Synergistic anti-proliferative and pro-apoptotic activities of 5F and cisplatin in human non-small cell lung cancer NCI-H23 cells

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Abstract. Two-drug combination chemotherapy, often including cisplatin and one other drug, remains the standard of care for patients with advanced non-small cell lung cancer (NSCLC). To improve the treatment of late-stage NSCLC and decrease the toxicity of combination chemotherapy, the search for novel drugs remains vigorous. Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid (5F), a bioactive compound isolated from the herb *Pteris semipinnata* L., has previously been shown to induce apoptosis and inhibit proliferation in various cancer cells. One outstanding property of 5F is its minimal side effects. In the present study, 5F was combined with cisplatin to treat NCI-H23 cells; proliferation, apoptosis and cell cycle arrest were measured by an MTT assay, Annexin V staining/flow cytometry and propidium iodide staining/flow cytometry, respectively. The messenger RNA levels of β-catenin, glycogen synthase kinase (GSK)-3β, c-Myc and cyclin D1 were determined by reverse transcription-quantitative polymerase chain reaction, and the protein levels of β-catenin and GSK-3β were measured by western blot analysis. The results revealed that 5F and cisplatin synergistically induced apoptosis and inhibited cell growth, arrested cell cycles in the G0/G1 phase, downregulated β-catenin, c-Myc and cyclin D1, and upregulated GSK-3β. These findings merit *in vivo* studies using animal models of NSCLC to confirm the addition of 5F as a third drug to cisplatin-based combination therapy for late-stage NSCLC.

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Introduction

Non-small cell lung cancer (NSCLC) accounts for 85% of all cases of lung cancer and is the leading cause of cancer-associated mortality worldwide (1). While surgery is the mainstay of treatment for early-stage and localized NSCLC, combination chemotherapy is considered the standard of care for patients with advanced NSCLC (1,2). Combination chemotherapy frequently uses two drugs, which often includes cisplatin plus one other drug (3-5). Cisplatin, also termed cis-diamminedichloroplatinum II (CDDP), is a platinum-containing compound that has also been used for the treatment of other human cancers, including head and neck, ovarian, breast, bladder, and testicular cancers (6-9). To improve the treatment of late-stage NSCLC, clinical trials of three-drug combinations have been performed (10). However, the results of these trials have demonstrated that adding a third drug may add little benefit as a result of the increased toxicity (11,12). Therefore, the search for novel drugs that are just as effective with less toxicity associated remains vigorous.

Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid (5F), a bioactive compound isolated from the herb *Pteris semipinnata* L., has been shown to induce cell apoptosis and inhibit cell proliferation in various cancer cells, including thyroid carcinoma, lung cancer, nasopharyngeal carcinoma and hepatocellular carcinoma cells (13-18). CDDP and 5F inhibit cancer cell growth by inducing cell apoptosis (9,14-16). In view of these findings, it was hypothesized that 5F and CDDP may have synergistic anticancer activity in human NSCLC cells. The present study was therefore conducted to examine the effects of 5F combined with CDDP on cell growth, cell apoptosis, cell cycle arrest and regulation of gene expression in NCI-H23 cells.

Materials and methods

Drugs. CDDP was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China). 5F was isolated from *Pteris semipinnata* L. as previously described (13), and the purity was >99%, as

analyzed by high-performance liquid chromatography (19). A stock solution of CDDP at 1 mg/ml was prepared with PBS (pH 7.4). A stock solution of 5F at 2 mg/ml was prepared by dissolving 5F in dimethyl sulfoxide (DMSO).

Cell growth inhibition analysis. Human NSCLC NCI-H23 cells (American Type Culture Collection; Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C under a humidified atmosphere containing 5% CO_2. Cells were detached with 0.25% trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc.), washed once with PBS and re-suspended at a density of $3x10^4$ cells/ml in RPMI-1640 medium. Cell suspension (100 μl) was seeded onto each well of 96-well plates and cultured at 37°C overnight. On day 2, the culture medium was replaced with fresh medium, and cells were divided into different groups and treated as follows: CDDP group, 5 μg/ml of CDDP (final concentration); 5F group, 40 μg/ml of 5F (final concentration); combination group, 5 μg/ml of CDDP and 40 μg/ml of 5F (final concentration); and control group, no drug added. Each group was analyzed in triplicate. Following the addition of drugs, cells were cultured at 37°C for 24 or 48 h, and an MTT assay was performed according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Haimen, China). Briefly, the culture medium was replaced with 100 μl of fresh culture medium, and 10 μl MTT (5 mg/ml) was added into each well. Following incubation for 4 h at 37°C, MTT was removed from the wells and 150 μl DMSO was added, followed by agitating the plate for 10 min. Subsequently, the absorbance of each well at 540 nm was measured using a microplate reader (Model 450; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell proliferation inhibition rate was calculated as: (Absorbance of control group-absorbance of treatment group)/absorbance of control group.

