A novel chenodeoxycholic derivative HS-1200 enhances radiation-induced apoptosis in MCF-7 cells

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Abstract. HS-1200, a synthetic chenodeoxycholic acid derivative, has cytotoxic activity in various human cancer cells. The present study was undertaken to examine whether HS-1200 sensitizes radiation-induced apoptosis in MCF-7 human breast carcinoma cells. Clonogenic assay elucidated that the combination treatment with HS-1200 and radiation induced more cytotoxic effects than the radiation treatment alone. Nuclear staining, DNA electrophoresis and Western blot analysis for poly(ADP-ribose) polymerase revealed that the increased cytotoxic effect by the combination treatment resulted from the augmentation of apoptosis. There was an increase in the expression level of Bax and its translocation onto the mitochondria, a reduction in the mitochondrial membrane potential in the earlier time-points, and the release of cytochrome c into the cytosol increased in the MCF-7 cells treated with radiation and HS-1200 compared to the cells treated only with radiation. Therefore, the synthetic bile acid derivative, HS-1200, could have the therapeutic potential as a radiosensitizer in MCF-7 cells.

Introduction

Bile acids are polar derivatives of cholesterol essential for the absorption of dietary lipids and they regulate the transcription of genes that control cholesterol homeostasis. Depending on the nature of chemical structures, each kind of bile acid exhibits distinct biological effects (1). Natural bile salts have been reported to inhibit cell proliferation and induce apoptosis in various cancer cells (2,3). Bile acid derivatives induced apoptosis (4) and inhibited angiogenesis in human hepatocellular carcinoma cells (5).

We have developed several ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) derivatives, and have been studying their biological activity. We have demonstrated that they have apoptosis-inducing effects on various cancer cells, such as leukemia and breast cancer cells (6-9). We have also reported that the combination treatment of the synthetic CDCA derivative, HS-1200, and the proteasome inhibitor, lactacystin, augmented prominently the extent of apoptosis on p815 mastocytoma cells (10).

Several apoptosis-inducing drugs have been shown to increase the therapeutic efficacy of irradiation or to reduce radiation-mediated side effects. TRAIL (11-13), synthetic phospholipids such as alkyllysophospholipids (14-16), the PKC inhibitor, PKC412 (17), other drugs such as Cox-2 inhibitors, betulinic acid, and proteasome inhibitors have all been suggested as radiosensitizers (18-21).

Although synthetic bile acid derivatives have been elucidated to induce apoptosis in various cancer cells, the possibility of HS-1200 as a radiosensitizer had been elusive before this study. In this study, we analyzed whether the synthetic CDCA, HS-1200, has the *in vitro* anticancer effects as a radiosensitizer. As will be shown, the combination treatment of HS-1200 and radiation significantly induced the apoptotic effects on MCF-7 cells, and this enhanced apoptotic efficacy was associated with the increase in the Bax protein level.

Materials and methods

Reagents. The following reagents were obtained commercially. The rabbit polyclonal anti-human poly(ADP-ribose) polymerase (PARP) antibody was from Oncogene (Cambridge, MA, USA). The polyclonal anti-mouse Bax, anti-human Bcl-2, Bak, cytochrome c, and the goat polyclonal anti-mouse AIF antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The goat FITC-anti-rabbit IgG antibody was from Vector (Burlingame, CA, USA). The TUNEL reaction mixture was from Boehringer Mannheim (Mannheim, Germany). The ECL Western blotting detection reagents were from Amersham International (Little Chalfont, UK).

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5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA).

Preparation of HS-1200. HS-1200 was synthesized as described previously (8). Bile acids were dissolved in 100% ethanol and stored at -20˚C before the experiments and dilutions were made in a culture medium. The maximal concentration of ethanol did not exceed 0.1% (vol/vol) in the treatment range, where there was no influence on the cell growth.

Cell culture. MCF-7 human breast carcinoma cells (ATCC, Rockville, MD, USA) were routinely cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 50 mg/ml gentamicin and 135 mg/ml glutamine at 37˚C with 5% CO_2 in air atmosphere.

HS-1200 treatment and irradiation. The original medium was removed 24 h after the MCF-7 cells were subcultured. The cells were washed with phosphate-buffered saline (PBS) and then incubated in the same fresh medium. HS-1200 from a stock solution was added to the medium in order to obtain various dilutions of the drug. Since the doses required for the half-maximal inhibition of viability were about 40 μM, as the results of preliminary studies on MCF-7 cells have revealed, (data not shown), this single concentration was used for the further *in vitro* assessment of apoptosis. The radiation dose was delivered at a rate of 200-300c Gy/min at room temperature using 2-8 Gy 6 MV X-ray (Clinac 1800C, Varian, CA, USA).

