Novel podophyllotoxin derivative exerts effects of an anti-cancer drug and a radiosensitizer

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1. Introduction

Podophyllotoxin (PPT) is a plant-derived natural product and has been used as therapeutics traditionally for treatment of several diseases. In view of the beneficial properties of PPT, many investigators in 20th century have attempted to synthesize various derivatives, and produced Etoposide and Teniposide that are representative semi-synthetic derivatives of PPT. These derivatives showed anti-cancer effect by targeting DNA topoisomerase II, and facilitate breakage of one or both DNA strands. However, both two agents have limitation of long-term usage due to strong side effects.[1] For that reason, investigators continuously tried to synthesize several new derivatives of PPT. We also synthesized several derivatives and identified JNC-1043 as a potential anti-cancer drug candidate. JNC-1043 has primarily evaluated against several cancer cell lines, lung, breast, gastric, blood, colorectal and other cancer cell lines, and showed anti-cancer activities.

Radiotherapy is common treatment for various cancer. However, the results of radiotherapy are reduced by the damaging normal cell and inducing radio-resistance against cancer cells.[2] This drawback of radio-resistance is the challenge that is overcome by combination of radiotherapy and radiosensitizer. This experiment aims to investigate whether JNC-1043 can act as a novel radiosensitizer to enhance cancer treatment efficacy of γ-ionizing radiation (IR) against colorectal cancer cell lines.

2. Materials and Methods

2.1 Cell cultures & chemical reagents

HCT116 and DLD-1 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Bothe cell lines were cultured in RPMI media of 10%Fetal bovine serum and 1% penicillin. Cells were grown at 37°C with 5% CO2. For harvest, cell was washed with cold PBS and scrapped into lysis buffer. JNC-1043 (JNC43) was synthesized from J&C Sciences (Daejeon, South Korea.)

2.2 MTT assay

HCT116 and DLD-1 cells were seeded on 96 well plates, exposed to diff erent concentrations of JNC43 for 48 h and 72 h at 37°C, and treated with 20 μL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution (2 mg/mL) for 1-2 h. Formazan crystals generated in living cells were lysed in 100 μL of DMSO, and absorbance of individual wells were measured at 545 nm using a microplate reader.

2.3 Clonogenic assay

HCT116 and DLD-1 cells were seeded in 60 mm dishes at cell concentrations estimated to yield 20–100 colonies/dish After 24 h of incubation, cells were treated with or without 110nM, 150nM JNC43, respectively, for 24 h and then irradiated using a 137Cs as a source of γ-ray (Atomic Energy of Canada, Ltd., Mississauga, ON, Canada) at different doses (1, 2, 3, or 4 Gy). Cells were fixed after at least 14 days and colonies larger than 200 μm in diameter were counted using a colony counter (Imaging Products, Chantilly, VA).

2.4 Cell cycle analysis

HCT116 and DLD-1 cells were incubated with or without 110nM, 150nM JNC43, respectively and exposed to 1 Gy irradiation, respectively, for 2or 4h. The cells were then harvested and collected by centrifugation at 1200rpm for 1 min. Subsequently, the cells were washed twice with ice-cold phosphate buffered salin (PBS) and fixed with ice-cold 70% ethanol. The fixed cells were stained with PI/RNase Staining Buffer (Becton Dickinson, Franklin Lakes, NJ, USA) for 15min, and then analyzed with a FACSort flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

2.5 Immunoblot analysis

Used to lysis buffer get to cytosolic extracts, and centrifuged at 13000 rpm for 20 min. To the next, supernatant was obtained. Total cell contents (20 mg per well) and immune-precipitated proteins were heated at 95℃ for 3 min, then separated on SDS-PAGE and transferred to nitrocellulose blotting membranes. After Blocking at 1 hour used 5% skim milk, primary antibody prepared with 5% BSA was incubated at 4℃ for 12 hours. Secondary antibody was incubated at room temperature for 1 hour. Then add the luminol enhancer and peroxide solution to measure the target protein. Primary antibodies were used against the following:

pro-caspase-3, pro-caspase-9, cleaved caspase-3, cleaved caspase-9, Bcl-2, Bcl-XL. An anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control.

2.6 Isolation of mitochondrial and cytosolic fraction

HCT116 and DLD-1 cells were treated with 110nM, 150nM JNC43, respectively and irradiated 3 Gy irradiation and incubated for 48 h at 37℃. The cells were incubated with trypsin-EDTA for 5 min, collected by centrifugation at 1200 rpm for 1 min, resuspended with extraction buffer, homogenized, and centrifuged at 12000g at 4 ℃ for 15 min. The supernatant was used as the cytosolic fraction and the pellet was resuspended with lysis buffer and used as the mitochondrial fraction. The fractions were immunoblotted with a cytochrome c antibody and VDAC was used as a loading control. These experiments are repeated in trice.

