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Article

Oridonin enhances radiation-induced cell death by promoting DNA damage in non-small cell lung cancer cells

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Abstract: Although many attempts have been made to improve the efficacy of radiotherapy to treat cancer, radiation resistance is still an obstacle in lung cancer treatment. Oridonin is a natural compound with promising antitumor efficacy that can trigger cancer cell death; however, its direct cellular targets, efficacy as a radiosensitizer, and underlying mechanisms of activity remain unclear. Herein, we report that oridonin exhibits additive cytotoxic and antitumor activity with radiation using H460 non-small cell lung cancer cell lines. We assessed the radiosensitizing effect of oridonin by MTT, clonogenic, reactive oxygen species (ROS) production, DNA damage, and apoptosis assays. *In vitro*, oridonin enhanced the radiation-induced inhibition of cell growth and clonogenic survival. Oridonin also facilitated radiation-induced ROS production and DNA damage and enhanced apoptotic cell death. *In vivo*, the combination of oridonin and radiation effectively inhibited H460 xenograft tumor growth, with higher caspase-3 activation and H2A histone family member X (H2AX) phosphorylation compared with that of radiation alone. Our findings suggest that oridonin possesses a novel mechanism to enhance radiation therapeutic responses by increasing DNA damage and apoptosis. In conclusion, oridonin may be a novel small molecule to improve radiotherapy in non-small cell lung cancer.

Keywords: oridonin; radiation; sensitization; ROS; apoptosis

1. Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide. Radiotherapy is an important treatment for unresectable advanced human lung cancers, as well as an adjuvant therapy after surgery and palliative treatment. It is used at every stage of clinical progression, with both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) forms of the disease [1]. According to epidemiological studies, over 60% of patients with NSCLC have been treated with radiotherapy [2, 3]. However, lung cancer radiotherapy is far from ideal due to problems associated with radiation resistance of the cancerous cells and severe cytotoxicity against noncancerous cells [4]. Despite recent advances in the delivery of lung cancer radiotherapy, most patients relapse and show poor survival [5, 6]. Therefore, it is necessary to develop new strategies to improve the efficacy of this treatment procedure.

Radiation resistance is a major impediment to the success of cancer therapy. There have been many attempts to reduce radioresistance to lung cancer treatment with the use of radiation sensitizers, which have the potential to overcome resistance and improve treatment outcomes. There have been many clinical trials examining the efficacy of enhancing radiotherapy; many of these trials

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tested agents that were cytotoxic chemotherapies such as taxol and cisplatin [7, 8]. In addition, kinase inhibitors targeting Akt, mammalian target of rapamycin (mTOR), and checkpoint kinase 1 (Chk1) have been extensively studied as potent radiosensitizers but their benefits are limited due to their broad biological activities and potential side effects [9, 10 , 11]. Due to a critical need for the discovery and development of new radiation enhancers, many investigations are ongoing on novel classes of small molecule radiation sensitizers that have high efficacy and low toxicity, including those targeting survivin [12, 13].

Oridonin is a natural diterpenoid compound that can be isolated from *Rabdosia rubescens* (*Isodon rubescens*) and other plants in the genus *Isodon*, which are Chinese and Japanese traditional medicines for the treatment of various human diseases [14, 15]. Oridonin has been reported to exhibits potent anticancer activity both *in vitro* and *in vivo* against various cancer cells, including human gastric cancer [16, 17], colorectal cancer [18], breast cancer [19], and leukemia [20]. Although many proteins, including extracellular signal-regulated kinase (ERK) [21], Bax/Bcl-xL [22], and nuclear factor (NF)-κB [23, 24] have been found to be involved in the anticancer activity of oridonin, its therapeutic effects remain largely unknown. Especially, a radiation sensitizing effect of oridonin has not been established for cancer treatment.

The aim of this study was to investigate oridonin as an effective radiosensitizer and explore its mechanism of action on human lung cancer cells. We found that oridonin could sensitize H460 NSCLC cells (both cultured and as xenograft tumors in mice) to radiation-induced cell death, most likely involving increased production of reactive oxygen species (ROS), DNA damage, and apoptosis.

2. Results

2.1. Oridonin inhibits H460 lung cancer cell growth

Oridonin (Figure 1A) is a multifunctional drug that demonstrates powerful anticancer effects. Accordingly, we determined the growth inhibitory effects of oridonin on H460 human lung cancer cells and L132 human lung epithelial cells using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were treated with different concentrations of oridonin (1, 2.5, 5, and 10 μM) for 48 h and the cell viability was measured. Oridonin inhibited H460 cell proliferation at 5–10 μM (Figure 1B). However, oridonin also showed cytotoxic effects on L132 human lung epithelial cells at 10 μM (Figure 1C). To reduce potential cytotoxicity toward normal cells, we restricted the oridonin treatment of the H460 cells to 5 μM for subsequent studies.

