A new 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one, synergistically enhances radiation sensitivity in human cervical cancer cells

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Radiation resistance can be overcome by a combination treatment with chemical modifiers. Here, we showed that treatment with 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP), a new 2-pyrone derivative, in combination with ionizing radiation enhances the sensitivity of human cervical cancer cells to ionizing radiation through overproduction of reactive oxygen species (ROS). The combined treatment with BHP and ionizing radiation caused a decrease in clonogenic survival and an increase in apoptotic cell death in cervical cancer cells. The combined treatment promoted conformational activation of Bax and led to mitochondrial apoptotic cell death. The combination treatment also induced a marked increase in intracellular ROS level. Inhibition of ROS attenuated the radiosensitizing effect of BHP, concurrent with a decrease in Bax activation, a decrease in mitochondrial cell death, and an increase in clonogenic survival. These results indicate that BHP synergistically enhances sensitivity of human cervical cancer cells to ionizing radiation through elevation of intracellular ROS and that ROS-dependent Bax activation is critically involved in the increase in apoptotic cell death induced by the combined treatment with BHP and ionizing radiation.

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Introduction

The use of chemical modifiers in conjunction with conventional radiation therapy may increase the effectiveness of ionizing radiation against cancer by overcoming high-cellular apoptotic thresholds [1–3]. However, combination treatment can increase toxicity and may therefore require a reduction in the dose of ionizing radiation, potentially worsening the treatment outcome. Moreover, acquisition of resistance to ionizing radiation and toxicity of chemical modifiers toward normal cells are the major causes of treatment failure in most solid tumors [4–6]. To overcome these problems, the development of additional combination treatments that are more selectively toxic to tumor cells is required.

It has become increasingly apparent that reactive oxygen species (ROS) are established modifiers of cellular functions affecting development, growth, aging, survival, and cell death [7–10]. Many stimuli, including tumor necrosis factor α, anti-cancer drugs, and chemopreventive agents, stimulate cells to produce intracellular ROS [7]. Moreover, overproduction of intracellular ROS by certain agents in combination with radiation can enhance a lethal chain reaction that results in damaged cellular integrity and cell death in human cancer cells [11]. This combined effect on cell death by overproduction of ROS has been exploited as a potentially effective strategy for treatment of various cancers [12].

2-Pyrone, a six-membered cyclic unsaturated ester that is highly abundant in bacterial, plant, and animal systems [13,14] is implicated in many different biological processes such as defense against other organisms [15]. Moreover, accumulating evidence indicates that microbial 2-pyrones, dihydro-2-pyrones, and secondary metabolites exhibit a wide range of antifungal, cytotoxic, neurotoxic, and phytotoxic properties [16]. These compounds further display antitumor and HIV-inhibiting qualities [17–19], suggesting medicinal importance of 2-pyrones. Especially, in a study aimed at investigating the anticancer properties of tricyclic 2-pyrones, the compounds prevented DNA synthesis and cell growth in human leukemic cells *in vitro* [20]. Although there is evidence that 2-pyrones display anticancer properties through prevention of DNA synthesis and cell growth in human leukemic cells, the effects of 2-pyrones on modulation of radiation resistance in human solid cancer cells are largely unknown.

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In this study, we show that 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP), a new 2-pyrone derivative, synergistically enhances the response of human cervical cancer cells to ionizing radiation. Moreover, we dissected the mechanisms underlying the modulation of radiation response by BHP. The results that we elucidated in this study may provide insights that aid in the design of future combination cancer therapies against cells that are intrinsically less sensitive to ionizing radiation treatment.

Materials and methods

Materials

Antibodies specific for polyclonal anti-apoptosis-inducing factor (AIF), anti-cytochrome *c*, anti-α-tubulin, and anti-HSP60 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Antibodies specific for polyclonal anti-cleaved caspase-3 and anti-poly (ADP-ribose) polymerase were from Cell Signaling Technology (Beverly, Massachusetts, USA). Monoclonal anti-Bax and anti-Bak antibodies were from Pharmingen (San Diego, California, USA).