2.7 Trypan blue cell counting assay

HCT116 and DLD-1 cells were incubated with or without 110nM, 150nM JNC43, respectively and exposed to 3 Gy irradiation, respectively. The cells were incubated for 72 h at 37℃. Then, the cells were then collected by trypsinization, washed twice with cold PBS, and stained with trypan blue as equal volume of cells. The cells were counted with hemocytometer. These experiments are repeated in trice.

2.8 Annexin V-Propidium iodide assay

HCT116 and DLD-1 cells were incubated with or without 110nM, 150nM JNC43, respectively and exposed to 3 Gy IR, respectively, and incubated for 72 h. The cells were then collected by trypsinization, washed twice with cold PBS, and stained with FITC Annexin V apoptosis detection kit I (Becton Dickinson, Franklin Lakes, NJ, USA) as described in manufacturer' protocol. A FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was used to measure the fraction of apoptotic cells (x-axis: FL1 channel and y-axis: FL-2 channel).

2.9 H2DCHDA-based assay

HCT116 and DLD-1 cells (5×105 cells/60 mm dish) were incubated with or without 110nM, 150nM JNC43, respectively, and exposed to 3Gy IR, respectively. After incubation for 24h, the cells were stained with 25mM H2DCFDA for 5 min and trypsinized. The cells were harvested by centrifugation and resuspended with cold PBS. A FACSort flow cytometer (Becton Dickinson) was used for detection and analysis of intracellular ROS (x-axis: FL1 channel).

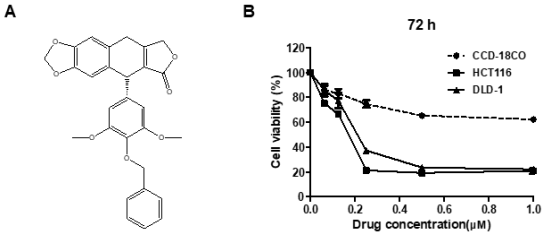
2.10 TUNEL assay

All animal experiments were performed using approved protocols of the Institutional Animal Care and Use Committee. The in vivo radiosensitization effects of JNC43 were measured by using a xenograft model constructed by subcutaneously injecting 1×107 HCT116 cells/mouse into 6 weeks old BALB/cAnNCrj-nu/nu mice (Envigo, Cambridgeshire, UK). The mice were divided into four groups (n=3 mice/group): control (mock treated), IR treatment only (IR only), JNC43 treatment only (JNC43 only), and co-treatment with JNC43 and IR (IR+JNC43). Mice of the IR only and IR+JNC43 groups were locally irradiated 1.5 Gy after 6 h from JNC43 treatment. Extracted tumors were fixed with formaldehyde, paraffin embedded and sliced. TUNEL assay for detection dUTP nick was performed by Super Bio Chips (Seoul, South Korea). The TUNEL-positive cells were measured for each group, and percentages were calculated relative to the value obtained from the control group.

3. Results

3.1 JNC43 inhibits cell-proliferation in CRC cell lines by DNA damaging

To investigate the anti-cancer effect of JNC43, human colon fibroblast cell CCD-18CO and CRC cell lines HCT116 and DLD-1 were treated with various concentrations of JNC43 for 48 or 72 h. Concentrations of JNC43 that caused 50% inhibition of the viability of NSCLC cell lines (IC50) including human colorectal fibroblast cell and CRC cell lines were determined using MTT assays. IC50 values of JNC43 against HCT116 and DLD-1 cells were calculated to be 0.1145 μM and 0.1570 μM, respectively. These results indicate that JNC43 can exert anti-cancer effect against CRC cell lines even at low concentrations and that the cytotoxicity of JNC43 is stronger to CRC cell lines than to human colon fibroblast cells. To determine the anti-cancer effect of JNC43, immunoblot assay of phosphorylation of H2AX was done. Results of immunoblot assay of H2AX activation indicated that the anti-cancer effect of JNC43 was by inducing DNA damage. As HCT116 has wild type of p53 and DLD-1 has mutant type of p53, these results also indicate that the effect of JNC43 is unrelated to the mutation state of p53.



