Compound K, a metabolite of ginseng saponin, enhances the susceptibility of osteosarcoma cells to cisplatin and induces cell apoptosis via mitochondrion-mediated pathways

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Abstract: Background: Compound K (20-O-beta-d-glucopyranosyl-20(S)-protopanaxadiol, CK), an intestinal bacterial metabolite of ginseng protopanaxadiol saponins, has been shown to inhibit cell growth in a variety of tumors. However, the precise mechanisms are not completely understood, especially in human osteosarcoma (OS). The aim of this research was to detect the antitumor effects of CK alone or in combination with cisplatin on OS cells, in vitro and in vivo, and to investigate CK as a potential novel therapeutic for treatment of OS. Material and methods: CK and cisplatin (a first-line chemotherapeutic agent for the treatment of OS) were administered to OS 143B cells alone or in combination. Viability of OS cells was performed by MTT assay. Flow cytometry was used to measure cell cycle and cell apoptosis. Western blotting was assessed to detect expression of proteins associated with cell cycle modulation and apoptosis induction. In addition, xenografts in nude mice were constructed to evaluate the antitumor efficacy of CK and cisplatin treatment, alone or in combination. Results: MTT assay showed that treatment of 143B cells with CK and/or cisplatin resulted in decreased tumor cell proliferation. Furthermore, the joint application of CK and cisplatin was significantly more effective than that of one agent alone (P<0.05). Similarly, cells more markedly accumulated in the G2/M phase following joint treatment with CK and cisplatin, compared to CK or cisplatin treatment alone. In addition, cell cycle signaling pathway Western blotting arrays were performed, identifying the marked downregulation of cyclin D1 and cyclin-dependent kinase 4 (CDK4) and upregulation of p21Cip1 and p27Kip1, following combined treatment with cisplatin and CK. Further studies also revealed that CK alone or combined with cisplatin downregulated Bcl-2, BCL-XL, and PARP and upregulated Bax. In in vivo studies, combined use of CK and cisplatin obviously inhibited the growth of xenografts generated from 143B cells. Conclusion: Results of the present study demonstrated that the concurrent administration of CK and cisplatin may result in enhanced antitumor efficacy, compared to that of one agent alone, providing a strong rationale for the use of CK as a therapeutic drug in combination with cisplatin in treating patients with OS.

Keywords: Compound K, osteosarcoma, apoptosis, cisplatin, mitochondria-mediated

Introduction

OS is the most common primary malignant bone tumor in children and adolescents, comprising 2.4% of all malignancies in pediatric patients. It usually occurs in the metaphyseal regions of the distal femur, proximal tibia, and proximal humerus, with a male predominance [1-3]. Over past decades, the five-year survival rate of OS patients has significantly improved to nearly 65%, due to the application of radiotherapy, neoadjuvant chemotherapy, or combined treatment [4-6]. However, these traditional treatment schemes have reached a plateau of efficacy. Despite the emergence of new reagents and schemes of chemotherapy, clinical outcomes of chemotherapy have not improved significantly: (1) Tumor cells have developed multi-drug resistance to chemotherapy drugs; (2) Toxic side-effects of chemotherapy treatment may cause systemic damage during medical practice. These side effects cause sys-
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

Systemic multi-organ damage to the blood system, circulatory system, and nervous system; (3) Such chemotherapeutic drugs cannot be used for cancer prevention [7]. In addition, patients with lung metastasis or recurrence only have a 10% chance of long-term survival despite the use of combined treatment [8, 9]. Thus, a greater understanding of OS pathogenesis is urgent in improving therapeutic regimens, discovering new therapeutic targets, and developing potential anti-Osteosarcoma drugs. One critical approach of increasing the efficacy of anticancer drugs and of decreasing toxicities or side effects is to explore traditional medicines, especially Chinese herbal medicines.

Cisplatin is one of the most effective chemotherapies for a series of cancers. Occurrence of drug resistance and considerable side effects make it imperative that less toxic and more effective approaches to overcoming these limitations are developed. Li et al. reported that CK could prominently strengthen cisplatin-induced p53 expression and activity in A549 lung cancer cells and that synergistic inhibition of cell growth was observed in their cotreatment group. In addition, the ability of cisplatin in apoptosis induction was similarly synergized by CK [10]. Moreover, both CK and cisplatin can inhibit proliferation and epithelial mesenchymal transition while inducing apoptosis in MCF-7 of human breast cancer cells. This may be related to the PI3K/Akt pathway [11]. Considering the fact that CK is well tolerated and has minimal side effects, in the present research, human OS 143B cells were used as the object to investigate the effects of CK alone and CK combined with cisplatin on proliferation, apoptosis, and xenografts of 143B cells. In addition, underlying molecular mechanisms were explored.