Trypan blue dye exclusion. The cells treated with HS-1200 over a time span of 7 h were harvested, stained with trypan blue and then counted using a hemocytometer.

Clonogenic assay. Appropriate numbers of cells were plated in 25-cm^2 plastic flasks and incubated in a medium overnight (about 14 h). To examine the efficacy of HS-1200 as a radiosensitizer, the cell culture medium containing 16 μM HS-1200 was removed 24 h after treatment and replaced with fresh medium without HS-1200. For the control, the original medium was replaced with fresh medium. The cells were exposed to 8 Gy X-ray, and cultured at 37˚C with 5% CO_2 in air atmosphere for 8-9 days. The cells were fixed with methanol/acetic acid (10:1) solution and then stained with 10% (wt/vol) crystal violet in methanol. The colonies of at least 50 cells were counted.

Hoechst staining. The cells were harvested and the cell suspension was centrifuged onto a clean fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/ml Hoechst 33342 at 37˚C for 30 min and fixed for 10 min in 4% paraform-aldehyde.

DNA fragmentation assay. Cells ($2x10^6$) were resuspended in 1.5 ml lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS] into which proteinase K (200 μg/ml) was added. After the samples were incubated overnight at 48˚C, 200 μl ice-cold 5 M NaCl were added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at -20˚C in 50% isopropanol and RNase A-treated for 1 h at 37˚C. The DNA from 10^6 cells (15 μl) was equally loaded on each lane of 2% agarose gels in Tris-acetic acid/EDTA buffer containing 0.5 μg/ml ethidium bromide at 50 mA for 1.5 h.

Immunofluorescent staining. The cytocentrifuged cells were fixed in 4% paraformaldehyde for 10 min, incubated with the anti-cytochrome c antibody for 1 h and then with the FITC-conjugated secondary antibody for 1 h at room temperature. The cells were mounted with PBS, and observed and photographed under an epifluorescence microscope.

Cell cycle phase analysis by flow cytometry. Ice-cold 95% ethanol with 0.5% Tween-20 was added into the cell suspension in a final 70% ethanol volume. The fixed cells were pelleted, and washed in 1% BSA-PBS solution. The cells were resuspended in 1 ml PBS containing 11 Kunitz U/ml RNase, incubated at 4˚C for 30 min, washed once with BSA-PBS, and resuspended in PI solution (50 μg/ml). After the cells were incubated at 4˚C for 30 min in the dark and washed with PBS, the DNA content was measured on an Epics XL (Beckman Coulter, FL, USA) and data were analyzed using the Multicycle software which allowed the simultaneous estimation of the cell-cycle parameters and apoptosis.

Assay of mitochondrial membrane potential (MMP). Alterations in MMP were determined by staining the cells with the indicator dye, JC-1. JC-1 was added directly to the cell culture medium (1 mM final concentration) and incubated for 15 min. The medium was then replaced with phosphate-buffered saline, and the cells were quantified for J-aggregated fluorescence intensity in a modular fluorimetric system (Spex, Edison, NJ, USA) using excitation and emission filters of 492 and 590 nm, respectively.

Western blot analysis. Cells ($2x10^6$) treated with bile acids were washed twice with ice-cold PBS, resuspended in 100 ml ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 1 ml protease inhibitor cocktail) (Sigma, St. Louis, MO, USA) and incubated at 4˚C for 30 min. The lysates were centrifuged at 14,000 rpm at 41˚C for 15 min. Protein concentrations of cell lysates were determined by the method of Bradford (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were subjected to 15 or 7.5% SDS-PAGE for caspase-3 and PARP, respectively, and transferred to a nitrocellulose membrane. Western blot analyses were carried out by using the appropriate antibody (as noted in the figure legends) and immunostaining with each antibody was performed using the SuperSignal West Pico enhanced chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA) and detected with LAS-3000PLUS (Fuji Photo Film Company, Kanagawa, Japan).