Construction of 2-pyrone derivative, 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one

A new 2-pyrone derivative, BHP, was constructed as described earlier (Fig. 1a) [15].

Cell culture and transfection of small-interfering RNA

Human cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and nonessential amino acids. The media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), and cells were incubated at 37°C in 5% CO_2. Cells were transfected with specific small-interfering RNA duplexes purchased from Ambion (Austin, Texas, USA), using lipofectamine agent (Invitrogen, California, USA) according to the manufacturer's recommendations.

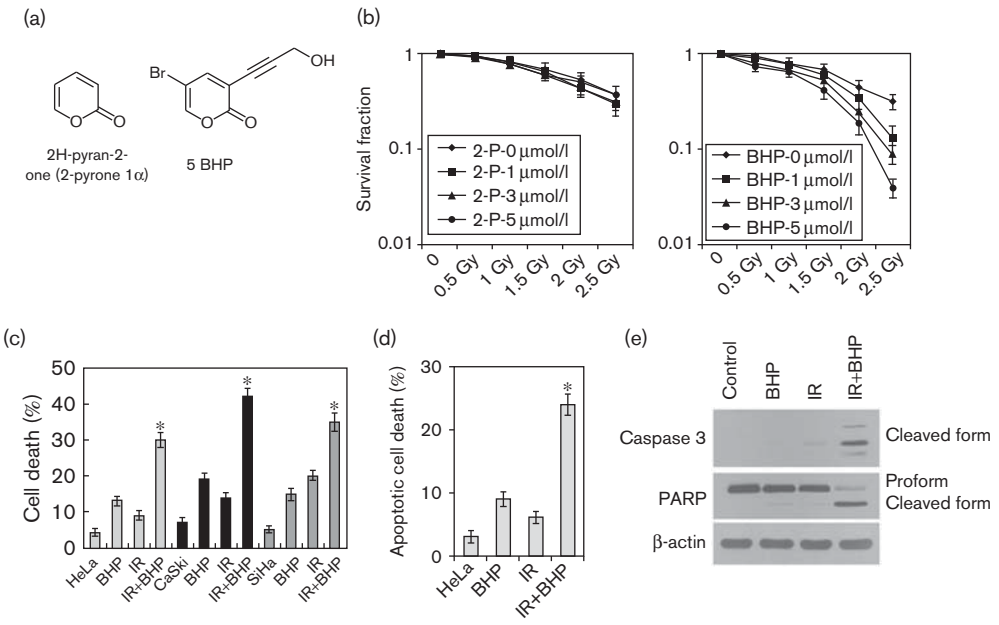
Clonogenic survival assay

Cells were plated in triplicate into 60-mm culture dishes at a density of 500 colonies per dish. Next day, cells were treated with BHP and then were exposed to ionizing radiation. After 7 days of incubation, the culture medium was decanted and the colonies were fixed with a mixture of 75% methanol and 25% acetic acid. Colonies were stained with 0.4% trypan blue dye and the number of colonies containing more than 5 mm cells was counted.

Quantification of cell death

Cell death was investigated by both propidium iodide staining, which detects cell death by means of the dye

Fig. 1



5-Bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP) synergistically enhances the response of human cervical cancer cells to ionizing radiation. (a) The chemical structure of 2-pyrone 1α and BHP. (b) HeLa cells were allowed to grow in the presence of 2-pyrone 1α (1, 3 or 5 μmol/l) or BHP (1, 3 or 5 μmol/l) in combination with indicated dose of ionizing radiation. After 7 days, colonies were stained with 0.4% trypan blue and scored for colony formation. Results from three independent experiments are presented as mean ± standard error of the mean (SEM). (c) HeLa, SiHa, and CaSki cells were treated with BHP (5 μmol/l) alone or in combination with ionizing radiation (5 Gy). After 48 h, cell death was measured as the percentage of propidium iodide (PI)-positive cells and determined by flow-cytometric analysis with annexin-V staining. Results from three independent experiments are presented as means ± SEM. Significantly different from control; $^*P<0.01$. (e) Total cell lysates were subjected to immunoblot analysis with anti-caspase-3, anti-poly (ADP-ribose) polymerase (PARP)-1, and anti-actin antibodies. IR, ionizing radiation.

