Phytosphingosine in combination with ionizing radiation enhances apoptotic cell death in radiation-resistant cancer cells through ROS-dependent and -independent AIF release*

Moon-Taek Park¹, Min-Jung Kim¹,², Young-Hee Kang¹, Soon-Young Choi¹,², Jae-Hoon Lee¹, Jung-A Choi¹, Chang-Mo Kang¹, Chul-Koo Cho¹, Seongman Kang², Sangwoo Bae¹, Yun-Sil Lee¹, Hee Yong Chung³, and Su-Jae Lee₁*¹

¹Laboratory of Radiation Effect, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea, ²Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea, ³Department of Microbiology, College of Medicine, Hanyang University, Seoul 133-791, Korea

Running Title: Phytosphingosine can overcome radiation resistance.

Key Words: γ-radiation, phytosphingosine, ROS, activation of PARP-1, Bax translocation, mitochondria, AIF release, apoptosis

¹Abbreviations used: AIF, apoptosis-inducing factor; ROS, reactive oxygen species; NAC, N-acetylcysteine; PARP, poly ADP-ribose polymerase; DiOC₆(3), 3,3’dihexylocarbocyanine iodide; DIQ, 1,5-Dihydroxyisoquinoline

*This work was supported by Nuclear R&D Program from the Ministry of Science and Technology in Korea.

*To whom correspondence should be addressed:
Su-Jae Lee
Laboratory of Radiation Effect
Korea Institute of Radiological and Medical Sciences
Gongneung-Dong, Nowon-Ku, Seoul 139-706, Korea
Phone: 82-2-970-1324
Fax: 82-2-970-2402
E-mail: sjlee@kcch.re.kr
Abstract

The use of chemical modifiers as radiosensitizers in combination with low-dose irradiation may increase the therapeutic effect on cancer by overcoming a high apoptotic threshold. Here, we showed that phytosphingosine treatment in combination with γ-radiation enhanced apoptotic cell death of radiation-resistant human T-cell lymphoma in a caspase-independent manner. Combination treatment induced an increase in intracellular reactive oxygen species (ROS) level, mitochondrial relocalization of Bax, PARP-1 activation, and nuclear translocation of apoptosis inducing factor (AIF). siRNA targeting of AIF effectively protected cells from the combination treatment-induced cell death. An antioxidant, NAC inhibited Bax relocalization and AIF translocation, but not PARP-1 activation. Moreover, transfection of Bax-siRNA significantly inhibited AIF translocation. Pre-treatment of PARP-1 inhibitor, DPQ, or PARP-1-siRNA also partially attenuated AIF translocation whereas the same treatment did not affect intracellular ROS level and Bax redistribution. Taken together, these results demonstrate that enhancement of cell death of radiation-resistant cancer cells by phytosphingosine treatment in combination with γ-radiation is mediated by nuclear translocation of AIF which is in turn mediated both by ROS-dependent Bax relocalization and ROS-independent PARP-1 activation. The molecular signaling pathways that we elucidated in
this study may provide potential drug targets for radiation sensitization of cancers refractive to radiation therapy.

**Introduction**

Radiation resistance is a significant problem in the treatment of malignant tumors (1). Many factors affect susceptibility of tumor cells to radiation. Among them intrinsic apoptosis sensitivity or resistancy seems to play an important role (2). The use of chemical modifiers as radiosensitizers in combination with low-dose irradiation may increase the therapeutic efficacy by overcoming a high apoptotic threshold (3).

Mitochondrial membrane permeabilization is considered to be one of the initial events of the apoptotic process induced by chemotherapeutic drugs (4, 5, 6). Opening of the mitochondrial permeability transition pore, which is under the control of members of the Bcl-2 family, can result in the permeabilization of the outer mitochondrial membrane and subsequent release of potentially apoptogenic proteins such as cytochrome c and AIF from the intermembrane space (7, 8, 9). Cytosolic cytochrome c binds to Apaf-1 in a ternary complex with caspase-9, leading to activation of caspase-9, which in turn activates caspase-3 (7). Cleavage of ICAD (inhibitor of the caspase-activated DNase) by caspase-3 leads to activation of CAD (caspase-activated DNase) and cleavage of DNA into characteristic oligonucleosomal-length fragments (10). AIF
(apoptosis-inducing factor) was more recently cloned and identified as a mitochondrial intermembrane space protein with homology to bacterial NADH oxidoreductases. In response to apoptotic stimuli, AIF is released from mitochondria and translocates to the nucleus and participates in the induction of chromatin condensation, the exposure of phosphatidylserine in the outer leaf of the plasma membrane, and the dissipation of the mitochondrial transmembrane potential. These effects seem to be caspase-independent, since none of them are prevented by the broad spectrum caspase inhibitor Z-VAD-fmk and are independent of the apoptosome complex (8, 11).

Recent studies suggest that reactive oxygen species (ROS) may play an important role during apoptosis induction (12). Many stimuli such as TNF-α, anticancer drugs, and chemopreventive agents stimulate cells to produce ROS (13-22). ROS can directly activate the mitochondrial permeability transition and result in mitochondrial membrane potential (MMP) loss (23, 24). Mitochondrial dysfunction such as loss of MMP results in cytochrome c release from mitochondrion into cytoplasm. This release of cytochrome c leads to activation of DEVD-specific caspases and subsequent nuclear fragmentation in vitro (25).

PARP-1, the most abundant protein of the poly (ADP-ribose) polymerase family members, is rapidly activated by DNA damage. PARP-1 utilizes NAD+ to form poly
(ADP-ribose) (PAR) polymers on specific acceptor proteins. PARP-1 activation appears to facilitate DNA repair under moderate stress conditions (26, 27). However, under conditions that cause extensive DNA damage such as excitotoxicity and ischemia, PARP-1 activation causes NAD+ depletion leading to ATP depletion (28, 29) and subsequent cell death. Although PARP-1 mediated cell death has been thought to be necrotic (28, 29), recent reports have demonstrated that PARP-1 mediated cell death also has many features in common with apoptotic forms of cell death (30, 31), such as nuclear translocation of AIF from mitochondria.