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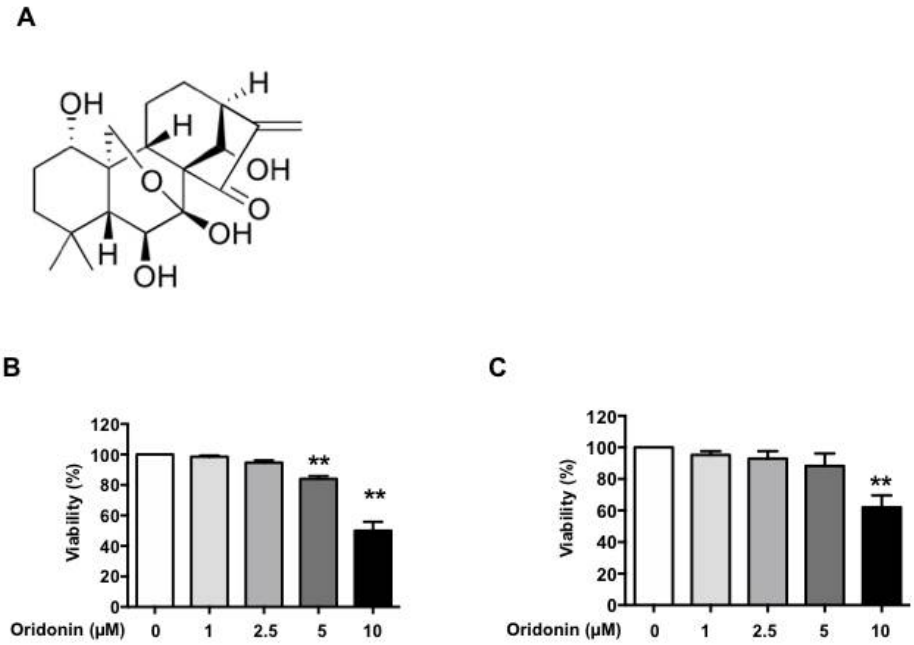

Figure 1. The cytotoxic effect of oridonin on human lung cancer cells and non-cancer cells (A) Molecular structure of oridonin. MTT assay in H460 cells (B) and L132 cells (C). 5×10^3 cells were seeded into 24-well plates and incubated for 48 h in the presence of 0-10 μM oridonin. All experiments were independently performed three times. The data are shown as means ± SEM; * $p<0.05$ and ** $p<0.01$ versus 0 μM oridonin.

2.2. Oridonin radiosensitizes lung cancer cells

To explore the effect of combining oridonin and radiation on H460 cell growth, cell viability was examined with oridonin and/or radiation using an MTT assay. Treatment of the cells with 5 μM oridonin or 4 Gy of gamma irradiation inhibited H460 cell growth by 12% and 23%, respectively; combining the two treatments inhibited their growth by 33% (Figure 2A and 2B). Next, we conducted clonogenic survival assays to determine the effect of oridonin on sensitizing the human lung cancer cells to radiation. The H460 cells were treated with 1 or 2 μM of oridonin for 1 h and then were exposed to different doses of gamma rays. The assessment of colony formation showed that the surviving cell fraction with combined oridonin and radiation treatment was significantly decreased compared to that with radiation alone (two-way ANOVA test with Tukey's multiple comparison, $p<0.01$, Figure 2C and 2D). These data indicate that oridonin enhances the cytotoxic effect of radiation in H460 lung cancer cells.

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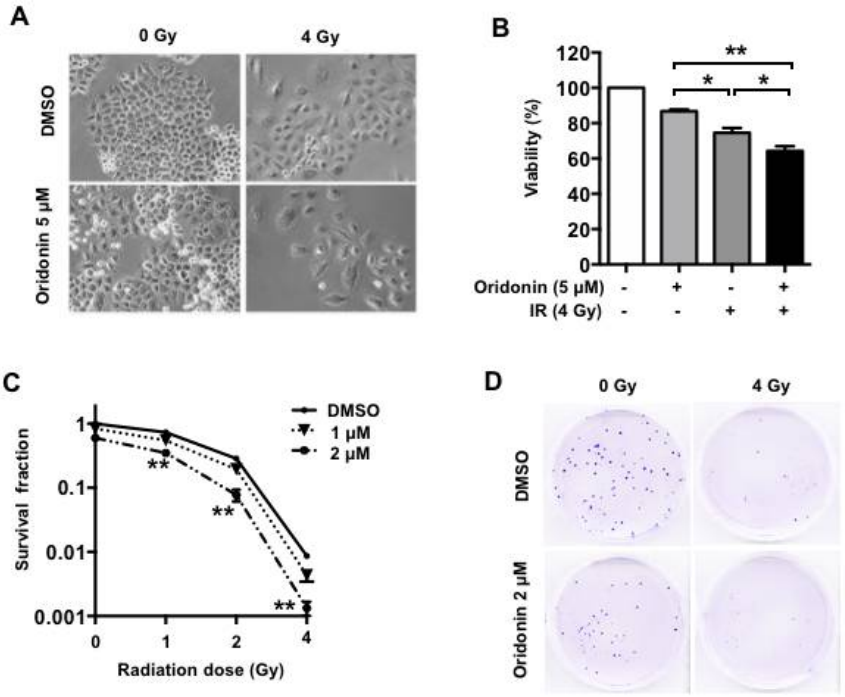

