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Effect of Compound K, a Metabolite of Ginseng Saponin, Combined with γ-Ray Radiation in Human Lung Cancer Cells in Vitro and in Vivo

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Pretreatment of NCI-H460 human lung cancer cells with compound K produced by intestinal bacteria enhances γ-ray radiation-induced cell death. Increases in apoptosis induced by combined treatment are made apparent in the observation of nuclear fragmentation, loss of mitochondrial membrane potential ($\Delta\psi$), and activation of caspase 3. Apoptosis induced by compound K and γ-ray radiation is associated with reactive oxygen species (ROS) generation. Furthermore, compound K, in combination with γ-ray radiation, has an enhanced effect in the regression of NCI-H460 tumor xenografts of nude mice. These results suggest that compound K has possible application for cancer therapy when used in combination with γ-ray radiation.

KEYWORDS: Compound K; radiation; apoptosis; cancer therapy; tumor xenograft; ROS

INTRODUCTION

Ginsenosides isolated from ginseng include protopanaxadiol types such as Rh_2 and Rg_3. These ginsenosides have been shown to inhibit tumor cell proliferation and tumor growth, to induce differentiation and apoptosis, and to inhibit tumor cell invasion and metastasis (*1*−*5*). Recently, intestinal bacteria have been shown to form ginsenoside metabolites after oral administration of ginseng extract in both humans and rats (*6*). One of the major metabolites detected in the urine and blood after administering the total ginsenoside in rats is compound K [20-*O*-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol, also known as IH-901; **Figure 1**]. Compound K, one of the derivatives resulting from the metabolism of ginsenosides, might be an active form in biological systems and is also known to inhibit glucose uptake in tumor cells (*7*), to possess chemopreventive activity against chemical carcinogens (*8*), to inhibit metastasis in vivo (*9*), and to inhibit tumor growth through inhibition of TPA-induced cyclooxygenase-2 expression (*10*, *11*). In previous studies, compound K was shown to have anticancer activities on different mechanisms and cell lines such as colon adenocarcinoma cells (*12*), breast cancer MCF-7, skin melanoma SK-MEL-2, human ovarian carcinoma B16 (*13*), human hepatoma Hep G2, pulmonary adenocarcinoma PC-14 (*14*), and human astroglioma U87MG and U373MG (*15*). Also, we recently reported that compound K exhibits cytotoxicity by the induction of apoptosis and cell cycle arrest at the G_1 phase (*16*), by caspase-dependent pathway via mitochondria disruption (*17*), and by inhibition of telomerase activity (*18*). These antitumor activities of compound K prompted us to further evaluate whether the combined treatment of compound K and radiation enhances human lung cancer cell death.

When ginsenosides are orally administered, the bioactive form of ginsenosides may be the aglycone or biotransformed one, not ginsenoside itself. The resulting form of ginsenosides by metabolism is a major constituent and plays a key role in the biological activity of ginsenoside. Thus, this biotransformation method for compound K might be one of the ways to produce valuable secondary metabolites of importance to the agricultural and food chemistry of ginseng. For example, there was a study comparing anticancer activity using ginsenosides and biotransformed ones, showing that ginsenosides had no effects (*13*).

In this study, we prepared compound K, a major metabolite of ginsenoside by intestinal bacteria, and evaluated whether compound K with cytotoxic properties enhances the sensitivity of human lung cancer cells to γ-ray radiation. To the best of our knowledge, this is the first report regarding combination treatment with compound K and radiation for anticancer activity.

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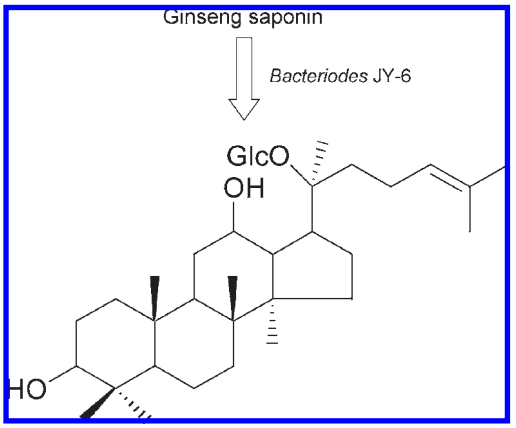


Figure 1. Chemical structure of compound K [20-*O*-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol].