entering the cells, and by annexin-V labeling using a kit according to manufacturer's directions (Sigma-Aldrich, St. Louis, Missouri, USA). For the assessment of cell death, the cells were plated in a 60-mm dish with a cell density of 2×10^5 cells/dish and treated with ionizing radiation and 2-pyrone derivatives the next day. After 48 h, cells were harvested and washed in phosphate-buffered saline (PBS). Annexin-V-positive early apoptotic cells or propidium iodide-positive cells were quantified by a FACScan flow cytometer fitted with CellQuestPro software (BD Biosciences, San Jose, California, USA).

Measurement of mitochondrial membrane potential and reactive oxygen species generation

In brief, cells were incubated in 3,3-dihexyloxacarboxyanine iodide (40 nmol/l) or 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; 10 μmol/l; Molecular Probes, Eugene, Oregon, USA) at 37°C for 15 min and washed with cold PBS three times. Retained 3,3-dihexyloxacarboxyanine iodide and DCFH-DA were analyzed by flow cytometry fitted with CellQuestPro software (Becton Dickinson, Seoul, Korea). For MitoSox Red staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After cell fixation, cells were incubated with MitoSox Red in PBS. Stained cells were then visualized with a fluorescence microscope (Olympus IX71, Seoul, Korea) or quantified with flow cytometry.

Preparation of cytosolic and mitochondrial fractions

The cells were washed with ice-cold PBS, left on ice for 10 min, and then resuspended in isotonic homogenization buffer [sucrose (250 mmol/l), KCl (10 mmol/l), $MgCl_2$ (1.5 mmol/l), Na-EDTA (1 mmol/l), Na-ethylene glycol tetra-acetic acid (1 mmol/l), dithiothreitol (1 mmol/l), phenylmethylsulfonylfluoride (0.1 mmol/l), and Tris–HCl (10 mmol/l, pH7.4)] containing proteinase inhibitor mixture (Roche, Basel, Switzerland). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at $30 \times g$ for 5 min. The mitochondria fractions were fractionated at $800 \times g$ for 10 min and $14\,000 \times g$ for 30 min, respectively, from the supernatant. For cytosolic fractionation, after 10 strokes with a loose homogenizer, the supernatant was collected after spinning at $800 \times g$ for 10 min and at $14\,000 \times g$ for 30 min.

Cross linking of Bax and Bak proteins

Cells were permeabilized at room temperature with 0.015–0.02% digitonin for 1–2 min in an isotonic buffer A [HEPES (10 mmol/l), NaCl (150 mmol/l), $MgCl_2$ (150 mmol/l), ethylene glycol tetra-acetic acid (1 mmol/l), pH 7.4] containing protease inhibitors. The permeabilized cells were shifted to 4°C, scraped, and collected into centrifuge tubes. The supernatants (digitonin/cytosol) were collected after centrifugation at $15\,000 \times g$ for 10 min at 4°C. The pellet was further extracted with ice-cold lysis buffer {[2% 3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in buffer A containing protease inhibitors} for 60 min at 4°C to obtain membrane fraction. Cells permeabilized with digitonin or membranes extracted with CHAPS were incubated with cross-linker (disuccinimidyl suberate with linker lengths of 11.4 Å) on a head-to-head rocker for 30 min at room temperature. After quenching the unreacted cross-linkers with 1/10 volume of Tris–HCl ((2 mol/l, pH 7.4), cells or extracts were incubated for another 30 min at room temperature with rocking. After cross linking, membranes were extracted with 2% CHAPS in buffer A and mixed with a nondenaturing loading buffer before SDS-polyacrylamide gel electrophoresis.

Statistical analysis

All experimental data are reported as mean and the error bars represent the experimental standard error. Statistical analysis was performed by the nonparametric Student *t*-test.