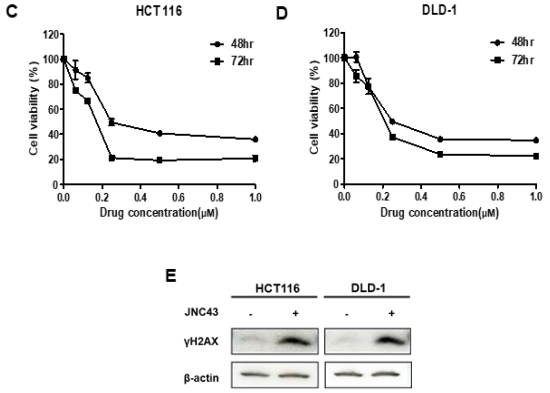


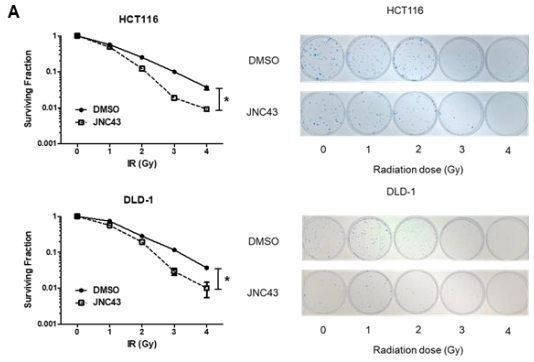
Fig. 1. Anti-cancer effect of JNC43 against colorectal cancer cells

Table Ⅰ. IC50 value

	IC50 value (μM)	
	48 h	72 h
HCT116	0.1719	0.1145
DLD-1	0.1598	0.1570

3.2 JNC43 acts as a radiosensitizer by inhibiting cell growth in vitro

Both clonogenic assay and cell counting assay revealed a radiosensitizing effect of JNC43. Clonogenic assays were performed by treating cells with JNC43 and 1, 2, 3, or 4 Gy IR. Its DER was found to be 1.528 for HCT116 cells and 1.25 for DLD-1 cells. Cell counting assay was also performed after co-treating cells with of 110 or 150 nM of JNC43 and 3Gy IR. JNC43 enhanced the effect of irradiation by inhibiting the growth of CRC cells. Additionally, immunoblot assay confirmed the activation of H2AX. Taken together, these results demonstrate that JNC43 has a radiosensitizing effect by inducing DNA damage.



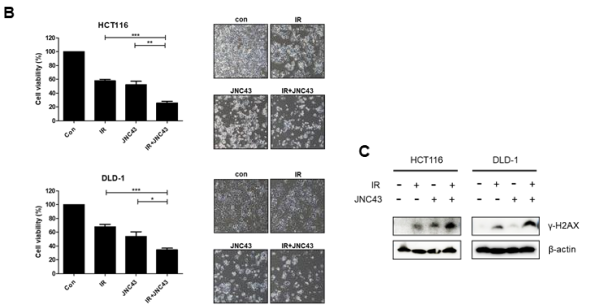


Fig. 2. JNC43 enhances the ability of irradiation to suppress the cell growth

3.3 Combination of JNC-1043 and Irradiation enhances apoptosis and DNA damage

Results showed that apoptosis of cells was induced by treatment with JNC43 only or irradiation only compared to the control. Apoptotic cell death was further induced by cotreatment with both JNC43 (at 110 or 150 nM) and irradiation compared to the control. Immunoblot assay showed that levels of Bcl-2 and bcl-xl were decreased while those of cleaved form of caspase-3, caspase-9, and PARP were increased in cells cotreated with JNC43 and irradiation than in cells treated with JNC43 or irradiation only. These results indicate that a combination of JNC43 and irradiation can enhance apoptosis of CRC cells by activating apoptosis related factors, including bcl-2, bcl-xl, cytochrome c, caspase 3, and caspase 9.

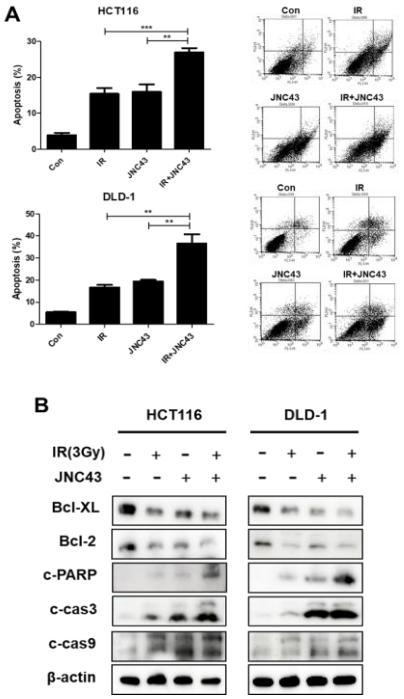


Fig. 3. Co-treatment with JNC-1043 and irradiation induces apoptotic cell death and DNA damage in CRC cells