Figure 2. Effect of oridonin on sensitivity of H460 cells to radiation (A) Representative images of cultured H460 cells in the presence of oridonin (5 μM) and/or radiation (IR, 4 Gy) (B) H460 cell viability was measured using MTT assay. The data are presented as means ± SEM (n=3); * $p<0.05$ and ** $p<0.01$. (C) Survival fractions obtained by colony forming assay. Three independent experiments were carried out. ** $p<0.01$; p-values represent two-way ANOVA results. (D) Representative photos of colony formation in the presence of 4 Gy radiation and/or 2 μM oridonin. DMSO, dimethyl sulfoxide vehicle.

2.3. Oridonin enhances radiation-induced ROS production

Oridonin has been reported to exhibit antitumor effects via ROS production [25]. Therefore, to explore further the mechanisms by which oridonin increases the radiation sensitivity of lung cancer cells, we investigated whether oridonin affects ROS production. We measured ROS production in H460 cells with increasing concentrations of oridonin from 2.5 to 10 μM. There was a significant increase in ROS production by ~2-fold at 10 μM oridonin, but concentrations ≤5 μM had no effect on ROS production (Figure 3A). Compared with that of irradiation alone, co-treatment with 5 μM oridonin significantly enhanced the radiation-induced ROS production ($p<0.01$, Figure 3B).

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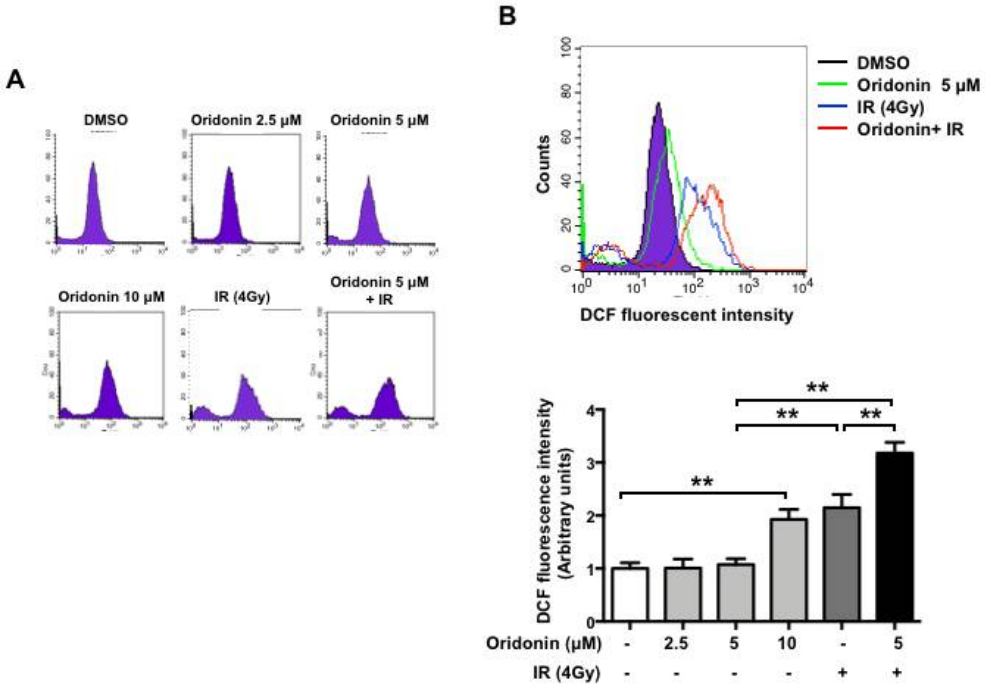

Figure 3. The effect of oridonin on radiation-induced ROS accumulation (A) Cellular ROS levels were determined by DCFH-DA staining 48 h after treatment with oridonin (0–10 μM) and/or irradiation (IR, 4 Gy). DCFH-DA fluorescence was measured by flow cytometry. (B) Upper panel. Univariate histogram plots represent DCF mean fluorescence of control (vehicle, 0.05% DMSO), oridonin (5 μM), IR (4 Gy), and combination of oridonin and IR. Lower panel. DCF fluorescence was quantified and values were normalized to the mean fluorescence of the control. Values are the average of three independent experiments (means ± SEM; ** $p< 0.01$). DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; DMSO, dimethyl sulfoxide.