Results

5-Bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one synergistically enhances the response of human cervical cancer cells to ionizing radiation

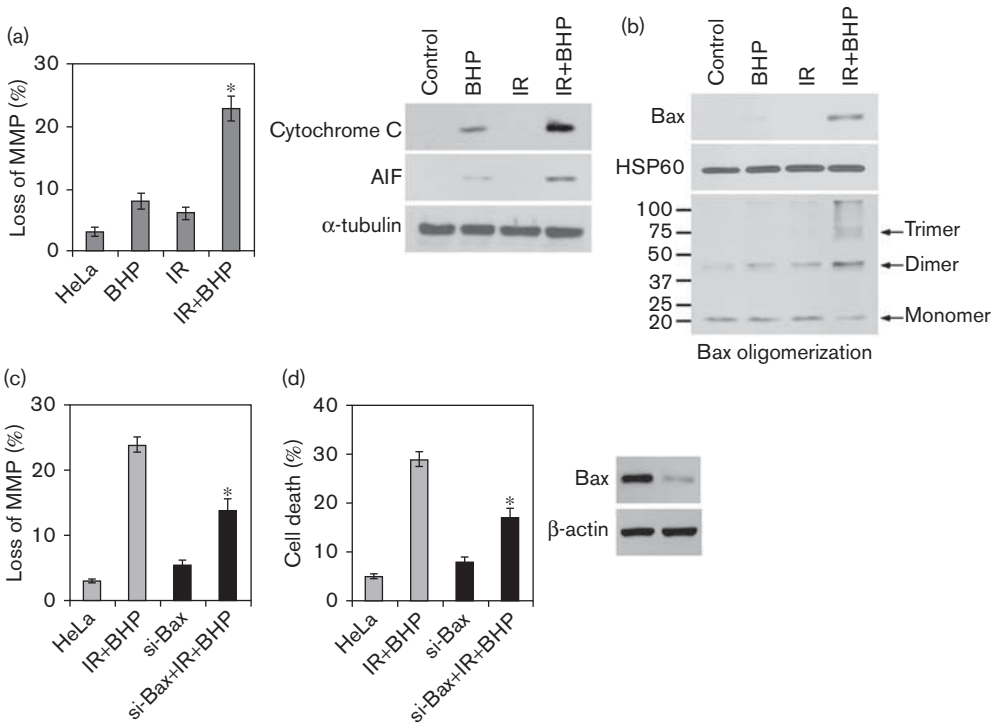
To examine whether a new 2-pyrone derivative, BHP, enhances sensitivity of human cervical cancer cells to ionizing radiation, we treated HeLa cells with 2-pyrone 1α or its derivative BHP together with ionizing radiation; we then analyzed clonogenic survival using the colony-forming assay and apoptotic cell death. As shown in Fig. 1b, clonogenic survival assay revealed that treatment of BHP (1, 3, or 5 μmol/l) in combination with ionizing radiation (0.5, 1, 1.5, 2, or 2.5 Gy) clearly enhanced the response of human cervical cancer cells (HeLa) to ionizing radiation, but the effects of 2-pyrone 1α in combination with ionizing radiation on clonogenic survival were subtle. To further examine whether treatment of BHP in combination with ionizing radiation induces cell death in human cervical cancer cells, three different human cervical cancer cell lines (HeLa, CaSki, and SiHa) were treated with 5 μmol/l of BHP in combination with 5-Gy ionizing radiation; cell death was measured by flow-cytometric analysis with annexin-V staining. The combined treatment synergistically induced cell death in three types of cancer cells, whereas treatment with BHP alone did not cause significant cell death (Fig. 1c). In addition, analysis of apoptotic cell death with annexin-V staining also clearly revealed that annexin-V-positive cell populations were markedly increased by the combined treatment of ionizing radiation with BHP (Fig. 1d). We next investigated whether caspase activities are required for the induction of cell death by the combination treatment. As shown in Fig. 1e, treatment of BHP in combination with ionizing radiation induced activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase-1. Moreover, a broad-spectrum caspase inhibitor, z-VAD-fmk, completely attenuated the combination treatment-induced cell death (data not

shown). These findings suggest that treatment of BHP in combination with ionizing radiation synergistically induces apoptotic cell death in human cervical cancer cells in a caspase-dependent manner.