MATERIALS AND METHODS

Preparation of Compound K and Reagents. Compound K was prepared by the incubation of the protopanaxadiol type ginsenosides with *Bacteroides* JY-6, a human intestinal bacterium, subcultured in a general anaerobic medium for 24 h at 37 °C. The incubated medium was extracted with *n*-butanol. The supernatant was concentrated in vacuo and processed using silica gel column chromatography with chloroform/methanol/H_2O (65:35:10, v/v). The isolated compound K was characterized by mass spectroscopy and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectrometry. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) and Hoechst 33342 were purchased from the Sigma Chemical Co. (St. Louis, MO). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine chloride (JC-1) was purchased from Molecular Probes (Leiden, The Netherlands). Anti-caspase 3 antibody was purchased from Cell Signaling Technology (Beverly, MA). The other chemicals and reagents used were of analytical grade.

Cell Culture and Irradiation. NCI-H460 human lung cancer cells were obtained from the American Type Culture Collection and maintained at 37 °C in an incubator at a humidified atmosphere of 5% CO_2. Cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, streptomycin, and penicillin. The cells were exposed to γ-rays at 1.5 Gy/min from a ^{60}Co γ-ray source (MDS Nordion C-188 standard source, Cheju National University, Jeju, Korea).

Cell Viability. The effect of compound K on cell viability was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay (*19*). Cells were treated with compound K at 30 μg/mL, and 1 h later, the plate was exposed to 10 Gy of γ-rays. Forty-eight hours later, 50 μL of the MTT stock solution (2 mg/mL) was added, and after incubation for 4 h, the absorbance at 540 nm was measured on a scanning multiwell spectrophotometer.

Nuclear Staining with Hoechst 33342. One and a half microliters of Hoechst 33342 (stock 10 mg/mL), which is a DNA-specific fluorescent dye, was added to each well, and the cells were incubated for 10 min at 37 °C. The stained cells were visualized under a fluorescent microscope, equipped with a CoolSNAP-Pro color digital camera.

Mitochondrial Membrane Potential (Δψ) Analysis. The cells were stained by JC-1 (10 μg/mL), and the stained cells were analyzed by flow cytometer (*20*). In addition, for image analysis, the JC-1-stained cells were mounted by mounting medium (DAKO, Carpinteria, CA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope.

Western Blot Analysis. The lysates (40 μg of protein) were electrophoresed, and blots were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated with primary rabbit monoclonal caspase 3 antibody and then with goat anti-rabbit immunoglobulin-G−horseradish peroxidase conjugates (Pierce, Rockford, IL). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Measurement of Intracellular ROS. The fluorescent probe DCF-DA was used for the assessment of intracellular ROS. The stained cells were mounted onto microscope slides in mounting medium. The microscopic images were collected using a confocal microscope. In addition, the fluorescence signal of 2′,7′-dichlorofluorescein was detected using a flow cytometer.

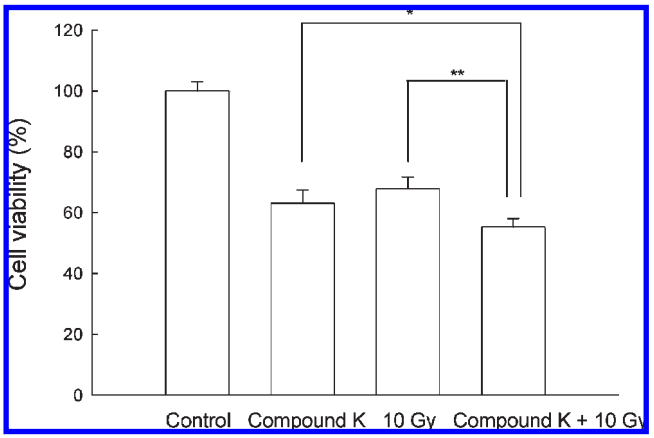
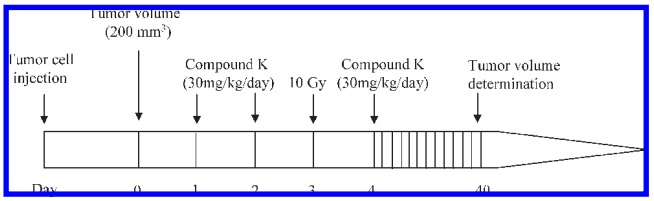


Figure 2. Combined effect of compound K and γ-ray radiation on cell death. Cells were treated with compound K at 30 μg/mL, followed by γ-rays at 10 Gy 1 h later. Next, the cells were incubated for 48 h. The cell viability was determined by MTT assay. ∗, significantly different from compound K treated group ($P < 0.05$); ∗∗, significantly different from 10 Gy irradiated group ($P < 0.05$).