Cell apoptosis assay. Cell detachment and wash were performed as aforementioned. Subsequently, cells were re-suspended at a density of $1x10^5$ cells/ml in RPMI-1640 medium. Cell suspension (500 μl) was seeded onto each well of 6-well plates. Following culture at 37°C for 24 h, cells were divided into four groups and treated with drugs at 37°C for 48 h as aforementioned. Cell apoptosis was assessed using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology, Haimen, China) as advised by the manufacturer. Briefly, cells were detached with PBS/1 mM EDTA, washed once with PBS and re-suspended in 195 μl Annexin V binding buffer from the kit, followed by addition of 5 μl Annexin V-fluorescein isothiocyanate and 10 min, 37°C incubation in the dark. The fluorescence of the apoptotic cells was then determined with a flow cytometer (FACSAria III; BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle arrest assay. Cells were treated with drugs for 48 h as previously described. Following treatment, cells were detached and washed once with PBS. A total of $1x10^6$ cells were fixed in cold 70% ethanol for 1 h at -20°C. Ethanol was then removed by centrifugation at 1,000 x g at 4°C for 5 min and cells were washed once with PBS. Subsequently, 200 μl of propidium iodide (PI; 50 μg/ml in PBS) was added to the cells and incubated at 4°C for 30 min in the dark. The cell cycle distribution was then determined with a flow cytometer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to determine the relative messenger RNA (mRNA) levels of β-catenin, glycogen synthase kinase (GSK)-3β, cyclin D1 and c-Myc in drug-treated and control cells. Total RNA of NCI-H23 cells was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's protocol and quantified by photospectrometry (BioSpectrometer; Eppendorf, Hamburg, Germany). Total RNA (2 μg) was reversed transcribed using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Complementary DNA (1 μl) was mixed with 5 μl of 2X SYBR Premix Taq buffer (Takara Biotechnology Co., Ltd.), 200 nM of each primer (final concentration) and nuclease-free H_2O, which was used to adjust the reaction volume to a final volume of 20 μl. Primers for RT-qPCR were designed according to the published gene sequences using Oligo software, version 5.0 (Molecular Biology Insights, Inc., Colorado Springs, CO, USA) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of all primers, which were based on sequences available from GenBank (https://www.ncbi.nlm.nih.gov/gene/), and relevant information are listed in Table I. The amplification was performed on the LightCycler 480 Instrument II (Roche Diagnostics, Basel, Switzerland) as follows: Stage 1, 95°C for 10 min; and stage 2, 95°C for 10 sec and 60°C for 20 sec. Stage 2 was repeated for 40 cycles. Relative mRNA levels against GAPDH were calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Western blotting. Total protein was extracted from H23 cells using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) and quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total protein (50 μg) was separated by 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked in TBS-Tween-20 (TBST) buffer [50 mM Tris-HCl, 150 mM NaCl (pH 7.5) and 0.1% Tween-20] containing 5% skimmed milk for 1 h at room temperature. Subsequently, the membrane was incubated with primary antibodies diluted in blocking buffer at 4°C overnight. The primary antibodies used were as follows: Mouse anti-human β-catenin monoclonal antibody (dilution 1:1,000, cat. no. 2698) and rabbit anti-human GSK-3β antibody (dilution 1:1,000, cat. no. 9315) (both Cell Signaling Technology, Inc., Danvers, MA, USA). Following incubation with the primary antibodies, the membrane was washed three times with TBST buffer, followed by incubation with horseradish peroxidase-conjugated anti-mouse (dilution 1:5,000, cat. no. L3032-2) or anti-rabbit secondary antibodies (dilution 1:5,000, cat. no. L3012-2) (both Signalway Antibody, College Park, MD, USA) at 4°C for 1 h. Subsequently, the membrane was washed three times with TBST buffer. The specific bands were visualized with an enhanced chemiluminescence western blot analysis detection kit (Beyotime Institute of Biotechnology), and densitometric analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD,

Table I. RT-PCR primer sequences.