2.4. Oridonin accelerates radiation-induced DNA damage

As it is known that an increase in ROS levels can result in DNA damage [26, 27], we determined the effect of oridonin on radiation-induced DNA damage using a comet assay. As shown in Figure 4A, oridonin treatment of H460 cells for 48 h resulted in concentration-dependent DNA damage. This DNA damage was determined by measurement of the DNA comet tail, photographed under a microscope, using OpenComet (the open-source software in ImageJ) [28]. Compared with that of both oridonin and IR individual treatments, the combination of 5 μM oridonin and irradiation showed a significant increase in the size of the DNA comet tail in the treated H460 cells ($p<0.01$, Figure 4B). Additionally, we evaluated the effect of oridonin on phospho-Histone H2A.X (γ-H2AX), a common marker of DNA damage. Five micromolar of oridonin alone did not alter γ-H2AX levels, but oridonin in combination with IR significantly increased γ-H2AX content ($p<0.05$, Figure 4C).

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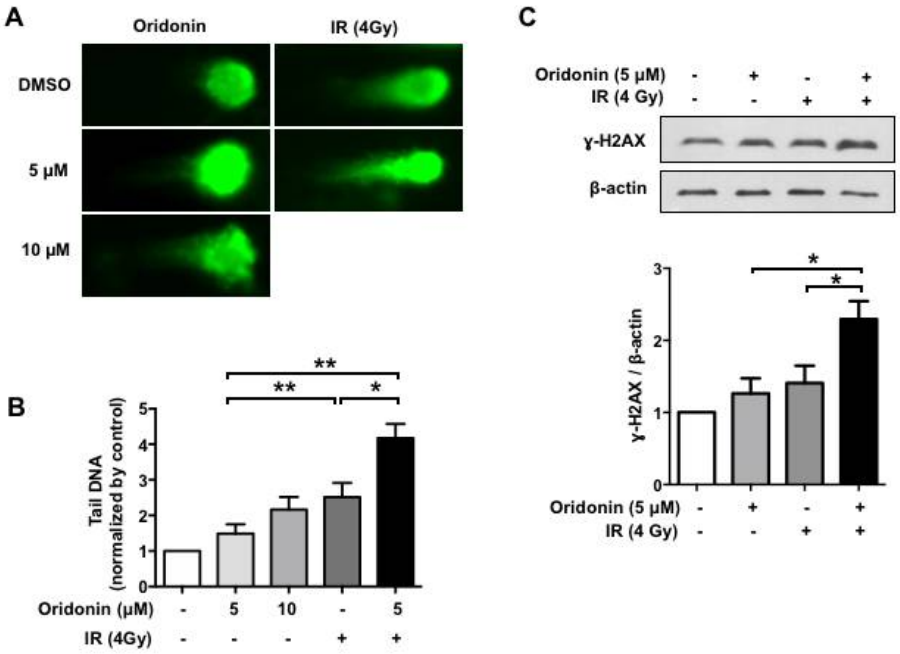

Figure 4. Effect of oridonin on radiation-induced DNA damage (A) Representative images show DNA comet assays. (B) Comet tail sizes were measured by OpenComet, an ImageJ program, and normalized to the tail intensity of the DMSO control. (C) γ-H2AX protein levels were analyzed by western blot. Ten micrograms of protein was loaded per lane (upper panel). Band intensity was quantified by ImageJ and normalized to control levels (white bar, bottom panel). Data are representative of more than three independent experiments and values are expressed as means ± SEM; * $p<0.05$ and ** $p<0.01$. DMSO, dimethyl sulfoxide.

2.5. Oridonin increases radiation-induced apoptotic cell death

To explore the mechanisms by which oridonin enhances the radiation sensitivity of lung cancer cells, we investigated whether it increased IR-induced apoptosis. Apoptotic cell death induced by oridonin and/or IR treatment was evaluated in H460 cells using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Figure 5A). Flow cytometry revealed that oridonin and IR alone induced apoptotic cell death in 29% and 52% of the cells, respectively; however, their combination significantly increased the proportion of apoptotic cells to 73% ($p<0.05$). Therefore, the treatment with oridonin was found to be effective in enhancing the apoptosis of H460 cells. Similarly, western blot analysis showed that the combination of oridonin and irradiation profoundly increased the cleavage of caspase-3 compared to that of irradiation alone ($p<0.01$, Figure 5B). These findings suggest that co-treatment with oridonin enhances radiation-induced apoptotic cell death.