Sphingolipid metabolites such as ceramides, sphingosines, sphingosine 1-phosphates, and phytosphingosine have emerged as key regulators of apoptosis (32). Sphingolipid metabolites also have been implicated as an important component of ionizing radiation-induced apoptosis of human cancer cells (33-36). This apoptotic pathway is initiated by hydrolysis of sphingomyelin, a membrane lipid, attributable to the activation of sphingomyelinases to generate ceramide. Ceramide, in turn, can activate several pathways important for the induction of apoptosis (37). Moreover, direct exposure of cells to the cell-permeable ceramide analogs also has been shown to sensitize cancer cells to ionizing radiation, which lends further support to the notion that ceramide generation might be an important step for radiation-induced apoptosis in
human cancer cells (38). Although many reports emphasized the contributions of ceramide to enhancement of the radiation response, the role of other sphingolipid metabolites in the modulation of radiation sensitivity and their precise action mechanisms were largely unknown.

In the present study, we investigated the mechanisms underlying modulation of radiation response by phytosphingosine in radiation-resistant human T-cell lymphoma. We demonstrated that combination treatment of phytosphingosine with radiation synergistically enhances caspase-independent apoptotic cell death of radiation-resistant Jurkat T cell clones. We showed that nuclear translocation of AIF from mitochondria is necessary for enhancement of cell death by combination treatment, and that ROS-mediated Bax translocation and ROS-independent PARP-1 activation are associated with AIF release from mitochondria. Our data provide a potential mechanism for radiosensitization effect of phytosphingosine, and suggest a potential clinical application of combination treatment of radiation-resistant cancers with ionizing radiation and phytosphingosine.

Materials and methods

Materials
Phytosphingosine was purchased from Avanti (Alabaster, AL). Antibodies specific for polyclonal anti-AIF, Hsp60 and Ref-1 were from Santa-Cruse (Santa Cruz, CA). β-actin was from Sigma (St Louis, MO). Anti-cleaved caspase-3 antibody and PARP were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-cytochrome c antibody was from Pharmingen (San Diego, CA). The specific PARP-1 inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) was from Calbiochem (Cambridge, MA). The broad spectrum caspase inhibitor, z-VAD-fmk was from Calbiochem (Cambridge, MA). Other chemicals were obtained from Calbiochem (Cambridge, MA).

**Cell culture**

Jurkat, human T cell lymphoma (Type II) was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Invitrogen), penicillin, and streptomycin at 37 °C in a humidified incubator with 5 % CO₂.

**Irradiation**

Cells were plated in 10 cm dishes and incubated at 37°C under humidified 5 % CO₂, 95 % air in culture medium until 70-80 % confluent. Cells were then exposed to γ-rays from a ³⁷Cs γ-ray source (Atomic Energy of Canada Ltd, Canada, located in Korea.
Establishment of ionizing radiation-resistant Jurkat clones

To establish ionizing radiation-resistant Jurkat clones, Jurkat T cells were first distributed into 96 well plates at a density of 0.2 cells per well. After 12 hr, individual wells were visually checked for the presence of a single cell. The clones were grown and tested for the radiation sensitivity, and those showing more than 95% survival rate at 48 hr after 10 Gy irradiation were chosen for further study as radiation-resistant clones. Among the 100 clones tested, clones #1, #6, #11 and #16 were chosen as radiation-resistant clones for further study. The average survival rate of parental Jurkat T cells at 48 hr after 10 Gy irradiation was about 64%.

Clonogenic survival assay in soft agar

Cells were plated onto 60-mm dishes at a density of $2 \times 10^6$ cells/dish and exposed to a range of doses of $\gamma$–radiation at 0 to 3 Gy. After irradiation, cells were harvested, and $10^4$ cells/dish were suspended in 2 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 2 ml of 0.8% agar-medium base layer in 60-mm dishes and followed by incubation for 14 days at $37^\circ C$ in 5% CO$_2$ incubator. Prior to counting colonies, the culture medium was
decanted and the cells were fixed in 95% methanol and stained with 0.5% crystal violet, and the numbers of colonies (> 50 cells) from triplicate dishes were counted. Mean colony numbers relative to unirradiated colony numbers were plotted.

**Small interfering RNA (siRNA) transfection**

RNA interferences of AIF or Bax were performed using a 21 base pair (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Ambion (Austin, TX, USA). The sense strand nucleotide sequence for AIF siRNA was GGAAUAUGGGAAAGAUCCdTdT. The sense strand nucleotide sequence for PARP-1 siRNA was GGCCAGGAUGGAUUGGUAdTdT. The coding strand for Bax siRNA was AACATGGAGCTGCAGAGGATGAdTdT. A control siRNA specific to the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG was used as a negative control. For transfection, γ-radiation resistant Jurkat clones were seeded in 6-well plates at 30 % confluency, and siRNA duplexes (200 nM) were introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations.

**Hoechst 33258 staining**

Hoechst 33258 staining was performed as described previously (39). Briefly, cells were fixed with 4 % paraformaldehyde for 30 min in room temperature and then washed
once with PBS. Hoechst 33258 (50 ng/ml) was added to the fixed cells, incubated for 30 min at room temperature, and washed with PBS. Cells were mounted and examined by fluorescence microscopy. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. At minimum, 500 cells were counted for each treatment.