Genes	GenBank number	Sequences	Product size, bp
β-catenin	X87838.1	F: 5'-GACAGATCCAAGTCAACGTC-3' R: 5'-CACAAGAGCCTCTATACCAC-3'	257
GSK-3β	NM_002093	F: 5'-TCCCTCAAATTAAGGCACATC-3' R: 5'-CACGGTCTCCAGTATTAGCATCT-3'	117
c-Myc	E01841.1	F: 5'-GAACTTACAACACCCGAGCAA-3' R: 5'-GCAGTAGAAATACGGCTGCAC-3'	205
Cyclin D1	BC023620.2	F: 5'-TACCCCAATAATCAACTCG-3' R: 5'-GATGCCTAGAACCCCACT-3'	245
GAPDH	NM_002046	F: 5'-ATGACATCAAGAAGGTGGTG-3' R: 5'-CATACCAGGAAATGAGCTTG-3'	177

F, forward; R, reverse; RT-PCR, reverse transcription-polymerase chain reaction; GSK-3β, glycogen synthase kinase-3β.

USA) to determine the relative levels of β-catenin and GSK-3β following normalization against GAPDH. GAPDH, serving as a loading control, was probed using a rabbit anti-human GAPDH monoclonal antibody (cat. no. 2118, dilution 1:3,000; Cell Signaling Technology, Inc.).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis of the differences between multiple groups was performed by one-way analysis of variance using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Additional two-group comparisons were performed using the least-significant difference test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Synergistic anti-proliferative effects of 5F and CDDP. At 24 h after drug treatment, the cell proliferation inhibition rate for the combined group was 64.5±3.6%, which was significantly higher than that of the 5F (44.6±1.6%; $P<0.05$; n=4) and CDDP (33.9±2.2%; $P<0.05$; n=4) groups (Fig. 1). When cells were treated for 48 h, the inhibition rates for the combined, 5F and CDDP groups were 85.5±1.2, 65.3±2.7 and 45.1±2.3%, respectively ($P<0.05$ compared with the combined group; n=4) (Fig. 1).

Synergistic pro-apoptotic effects of 5F and CDDP. Representative histograms of cell apoptotic analysis by flow cytometry for the control, 5F, CDDP and combined groups are shown in Fig. 2A-D, respectively. The apoptotic rates for the control, 5F, CDDP and combined groups were 4.9±1.8, 27.4±1.4, 23.7±1.4 and 71.3±3.3%, respectively. Treatment with 5F or CDDP alone markedly increased cell apoptosis compared with that of no-drug treatment ($P<0.05$). Statistical analysis revealed that the combined group had a significantly higher apoptotic rate than the 5F ($P<0.001$; n=6) and CDDP ($P<0.001$; n=6) groups (Fig. 2E).

Synergistic cell cycle arrest effects of 5F and CDDP. PI staining and flow cytometric analysis were employed to
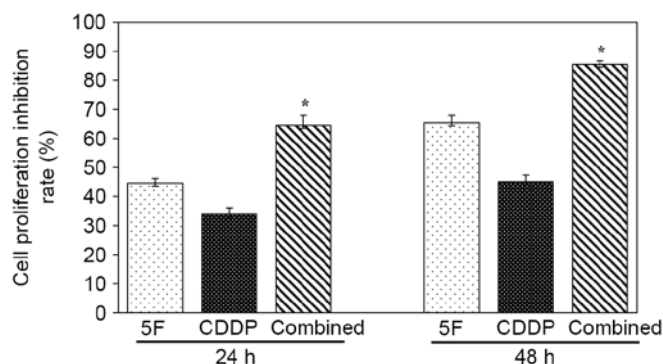

Figure 1. Synergistic anti-proliferative effects of 5F and CDDP. Cell proliferation inhibition rates for different groups of cells at 24 and 48 h after drug treatment. *$P<0.05$ compared with 5F or CDDP groups at the same time-point. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid; CDDP, cisplatin.

determine cell numbers at each phase of the cell cycle following drug treatment. Fig. 3A-D shows representative histograms of cytometric analysis of the cell cycle for the control, 5F, CDDP and combined groups, respectively. As demonstrated in Fig. 3E, treatment with 5F or CDDP alone caused cell cycle arrest at the G0/G1 phase. When cells were incubated with 5F combined with CDDP, the percentage of cells at the G0/G1 phase was 56.4±5.2%, which was significantly higher than that of the 5F (46.2±2.5%; $P<0.05$; n=6) and CDDP (41.9±3.1%; $P<0.05$; n=6) groups.