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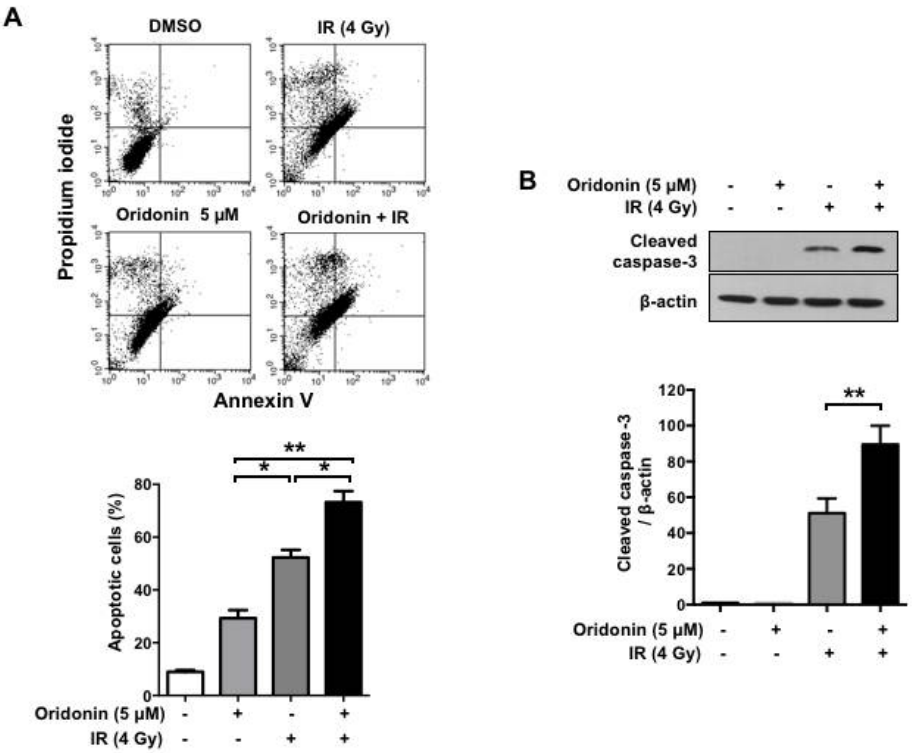

Figure 5. Effect of oridonin on radiation-induced cell death and cleavage of caspase-3. Cells were treated with oridonin (5 μM) and/or IR for 72 h. (A) Cells were stained with Annexin V-FITC/PI prior to flow cytometry analysis. Apoptotic cells were estimated by the sum of upper left, upper right, and lower right quadrants in each dot plot. Plotted values represent the percentages of apoptotic cells. (B) Representative western blots (upper) and plotted, quantified data (lower) for cleaved caspase-3. All plotted data represent the means ± SEM of three independent experiments * p<0.05 and ** p< 0.01. PI, propidium iodide.

2.6. Oridonin enhances radiation-induced tumor growth inhibition

To investigate the radiosensitizing effect of oridonin *in vivo*, we established an H460-bearing tumor model. Once the tumors reached 150 mm^3, the mice were treated with radiation (6 Gy) and/or oridonin (15 mg/kg) as shown in Figure 6A. Oridonin alone (15 mg/kg) showed a weak inhibitory effect on tumor growth (up to 11%, p<0.05). IR alone (6 Gy) led to 44% growth inhibition when compared to that of control (p<0.05), while the combination of oridonin and IR decreased tumor volume by 65% (p<0.01 versus control) And combination treatment of oridonin and IR significantly reduced the tumor volume compared to oridonin alone (p<0.01) and IR alone (p<0.01) (Figure 6B). IR treatment alone led to a slight decrease in body weight; however, there was no significant difference in body weights among the groups at the end of treatment (Figure 6C). Immunohistochemical analysis showed that co-treatment with oridonin increased cleaved caspase-3 and γ-H2AX levels up to 2.2-fold (p<0.05) and 2.6-fold (p<0.05), respectively, compared to the values with IR alone (Figure 7). These findings suggest that the mechanism of oridonin-mediated radiosensitization involves increased DNA damage and apoptotic cell death.

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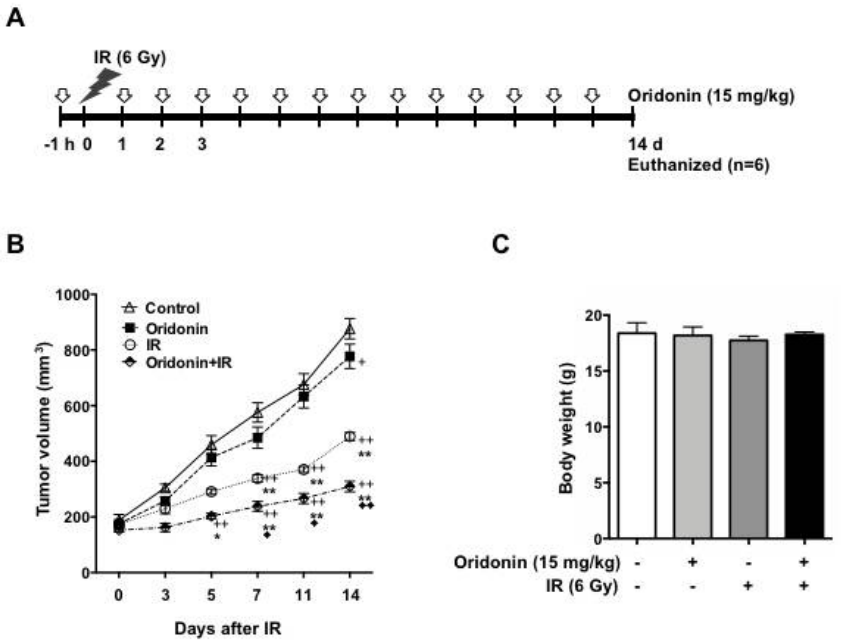