Tumor Xenograft in Nude Mice. A single cell suspension (3×10^6 cells) was subcutaneously injected into the hind legs of 5-week-old BALB/c athymic nude mice (Charles River Laboratories, Wilmington, MA). When the tumor reached a minimal volume of 200 mm^3, compound K (30 mg/kg/day) was injected subcutaneously at the tumor site for 2 days. Twenty-four hours later, the mice were exposed to 10 Gy of γ-rays, and 24 h later, compound K (30 mg/kg/day) was again administered at the tumor site as follows.



Each group consisted of five mice, and tumor volumes were determined according to the formula $(L \times l^2)/2$ by measuring tumor length (L) and width (l) with a caliper. All animal work was carried out in accordance with the policies of the Korea Institute of Radiological and Medical Sciences.

Statistical Analysis. All of the measurements were represented as means ± SE. Differences were determined by ANOVA using Tukey's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Combined Effect of Compound K and γ-Ray Radiation on Cell Death in Vitro. We investigated whether a combination of compound K and γ-ray radiation enhances cancer cell death. Cell survival with compound K was 63%, and cell survival with γ-ray radiation was 68%. Cell survival in combined treatment was only 55%. These data suggest that compound K showed an enhancing effect with γ-ray radiation (**Figure 2**).

Combined Effect of Compound K and γ-Ray Radiation on Apoptosis Induction. Whether the enhancing effect of compound K to cell death exposed to γ-ray radiation is associated with induction of apoptosis, the nuclei of NCI-H460 cells were stained with Hoechst 33342 for visualization by microscopy. The microscopic images in **Figure 3A** show that the control cells had intact nuclei, whereas compound K-treated and radiation-exposed cells

