaneously injected with 2×10^6 A549 cells. After 7 days, the mice were treated with GEM plus DDP (GEM: 50 mg/kg and DDP: 2 mg/kg) for 5 weeks with twice in one day. As in[15], tumor size was measured every 3-4 days (about 1 to 2 weeks) after tumor formation. After 45 days, the mice were sacrificed and photographed, and the tumor was weighed on a digital balance.

Immunohistochemistry

Tumor tissues from lung cancer mice were extracted and prepared for immunohistochemical studies as in previous study[16]. The sections were stained with anti-CD34 primary antibodies (optimal dilution of 1:125, TransGenic, Kumamoto, Japan). For negative controls, Tris-buffered saline (TBS) was used instead of primary antibody. A microscope (× 400) to check the fields from each slide was used. An optical microscope (Olympus BX-50; Olympus Optical, Tokyo, Japan) was used to take pictures.

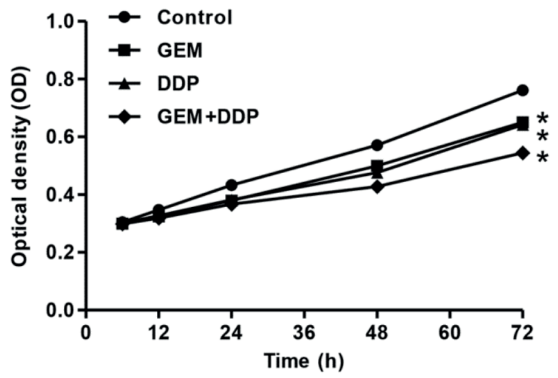

Figure 1. Gemcitabine and cisplatin inhibit the viability of A549 cells. Cell viability was measured by the Cell Count Kit-8 (CCK-8). Gemcitabine (GEM) and cisplatin (DDP) inhibited A549 cells viability when compared with control group, with the significant inhibition detected in GEM plus DDP combination. Data are presented as mean ± SD (n=3). *p<0.01.

Statistical Analysis

By GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) analysis, the experimental data were expressed as at least three independent repetitions of the mean ± SE and were analyzed by Kaplan-Meier method and logarithmic rank test. The paired two-tailed Student *t*-test was applied to analyze the significance of differences among groups. A significant difference was observed at a value of p<0.05.

Results

Gemcitabine and Cisplatin Inhibit A549 Cells Proliferation

To investigate the inhibitory impacts of GEM and DDP on the growth of A549 cells, cell viability was assessed by CCK-8 after treatment with GEM, DDP, and GEM plus DDP for 6, 12, 24, 48, and 72 h. As Figure 1 shows, GEM plus DDP had great growth inhibition impact on the A549 cells compared with GEM or DDP single-handed groups and control group (p<0.01).

Gemcitabine and Cisplatin Induce A549 Cells Apoptosis

An annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining and flow cytometry analysis were applied to confirm the apoptosis induced by GEM, DDP, and GEM plus DDP treatment. As shown in the lower quadrant of the histogram, the number of apoptotic cells is counted as early apoptotic cells. In Figure 2A, treatment of GEM plus DDP greatly increased the number of early apoptotic cells (56.9±0.15%) compared with GEM or DDP single-handed groups (31.2±1.5% and 31.7±1.07%) and control group (2.6±0.35%) (p<0.01). Moreover, A549 cells with GEM, DDP, and GEM plus DDP treatment become membrane-bound small particles, so-called apoptotic bodies (Figure 2B).