3.4 Combination of JNC43 and Irradiation induce cell cycle arrest at G2/M phase

It is well-known that radiation can induce cell cycle arrest. Results showed that the G2/M phase population of treated CRC cells was increased in samples treated with both 1 Gy and 110 or 150 nM JNC43. Thus, co-treatment with irradiation and JNC43 increased G2/M phase arrest than in other samples. These results indicate that JNC43 can also induce cell cycle arrest and more arrest, eventually leading to cell death.

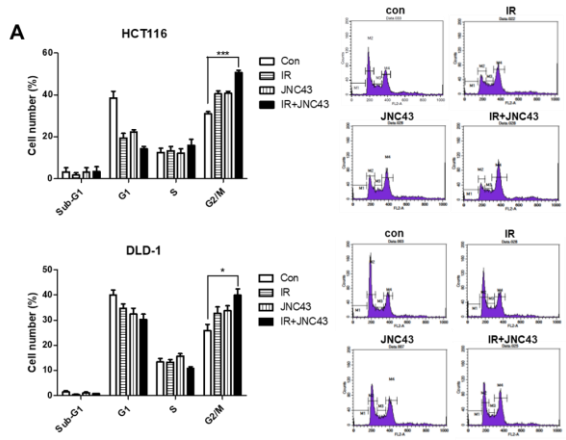


Fig. 4. JNC43 and irradiation arrest the cell cycle at G2/M phase

3.5 Co-treatment of JNC43 and irradiation enhanced accumulation of ROS in CRC cells.

Results of H2DCFDA-based intracellular ROS detection assay showed that combination of JNC43 and irradiation induced the production of ROS. Treatment with ROS scavenger N-acetyl cysteine (NAC) abolished such increases of both intracellular and mitochondrial ROS production induced by co-treatment of JNC43 and irradiation. Therefore, combination of JNC43 and irradiation can induce ROS production by targeting mitochondria and affecting the homeostasis of ROS level.

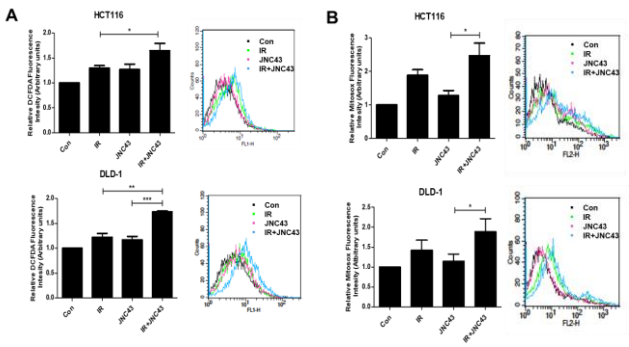


Fig. 5. Improvement of ROS accumulation by JNC43 and irradiation in CRC cells

3.6 ROS is an important factor in the radiosensitizing effect of JNC43 against CRC cells

As JNC43 and irradiation appeared to affect ROS homeostasis in CRC cells, assays were performed to determine whether NAC-associated reduction in ROS could reduce cell death triggered by cotreatment with JNC43 and irradiation. Pre-treatment with NAC reduced cell death caused by co-treatment with JNC43 and irradiation than the control. The reduction of apoptosis by NAC was related to bcl-2, bcl-xl, caspase activation, and cytochrome c release. Decrease of ROS also affected DNA damage induced by JNC43 and irradiation. Taken together, these results indicate that the combination of JNC43 and irradiation can improve apoptotic cell death through mitochondrial ROS accumulation and that this apoptotic cell death can be suppressed by NAC pre-treatment.