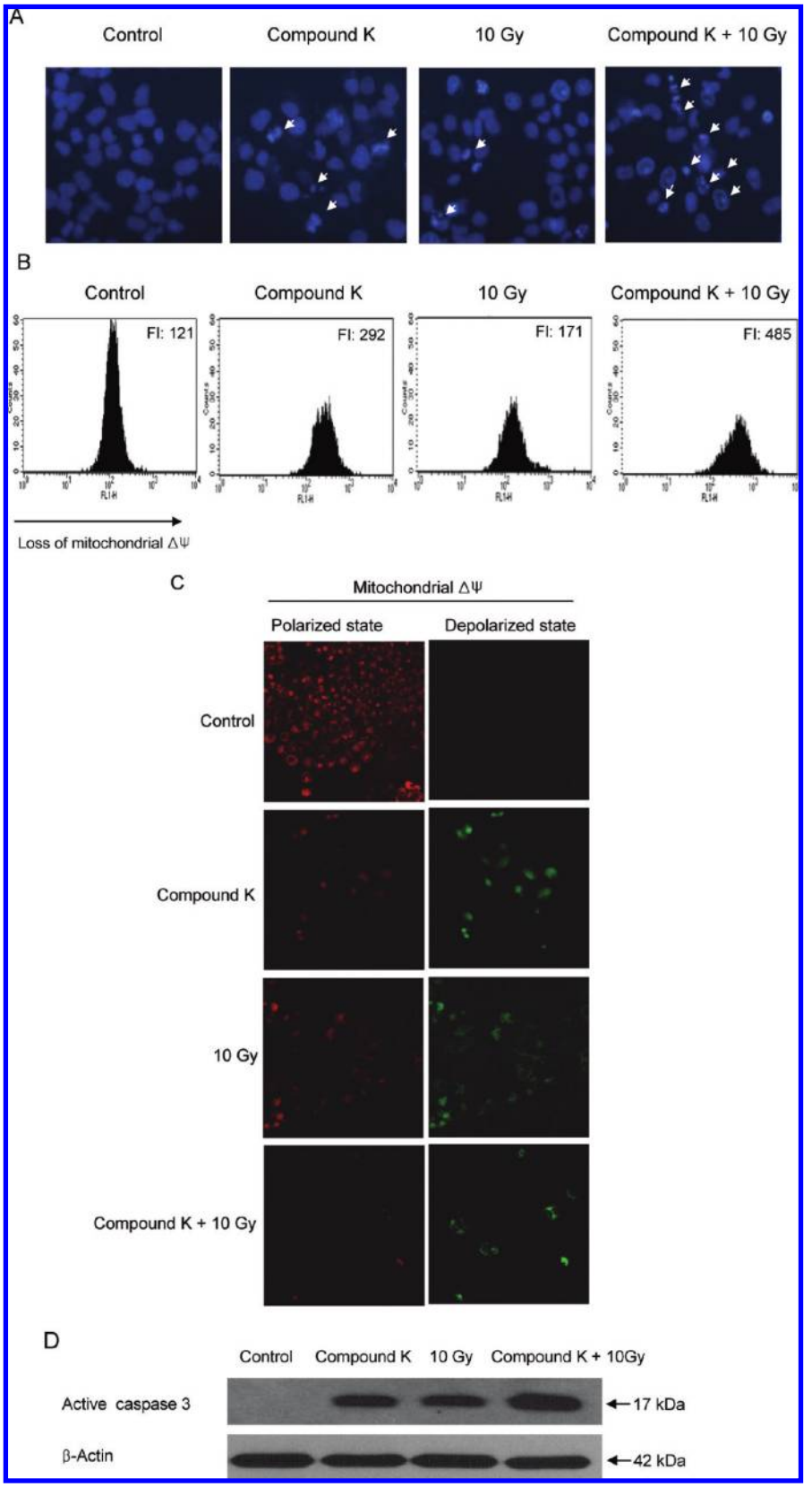


Figure 3. Combined effect of compound K and γ-ray radiation on apoptosis induction. Cells were treated with compound K at 30 μg/mL alone, radiation at 10 Gy alone, and combination of both compound K at 30 μg/mL and radiation at 10 Gy. After 48 h, (**A**) apoptotic body formation was observed under fluorescent microscope after Hoechst 33342 staining, and apoptotic bodies are indicated by arrows. The mitochondrial membrane potential ($\Delta\psi$) was analyzed using a (**B**) flow cytometer and (**C**) confocal microscope after cells had been stained with JC-1. FI is described as fluorescence intensity. (**D**) Cell lysates were prepared and Western blot analysis was performed using anti-caspase 3 antibody.

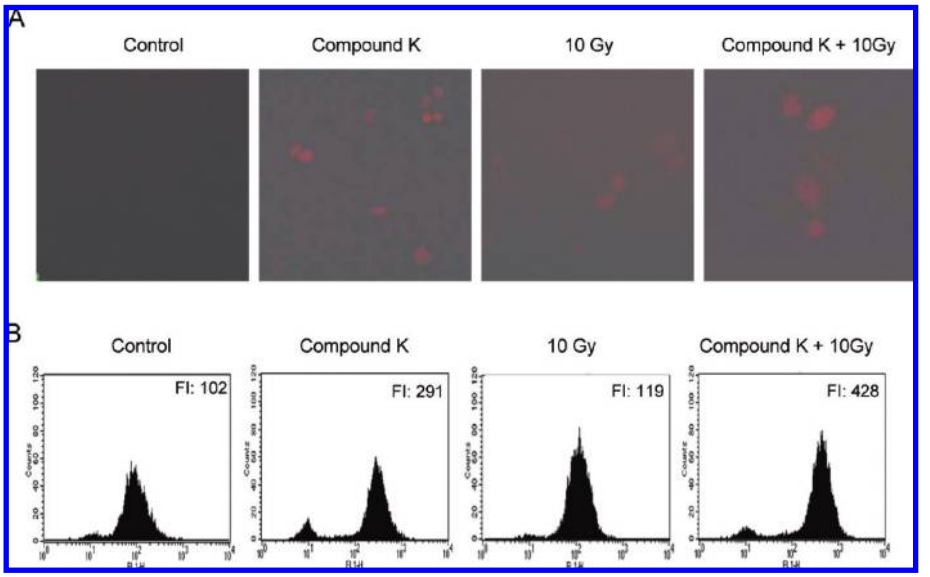


Figure 4. Combined effect of compound K and radiation on ROS generation. Cells were treated with compound K at 30 μg/mL alone, radiation at 10 Gy alone, and combination of both compound K at 30 μg/mL and radiation at 10 Gy. After 24 h, the intracellular ROS were detected using a confocal microscope (**A**) and a flow cytometer (**B**) after DCF-DA staining.