**Measurement of mitochondrial membrane potential and ROS Generation**

The measurement of mitochondrial transmembrane potential ($\Delta \psi_m$) and reactive oxygen species (ROS) generation were performed as described by Marchetti et al. (40). Briefly, cells ($2 \times 10^6$/ml) were exposed to 10 Gy radiation and/or 5 µg/ml phytosphingosine for the indicated times. After exposure, cells were incubated in 30nM 3,3'-dihexyloxacarboxyanine iodide (DiO6(3)) (Molecular Probes, Eugene, OR), or 10 µM DCFH-DA (Molecular Probes, Eugene, OR) at 37 °C for 30 min to measure the mitochondrial membrane potential and ROS level respectively. The cells were harvested and washed with cold PBS solution for three times, and the ROS level and $\Delta \psi_m$ were determined by FACS analysis.

**Confocal Microscopy**

The $\gamma$-radiation-resistant Jurkat #6 clones treated with 10 Gy radiation and/or 5
µg/ml phytosphingosine were washed twice with ice-cold PBS and fixed with ice-cold methanol. After blocking with 2% bovine serum albumin in PBS containing 0.2% Triton X-100, cells were incubated with the primary antibodies against AIF or Bax for 1 h. Cells were washed with blocking solution three times and incubated with the secondary antibodies conjugated with FITC (Molecular Probes) for 1 hr. Nuclei were stained with 2.5 µg/ml propidium iodide (PI) (Sigma, St. Louis, MO) for 10 min, and the mitochondria were stained with 25 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min at room temperature. After washing three times with PBS, coverslips were mounted onto microscopic slides using ProLong antifade mounting reagent (Molecular Probes). The slides were analyzed by a confocal laser-scanning microscope (Leica Microsystems).

**Western Blot Analysis**

Western blot analysis was performed as described (41). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mM Tris-Cl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tris buffered saline and then incubated with primary antibodies for 1 hr at room temperature. Blots were developed
by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham Biosciences) according to the manufacturer's recommendations.

**Preparation of nuclear and mitochondrial fractions**

The cells were washed with ice-cold PBS, left on ice for 10 min and then resuspended in isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 10 mM Tris-HCl, pH 7.4) containing a proteinase inhibitor cocktail (Roche). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 x g for 5 min. The nuclei and heavy mitochondria fractions were fractionated at 750 x g for 10 min and 14,000 x g for 20 min, respectively from the supernatant. The nuclei fraction was washed three times with homogenization buffer containing 0.01% NP-40.

**Cellular ELISA method for detection of PARP-1 activation**

PARP enzyme activity was measured using a commercial kit (Oncogene, San Diego, CA, USA). Briefly, cell extracts were incubated in reaction buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM MgCl2) containing 0.01% digitonin and 10 µM biotinylated NAD+. After incubating at 37 °C for 30 min, reactions were developed with
TACS-Saphire (Trevigen) substrate (100 µl/well). The optical density was measured with a microplate spectrophotometer (Molecular Devices; Sunnyvale, CA). Data were expressed as mean ± SD of quadruplicate samples.

Results

Phytosphingosine treatment in combination with ionizing radiation enhances apoptotic cell death of radiation-resistant cancer cells.

We first selected variant clones from Jurkat, human T cell lymphoma, for resistance to ionizing radiation-induced apoptotic cell death. As shown in Fig. 1A, clonogenic survival assay revealed that clones #1, #6, #11 and #16 showed resistance to radiation compared with the parental Jurkat T cells. We next investigated whether the radiation resistant cells showed an increased clonogenic survival following radiation had also resistance to the radiation-induced apoptotic cell death. To examine radiation-induced apoptosis, the cells were treated with 10 Gy radiation, and were stained with Hoechst 33258 at 24 or 48 hr. In Fig. 1B, ionizing radiation at a single dose of 10 Gy effectively killed parental Jurkat T cells, but failed to induce cell death in clones #1, #6, #11 and #16 at 48hr after γ-irradiation. Previously we have reported that phytosphingosine can potently induce apoptotic cell death in Jurkat T cells (42, 43). To examine whether there
is a cross-resistance to the phytosphingosine in radiation-resistant Jurkat clones, we measured apoptotic cell death in parental Jurkat and radiation-resistant clones at 3hr after phytosphingosine treatment. A dramatic decrease in the number of apoptotic cells was observed in radiation-resistant clones, compared to the parental cells, suggesting that the radiation-resistant Jurkat clones have a cross-resistance to the phytosphingosine (Fig. 1C).

We next examined whether phytosphingosine treatment in combination with radiation had a sensitizing effect on cell death in radiation-resistant Jurkat clones. As shown in Fig. 1D, the combination treatment with phytosphingosine and γ-radiation indeed synergistically enhanced the apoptotic cell death of radiation-resistant Jurkat clones. However in parental Jurkat T cells, phytosphingosine did not show synergistic effect on cell death when simultaneously treated with γ-radiation. Instead, phytosphingosine treatment alone showed a significant cytotoxic effect. To determine the contribution of the mitochondrial pathway to the induction of apoptotic cell death seen after the combination treatment of phytosphingosine with γ-radiation, we examined changes in mitochondrial membrane potential ($\Delta\psi_m$). Fig. 1E shows a marked loss of $\Delta\psi_m$ in radiation-resistant cells treated with γ-radiation and phytosphingosine. We next investigated whether caspase activities are required for enhancement of apoptotic cell
death of radiation-resistant cells treated with \( \gamma \)-radiation and phytosphingosine. As shown in Fig. 1F, the combination treatment with \( \gamma \)-radiation and phytosphingosine did not affect the caspase activities. Moreover, a broad-spectrum caspase inhibitor, z-VAD-fmk did not attenuate the cell death induced by combination treatment (Fig. 1G). These findings suggest that phytosphingosine in combination with ionizing radiation synergistically enhances apoptotic cell death in ionizing radiation-resistant cells in a caspase-independent manner.