Activation of Bax is involved in mitochondrial cell death induced by the combined treatment

To determine whether the mitochondrial pathway is involved in the induction of cell death by combined treatment with BHP and ionizing radiation, we examined changes in mitochondrial membrane potential and the release of proapoptotic molecules from the mitochondria. As shown in Fig. 2a, the combination treatment induced a significant dissipation of mitochondrial membrane potential and promoted subsequent cytosolic redistribution of cytochrome *c* and AIF, indicating that combination treatment-induced cell death is accompanied by mitochondrial dysfunction. It has been demonstrated that proapoptotic Bcl-2 family members, especially Bax and Bak, are crucial to the mitochondrial cell death pathway [21–23]. Therefore, we next analyzed conformational activation of Bax and/or Bak after the combination treatment. To identify oligomerization of Bak and Bax, we fractionated the membrane fraction followed by chemical cross-linking with disuccinimidyl suberate. The combination treatment induced activation of Bax (Fig. 2b), but not Bak (data not shown), manifested as western blot analysis of cross-linked proteins. It also induced the subsequent relocalization of Bax from the cytosol to mitochondria (Fig. 2b). Moreover, targeted suppression of Bax by small-interfering RNA significantly attenuated mitochondrial membrane potential loss (Fig. 2c) and cell death (Fig. 2d) by the combination treatment. These results indicate that selective activation and mitochondrial relocalization of Bax plays a crucial role in the synergistic enhancement of mitochondrial cell death by combined treatment with BHP and ionizing radiation.

Fig. 2



Activation of Bax is involved in mitochondrial cell death induced by the combined treatment. (a) HeLa cells were treated with 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP, 5 μmol/l) in combination with ionizing radiation (5 Gy). After 48 h, mitochondrial membrane potential of the cells was determined by retention of 3,3-dihexyloxacarboxyanine iodide [$DiOC_6(3)$] as described in Materials and Methods. Results from three independent experiments are presented as mean ± standard error of the mean (SEM). Significantly different from control; $^*P<0.01$. Cytosolic fractions were subjected to immunoblot analysis with anti-cytochrome *c*, anti-apoptosis-inducing factor (AIF), and anti-α-tubulin antibodies. (b) Mitochondrial fractionations were subjected to Bax oligomerization as described in Materials and Methods. Mitochondrial fractions were subjected to immunoblot analysis with anti-Bax and anti-HSP60 antibodies. (c) HeLa cells were transfected with si-control or si-Bax and then treated with BHP in combination with ionizing radiation. After 48 h, mitochondrial membrane potential of the cells was determined by retention of $DiOC_6(3)$ as described in Materials and Methods. Results from three independent experiments are presented as means ± SEM. Significantly different from control; $^*P<0.01$. (d) After 48 h, cell death was measured as the percentage of propidium iodide (PI)-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± SEM. Significantly different from control; $^*P<0.01$. Total cell lysates were subjected to immunoblot analysis with anti-Bax and anti-actin antibodies.