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177 **Figure 6.** Effect of oridonin on radiotherapy (A) Schema of the timeline for oridonin and irradiation
178 treatment. Daily oridonin (15 mg/kg, intraperitoneal injection) administered up to the indicated time
179 points pre- and post-irradiation (6 Gy). (B) Growth curves of H460 tumors following oridonin and/or
180 IR treatment. (C) Mean body weights of experimental groups at study termination. The data represent
181 the means ± SEM of 6 mice per group. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ compared to control; * $p<0.05$ and ** $p<0.01$
182 compared to oridonin; $^{\diamond}$ $p<0.05$ and $^{\diamond\diamond}$ $p<0.01$ compared to IR.

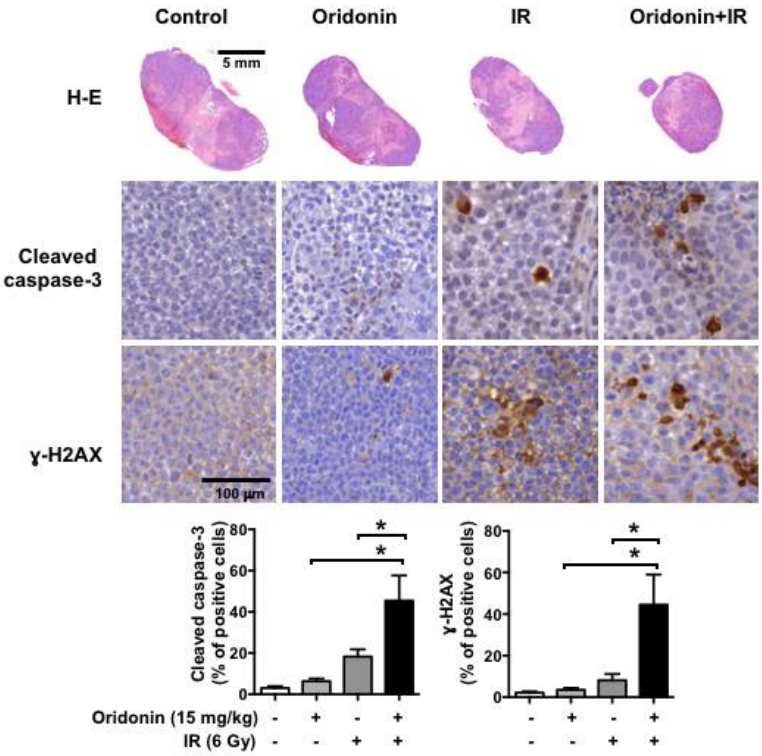

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Figure 7. Histopathological analysis of H460 tumors following combination treatment with oridonin and radiation. Hematoxylin and eosin (H-E) staining and immumohistochemistry for cleaved caspase-3 and γ-H2AX were performed on tumors harvested at 14 days after IR. Representative image of cleaved caspase-3 and γ-H2AX positive cells (brown staining) and quantification of cleaved caspase-3 and γ-H2AX-positive staining from 6 mice in each group (means ± SEM); * $p<0.05$.

3. Discussion

Radiotherapy is an important modality in lung cancer patients during the course of cancer treatment both as a curative modality and for palliation. However, tumor radioresistance and toxic side effects toward normal tissue, which impede dose escalation, are major obstacles to the success of radiation therapy [29, 30]. Thus, strategies for optimizing the response of cancer to increase the therapeutic efficiency of radiotherapy are needed. There is an increasing interest in combining radiation and natural compounds to enhance the efficacy of radiotherapy. The use of natural products as antitumor agents or radiosensitizers for the management of human cancers is an attractive idea because they are readily available and exhibit little or no toxicity [31-33]. Oridonin, a natural tetracyclic diterpenoid compound, has well-known potent anticancer activity against a wide range of cancer cell types, including prostate [34] and breast cancers [19] and acute leukemia [20]. It has been reported that oridonin elicits an antiproliferative effect on lung cancer cell lines *in vitro* and *in vivo* [35, 36]. However, the effects of oridonin on the radiosensitization of lung cancer cells remain poorly understood. In this study, we found a novel potential of oridonin to sensitize the anticancer effect of radiation via increases in ROS accumulation, DNA damage, and apoptosis in H460 cells. To the best of our knowledge, this is the first report to support the potential beneficial effect of oridonin to improve responses to radiotherapy in NSCLC patients.