**Combination treatment of \( \gamma \)-radiation with phytosphingosine enhances apoptotic cell death through AIF translocation to nucleus.**

Since apoptosis inducing factor (AIF) is known to be involved in induction of apoptotic cell death through a caspase independent pathway (8, 44), we next examined whether AIF plays a role in apoptotic cell death induced by the combination treatment. AIF is a mitochondria-localized flavoprotein that is released and translocated to the nucleus, and causes nuclear condensation in response to death stimuli (8, 44). Subcellular fractionation showed that combination treatment of radiation-resistant cells with \( \gamma \)-radiation and phytosphingosine dramatically redistributed AIF from mitochondria to the nucleus (Fig. 2A). Confocal microscopy also clearly revealed that AIF was translocated to the nucleus and caused nuclear condensation after the
combination treatment (Fig. 2B). To test whether redistribution of AIF occurs in a caspase-independent manner, we examined the effect of z-VAD-fmk, a broad spectrum caspase inhibitor, on AIF translocation to nucleus. The z-VAD-fmk treatment did not affect intracellular redistribution of AIF and nucleus condensation seen after the combination treatment (Fig. 2B). Furthermore, experiment with small interfering RNA (siRNA) showed that siRNA targeting of AIF effectively attenuated the combination treatment-induced cell death (Fig. 2C). These results suggest that translocation of AIF from mitochondria to nucleus is required for caspase-independent cell death in radiation-resistant cells induced by combination treatment of γ-radiation with phytosphingosine.

**Combination treatment of γ-radiation with phytosphingosine induces Bax translocation to mitochondria, dissipation of mitochondrial membrane potential ($\Delta \psi_m$) and AIF translocation to nucleus.**

Since it has been shown that translocation of Bax from the cytosol to the mitochondria causes a decline of $\Delta \psi_m$ (45, 46, 47), we investigated whether the combination treatment with γ-radiation and phytosphingosine induces mitochondrial translocation of Bax. As shown in Fig. 3A, the combination treatment redistributed Bax from cytosol to the mitochondria without changing the protein expression levels of Bel-
2 and Bax (data not shown). Confocal microscopy also clearly revealed that Bax was translocated to the mitochondria (Fig. 3B). Furthermore, siRNA targeting of Bax effectively attenuated combination treatment-induced depolarization of $\Delta \psi_{m}$ and cell death (Fig. 3C and D). We also showed that siRNA targeting of Bax effectively inhibited nuclear translocation of AIF induced by the combination treatment (Fig. 3E). These results suggest that combination treatment with radiation and phytosphingosine induces Bax redistribution to mitochondria and subsequently promotes the loss of $\Delta \psi_{m}$ and translocation of AIF to nucleus.

**Role of ROS in apoptotic cell death of radiation-resistant cancer cells induced by combination treatment with $\gamma$-radiation and phytosphingosine**

Since sphingolipid metabolites have been implicated in intracellular ROS accumulation, which, in turn, can activate several pathways important for the induction of apoptosis (48, 49, 50), we examined involvement of ROS in combination treatment-induced cell death of radiation-resistant cells. As shown in Fig. 4A, ROS levels were dramatically increased after the combination treatment with $\gamma$-radiation and phytosphingosine in radiation-resistant clones and the increase in ROS level was effectively blocked by NAC. To determine the direct relationship between the increased intracellular ROS level and mitochondrial activation-mediated cell death pathway, cells
were pretreated with antioxidant N-acetyl-L-cysteine (NAC) before combination treatment. As shown in Fig. 4B and 4C, NAC significantly attenuated the combination treatment-induced loss of Δψₘ and cell death, suggesting that ROS plays an important role in enhancement of γ-radiation-induced cell death by phytosphingosine. Furthermore, NAC effectively blocked nuclear translocation of AIF induced by combination treatment with γ-radiation and phytosphingosine in radiation-resistant cells (Fig. 4D). Moreover, NAC also effectively blocked the combination treatment-induced Bax translocation to mitochondria (Fig. 4E). These observations suggest that ROS contributes to Bax-mediated AIF release and cell death induced by the combination treatment.

**Phytosphingosine treatment in combination with radiation induces PARP-1 activation leading to AIF-mediated cell death.**

Since it has been shown that ROS-mediated DNA damage triggers activation of the poly(ADP-ribose) polymerase-1 (PARP-1) and subsequent cell death (30, 51), we investigated whether PARP-1 is involved in combination treatment-induced apoptotic cell death. Intracellular PARP-1 enzyme assay and immunoblot analysis with the antibody against PARP-1 shows that combination treatment with radiation and phytosphingosine dramatically enhanced PARP-1 activity and poly (ADP-ribose)
(PAR) polymer formation in radiation-resistant cells (Fig. 5A and B). Pretreatment of DPQ, a specific PARP-1 inhibitor, or siRNA targeting of the PARP-1 effectively blocked PARP-1 activation and PAR polymer formation induced by the combination treatment. Furthermore, pretreatment of DPQ or PARP-1 siRNA attenuated the combination treatment-induced loss of $\Delta \psi_m$ and subsequent cell death (Fig. 5C and D) as well as PARP-1 activation and PAR formation (Fig. 5A and 5B). In addition, translocation of AIF to nucleus was effectively blocked by DPQ pretreatment (Fig. 5E). Nevertheless, DPQ pretreatment did not affect the Bax translocation to mitochondria (Fig. 5E). Moreover, the pretreatment of antioxidant NAC has no effect on combination treatment-induced PARP-1 activation (Fig. 6 A and B). These observations indicate that the PARP-1 activation, similarly to intracellular ROS, induces mitochondrial membrane potential loss, AIF translocation and cell death in a ROS-independent manner upon combination treatment. Taken together, these results suggest that apoptotic cell death induced by the combination treatment of phytosphingosine with $\gamma$-radiation is regulated by two pathways: one is ROS-dependent Bax redistribution that mediates AIF release and translocation to nucleus, and the other is ROS-independent PARP-1 activation that mediates nuclear translocation of AIF.