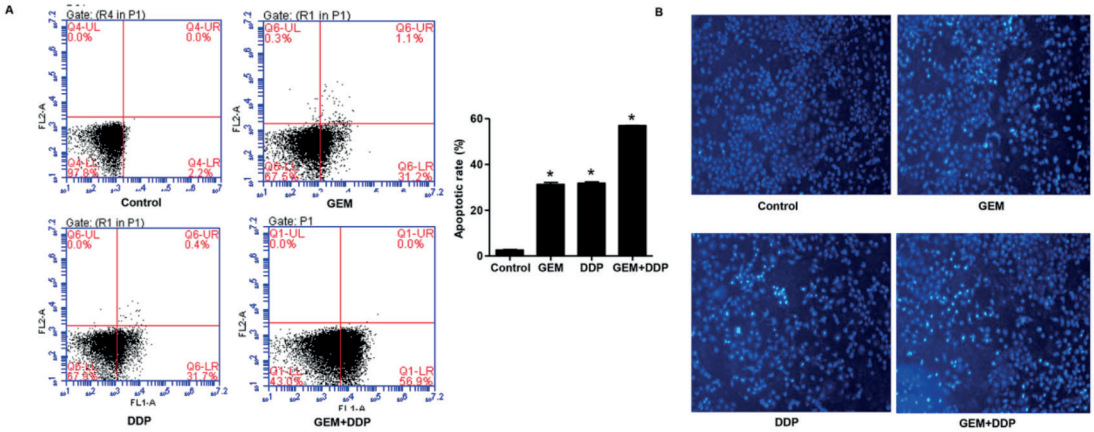

Figure 2. Gemcitabine and cisplatin induce cell apoptosis and apoptotic bodies formation of A549 cells. **A,** Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis. GEM and DDP increased the apoptotic percentage of A549 cells, with the significant increase detected in GEM plus DDP combination. **B,** Hoechst staining was used to stain apoptotic cells treated with GEM and DDP. *p<0.01.

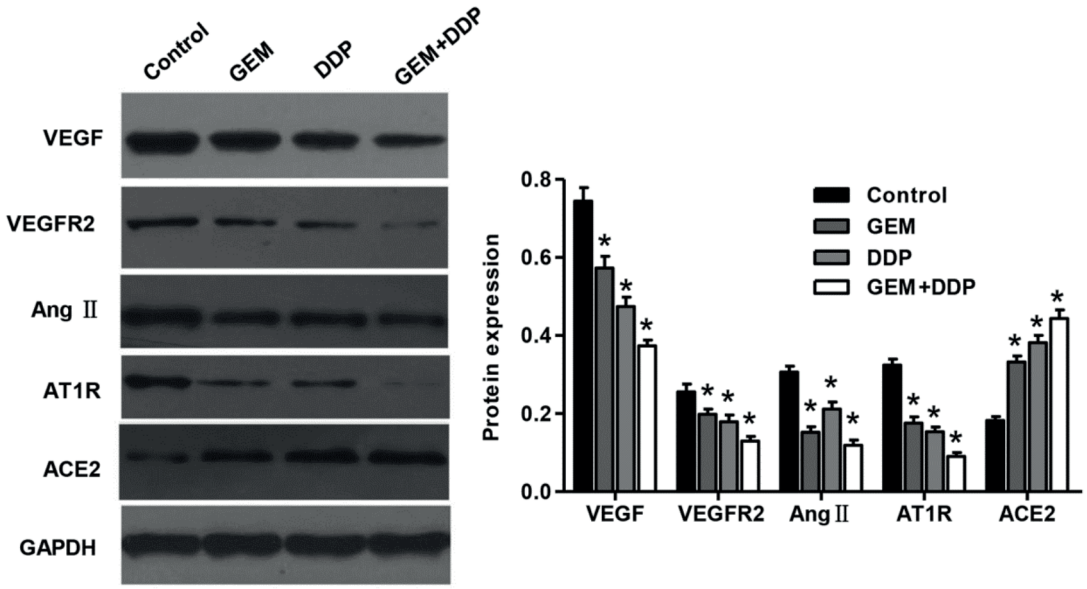

Figure 3. Effects of gemcitabine and cisplatin on the expression of key proteins involved in cell apoptosis and angiogenesis. Treatment with GEM and DDP decreased expression of VEGF, VEGFR2, Ang II and AT1R protein, and increased ACE2 protein expression, with the significant changes detected inGEM plus DDP combination. *p<0.01.

Gemcitabine and Cisplatin Inhibit Angiogenic Proteins Expression in A549 Cells

To elucidate the mechanism of GEM and DDP-induced angiogenesis in A549 cells, the expression of angiogenesis-related proteins was detected by Western blotting. As The treatment with GEM, DDP, and GEM plus DDP decreased angiogenesis-related proteins VEGF, VEGFR2, Ang II, and AT1R, while ACE2 was significantly increased with GEM plus DDP treatment (p<0.01) (Figure 3).