Statistical analysis. All the data are expressed as the means ± SD from three independent experiments. The results of the experimental and control groups were tested for statistical

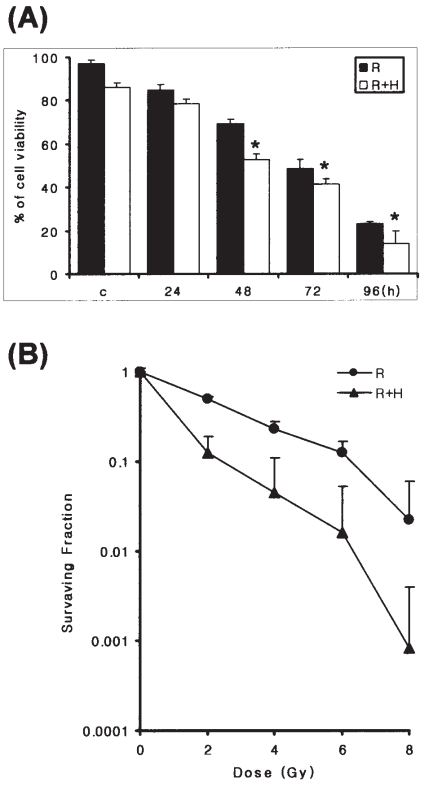

Figure 1. Enhancement of cytotoxicity and the anti-proliferative effect in HS-1200 plus radiation-treated MCF-7 cells. (A) Trypan blue assay on MCF-7 cells. Cells treated with HS-1200 or HS-1200/radiation over a time span of 24 h were harvested, stained with trypan blue and then counted using a hemocytometer. The asterisks indicate a significant difference compared to the control (0 μM) ($p<0.05$). ■, Radiation alone; ⊐, HS-1200 plus radiation. (B) Clonogenic assay on MCF-7 cells. The increased anti-proliferative effect of HS-1200 plus radiation treatment on MCF-7 cells. Exponentially growing MCF-7 cells were exposed to 16 μM HS-1200 for 24 h before radiation. Following the HS-1200 treatment, the cells were irradiated at a dose rate of 0-8 Gy/min. ●, Radiation alone; ▲, HS-1200 plus radiation. Four independent assays were performed and the data shown are the means ± SD of the means obtained from triplicates of each assay.

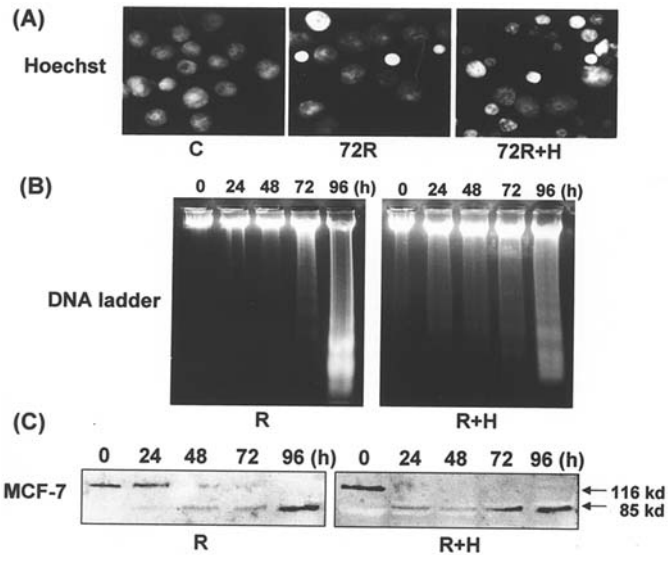

Figure 2. Increased apoptotic effects on co-treated MCF-7 cells with HS-1200 and radiation. (A) Demonstration of nuclear condensation in MCF-7 cells. Immunofluorescent micrographs after hoechst staining (x400). C, control cells; 72R, cells exposed to radiation alone at a dose of 8 Gy; 72R+H, cells treated with 40 μM HS-1200 for 24 h before irradiation and then irradiation at a dose of 8 Gy. (B) Enhanced DNA fragmentation in HS-1200 plus radiation-treated MCF-7 cells. The cells were exposed to radiation alone at a dose of 8 Gy (R) and to HS-1200 at 40 μM for 24 h before irradiation and then irradiation at a dose of 8 Gy (R+H). (C) Demonstration of degradation and production of cleavage products of PARP. The inactivated PARP protein (116 kD) and the cleaved product (85 kD) are indicated. An enhanced PARP cleavage is shown in HS-1200 plus radiation-treated MCF-7 cells. Data shown are representative of four independent experiments.

significance by the Kruskal-Wallis nonparametric test. P-values <0.05 were considered significant.

Results

Combination treatment with HS-1200 and radiation enhanced cytotoxicity and the anti-proliferative effect on MCF-7 cells. Combination treatment with HS-1200 and radiation produced a significant decrease in the cell viability and proliferation of MCF-7 (Fig. 1). The trypan blue assay showed that the co-treatment of HS-1200 and radiation for 0-96 h induces more severe cytotoxicity on MCF-7 cells (Fig. 1A). As determined by the clonogenic assay, the co-treatment also showed a greater inhibitory effect on the proliferation of MCF-7 cells (Fig. 1B).