To confirm the hypothesis that combination treatment with $\gamma$-radiation and
phytosphingosine utilize two separate pathways to induce cell death in the \( \gamma \)-radiation-resistant cells, we treated NAC to the cells transfected with PARP-1 siRNA before combination treatment with \( \gamma \)-radiation and phytosphingosine. As shown in Fig. 7, the cells simultaneously treated with PARP-1 siRNA and NAC show more dramatic attenuation of the apoptotic cell death than the cells treated with either reagent alone.

**Discussion**

Previously, we have shown that phytosphingosine, a member of sphingolipid metabolites, had an anticancer effect on Jurkat T cell lymphoma and NCI-H460, a human non-small-cell lung cancer cells (42, 43). One of the important points in the development of a new anti-cancer drug is the understanding of its potential for inclusion in combination treatment regimens. In the present study, we examined whether the use of phytosphingosine in combination with \( \gamma \)-radiation may increase the therapeutic effect on cancer by overcoming a high apoptotic threshold. We demonstrated that combination treatment of phytosphingosine with \( \gamma \)-radiation synergistically enhanced caspase-independent apoptotic cell death of radiation-resistant Jurkat T cell clones. We also showed that nuclear translocation of AIF from mitochondria was necessary for enhancement of cell death by combination treatment, and that ROS-mediated Bax
translocation and ROS-independent PARP-1 activation were associated with AIF release from mitochondria.

We first established radiation-resistant variant clones from Jurkat cells, which showed resistance to radiation compared with the parental Jurkat T cells in both clonogenic survival and apoptotic cell death. The greatest difference in radiation-induced cell death between parental Jurkat and resistant cells was observed at 48 hr. Previously we have reported that phytosphingosine can potently induce apoptotic cell death in Jurkat T cells (42, 43). Consistently, phytosphingosine treatment alone showed a strong induction of cell death (close to 30 %) in parental Jurkat cells at 3 hr. However, in γ-radiation-resistant Jurkat T cell clones, phytosphingosine did not effectively induce cell death, which means that radiation-resistant clones show cross-resistance to phytosphingosine. Nevertheless, interestingly, combination treatment with γ-radiation and phytosphingosine strongly enhanced apoptotic cell death and disruption of mitochondrial membrane potential measured at 3 hr after the treatment in radiation-resistant Jurkat clones. However, phytosphingosine did not show synergistic effect on cell death in parental Jurkat T cells upon combination treatment with γ-radiation at the same experimental condition as above. Moreover, in the previous study, we have shown that down-regulation of the ERK pathway is critical in the death receptor-independent
activation of caspase-8, and activation of p38 MAPK is essential for mitochondria-mediated caspase-9 activation dependent cell death in phytosphingosine-treated Jurkat cells (43), in marked contrast, we failed to detect any changes in ERK and p38 MAPK activity in radiation-resistant Jurkat clones treated with phytosphingosine alone or in combination with γ-radiation (data not shown). These results suggest that differential regulatory mechanisms of cell death are operating in parental Jurkat cells compared to radiation-resistant Jurkat clones. Moreover, the combination treatment with radiation and phytosphingosine did not affect the activity of caspases in radiation-resistant clones, and the caspase inhibitors failed to attenuate cell death, suggesting that the apoptotic cell death induced by the combination treatment is caspase-independent in these variant clones, thus explaining the different outcomes of combination treatment between the parental cells and variant resistant clones.

It has been reported that apoptosis inducing factor (AIF) mediates cell death through a caspase-independent pathway. Mitochondrial AIF translocates to the nucleus upon death stimuli and initiates nuclear condensation (8, 11). Once nucleus condenses, this leads to large-scale chromatin fragmentation followed by the cell death (8, 11). Consistent with these findings, we found translocation of AIF from mitochondria to the nucleus and nuclear shrinkage after the combination treatment of phytosphingosine with
radiation in radiation-resistant clones. Furthermore, siRNA knockdown of AIF in radiation-resistant clones effectively attenuated cell death induced by the combination treatment. Recently, several reports proposed that AIF is indeed a caspase-dependent death effector (52), implicating that the translocation of AIF to the nucleus is caspase-dependent. However, we found that caspase inhibitors failed to attenuate AIF translocation and nuclear shrinkage, suggesting that the combination treatment of phytosphingosine with γ-radiation induces nuclear redistribution of AIF in a caspase-independent manner during apoptotic process.

In response to stimuli such as TGF-β, etoposie, NO, staurosporine and UV, which require mitochondria-dependent pathway for apoptosis, Bax become activated, translocated to the outer membrane of mitochondria and oligomerized therein (53-57). The mitochondrial membrane permeabilization and the release of mitochondrial apoptogenic molecules into the cytosol ensue. We also found the combination treatment with γ-radiation and phytosphingosine redistributed Bax from cytosol to the mitochondria. Moreover, siRNA targeting of Bax effectively attenuated mitochondrial membrane potential (Δψm) loss and AIF translocation to the nucleus seen after the combination treatment. These results suggest that mitochondrial redistribution of Bax may triggers Δψm loss and causes subsequent AIF translocation to the nucleus.
following the combined treatment with γ-radiation and phytosphingosine in radiation-resistant cancer cells.

ROS production frequently occurs in cells exposed to UV light, ionizing radiation, H₂O₂, or cytokines (58, 59, 60) and accumulation of intracellular ROS leads to disruption of the Δψₘ and subsequent activation of cell death machinery (61, 62). Moreover, experiments using antioxidants indicated that ROS act upstream of Bax relocalization and mitochondrial membrane depolarization (58). Similarly, an early increase in ROS levels has been found to precede mitochondrial membrane permeabilization and in some cases to be independent of caspases, in various models including apoptosis induced by Fas (63), p53 (64), ischemia (65), DNA alkylation (66) or etoposide (54). In this study, we provided further evidence that increased level of ROS is involved in the Bax translocation to the mitochondria, Δψₘ loss, AIF release and cell death triggered by the combination treatment of phytosphingosine with γ-radiation in radiation-resistant Jurkat clones. Complete inhibition of Bax translocation to the mitochondria by thiol-containing antioxidant, NAC, suggests that increased intracellular ROS level is critical for the Bax relocalization after the combination treatment. However, as the AIF translocation to the nucleus and cell death induced by the combination treatment is not fully inhibited by NAC, it is possible that undefined
signals other than ROS also play an important role in combination treatment-induced AIF translocation and subsequent cell death.