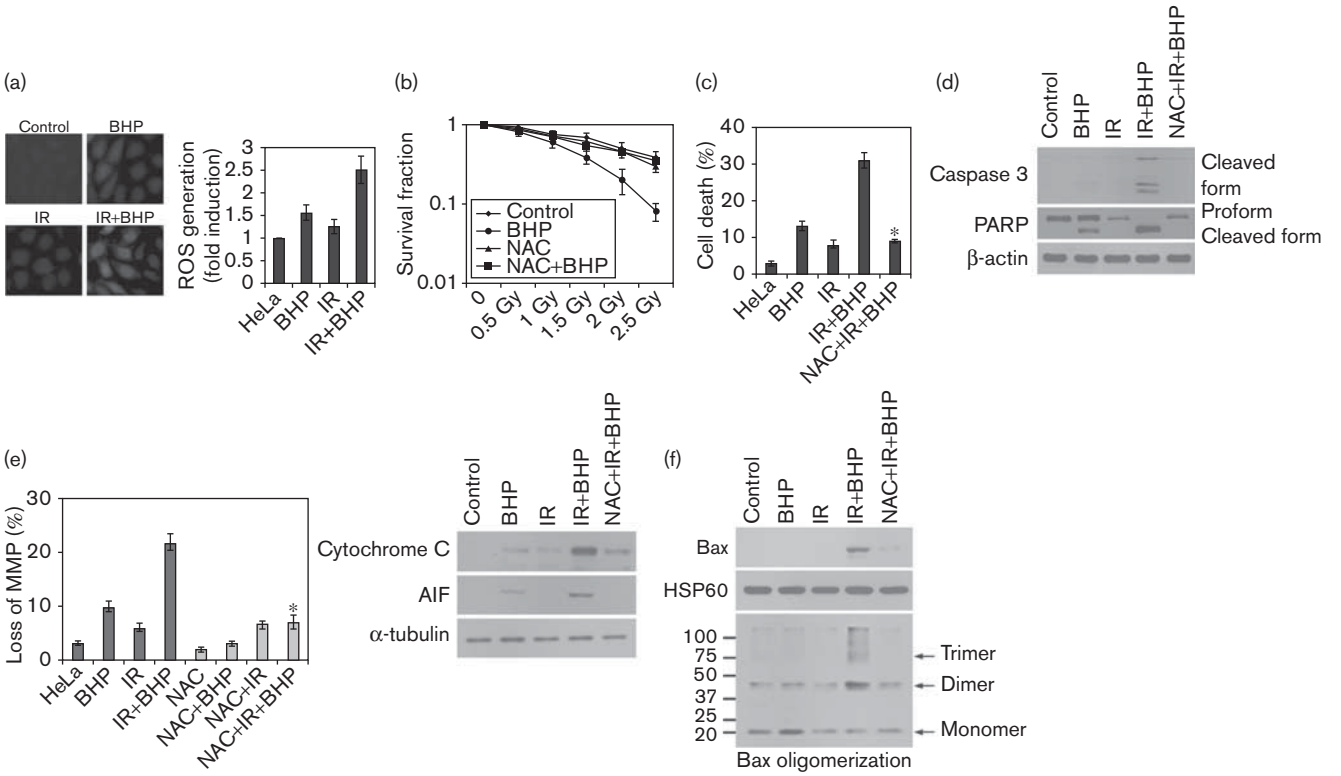
Elevation of intracellular reactive oxygen species is required for the activation of mitochondrial cell death pathway by the combined treatment

Oxidative damage has been shown to play an important role in the anticancer action of ionizing radiation and/or chemotherapeutic drugs [24,25]. Thus, we examined changes in intracellular ROS levels induced by combined treatment with BHP and ionizing radiation. As shown in Fig. 3a, the combination treatment led to approximately a 3-fold increase in mean DCF fluorescence, indicating an increase in intracellular ROS; however, treatment with BHP alone had no effect on intracellular ROS levels. To further establish a link between elevation of intracellular ROS and synergistic enhancement of response to ionizing radiation, we incubated cells with the antioxidant, *N*-acetyl-L-cysteine (NAC), before treatment with BHP and ionizing radiation. As shown in Fig. 3b, NAC clearly reversed inhibition of clonogenic survival by the combined treatment and protected cells from the combination treatment-induced cell death (Fig. 3c). Moreover, inhibition of ROS with NAC completely prevented the oligomerization and mitochondrial redistribution of Bax (Fig. 3f), the induction of mitochondrial membrane potential loss (Fig. 3d), and release of AIF and cytochrome *c* (Fig. 3e) induced by combined treatment with BHP and ionizing radiation. These observations suggest that an increase in intracellular ROS is required for the synergistic enhancement of response to ionizing radiation by combination treatment with BHP and ionizing radiation.