Synergistic effects of 5F and CDDP on the regulation of β-catenin, GSK-3β, c-Myc and cyclin D1. Fig. 4 shows the RT-qPCR data (n=6 for each gene). The results revealed that treatment with 5F or CDDP alone resulted in reduced mRNA expression of β-catenin, c-Myc and cyclin D1, but increased mRNA expression of GSK-3β. When compared with that of the 5F or CDDP group, the combined group had a significantly lower level of β-catenin, c-Myc and cyclin D1, but a markedly higher level of GSK-3β (Fig. 4A-D). Western blot analysis of β-catenin and GSK-3β was performed (Fig. 5A and B, respectively), and statistical analysis of β-catenin and GSK-3β protein
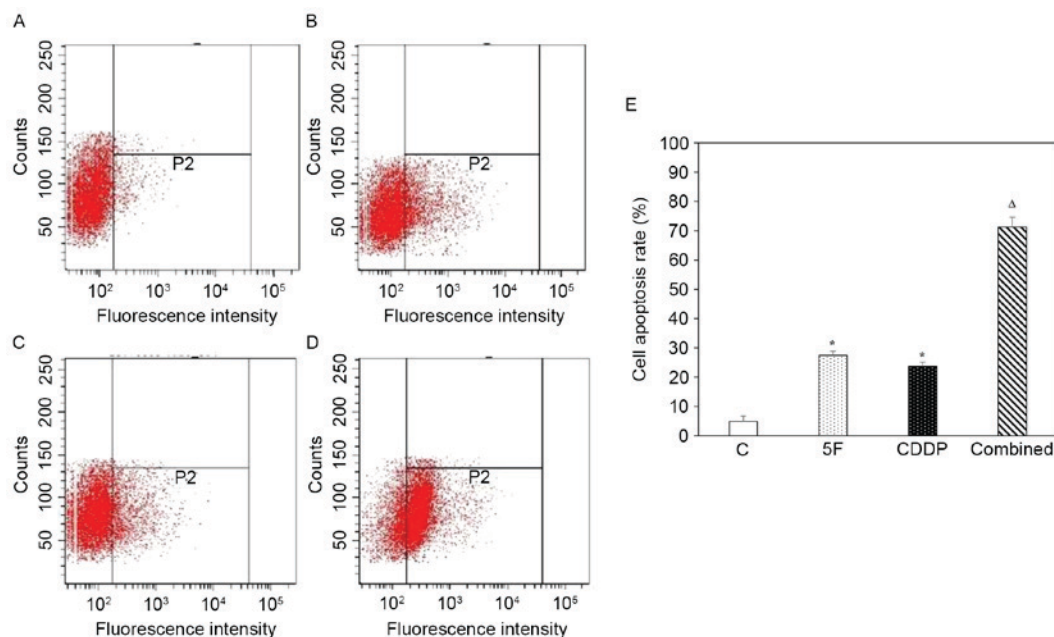
Figure 2. Synergistic pro-apoptotic effects of 5F and CDDP. (A-D) Representative flow cytometric histogram of cell apoptosis for the (A) control, (B) 5F, (C) CDDP and (D) combined groups. Treatment with 5F or CDDP alone markedly increased cell apoptosis compared with that of no-drug treatment. (E) Statistical analysis revealed that the combined group had a significantly higher apoptotic rate compared with that of the 5F and CDDP groups. *$P<0.05$ compared with control; ^$P<0.001$ compared with 5F or CDDP groups. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid; CDDP, cisplatin; C, control.
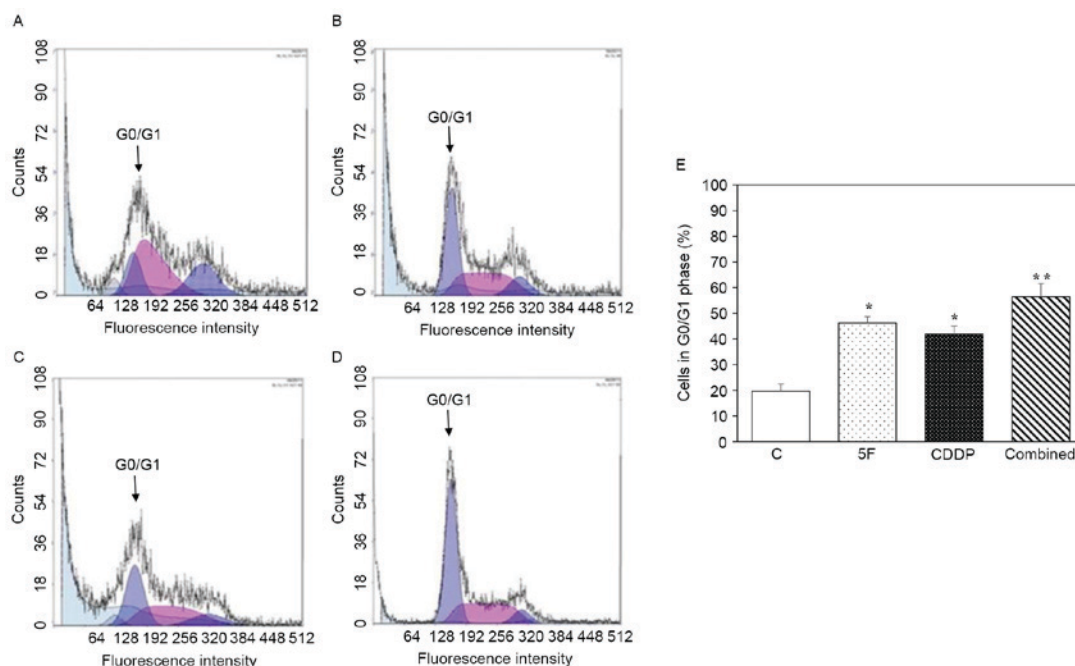