PARP-1 is a nuclear enzyme that facilitates DNA repair in response to DNA damage (26, 27). Recently it has been shown that excessive activation of PARP-1 results in depletion of cellular NAD and ATP, eventually leading to cell death (30, 31). Although PARP-1-mediated cell death is thought to be necrotic (67, 68), recent reports have demonstrated that PARP-1 mediated cell death also has many features in common with apoptotic forms of the cell death (27, 30). These studies suggested that translocation of AIF from mitochondria to the nucleus is required for PARP-1-mediated cell death. We also found that PARP-1 activation is involved in the nuclear translocation of AIF and subsequent cell death induced by the combination treatment with \(\gamma\)-radiation and phytosphingosine in radiation-resistant Jurkat clones. Pretreatment of cells with PARP-1 inhibitor or transfection of PARP-1 siRNA efficiently attenuated the disruption of mitochondrial membrane potential, AIF translocation, nuclear condensation and subsequent cell death induced by the combination treatment. However, Bax translocation to mitochondria was not blocked by inhibition of PARP-1, indicating that the Bax translocation is independent of PARP-1 activation during the apoptotic process induced by the combination treatment. It is possible that PARP-1 activation by
combination treatment in our experimental system is caused by ROS-dependent DNA damage. Recently it has been shown that ROS-mediated DNA damage triggers activation of PARP-1 and subsequent cell death (69). Consistent with these findings, we found that combination treatment induces significant degree of DNA damage as assessed by the phosphorylation level of histone H2AX (γ-H2AX) and its foci formation (data not shown), an established marker for double strand breakages (DSBs) in chromosomal DNA (70, 71). However, a thiol-containing antioxidant, NAC failed to block PARP-1 activation and DNA damage (data not shown) induced by the combination treatment, indicating that PARP-1 activation and DNA damage by combination treatment in our experimental system is independent on ROS production. In this regard, it has been reported that ROS generation by UVB is not involved in UVB-induced DNA damage (73). However, mechanism by which ROS-independent DNA damage and activation of PARP-1 occur remains unclear.

Taken together, we have shown that combination treatment with γ-radiation and phytosphingosine can overcome the radiation-resistance through the caspase-independent cell death pathway by enhancing ROS generation and PARP-1 activation. The enhancement of intracellular ROS level by combination treatment promotes mitochondrial translocaion of Bax leading to the collapse of mitochondria membrane
potential and subsequent AIF release. PARP-1 activation also affects mitochondria membrane potential loss and subsequent AIF release. Interestingly, phytosphingosine appears to trigger two independent cell death pathways to overcome radiation-resistance in Jurkat variant clones. Our results suggest that the combination treatment of phytosphingosine and γ-radiation is potentially an effective way of treating cancers refractive to conventional radiation therapy.

**Acknowledgements**

This work was supported by a Nuclear R&D Program from the Ministry of Science and Technology in Korea.

**Reference**


18. Simizu S, Takada M, Umezava K, Imoto M. Requirement of caspase-3(-like) proteases-mediated hydrogen peroxide production for apoptosis induced by various


40. Marchetti P, Castedo M, Susin SA, et al. Mitochondrial permeability transition is a


Figure legends

Figure 1. Phytosphingosine in combination with ionizing radiation enhances apoptotic cell death in radiation-resistant cancer cells.

A) Selection of radiation-resistant Jurkat clones. Single cells in limiting dilution condition (0.2 cells/well) were incubated in 96 well plates for 2 months and then individual clones were irradiated with increasing doses of γ-radiation. Cells were allowed to grow on soft agar for 10–14 days and were stained with 0.5% crystal violet and scored for colony formation. Results are given as means ±S.E.M. of three independent experiments. B) Individual clones were treated with 10 Gy of γ-radiation and cultured for 24 or 48hr. Cells were stained with Hoechst 33258, and apoptotic cells were analyzed by fluorescence microscopy. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell numbers measured. Results from three independent experiments are shown as means ±S.E.M. C) Cross-resistance to the phytosphingosine in radiation-resistant clones. Radiation-resistant Jurkat clones were treated with 5 μg/ml of phytosphingosine. After 3 hr, cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ±S.E.M. D) Phytosphingosine sensitizes radiation-resistant Jurkat clones to
radiation-induced apoptotic cell death. Radiation-resistant Jurkat clones were treated with 10 Gy of γ-radiation alone, 5 µg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 µg/ml). After 3 hr, cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M. E) Mitochondrial transmembrane potential was determined by retention of DioC₆(3) added during the last 30 min of treatment. After removal of the medium, the amounts of retained DioC₆(3) were measured by flow cytometry. F) Cell lysates of clone #6 treated with 10 Gy of γ-radiation and/or 5 µg/ml of phytosphingosine were subjected to western blot analysis with anti-caspase-8, -Bid, -caspase-9, -caspase-3 and -PARP antibodies. The data represent a typical experiment conducted at least three times with similar results. G) Cells were treated with 10 Gy of γ-radiation and/or 5 µg/ml of phytosphingosine in the presence or absence of 30 µM z-VAD-fmk. After 3 hr, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M.