Combined treatment with 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one and ionizing radiation induces reactive oxygen species generation from mitochondria

To further determine whether combination treatment with BHP and ionizing radiation increases the intracellular

Fig. 3



Elevation of intracellular reactive oxygen species (ROS) is required for the activation of mitochondrial cell death pathway by the combined treatment. (a) HeLa cells were treated with 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP) in combination with ionizing radiation. After 24 h, cells were loaded with dichlorofluorescein-diacetate (DCFH-DA) for 30 min. The dichlorofluorescein (DCF) fluorescence was visualized using fluorescence microscopy, and the amount of retained DCF was measured using flow cytometry as described in Materials and Methods. Results from three independent experiments are presented as means ± standard error of the mean (SEM). Significantly different from control; $^*P < 0.01$. (b) HeLa cells were allowed to grow after treatment of BHP with indicated dose of ionizing radiation in the presence of *N*-acetyl-L-cysteine (NAC). After 7 days, colonies were stained with 0.4% trypan blue and scored for colony formation. Results from three independent experiments are presented as means ± SEM. (c) HeLa cells were treated with BHP in combination with ionizing radiation in the presence of NAC. After 48 h, cell death was measured as the percentage of propidium iodide (PI)-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± SEM. Significantly different from control; $^*P < 0.01$. (d) Mitochondrial membrane potential of cells was determined by retention of 3,3-dihexyloxacarboxyanine [$DiOC_6(3)$, (40 μmol/l)] as described in Materials and Methods. Results from three independent experiments are presented as means ± SEM. Significantly different from control; $^*P < 0.01$. Cytosolic fractions were subjected to immunoblot analysis with anti-cytochrome *c*, anti-apoptosis-inducible factor (AIF), and anti-α-tubulin antibodies. (e) Mitochondrial fractionations were subjected to Bax oligomerization as described in Materials and Methods. Mitochondrial fractions were subjected to immunoblot analysis with anti-Bax and anti-HSP60 antibodies.

ROS levels through mitochondria, we used the mitochondrial ROS detection reagent, MitoSOX Red, in conjunction with fluorescence microscopy.

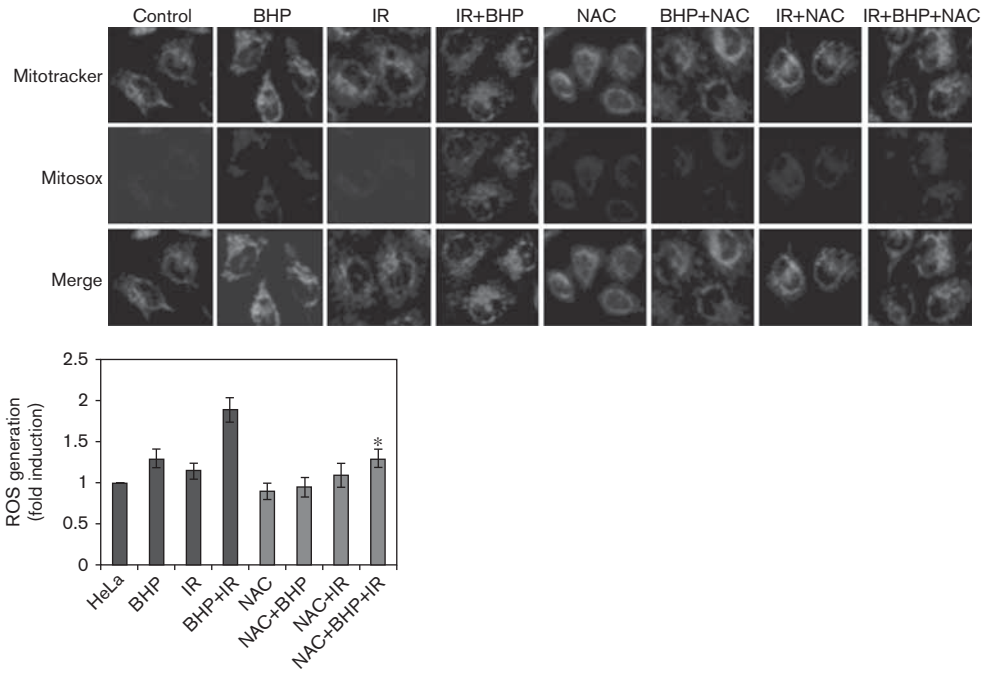
As shown in Fig. 4, combined treatment with BHP and ionizing radiation induced an increase in mitochondrial fluorescence, similar to the observed increase in CM-H_2DCFDA fluorescence. The stippled linear pattern of fluorescence in HeLa cells loaded with MitoSOX Red is consistent with mitochondrial localization of ROS. These results suggest that mitochondria contribute to the overall elevation of ROS levels in HeLa cells treated with the combination of BHP and ionizing radiation.