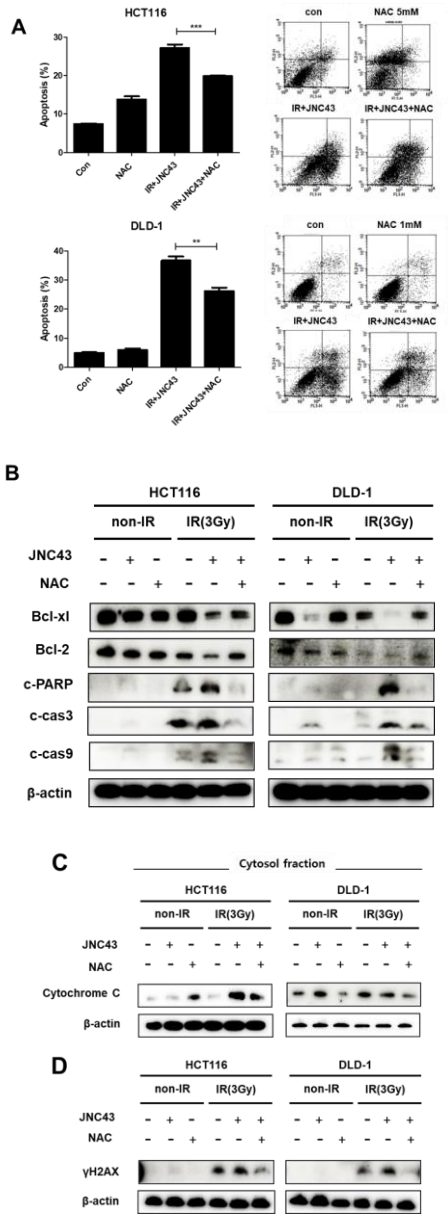


Fig. 6. ROS effects the radiosensitizing ability of JNC43

3.7 In vivo radiosensitization effect of JNC43

Xenograft mice models were prepared using HCT116 cells. Co-treatment with JNC43 and irradiation diminished tumor growth than the control (no treatment) and treatment with either IR only or JNC43 only. These results indicate that JNC43 has a radiosensitization effect in vivo as well as in vitro. Furthermore, TUNEL assay was performed to determine numbers of apoptotic cells in tumor tissue samples from control, IR only, JNC43 only, and IR+JNC43 groups of mice. It was revealed that percentage of apoptotic cell death of the JNC43+IR group was about 2-fold higher than that of the group treated with IR only or JNC43 only of HCT116 cell-derived xenografts. These results demonstrate a radiosensitization effect of JNC43 against CRC cells both in vitro and in vivo via the induction of apoptosis.

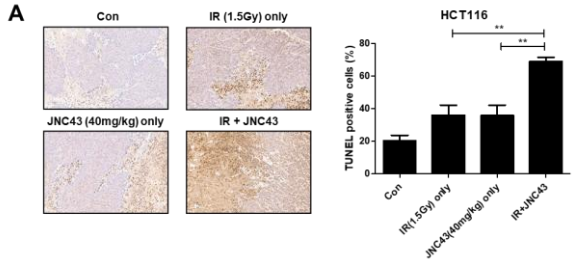


Fig. 7. Combination of JNC43 and irradiation improves apoptosis *in vivo*

4. Conclusions

JNC43 is a derivative of podophyllotoxin that is extensively distributed in several plant genera including Podophyllum. [3] In this study, JNC43 showed anti-cancer effects against CRC cells, and also exerted its anti-cancer effects on CRC cells by damaging DNA.

JNC43 also showed a radiosensitizing effect on CRC cells. Co-treatment with JNC43 and irradiation further inhibited cell growth and enhanced apoptosis by activation caspase3 and 9 than single treatment with JNC43 or irradiation alone.

It is well-known that exposure to penetrating radiation can directly or indirectly lead to cellular stress due to the generation of ROS. [4] Therefore, this study investigated whether ROS play a role in the cell death mechanism involved in the effect of cotreatment with JNC43 and irradiation on CRC cells. Results revealed that co-treatment with JNC43 and irradiation enhanced intracellular and mitochondrial ROS levels in CRC cells than treatment with either JNC43 only or irradiation only. As an ROS scavenger, N-acetylcysteine was used for treatment to investigate whether apoptotic cell death might lay downstream of ROS.[5] Results revealed that apoptotic cell death, which was triggered by JNC43and irradiation, was recovered by NAC treatment.

Results of this study demonstrate that JNC43 has a strong anti-cancer effect on CRC cells while it has a low cytotoxicity to normal cells. It works as radiosensitizer by enhancing ROS accumulation, eventually leading to apoptotic cell death.

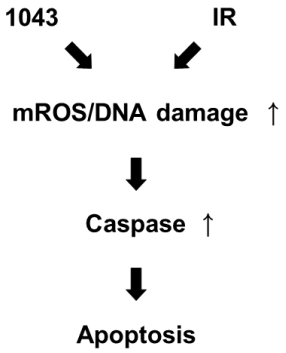


Fig. 8. Scheme of JNC43 and irradiation effects on CRC cells

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