demonstrated significant nuclear fragmentation, which is characteristic of apoptosis. Combined treatment increased the degree of nuclear fragmentation. We next examined changes in $\Delta\psi$. The flow cytometric data showed that compound K or γ-ray radiation resulted in the loss of $\Delta\psi$ compared to control, as substantiated by an increased fluorescence of JC-1 dye (**Figure 3B**). Combined treatment increased the loss of $\Delta\psi$ compared to compound K or radiation treatment. These data were consistent with data of the confocal microscope; JC-1-stained control cells exhibited red fluorescence in the mitochondria. JC-1-stained cells treated with compound K or γ-ray radiation showed decreased red fluorescence (polarized state of $\Delta\psi$) in the mitochondria and increased green fluorescence (depolarized state of $\Delta\psi$), suggesting that compound K and γ-ray radiation disrupt the mitochondrial $\Delta\psi$. The loss of $\Delta\psi$ was enhanced by combined treatment (**Figure 3C**). To assess the induction of apoptosis at the biochemical level, changes in the activation of caspase 3, the major executive caspase during the apoptotic process, were determined. The enhanced activation of caspase 3 (17 kDa) in combination treatment was shown by comparison to compound K or radiation alone (**Figure 3D**). These findings suggest that combination treatment modulates caspase 3 activity following loss of $\Delta\psi$, thereby enhancing apoptotic cell death.

Combined Effect of Compound K and γ-Ray Radiation on ROS Generation. γ-Ray radiation is known to generate ROS in cells and to induce cell death via apoptosis (*21*). To investigate the relationship between ROS production and enhancement of radiation-induced apoptosis by compound K, ROS generation was detected by a confocal microscope and flow cytometry after staining with DCF-DA. Analysis by confocal microscope revealed that γ-ray radiation with compound K-treated cells increased the red fluorescence intensity compared to compound K- and γ-ray radiation-treated cells (**Figure 4A**). Moreover, the level of ROS detected using a flow cytometer revealed a fluorescence intensity value of 428 for the ROS stained with the DCF-DA fluorescence dye in γ-ray radiation with compound K-treated cells compared to fluorescence intensity values of 291 in compound K-treated cells and 119 in γ-ray radiation-treated cells. These findings suggest that ROS generated by combined treatment might regulate the apoptotic process (**Figure 4B**).

Combined Effect of Compound K and γ-Ray Radiation on Tumor Regression in Vivo. To determine whether the in vitro effects of compound K extend to an in vivo xenograft model, the effect of combined treatment was tested in vivo against tumors derived from NCI-H460 cells injected into the hind legs of nude mice. Treatment with compound K or radiation alone resulted in partial tumor growth regression, whereas compound K increased the antitumor effect of radiation. Taken together, these results demonstrate that compound K augments cell death in human lung cancer cells in response to radiation (**Figure 5**).

DISCUSSION

The aim of the present study was to determine whether compound K could sensitize NCI-H460 human lung cancer cells to radiation therapy. Our results suggest that compound K enhances the antitumor effects of radiation therapy in vivo by suppressing tumor growth in tumor xenografts. Tumor growth delay was related to the apoptotic activity of compound K in vitro. Combined treatment was associated with ROS generation and loss of $\Delta\psi$, which resulted in the activation of caspase 3. Ionizing radiation is believed to cause cell damage via the production of ROS and subsequently to induce oxidative stress (*22*) and to induce apoptotic signaling via mitochondrial pathway (*23*). Apoptosis was induced either by the extrinsic pathway involving cell surface death receptors or by the intrinsic pathway induced by intracellular stimuli that stimulate the mitochondria (*24*). The intrinsic pathway of apoptosis requires an alteration of $\Delta\psi$ leading to mitochondrial membrane permeabilization and followed by a release of cytochrome *c* and activation caspases (*25*, *26*). Therefore, apoptosis induced by combined treatment with compound K and γ-ray radiation suggests involvement of the intrinsic pathway via mitochondria.