Discussion

2-Pyrones, dihydro-2-pyrones, and secondary metabolites exhibit a wide range of antifungal, cytotoxic, neurotoxic, and phytotoxic properties [16], including antitumor activities [26,27]. 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 this study, we examined whether the use of 2-pyrone derivatives, 2-pyrone-1α and BHP in combination with γ-radiation increase the therapeutic effect on cancer by overcoming a high apoptotic threshold. We found that BHP, a new 2-pyrone derivative, in combination with ionizing radiation synergistically enhances response of human cervical cancer cells to ionizing radiation, whereas either 2-pyrone-1α or BHP treatment alone does not effectively induce cell death. The combination treatment with γ-radiation and BHP, but not 2-pyrone-1α, strongly enhanced apoptotic cell death accompanied with disruption of mitochondrial membrane potential in human cervical cancer cells. We also found that the combination treatment induced caspase-3 activation, and pretreatment with caspase inhibitor clearly attenuated apoptotic cell death induced by the combination treatment (data not shown); this suggested that the combination treatment with BHP and γ-radiation induces apoptotic cell death in a caspase-dependent manner.

In response to stimuli such as etoposide, staurosporine, TGF-β, and UV, which require mitochondria-dependent pathway for apoptosis, Bax becomes activated, translocated to the outer membrane of mitochondria, and oligomerized therein [28–31]. Thus, Bax activation results in mitochondrial membrane permeabilization and the release of mitochondrial apoptogenic molecules into the cytosol and finally leads to cell death. In this study, the combination treatment with γ-radiation and BHP redistributed Bax from cytosol to the mitochondria. Moreover, small-interfering RNA targeting of Bax effectively attenuated mitochondrial membrane potential loss

Fig. 4



Combined treatment with 5-bromo-3-(3-hydroxyprop-1-ynyl)-2H-pyran-2-one (BHP) and ionizing radiation induces reactive oxygen species (ROS) generation from mitochondria. HeLa cells were treated with BHP in combination with ionizing radiation. After 48 h, cells were loaded with mitochondrial ROS detection reagent. The MitoSOX Red fluorescence was then visualized using fluorescence microscopy, and the amount of retained fluorescence was measured using flow cytometry. Results from three independent experiments are presented as mean ± standard error of the mean. Significantly different from control; $*P<0.01$.

and apoptotic cell death seen after the combination treatment. These results suggest that mitochondrial redistribution of Bax may trigger mitochondrial membrane potential loss and causes subsequent apoptotic cell death after the combined treatment with γ-radiation and BHP.

It has been reported in a variety of eukaryotic cells that overproduction of intracellular ROS by certain agents in combination with radiation can enhance a lethal chain of reactions that results in cell death in human cancer cells [32]. This overproduction of intracellular ROS can lead directly or indirectly to the activation of the mitochondrial apoptotic cell death [33,34]. Several studies provided further evidence of the role of ROS as the potential inducers of the proapoptotic molecules Bax and/or Bak activation during apoptotic cell death [35]. Consistent with these findings, we also found that the ROS-dependent activation and mitochondrial relocalization of Bax are required for the enhancement of mitochondrial apoptotic cell death by the combination treatment. Complete inhibition of Bax translocation to the mitochondria by the thiol-containing antioxidant NAC suggests that an increased intracellular ROS level is critical for the Bax relocalization after the combination treatment.

In summary, we demonstrate here that BHP, a new 2-pyrone derivative, synergistically enhances sensitivity of human cervical cancer cells to ionizing radiation through elevation of intracellular ROS and that ROS-dependent Bax activation is critically involved in the combination treatment-induced enhancement of cell death. These results suggest that BHP may be beneficial to enhance radiation sensitivity against cancer cells that are intrinsically less sensitive to ionizing radiation treatment.

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Conflicts of interest

There are no conflicts of interest.

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