Materials and methods

Cell lines, reagents, and antibodies

Human osteosarcoma 143B cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). CK was purchased from ApexBio Technology (Apexbio Technology LLC, Houston, TX, USA). MTT (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) was dissolved in phosphate-buffered saline (PBS). Antibodies were as follows: mouse monoclonal anti-PARP (SantaCruz, sc-493, 1:1,000); polyclonal rabbit anti-Bcl-2 (SantaCruz, sc-492, 1:1,000); rabbit Polyclonal anti-p21Waf1 antibody (GeneTex, GTX-100444, 1:1,000); rabbit Polyclonal anti-p27kip1 antibody (GeneTex, GTX100446, 1:1,000); rabbit polyclonalanti-cyclin D1 (Abcam, ab24249, 1:1,000); goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., ZB-2306, 1:1,000), Horseradish peroxidase-conjugated anti-rabbit immnoglobulin (lg)G and anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Measurement of cell proliferation

143B cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS: HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), at 37°C in a 5% CO2 humidified incubator. In all experiments, cells were used in the logarithmic growth phase. For cell proliferation detection, 143B cells were seeded at a density of 3000 cells/well into 96-well flat-bottom cell culture plates (Beyotime Institute of Biotechnology, Haimen, China), in a medium containing 10% FBS. MTT assay was used to detect the effects of CK on proliferation. Subsequently, cells were then treated with indicated concentrations of CK and/or cisplatin for 48 hours, whereas only DMEM medium was added for the control group. MTT was added to the cells and incubated for 2 hours at 37°C before the absorbance was read at 450 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Cells were incubated under similar conditions for variable times ranging from 24 hours to 72 hours. All experiments were performed at least 3 times.

Cell morphological studies

143B cells were treated with 10 µM CK or 0.1% dimethyl sulfoxide (control) for 48 hours. Cells were then incubated with 10 µg/mL Hoechst 33342 and observed by fluorescence microscope (DMIRB; Leica, Wetzler, Germany).

Cell cycle analysis by FCM

Different phases of the cell cycle are characterized by differing DNA content. PI is a fluores-
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

cent dye that binds strongly to DNA at a ratio of 1:1. Hence, the change in PI intensity represents the different DNA content of each phase of the cell cycle. 143B cells (~5×10^5 cells/well) were seeded into a 6-well plate overnight at 37°C. Cells were washed once with PBS, the medium was replaced with fresh medium, and cells were subsequently treated with indicated concentrations of CK and cisplatin, individually or in combination, for 48 hours. Next, the cells were harvested, fixed with 70% ethanol, incubated with 25 µg/mL ribonuclease A, and stained with 50 µg/mL PI for at least 30 minutes at room temperature in the dark. This experiment was repeated at least three times. 143B cells were analyzed by a fluorescence-activated cell sorting (FACS) caliber II sorter and Cellquest FACS system (BD Biosciences, USA). The percentage of 143B cells in G0/G1, S, and G2/M phases was determined by FACSCalibur, respectively.

**Cell apoptosis assay by FCM analysis**

Flow cytometric analysis was performed to determine percentages of apoptotic cells. Following treatment with indicated concentrations of CK and cisplatin, individually or in combination for 48 hours, 143B cells were collected by trypsinization, washed twice with PBS, and resuspended in trypsin-EDTA solution (containing 0.25% trypsin and 0.02% EDTA; Beyotime Institute of Biotechnology, Haimen, China) binding buffer (~1×10^5 cells/mL). Cells were then incubated with 2 µl Annexin V and 5 µl PI for 15 minutes at room temperature. The distribution of viable, early apoptotic, late apoptotic, and necrotic cells was calculated using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), according to manufacturer instructions. Cells that were negative for Annexin V-FITC and PI were considered viable cells. The sum of the early and late apoptotic cells constituted the total number of apoptotic cells, presented as the percentage of total cells. Each group was detected at least three times.