Either chemotherapy or radiotherapy treatment often results in therapeutic resistance and side effects in all types of cancers. The combination of radiation and chemotherapy is based on the theory that two types of cancer treatment act via different mechanisms (*27*, *28*). Combined treatment for cancer is an important part of offering hope of improved cancer patient survival, not only to enhance the therapeutic effect but also to use the advantages of each treatment, minimizing treatment doses

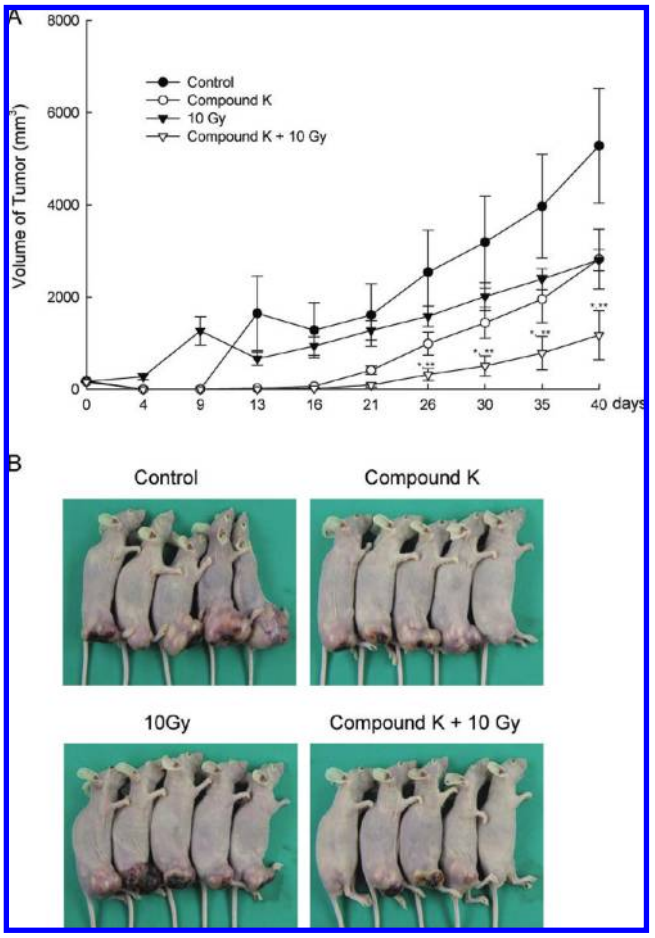


Figure 5. Compound K sensitizes NCI-H460 cells to γ-ray radiation in nude mice: (**A**) tumor volume measured in different time intervals using vernier calipers and calculated as described under Materials and Methods. (**B**) photographs of mice bearing NCI-H460 cells after treatment with compound K, γ-ray radiation, and combination of compound K and γ-ray radiation. $*$, significantly different from compound K treated group ($P < 0.05$); $**$, significantly different from 10 Gy irradiated group ($P < 0.05$).

and side effects on the patient, simultaneously. An ideal radiation sensitizer should be effective in increasing apoptotic cell death and should be less toxic to normal cells. However, an ideal radiation sensitizer does not yet exist (*27*). Therefore, many new promising candidate compounds are being investigated (*29*), whether they are naturally occurring or synthetically made.

In radiation, apoptosis-related proteins such as p53, Bax, p21, Bcl-2, or caspases are involved in the radiation-induced apoptosis of cancer cells (*28*, *30*). These proteins might be responsible for the synergistic mechanism and target of radiation and chemoagent. A possibly synergistic effect of radiation might be related with the fact that chemoagent can enhance radiation-induced cell death by disrupting physiological phenomena such as inhibition of angiogenesis, cell cycle arrest, induction of apoptosis, and suppression of cell survival signaling pathway. It is known that abolition of mitochondrial membrane results in the disruption of $\Delta\psi$ and the release of cytochrome *c*. During this process, Bcl-2 plays an antiapoptotic role, and the down-regulation of Bcl-2 induces the apoptotic process (*28*). In this study, radiation and compound K showed apoptosis-related collapsing $\Delta\psi$ and caspase 3 activation. A molecular approach to the relationship between the major factor for inducing apoptosis-related proteins including Bcl-2 and cell survival signaling pathways of NCI-H460 cells treated by compound K and/or radiation remains for further study.

In summary, compound K produced by biotransformation enhances the efficacy of radiation therapy in NCI-H460 human lung cancer cells. Compound K enhances apoptotic cell death by enhancing intracellular ROS generation, collapsing $\Delta\psi$, and activating caspase 3. Thus, compound K may provide strategies for clinical trials in radiation cancer therapy and a potential source for chemopreventive functional food.

ABBREVIATIONS USED

ROS, reactive oxygen species; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide; ANOVA, analysis of variance; SE, standard error; RPMI 1640 medium, Roswell Park Memorial Institute 1640 medium.

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