Gemcitabine Plus Cisplatin Treatment Inhibits CD34 Expression and Tumor Growth of Lung Cancer In Vivo

CD34 is an endothelial antigen that has been used to highlight the density of blood vessels within the tumor as a direct marker of neovascularization. The total number of vascular endothelial cells from lung cancer mice was determined by immunohistochemistry for CD34 (Figure 4A). The results revealed a decreased expression of CD34 in mice treated with GEM plus DDP (p<0.01). Next, we determine whether GEM and DDP can reduce the growth of tumors *in vivo*. A549 cells were injected into nude mice subcutaneously without thymus and treated with GEM plus DDP. The tumor volume was measured for 46 days. In Figure 4B, GEM plus DDP treatment decreased tumors growth in mice compared with control tumors in lung cancer mice. After 46 days, tumor weight and volume in mice treated with GEM plus DDP was half of those in control mice (Figure 4C and 4D, p<0.01). The survival time of lung cancer mice showed that GEM plus DDP treated mice notably lived longer than control mice (Figure 4E). These data suggested that GEM plus DDP treatment reduce the growth of tumors in nude mice.

Discussion

Chemotherapy in the treatment of lung cancer has become a hotspot for decades. Until recent years, cisplatin (DDP)-based combination chemotherapy suggested a small but definite survival benefit in a significant proportion of patients with advanced lung cancer. Moreover, it also improved symptoms, performance status and life quality of patients with lung cancer. We demonstrated that chemotherapy with gemcitabine (GEM) plus DDP induced cell apoptosis and inhibited angiogenesis in lung cancer *in vitro* and *in vivo*.

Gemcitabine is a new cytarabine analog with antitumor activity, whose primary role is preventing cell progression in the cell cycle. It has been demonstrated that GEM plus DDP is a reasonable combination therapy, which gives them non-overlapping toxicity and synergistic cytotoxic activity