**SDS-PAGE and Western blot analysis**

143B cells were treated with indicated concentrations of CK and cisplatin, individually or in combination for 48 hours. Protein collections from the above treated cells were harvested subsequently in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium vanadate, and 0.2 U/ml aprotinin), containing protease inhibitor cocktail (Santa Cruz Biotechnology; Santa Cruz, CA). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). For each lane, 30 µg of protein was separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were then blocked with fat-free milk at room temperature for 1 hour and incubated with indicated specific primary antibodies for 3 hours. Proteins were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies. To corroborate equal loading, membranes were stripped and re-probed using an antibody specific for β-actin. Finally, antigen-antibody complexes were detected using the ECL system and signals were quantified by densitometry (Quantity One software, version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**143B tumor xenografts**

All animal experiments were done according to Institutional Animal Care and Use Committee-approved protocol. Institutional guidelines for proper and humane use of animals in research were followed. A total of 30 male nude mice were used between the ages of 5 and 6 weeks, supplied by Experimental Animal Department of Binzhou Medical College. 143B cells were implanted by subcutaneous injections into the right front legs of mice (~2×10^6/mouse). Ten days later, 20 mice with sizes of ~170 mm^3 were randomly distributed into four groups, control group (normal saline), CK (10 mg/kg/day) group, cisplatin (10 mg/kg/day) group, and CK (10 mg/kg/day) plus cisplatin (10 mg/kg/day) group. Tumor volume (mean values and 95% confidence intervals) was measured at various times after the initial intraperitoneal injection. Mice were then sacrificed and tumor weights were measured 4 weeks after inoculation of the cancer cells.

**Statistical analysis**

Data are presented as mean ± standard deviation. All experiments were repeated at least three times. Statistical significance was determined by Student’s t-test, with P<0.05 defined as statistically significant.
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

**Results**

**CK synergizes cisplatin to inhibit 143B cell viabilities in vitro**

Morphological changes, due to apoptosis, occurring in the nuclei of the cells were observed under a fluorescence microscope. Untreated 143B cells exhibited a pale blue fluorescence, demonstrating an even pattern of distribution of the chromatin in the nucleolus, whereas those treated with CK were shown to manifest brighter, granular, blue fluorescence, and more apoptotic bodies (Figure 1A). In addition, cell proliferation inhibition was detected by treating 143B cells with 10 µM CK and 5 µM cisplatin, either alone or in combination, at different points in time. As shown in Figure 1B, CK in combination with cisplatin inhibited 143B cell proliferation in a time-dependent manner. Furthermore, a significant decrease in proliferation of 143B cells was observed in the combined treatment group, compared to that of the CK and cisplatin alone treatment groups, at 72 hours ($P<0.05$ or $P<0.01$).

**CK synergizes cisplatin to alter 143B cell cycle distribution**

Treatment with CK or cisplatin, either alone or in combination, for 48 hours resulted in an increase in OS cells in the G2/M population to $26.5 \pm 2.77\%$, $41.6 \pm 3.63\%$, and $54.3 \pm 3.49\%$, respectively, compared to $24.0 \pm 2.26\%$ in the control group. Moreover, the number of 143B cells in the G0/G1 and S phases correspondingly decreased (Figure 2A, 2B). Therefore, the data showed that CK could induce G2/M cell-cycle arrest in 143B cells. In addition, Western-blotting showed that expression of CDK4 and its downstream kinase, cyclin D1, in 143B cells, decreased significantly. In contrast, a marked reduction in levels of p21$^{CIP1}$ and p27$^{KIP1}$ was observed in 143B cells following different treatment for 48 hours (Figure 2C). Taken together, these results suggest that CK plays an important role in inhibiting cell growth by inducing G2/M cell cycle arrest, followed by downregulation of expression of cyclin D1 and CDK4 and upregulation of expression of p21$^{CIP1}$ and p27$^{KIP1}$ in 143B cells.

**CK synergizes cisplatin to induce apoptosis of 143B cells**

To detect the antitumor effects of CK in combination with cisplatin, Annexin-V/PI staining was carried out after cells were treated with CK or cisplatin, either alone or in combination, for 48 hours. Percentage of apoptotic cells was determined by cell flow cytometric analysis, following PI staining. Compared to the control group, these other drug treatment methods lead to a moderate induction of apoptosis in 143B cells (5.0% untreated cells, 28.0% at CK group, and 32.0% at CDDP group).
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

Figure 2. CK synergizes cisplatin to alter 143B cell cycle distribution. A, B. Flow cytometry analysis of proliferating 143B cells 48 hours after the treatment with CK and/or cisplatin. Fractions of cells in the G0/G1, S, and G2/M phases of the cell cycle are indicted. Untreated cells were used as controls. C. Western-blotting assay showed that expression of CDK4 and its downstream kinase, cyclin D1, in the 143B cells decreased significantly. In contrast, a marked reduction in levels of p21Cip1 and p27Kip1 was observed in the 143B cells following different treatment for 48 hours.