Many recent studies have focused on the antitumor effects of oridonin, and its derivatives have been developed to be used in combination with other anticancer drugs. Guo et al. reported that oridonin synergizes the anti-leukemia effect of imatinib via the LYN/mTOR pathway [37]. Zhang et al. also reported that oridonin treatment could overcome ciplastin-resistance in human acute leukemia cells [38]. Wu et al. reported that an analog of oridonin, named CYD-6-28, effectively suppressed triple-negative breast cancer cell growth via induction of death receptor 5 [39]. Therefore, our findings and those from other investigators strongly support the potential of oridonin as an anticancer agent.

An ideal radiosensitizer enhances the ability of radiation to kill tumor cells while not altering the radioresponse of normal tissues [40]. We investigated the ability of oridonin, a natural small molecule, to act as a radiosensitizer in human non-cancer cells. As previously reported, we found that oridonin has a potent anticancer effect to inhibit the proliferation and clonogenic ability of cancer cells. However, we also identified cytotoxicity against non-cancerous cells that was not considered in previous studies. Specifically, oridonin demonstrated cytotoxicity against both normal lung epithelial cells and lung cancer cells. To diminish the toxic side effects of oridonin, we used a lower concentration of oridonin (5 μM), which showed a weaker anticancer effect compared to that in previous studies, to observe its beneficial effects on radiotherapy. Five micromolar of oridonin alone did not alter ROS production and DNA damage in the comet assay, but still showed mild effects on growth inhibition and cell death of the H460 cells. However, notably, the combination of 5 μM oridonin and radiation greatly enhanced ROS production, DNA damage, and apoptotic cell death of H460 cells. In addition, H460 tumors were sensitized to the effect of radiation by oridonin treatment, which was assessed by the inhibition of tumor growth (i.e., tumor volume). Although the dose of oridonin used in this study (15 mg/kg) is a low to moderate dose compared to that used in previous studies (7.5–40 mg/kg) [41-43], the combination of oridonin and radiotherapy significantly inhibited tumor growth in the experimental time period. Our results suggest carefully considering dose reduction to minimize side effects while promoting therapeutic efficacy. Therefore, further studies will be needed to optimize the dosage and assess the toxicity of oridonin in various types of cancer.

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In the present study, we demonstrated that oridonin enhanced the therapeutic effect of radiation by augmenting radiation-induced apoptotic death. Our results identify and highlight a new role of oridonin as a radiosensitizer and we propose that oridonin be used as a novel adjuvant treatment during radiotherapy.

4. Materials and Methods

4.1. Cells and treatments

The NCI-H460 NSCLC cell line was cultured in RPMI medium (WELGENE, INC., Gyeongsangbuk-do, Korea) with 10% fetal bovine serum (FBS, 35-015-CV, CORNING, NY, USA) and 1% Gibco® antibiotic-antimycotic (15240-062, Thermo Fisher Scientific, UK) at 37°C in a 5% CO_2 humidified incubator. The cells were seeded at the indicated numbers and incubated overnight before treatment with various concentrations of oridonin and doses of IR. Oridonin was purchased from Selleck Chemicals (S2335, TX, USA).

4.2. MTT assay

H460 cells (5×10^3) were seeded into 24-well plates and incubated overnight. Oridonin was added at various concentrations and the cells incubated for 48 h. After incubation, MTT stock solution (5 mg/mL) was added at 10% of the culture medium volume and the cells were incubated for 2 h at 37°C. Then, the medium was discarded and DMSO (200 μL) was added to each well and incubated for 10 min to dissolve the insoluble formazan precipitate. The solution was then transferred to a 96-well plate and its absorbance read at 560 nm.

4.3. Clonogenic assay

The cells were seeded in 60-mm dishes according to radiation dose (0 Gy 100 cells, 1 Gy 200 cells, 2 Gy 500 cells, and 4 Gy 2000 cells) and incubated overnight. Then, the cells were treated with oridonin (0, 1, and 2 μM and 0.05%) for one hour prior to radiation exposure. The cells were then exposed to radiation using ^{137}Cs as a radiation source (BIOBEAM GM 8000, Gamma Service Medical GmbH, DE, UK). After treatments for 7 days, the dishes were washed with PBS and incubated with 0.5% crystal violet in 5% neutral-buffered formalin. Then, the dishes were washed and the colonies were counted.

4.4. Determination of ROS production

Cellular ROS levels were estimated using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) purchased from Cayman Chemical (85155, MI, USA). The cells were trypsinized and mixed with 1% FBS in PBS containing 10 μM DCFH-DA for 15 min, protecting from light. The fluorescent cells were analyzed on a FACSCalibur™ flow cytometer (Becton-Dickinson, ON, Canada).