Figure 3. Synergistic cell cycle arrest effects of 5F and CDDP. Representative cytometric histogram of cell cycle for the (A) control, (B) 5F, (C) CDDP and (D) combined groups. Treatment with 5F or CDDP alone caused cell cycle arrest at G0/G1 phase. (E) When cells were incubated with 5F combined with CDDP, the percentage of cells at the G0/G1 phase was significantly higher compared with that of the 5F and CDDP groups. *$P<0.05$ compared with control; **$P<0.05$ compared with 5F or CDDP groups. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid; CDDP, cisplatin; C, control.

levels (Fig. 5C and D, respectively) confirmed the mRNA data for β-catenin and GSK-3β.

Discussion

CDDP-based combination chemotherapy has been shown to be effective in improving survival and quality of life in patients with advanced NSCLC (4,5). Recently, the identification of abnormal molecular pathways in a number of NSCLC cases has led to the development of targeted therapies for a subset of patients (20,21). However, >50% of patients with advanced NSCLC are usually treated with combination chemotherapy (20). In the past two decades, several anti-cancer drugs, including gemcitabine, vinorelbine, paclitaxel,
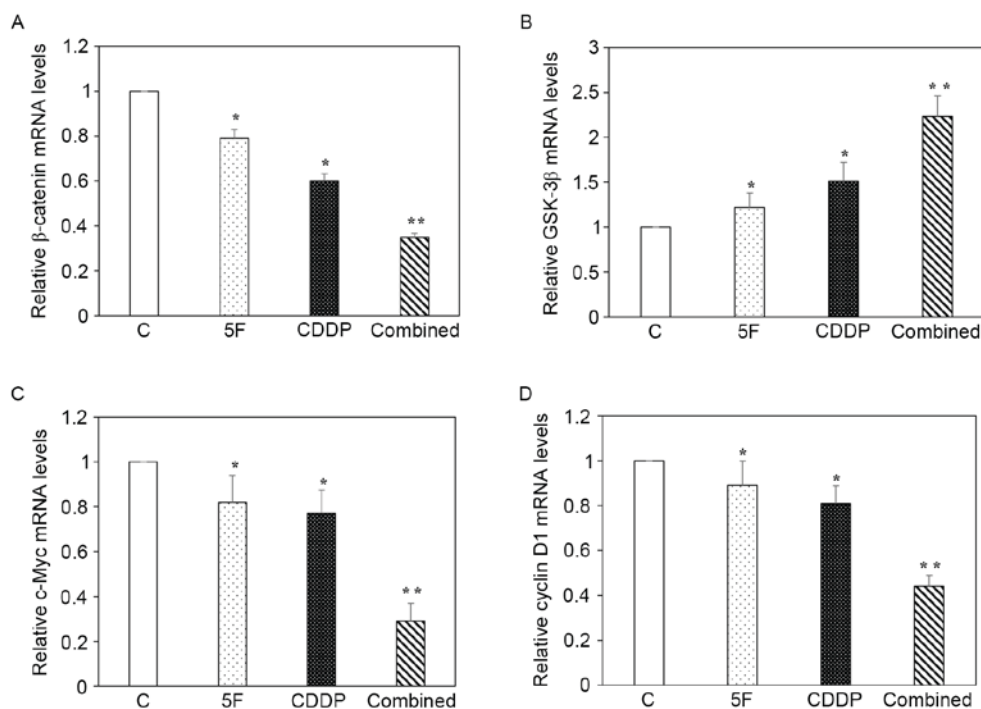
Figure 4. Effects of 5F and CDDP on regulating the mRNA expression of β-catenin, GSK-3β, c-Myc and cyclin D1. Relative mRNA levels of (A) β-catenin, (B) GSK-3β, (C) c-Myc and (D) cyclin D1. The combined group had significantly lower mRNA levels of β-catenin, c-Myc and cyclin D1, but markedly higher mRNA levels of GSK-3β, when compared with those of the 5F or CDDP groups. *P<0.05 compared with control; **P<0.05 compared with 5F or CDDP groups. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid; CDDP, cisplatin; C, control; GSK-3β, glycogen synthase kinase-3β; mRNA, messenger RNA.
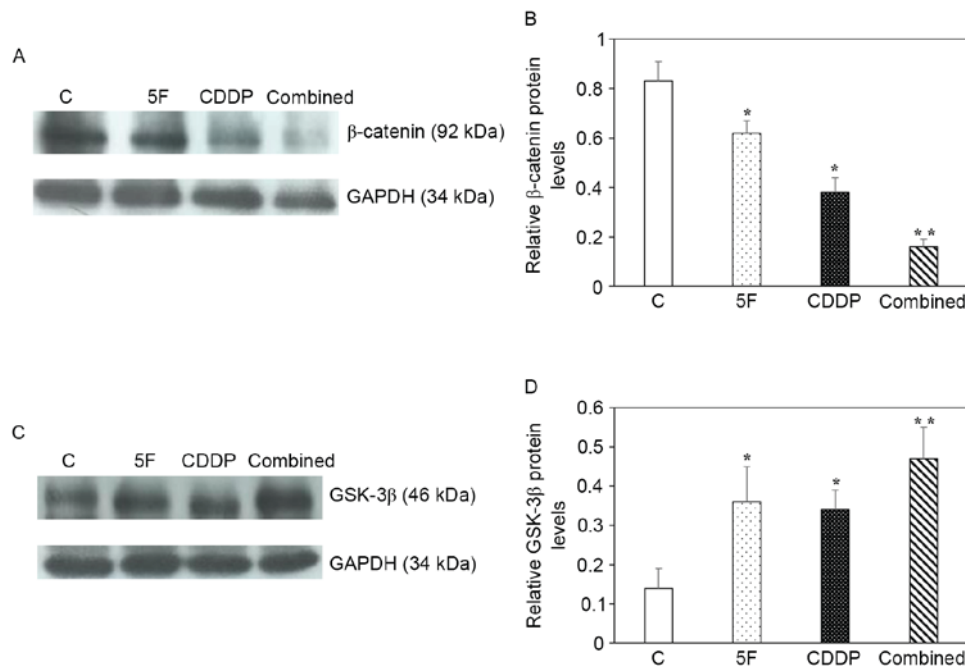