Figure 3. CK synergizes cisplatin to induce apoptosis of 143B cells. A, B. 143B cells were treated with CK and/or cisplatin for 48 hours and stained with Annexin V-propidium iodide. Percentage of apoptotic cells was measured by FACSCalibur analysis. Untreated cells were used as controls. PI, propidium iodide. *P<0.05 vs. control group.
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

48.5% at CDDP group, and 64.8% at co-treatment group). A significant difference was observed between them (P<0.05, Figure 3). Current results indicated that pro-apoptotic effects of CK combined with cisplatin are greater than that of CK and cisplatin treatment alone.

**Synergistic anti-tumor effects of CK and cisplatin are mitochondria mediated**

In mammalian cells, there are two classic apoptotic pathways, the death receptor-mediated apoptotic pathway and mitochondria-mediated apoptotic pathway. Previous studies have indicated that both pathways cross-talk and are involved in CK-mediated apoptosis of tumor cells [12]. To determine whether mitochondria plays an important role in mediating the synergistic effects of CK and cisplatin, this study tested the cell apoptosis induction of these two drugs on 143B using Western-blotting assay. Results indicated that treatment of CK and/or cisplatin decreased expression of Bcl-2 and Bcl-xl and increased expression of Bax, in a...
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

dose dependent manner. In the downstream of apoptosis signaling pathways, there is a significant cleavage activation of PARP. A significant release of cytochrome c from mitochondria to cytosol can be seen after cells are treated with CK and/or cisplatin. However, expression of Fas and Fas-L were not changed. These results indicate that CK induces apoptosis mainly through mitochondrion-mediated internal pathways in OS 143B cells (Figure 4). These results demonstrated that the synergistic anti-tumor effects of CK and cisplatin are mainly through mitochondria-mediated internal pathways in 143B cells.

**CK suppresses 143B tumor growth in vivo**

To determine whether CK and/or cisplatin could curtail tumor growth in nude mice, a model of human OS xenografts was established in nude mice by injecting 143B cells. Volumes and weights of transplanted tumors from the nude mice, treated with indicated concentrations of CK and cisplatin, individually or in combination, were assessed. As shown in Figure 5, tumor proliferation was not obviously inhibited in CK-treated groups. However, compared to the control group, tumor xenografts in the CDDP group or in the CK+CDDP group were all significantly decreased in size and weight ($P<0.05$ or $P<0.007$, Figure 5). Throughout this study, no significant body weight loss was observed during the three-week treatment process (data not shown), indicating that the treatment strategies were well-tolerated with no obvious toxicity. This data is consistent with present in vitro results and demonstrates, for the first time, that CK synergizes cisplatin to restrain the growth of human OS xenografts in nude mice.

**Discussion**

Compound K is the main metabolite of protopanaxadiol-type ginsenoside formed in the intestines after oral administration. It has been reported to exert anti-cancer effects in a variety of tumors, such as hepatocellular carcinoma, colorectal cancer, myeloid leukemia, gastric cancer, and lung carcinoma [13-16]. The anti-cancer effects of CK have been shown due to inhibition of glucose uptake by cancer cells and stimulation of apoptosis. For instance, recent studies have reported that Compound K could exhibit cytotoxicity through the induction of cell apoptosis, cell cycle arrest at the G1 phase, and inhibition of telomerase activity in human leukemia cells [17]. Compound K induced apoptosis in MCF-7 breast cancer cells through the modulation of AMP-activated protein kinase [18]. Pretreatment of lung tumor cells with CK could lead to gamma-ray radiation-induced cell apoptosis. Mechanisms involved include nuclear fragmentation, loss of mitochondrial membrane potential, and activation of caspase 3 [19]. The present study demonstrated that CK and CDDP, individually or in combination, reduces cell viability of OS 143B cells. For instance, 143B cells treated with 10 µM CK and/or 5 µM of cisplatin, at different points in time (24, 48, and 72 hours), led to cell proliferation inhibition with significant decline of cell viability. It was hypothesized that CK