4.5. Comet assay

The CometAssay™ (4250-050-K, TREVIZEN, MD, USA) was used for DNA damage assessment. Cells (1×10^5) were collected in 50 μL ice-cold PBS. 10 volumes of completely melted low-melting agarose (LMA) was mixed with 10 volumes of suspended cells and 75 μL cell/agarose mixture was spread on the comet slide and incubated at 4°C for 30 min to solidify the agarose. After congelation, the slides were incubated in lysis solution (4250-050-01, TREVIZEN, MD, USA) at 4°C for 60 min. Then, the slides were moved carefully into alkaline solution (pH >13) and incubated at room temperature for 30 min. For neutralization, the slides were washed once in 1×TBE buffer and electrophoresis was performed at 31 V for 40 min. The slides were washed with 70 % ethanol and air dried after removing excess liquid. Fifty microliters of SYBR® Green I was placed on each sample

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and the slides were analyzed by fluorescence microscopy. The signal of the tail DNA of 50 - 80 cells from five high magnification fields (x100) was analyzed to estimate DNA damage in each group. The values were measured using OpenComet in ImageJ software.

4.6. Western blots

The cells (2×10^4) were seeded in 60-mm dishes and incubated for 72 h in RPMI. The cells were trypsinized and lysed in 100 μL of ice-cold RIPA buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM Na_3VO_4, 5 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). The cell suspension was sonicated and incubated on ice for 30 min. After centrifugation at 13,000 rpm for 10 min, the supernatants were collected and protein concentrations were quantified by the bicinchoninic acid (BCA) method (23227, Pierce Biotechnology, Inc., IL, USA). For western blot analysis, proteins (60 μg) were elctrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The specific antibodies were cleaved caspase-3 (1:1000, #9661, Cell Signaling Technology, Inc., MA, USA), γ-H2AX (1:3000, 05-636, Merck KGaA, Darmstadt, Germany), and β-actin (1:5000, sc-47778, Santa Cruz Biotechnology, Inc., CA, USA).

4.7. Apoptotic cell death by flow cytometry

The Annexin V-FITC Apoptosis Detection Kit (K101-25, BioVision Incorporated, CA, USA) was used for detecting apoptotic cell death. The cells were seeded (1×10^4 per well) into 6-well plates and incubated with various concentrations of oridonin before irradiation (IR). The cells were exposed to IR (4 Gy) and incubated for 72 h. The cells were then washed with cold PBS and resuspended in 500 μL of 1× binding buffer containing 5 μL of annexin-FITC and 5 μL of propidium iodide. After mixing, the apoptotic cells were analyzed immediately using the FACSCalibur™ flow cytometer (Becton-Dickinson).

4.8. Animal experiments

All protocols in this study were approved by the Institutional Animal Care and Use Committee of the Korean Institute of Radiological and Medical Sciences (IACUC permit number: KIRAMS2017-0007). Six-week-old female BALB/c nude mice were purchased from Orient Bio Inc. (Seoul, Korea). The animals were maintained at 23 ± 2°C with humidity of 50 ± 5%, lighting cycle of 08:00 to 20:00, and 13–18 air changes per hour. The H460 cells were cultured in RPMI containing 10% FBS and 1% antibiotic/antimitotic at 37°C in a 5 % CO_2 humidified incubator. A xenograft tumor was created by subcutaneous injection of 1×10^6 cells to the right hind leg. When the tumor volume reached 100–120 mm^2, oridonin treatment (15 mg/kg) was initiated by intraperitoneal injection one hour before IR treatment. Each mouse was anesthetized with tiletamine/zolazepam (Zoletil 50®, Virak Korea, Seoul, Korea) and exposed to 6 Gy of IR using an X-RAD 320 system (Precision X-ray, Inc., North Branford, CT, USA) at 250 kV and 10 mA with 420 mm of aluminum shielding, resulting in a dose rate of 2 Gy/min. Afterward, oridonin was injected daily until 14 days, when the mean tumor volume reached approximately 1000 mm^2 in the control group. Tumor tissue was then harvested from each mouse for histological analysis.

4.9. Immunohistochemistry and quantification

The tumor tissue was fixed in 10% formaldehyde and embedded in paraffin wax. For histological experiments, the embedded tissues were sectioned at 5 μM, mounted on slides, deparaffinized, and rehydrated by graded ethanol washes. For antigen retrieval, the sections were boiled in citrate buffer (pH 6.0). Immunohistochemistry was performed using a Vectastain Elite ABC Kit (Vector Laboratories Inc., CA, USA) following the manufacturer's protocol. The sections were incubated with anti-cleaved caspase-3 (1:100, #9661, Cell Signaling Technology, Inc.), anti-γ-H2AX (1:100, 05-636, Merck KGaA), or anti-Ki-67 (1:200, DRM004, Acris Antibodies, Germany) antibodies at 4°C overnight

and then washed with PBS containing 0.05% Triton X-100. The sections were incubated with horseradish peroxidase-conjugated secondary antibody for 30 min and counterstained with hematoxylin. The percentage of Ki-67, cleaved caspase-3, and γ-H2AX positive cells were quantified at five randomly selected fields at 400× magnification.

4.10. Statistical analysis

All experimental data are shown as the means ± standard errors of the mean (SEM). The data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests or two-way ANOVA using GraphPad Prism version 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). *P*-values less than 0.05 indicated statistical significance.

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Conflicts of Interest: The authors declare no conflict of interest.

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