Combination treatment with HS-1200 and radiation augmented the apoptosis-inducing effect on MCF-7 cells. DNA fragmentation assay and Western blotting for the PARP protein were performed. Hoechst staining showed more MCF-7 cells with the nuclear condensation in the co-treated group (Fig. 2A), compared to the radiation only treated group. In the DNA electrophoresis experiment, a DNA ladder was observed in the combination-treated cells after 24 h, whereas it was not evident until 72 h after radiation only treatment (Fig. 2B). The PARP cleavage was also demonstrated in the earlier time-point (24 h) on the co-treated MCF-7 cells, compared to the radiation only-treated cells (Fig. 2C).

Expression level of Bax was more evidently increased in co-treated MCF-7 cells. Western blot assay for Bax, Bcl-2, Bak, and AIF showed that the increased expression of Bax was prominently evident in the co-treated MCF-7 cells. However, there was no remarkable difference in the expression level of other proteins between the radiation only- and HS-1200/radiation-treated groups (Fig. 3).

Enhanced apoptotic effect of HS-1200 plus radiation on co-treated MCF-7 cells is involved with mitochondrial controls. To investigate the association with the mitochondria, immunofluorescent staining for cytochrome c, Bax, and AIF proteins was performed. The release of cytochrome c and the Bax translocation were more prominent in the co-treated cells than in the radiation only-treated ones. However, no significant difference in the translocation of AIF to the nucleus was observed between the co-treated and the radiation only-treated cells (Fig. 4A). Flow cytometry demonstrated that the portion of cells showing the depolarization of MMP was higher in the co-treated group as opposed to the radiation only-treated cells at the same time-points (Fig. 4B).

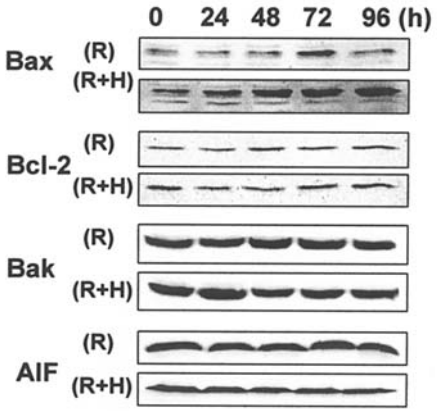

Figure 3. Effect of combination treatment with HS-1200 and irradiation on the expression of Bax, Bcl-2, Bak and AIF. The cells were exposed to radiation alone at a dose of 8 Gy (R) and to HS-1200 at 40 μM for 24 h before irradiation and then irradiation at a dose of 8 Gy (R+H). The prominently increased Bax expression in the R+H group is shown. Data are representative of four independent experiments.