**Figure 5. CK suppresses 143B tumor growth in vivo.** A. Tumors from 143B xenografts administered different treatments for 18 days; B. Weight of tumor samples from nude mice. C. Tumor growth curves in different treatment groups. Tumor volume ($mm^3$) was calculated as ($tumor$ length ($mm$) × $tumor$ width ($mm$)$^2$)/2. Each point represents the mean ± standard deviation of 5 animals. * and ** indicate $P<0.05$ and $P<0.01$ compared with control group, respectively.
Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

could induce cell cycle arrest in 143B cells. To confirm this hypothesis, proliferating 143B cells were dealt with CK and cisplatin, either alone or in combination, for 48 hours. As shown in Figure 2, an increasing number of cells accumulated in G2/M. In parallel, a reduction percentage of cells in S and G0/G1 phases was observed. Consistent with the present study, Hu's group also indicated that CK induced cell cycle arrest in the G2 phase in human gastric cancer cells [20]. Furthermore, according to present research, treatment of 143B cells with CK and cisplatin either alone or in combination evidently decreased expression of CDK4 and its downstream kinase, cyclin D1. Moreover, an obvious increase in protein expression levels of p27Kip1 and p21Cip1 in 143B cells was observed following different methods of drug treatment. Taken together, these results indicate that CK and/or cisplatin may induce G2/M phase arrest in 143B cells by regulating several key proteins [21-23].

To determine whether CK combined with cisplatin produces a synergistic effect in inducing apoptosis in OS 143B cells, an annexin V-FITC labeling assay was performed. It was found that CK combined with cisplatin increased a substantially greater percentage of cell positives for annexin V, compared with CK or cisplatin treatment alone. Apoptosis was induced either by the extrinsic pathway involving cell surface death receptors or by the intrinsic pathway induced by intracellular stimuli that stimulates the mitochondria. To further clarify apoptotic molecular mechanisms of human OS cells induced by CK, total proteins of 143B cells treated with CK and cisplatin, either alone or in combination, were analyzed using Western blot assay. Data analysis concluded that CK synergistically enhances apoptosis-induced effects of cisplatin in 143B cells, mainly through mitochondrion-mediated internal pathways. This is initiated by a variety of apoptosis-inducing signals that break the balance of the major apoptosis regulator and proteins of the Bcl-2 family, such as Bcl-2, Bcl-xl, and Bax. Moreover, the pro-apoptotic protein Bax accumulates on mitochondria after being activated and triggers an increase in the permeability of the outer mitochondrial membrane. Consequently, the mitochondria release cytochrome c. This, in turn, activates downstream death programs, such as PARP. These observations suggest that modulation of mitochondrion-mediated pathways may be an important mechanism underlying the biological effects of CK. However, expression levels of Fas and FasL could not be changed after treatment of CK, indicating that Fas-mediated extrinsic pathways may not be involved in the compound K-induced apoptosis in 143B cells. Differing from current findings, Kang et al. [24] reported that Compound K inhibits colorectal cancer cell growth and induces apoptosis through inhibition of histone deacetylase activity. While KIM's group confirmed that compound K induces autophagy and apoptosis via generation of reactive oxygen species and activation of JNK in human colon cancer cells [25]. These data indicate that the antitumor mechanisms of CK may be diverse because of different types of tumors [26]. In vivo, xenografts in nude mice were constructed to evaluate the antitumor efficacy of CK and cisplatin treatment, alone or in combination. Data showed that CK used alone could not inhibit the growth of xenografts obviously. Tumor weights and volumes were further reduced in CK and cisplatin combination treatments, compared with cisplatin-alone-treated mice, indicating that concurrent administration of CK and cisplatin may result in enhanced antitumor efficacy compared with that of one agent alone. There were no obvious side effects, such as weight loss, hair loss, dysphoria, or lethargy, during drug treatment in each group.

In conclusion, the present study is a preliminary one, suggesting that CK selectively induces apoptosis of OS 143B cells by mitochondrion-mediated internal pathways. Although CK has been shown to inhibit proliferation in vitro in breast, prostate, colon, and ovarian cancer cell lines, its effects on osteosarcoma cells in vivo have been demonstrated for the first time. Present results support the development of pre-clinical experiments to further evaluate the potential role of combining CK with chemotherapy as a new treatment for OS. However, the present conclusions are based on preliminary experimental research. Large-scale clinical trials are required to validate these preclinical findings.

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Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

Disclosure of conflict of interest

None.

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Compound K enhances the susceptibility of osteosarcoma cells to cisplatin