Figure 5. Synergistic regulation of β-catenin and GSK-3β protein expression by 5F and CDDP. Western blot analysis was performed to measure the relative protein levels of (A) β-catenin and (C) GSK-3β. Statistical analysis demonstrated that 5F and CDDP synergistically (B) reduced β-catenin protein production but (D) increased GSK-3β protein levels. *P<0.05 compared with control; **P<0.01 compared with 5F or CDDP groups. 5F, ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid; CDDP, cisplatin; C, control; GSK-3β, glycogen synthase kinase-3β.

docetaxel, pemetrexed and vinblastine, have been developed and combined with CDDP to form a doublet therapy regimen (22). Over the last decade, the 5-year survival rate of patients with advanced NSCLC has only marginally improved with combination therapy (20). To improve the treatment of

advanced NSCLC, clinical trials using three-drug combinations have been performed (10). However, the results show that adding a third drug does not add much benefit due to increased toxicity (11,12). Therefore, the search for novel drugs that are just as effective but with less toxicity remains vigorous (2,22).

5F has been shown to induce apoptosis and inhibit cell proliferation in various cancer cells (14-18). One outstanding property of 5F is its minimal side effects (14), making it a promising anticancer agent. In a preliminary experiment, the half-maximal inhibitory concentration (IC_{50}) for CDDP and 5F was determined in H23 cells (data not shown). In the present study, a final concentration of 5F of 40 μg/ml and of CDDP of 5 μg/ml, which was close to their IC_{50}, was selected to treat H23 cells. It was observed that 5F and CDDP synergistically induced apoptosis and inhibited cell growth, arrested cell cycles in the G0/G1 phase, and regulated the expression of β-catenin, GSK-3β, c-Myc and cyclin D1 genes.

Liu *et al* (15) observed that treatment of thyroid carcinoma cells with 5F led to the translocation of B-cell lymphoma-2-associated X protein into mitochondria, and the release of cytochrome *c* and apoptosis-inducing factor from mitochondria into the cytosol, indicating that the cell death induced by 5F was through a mitochondrial-mediated pathway. It is known that the anticancer activity of CDDP is associated with its ability to interact with the purine bases of DNA, causing DNA damage and subsequently inducing apoptosis in cancer cells (9,23). Therefore, 5F and CDDP likely exert synergistic anticancer effects in H23 cells through targeting different pathways, although the mechanisms behind these interactions were not elucidated in the present study.

The Wnt signaling pathway was first identified due to its role in carcinogenesis (24). Activation of the canonical or Wnt/β-catenin-dependent signaling pathway in numerous cases promotes cell growth. It has been reported that the Wnt signaling pathway is activated in ~50% of human NSCLC cell lines and primary tumors, and downregulation of activated Wnt signaling inhibits NSCLC proliferation and induces a more differentiated phenotype (21). Wnt ligands initiate pathway activation by binding to the Frizzled receptor on the cell membrane. Subsequently, the signal is transduced to the cytoplasmic phosphoprotein Dishevelled (Dsh) (25,26). Once Dsh is activated, it leads to the accumulation and stabilization of β-catenin. Subsequently, β-catenin is translocated into nuclei, where it modulates target gene expression, including upregulation of cyclin D1 and c-Myc, to stimulate cell proliferation (27,28). GSK-3β negatively regulates the Wnt signaling pathway by destabilizing β-catenin and inhibiting β-catenin accumulation (28). Overexpression of cyclin D1 and c-Myc has been revealed to be associated with cancer onset and progression (29-32). The present study revealed that 5F and CDDP concurrently downregulated β-catenin but upregulated GSK-3β in H23 cells, leading to reduced expression of cyclin D1 and c-Myc, which may represent one of the mechanisms for the synergistic anticancer activity of 5F and CDDP. In addition, as cyclin D1 is required for progression through the G1 phase of the cell cycle, reduced cyclin D1 levels may also be responsible for G0/G1 cell cycle arrest resulting from 5F and CDDP treatment.

Considering that 5F exerts anticancer activity with minimal side effects, future studies should investigate whether combination of 5F and CDDP will have the same or greater anticancer activity but with less side effects compared with that of CDDP plus one other drug, and whether addition of 5F as a third drug to CDDP-based two-drug combinations will enhance the anticancer effects without increasing toxicity. To address these questions, *in vivo* studies using animal models of NSCLC are required.

Acknowledgements

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