**Figure 2. Combination treatment of phytosphingosine with ionizing radiation induces AIF translocation to nucleus.**

A) Analysis of AIF translocation by subcellular fractionation. Nuclear fraction was
obtained from the Jurkat clones treated with 10 Gy of γ-radiation alone, 5 µg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 µg/ml), and was subjected to Western blot analysis with anti-AIF and -Ref-1 antibodies. Ref-1 was used as a nuclear marker protein. B) Representative confocal images for translocation of AIF to the nucleus and nuclear condensation after combination treatment in clone #6 in the absence or presence of 30 µM z-VAD-fmk. The nuclear translocation of AIF is demonstrated by the overlap of AIF (green) and nuclear staining (red), as noted by yellow color. C) siRNA targeting of AIF attenuates combination treatment-induced cell death. The clone #6 transfected with AIF siRNA was treated with 10 Gy of γ-radiation and/or 5 µg/ml of phytosphingosine. After 3 hr, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M.

**Figure 3. Combination treatment of ionizing radiation with phytosphingosine enhances Bax translocation to mitochondria**

A) Analysis of Bax translocation by subcellular fractionation. Mitochondrial fractionation was performed with Jurkat clones treated with 10 Gy of γ-radiation alone, 5 µg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 µg/ml). After 3 hr, proteins were subjected to Western blot analysis
with anti-Bax and HSP 60 antibodies. HSP 60 was used as a mitochondrial marker protein. B) Representative confocal images for translocation of Bax to the mitochondria. Clone #6 was incubated with Mitotracker and stained with anti-Bax antibody and analyzed by confocal laser scanning microscopy. Mitochondrial localization of Bax was defined by *yellow spots*, indicating overlap of fluorescein isothiocyanate (Bax) and Mitotracker-Red. C) siRNA targeting of the Bax attenuates combination treatment-induced cell death. The clone #6 transfected with Bax siRNA was treated with 10 Gy of γ-radiation alone and/or 5 µg/ml of phytosphingosine. After 3 hr, cells were stained with Hoechst 33258 and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M. D) The clone #6 transfected with Bax siRNA was treated with 10 Gy of γ-radiation and/or 5 µg/ml of phytosphingosine. After 3 hr, mitochondrial transmembrane potential of these cells was determined by retention of DioC6(3) added during the last 30 min of treatment. After removal of the medium, the amount of retained DioC6(3) were measured by flow cytometry. E) The clone #6 transfected with Bax siRNA was treated with 10 Gy of γ-radiation and/or 5 µg/ml of phytosphingosine. After 3 hr, nuclear fraction was prepared, and was subjected to Western blot analysis with anti-AIF and -Ref-1 antibodies. Ref-1 was used as a nuclear marker protein.
Figure 4. Enhancement of ROS production by combination treatment of ionizing radiation with phytosphingosine.

Radiation-resistant Jurkat clones were treated with 10 Gy of γ-radiation alone, 5 μg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 μg/ml) in the presence or absence of 10 mM NAC. A) After 3 hr, cells were incubated with 10 μM of H2DCF-DA for 30 min and analyzed by flow cytometry as described under “Materials and methods.” B) Mitochondrial transmembrane potential was determined by retention of DioC₆(3) added during the last 30 min of treatment. After removal of the medium, the amount of retained DioC₆(3) were measured by flow cytometry. C) Cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M. D) Clone #6 was treated with 10 Gy of γ-radiation and/or 5 μg/ml of phytosphingosine in the presence or absence of 10 mM NAC. After 3 hr, nuclear fraction was prepared and subjected to Western blot analysis using anti-AIF or -Ref-1 antibody. Ref-1 was used as a nuclear marker protein. E) Clone #6 was treated with 10 Gy of γ-radiation and/or 5 μg/ml of phytosphingosine in presence or absence of 10 mM NAC. After 3 hr, mitochondrial fraction was prepared, and was subjected to Western blot analysis with anti-Bax and -HSP60 antibodies. HSP 60 was used as a
mitochondrial marker protein.

**Figure 5. Enhancement of PARP-1 activation by combination treatment of ionizing radiation with phytosphingosine.**

A) Clone #6 was treated with 10 Gy of \( \gamma \)-radiation alone, 5 \( \mu \)g/ml of phytosphingosine alone, or combination of \( \gamma \)-radiation (10 Gy) and phytosphingosine (5 \( \mu \)g/ml) in the presence or absence of 30 \( \mu \)M DPQ, or PARP-1 siRNA. After 3 hr, PARP enzyme activity was measured using a commercial kit under guidance of manufacturer (see Material and Methods). B) Radiation-resistant cells were treated with 10 Gy of \( \gamma \)-radiation and/or 5 \( \mu \)g/ml of phytosphingosine in the presence or absence of 30 \( \mu \)M DPQ, or PARP-1 siRNA. After 3 hr, cell lysates were subjected to Western blot analysis with anti-PARP-1 and \( \beta \)-actin antibodies. The data represent a typical experiment conducted at least three times with similar results. C) Radiation-resistant cells were treated with 10 Gy of \( \gamma \)-radiation and 5 \( \mu \)g/ml of phytosphingosine in the presence or absence of 30 \( \mu \)M DPQ. After 3 hr, mitochondrial transmembrane potential of these cells was determined by retention of DioC\(_6\)(3) added during the last 30 min of treatment. After removal of the medium, the amounts of retained DioC\(_6\)(3) were measured by flow cytometry. D) Radiation-resistant cells were treated with 10 Gy of \( \gamma \)-radiation and/or 5 \( \mu \)g/ml of phytosphingosine in the presence or absence of 30 \( \mu \)M DPQ. After 3 hr, cells were
stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M.

E) Clones #6 was treated with 10 Gy of γ-radiation and/or 5 μg/ml of phytosphingosine in the presence or absence of 30 μM DPQ and 10mM NAC. After 3 hr, mitochondrial or nuclear fractions were prepared. Mitochondrial protein fraction was subjected to Western blot analysis with anti-Bax and -HSP60 antibodies, and nuclear protein fraction was subjected to Western blot analysis with anti-AIF and -Ref-1 antibodies. HSP 60 and Ref-1 were used as mitochondria and nuclear marker proteins, respectively.