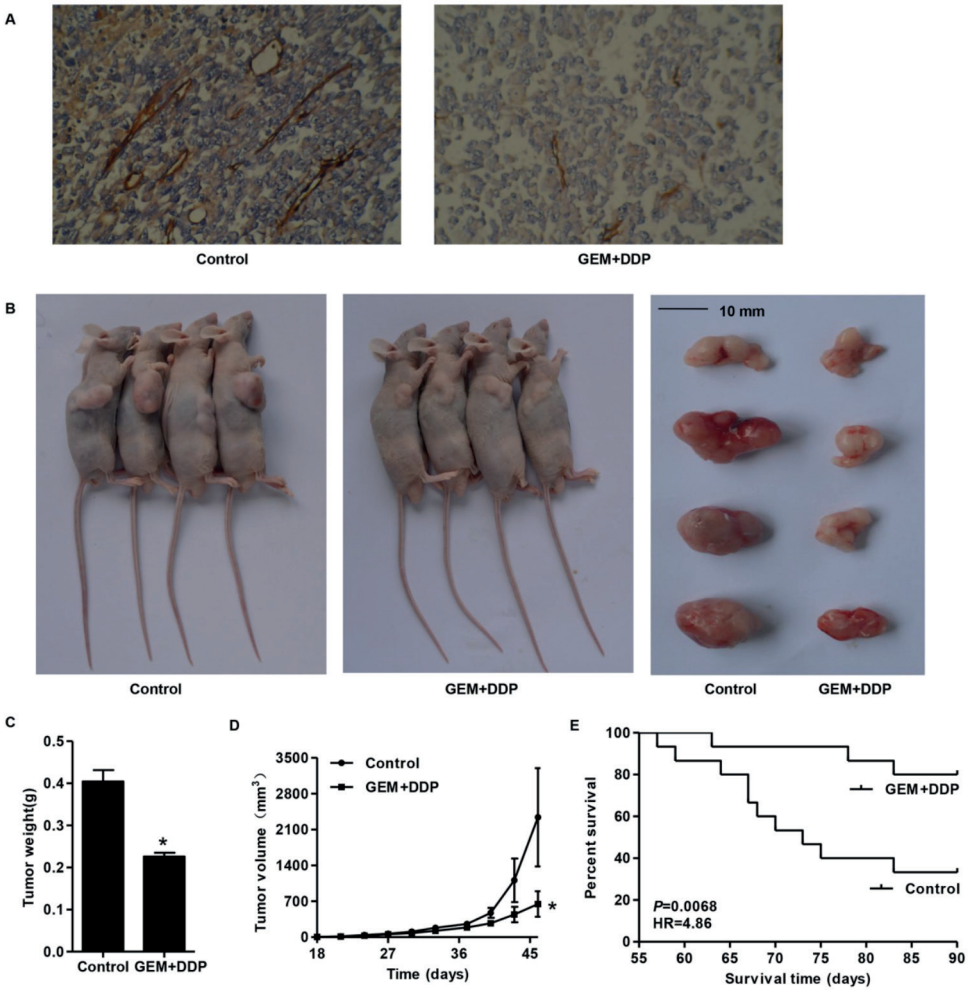

Figure 4. Gemcitabine plus cisplatin treatment inhibits CD34 expression and tumor growth *in vivo*. ***A,*** Example of immunohistochemical staining for antibody against CD34. B: Tumor diameter was evaluated for 46 days. ***C,*** At day 46, mice were sacrificed and tumors were weighted. ***D,*** Tumor growth was significantly reduced in GEM plus DDP combined tumors. ***E,*** Effect of GEM plus DDP combination on the overall survival of mice with lung cancer. The GEM plus DDP combination tumors have a favorite prognosis compared to control tumors. **p*<0.01.

in vitro[13]. When DDP-containing combinations have failed, GEM also presented to be active in rescue therapy[17]. It is believed that an attempt to repair DDP-induced DNA damage might eventually lead to triggering of apoptosis[18]. It is confirmed that DDP-induced protein damage, rather than DNA damage, is important in triggering an apoptotic pathway[19]. By the 1990s, it was believed that most of the clinically anticancer agents bind to DNA kill cancer cells by apoptosis[20]. The apoptotic process is usually divided into 3 stages[7], the final result is the formation of apoptotic bodies to decompose cells. Similar results of cell apoptosis and formation of apoptotic bodies were also found in our study.

Tumor angiogenesis is an important step in the growth of tumor and metastasis. Identification of new pathologic angiogenesis inhibitors will benefit drug discovery in tumors and other diseases related to angiogenesis[21-23]. In this work, it is demonstrated that GEM and DDP could inhibit angiogenesis *in vitro* through decreasing expression of angiogenesis-related proteins in lung cancer. Angiogenesis is a multi-step process, including cell proliferation, migration, and tube formation, where cell migration and tube formation are essential steps[24,25]. We show that GEM and DDP not only inhibit the proliferation and induction of apoptosis in A549 cells, but also inhibit angiogenesis and tumor formation, indicating that GEM

and DDP can inhibit tumor by affecting multiple aspects. Inhibition of tumor angiogenesis might be an essential aspect of the antitumor activity of GEM and DDP at an effective dose for treatment of lung cancer.

To increase the clinical outcome of GEM, Phase II-III trials have recently been evaluated and showed that GEM exhibited synergistic effects *in vitro* with other agents, including 5-fluorouracil (5-FU), DDP, topotecan, etc.[26,27]. Major meta-analysis showed that GEM combinations produced important survival advantages[28]. Similar results were found in our study. In another meta-analysis, we found that in patients with advanced pancreatic cancer, the program GEM plus DDP is not better than GEM, which produces more side effects[29]. Also, subgroup analysis has not shown any distinguished survival advantage in most GEM combinations (e.g., GEM plus 5-FU, GEM plus topoisomerase I inhibitors, etc.). This suggests that not all GEM combined chemotherapy has therapeutic advantages.

Conclusions

We showed that GEM plus DDP combined administration can be an effective chemotherapy through inducing cell apoptosis and inhibiting angiogenesis of lung cancer *in vitro* and *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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