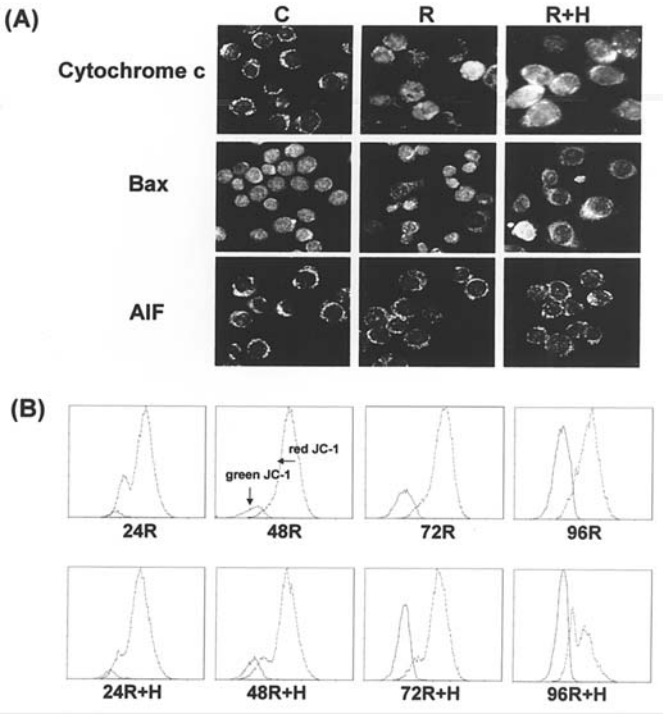

Figure 4. Mitochondrial involvement with the enhanced apoptotic effect of the co-treatment on MCF-7 cells. (A) An immunofluorescent micrograph showing the localization of the mitochondrial proapoptogenic factors. The cells were exposed to radiation alone at a dose of 8 Gy (R) and to HS-1200 at 40 μM for 24 h before irradiation and then irradiation at a dose of 8 Gy (R+H) (x400). Data shown are representative of four independent experiments. (B) Effect of co-treatment of HS-1200 and irradiation on the loss of mitochondria membrane potential ($\Delta\Psi_m$) in MCF-7 cells. The cells were exposed to radiation alone at a dose of 8 Gy (R) and to HS-1200 at 40 μM for 24 h before irradiation and then irradiation at a dose of 8 Gy (R+H). $\Delta\Psi_m$ was quantitated by the measurement of the J aggregate (JC-1) fluorscence in the cells. The percentage of control was calculated by the $\Delta\Psi_m$ of treated cells/$\Delta\Psi_m$ of control cells. R, 24 h-97%, 48 h-92%, 72 h-86%, 96 h-59%; R+H, 24 h-96%, 48 h-82%, 72 h-64%, 96 h-51%. The arrows indicate green and red JC-1. Data shown are representative of four independent experiments.

Discussion

Numerous apoptosis-inducing drugs have been shown to increase the therapeutic efficacy of irradiation or to reduce radiation-mediated side effects. TRAIL is of high potential value for a combination with ionizing radiation in several tumor therapies. This efficacy has been proven *in vitro* and in preclinical mouse models (11-13). Synthetic phospholipids such as alkyllysophospholipids have also been shown to act as radiosensitizers and to enhance radiation-induced apoptosis *in vitro* (14-16). The PKC inhibitor, PKC412, has also been shown to increase the apoptotic sensitivity of cancer cells through the inhibition of the PI3K/Akt signaling pathway (17).

In addition, the COX-2 inhibitor, SC-236, significantly enhanced the radiation response of a murine model of sarcoma as well as human glioma U251-cells *in vitro* and in a tumor xenograft model without increasing normal tissue toxicity (18,19). Not only did the combination treatment of betulinic acid and irradiation also induce the apoptotic effect in human melanoma cells (20), but proteasome inhibitors and the human immunodeficiency virus protease inhibitor, saquinavir, have been demonstrated to modulate the apoptotic response through the inhibition of NF-κB activation (21-23). Several publications have provided evidence that signaling cascades originating at the epidermal growth factor receptor (EGFR) activate anti-apoptotic signaling pathways and increase the radiation resistance of tumor cells (24-26). Furthermore, nitric oxide has been proposed as an intrinsic radiosensitizer *in vivo*. (27). It has been reported that the adenoviral gene transfer of the iNOS gene enhances the radiation response of human colorectal cancer associated with alterations in tumor vascularity (28).

We developed several UDCA and CDCA derivatives, and have been studying their biological activity. They have apoptosis-inducing effects in various cancer cells, such as leukemia and breast cancer cells (6-9). We have demonstrated that the synthetic CDCA derivative, HS-1200, has an apoptotic effect on p815 mastocystoma cells when used in combination therapy with lactacystin (10). Although natural bile salts and derivatives have been reported to inhibit cell proliferation and induce apoptosis in various cancer cells (2-4), no bile acid has yet been demonstrated to have an efficacy as a radiosensitizer.

In this study, we investigated whether HS-1200 has the efficacy as a radiosensitizer. Since the data of clonogenic assay employing two cancer cell lines, MCF-7 and HT-29, showed that MCF-7 cells were more sensitive to radiation or combination treatment than HT-29 cells (HT-29 cells data not shown), further analysis to reveal the underlying mechanism of this increased cytotoxicity was undertaken for the MCF-7 cells. Among our data, it is noticeable that the expression level of Bax was substantially increased and that it translocated onto the mitochondria in the combination treatment group. Moreover, a greater cytochrome c release was also demonstrated. The co-treatment of MCF-7 cells with radiation and HS-1200 caused the reduction of MMP at the earlier timepoints. These data suggest that this augmented apoptotic effect of the combination treatment is mediated via the mitochondria.

In conclusion, the combination treatment with the synthetic CDCA derivative, HS-1200, and irradiation enhanced the

radiation-induced apoptosis of MCF-7 cells. Particularly, the radiosensitive effect of the combination treatment on MCF-7 was associated with the increased Bax protein and the enhanced release of cytochrome c from the mitochondria. We propose that the synthetic bile acid derivative, HS-1200, has the potential as a radiosensitizer in anticancer therapeutics.

Acknowledgements

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