**Figure 6. PARP-1 activation induced by combination treatment of ionizing radiation with phytosphingosine is independent from ROS-generation.**

A) Radiation-resistant cells were treated with 10 Gy of γ-radiation alone, 5 μg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 μg/ml) in the presence or absence of 10 mM NAC. After 3 hr, cell lysates were subjected to Western blot analysis with anti-PAR and β-actin antibodies. The data represent a typical experiment conducted at least three times with similar results. B) Clone #6 was treated with 10 Gy of γ-radiation alone, 5 μg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 μg/ml) in the presence or absence of 10 mM NAC. After 3 hr, PARP enzyme activity was measured.
using a commercial kit under guidance of manufacturer (see Material and Methods).

Figure 7. **Enhancement of cell death after combination treatment of ionizing radiation with phytosphingosine is mediated both by ROS generation and PARP-1 activation pathways.**

Clone #6 was treated with 10 Gy of radiation alone, 5 µg/ml of phytosphingosine alone, or combination of γ-radiation (10 Gy) and phytosphingosine (5 µg/ml) in the presence or absence of 10 mM NAC or siRNA for PARP-1. After 3 hr, cells were stained with Hoechst 33258, and apoptotic cells were quantitated by fluorescence microscopy. Results from three independent experiments are shown as means ± S.E.M.
Figure 1

A

B

C
Figure 1 continued

D

Apoptotic cells (%)

Jurkat  #1  #6  #11  #16

Cont  IR  PS  IR+PS

E

Loss of MMP (%)

Jurkat  #1  #6  #11  #16

Cont  IR  PS  IR+PS

F

IR (10Gy)  -  +  -  +
PS (6 μg/ml)  -  -  +  +

Caspase-8  proform
Bid  proform
Caspase-9  proform
Caspase-3  proform
PAPR  proform
β-actin  proform
Figure 1 continued

![Graph showing apoptotic cell percentages for different conditions and patient samples.](image)

- Cont
- IR
- PS
- IR+PS
- z-VAD-fmk+PS
- z-VAD-fmk+IR+PS

Apoptotic cells (%) vs. Patient sample #1, #6, #11, #16.
Figure 2

A

<table>
<thead>
<tr>
<th>Nuclear fraction</th>
<th>IR (10Gy)</th>
<th>PS (5 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ref-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AIF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ref-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AIF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#11</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ref-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AIF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#16</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ref-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AIF</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B

FITC-AIF

PI

Merge

C

<table>
<thead>
<tr>
<th>si:Ref-1</th>
<th>si:Alf</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td></td>
</tr>
</tbody>
</table>

AIF

Apoptotic cells (%) vs. IR (10Gy) vs. PS (5 μg/ml) vs. si:Alf
**Figure 3**

### A

<table>
<thead>
<tr>
<th>Mitochondrial fraction</th>
<th>IR (10 Gy)</th>
<th>PS (5 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>#1</th>
<th>#6</th>
<th>#11</th>
<th>#16</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- Bax
- HSP 60

### B

- FITC-Bax
- Mitotracker
- Merge

### C

![Graph](image5.png)

- Apoptotic cells (%)
- Conditions: cont, IR, PS, IR+PS, siBAX, siBAX+IR, siBAX+IR+PS
Figure 3 continued

**D**

![Graph showing Loss of MMP (ΔΨm)]

**E**

<table>
<thead>
<tr>
<th>Condition</th>
<th>AIF (nuclear)</th>
<th>Ref-1 (nuclear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Cont)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IR (10Gy)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PS (6 μg/ml)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>siBax</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>siBax + IR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>siBax + PS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>siBax + PS + IR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>siBax + IR + PS</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend:
- : present
- : absent
Figure 4

A

B

C

52
Figure 4 continued

D

<table>
<thead>
<tr>
<th>Condition</th>
<th>AIF (nuclear)</th>
<th>Ref-1 (nuclear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (10 Gy)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PS (5 μg/ml)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NAC (10 μM)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bax (Mitochondrial)</th>
<th>HSP 60 (Mitochondrial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (10 Gy)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PS (5 μg/ml)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NAC (10 μM)</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5

A

PARP activity

IR (10 Gy) - + + + + +
PS (5 μg/ml) - + + + + +
DPQ (30 μM) - - - + - +
si-PARP-1 - - - + + +

B

IR (10 Gy) - + + + + +
PS (5 μg/ml) - - + + + +
DPQ (30 μM) - - - + - +
si-PARP-1 - - - + + +

#1 PAR β-actin
#6 PAR β-actin
#11 PAR β-actin
#16 PAR β-actin
Figure 5 continued

C

![Bar chart showing loss of MMP (%) for different treatments.]

D

![Bar chart showing apoptotic cells (%) for different treatments.]

E

<table>
<thead>
<tr>
<th>Treatment</th>
<th>#1</th>
<th>#6</th>
<th>#11</th>
<th>#16</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (10 Gy)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PS (5 μg/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DPQ (30 μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NAC (10 mM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Bax (mitochondrial)  
HSP60 (mitochondrial)  
AIF (nuclear)  
Ref-1 (nuclear)
Figure 6

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (10 Gy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS (5 μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAC (10 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#1
- PAR
- β-actin

#6
- PAR
- β-actin

#11
- PAR
- β-actin

#16
- PAR
- β-actin

B

![Graph showing PARP-1 activity](image)
Figure 7
Phytosphingosine in combination with ionizing radiation enhances apoptotic cell death in radiation-resistant cancer cells through ROS-dependent and -independent AIF release

Moon-Taek Park, Min-Jung Kim, Young-Hee Kang, Soon-Young Choi, Jae-Hoon Lee, Jung-A Choi, Chang-Mo Kang, Chul-Koo Cho, Seongman Kang, Sangwoo Bae, Yun-Sil Lee, Hee Y Chung and Su-Jae Lee