Hochu-ekki-to (Bu-zhong-yi-qi-tang), a herbal medicine, enhances cisplatin-induced apoptosis in HeLa cells

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Abstract. Hochu-ekki-to (HET), a Kampō herbal medicine composed of ten medicinal plants, is traditionally used to improve the general state of patients with malignant diseases such as cancer. Recent studies showed that HET had an anti-cancer effect against several cancer cell lines *in vitro* by inducing apoptosis. However, high doses of HET may have cytotoxic effects attributed to saponins or detergent-like compounds. Therefore, the present study used low doses of HET (50 μg/ml), which did not affect cell viability, to evaluate its synergistic anti-cancer effects with cisplatin. HeLa cells were cultured for 24 h with 50 μg/ml HET, followed by cisplatin treatment for 24 h at various concentrations. Subsequently, the sensitivity of the cells to cisplatin was assessed using a colony survival and a crystal violet cell viability assay. Furthermore, cisplatin-induced apoptosis was analyzed by flow cytometry. Proteins associated with cell viability and apoptosis, including phosphorylated (p-) Akt, p53, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and active caspase-3 were analyzed by immunoblotting. The present study revealed that cell survival was decreased and apoptosis was increased in HeLa cells pre-treated with HET prior to cisplatin treatment compared with HET-untreated cells. Furthermore, protein expression of p53 and active caspase-3 was increased, while the expression of p-Akt as well as the Bcl-2/Bax ratio, an index of survival activity in cells, were decreased in the HET-pre-treated cells compared with those in HET-untreated cells following incubation with cisplatin. In conclusion, the present study indicated that HET enhanced cisplatin-induced apoptosis of HeLa cells and that the administration of HET may therefore be clinically beneficial alongside apoptosis-inducing chemotherapy.

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Introduction

Hochu-ekki-to (HET) (bu-zhong-yi-qi-tang in Chinese) is a Kampō herbal medicine. Kampō medicines are traditional remedies composed of several herbs, and have been used for several hundred years in Japan (1). To date, the Japanese Ministry of Health, Labour and Welfare has approved >120 Kampō prescriptions for clinical use (2). These Kampō medicines have been considered to be clinically effective since antiquity in Japan. However, studies on the biochemical and pharmacological mechanisms of action of Kampō medicines are not extensive.

HET is mainly used for the treatment of appetite loss (3), general fatigue (4) and loss of vigor (1). It is particularly used for treating elderly people and, more recently, patients with infectious and malignant diseases (5,6), or after chemotherapy or radiation therapy of malignant tumors (7,8). It has been reported that HET activates macrophages (9) and natural killer cells (10) and restores impaired immune function, which is beneficial for the prevention of cancer (11,12). In studies on mice, HET suppressed the development and metastasis of several malignant tumor types, including biliary carcinoma and uterine cancer (3,13). In an *in vitro* study, inhibition of viability measured by MTT assay was observed when Hep3B hepatocellular carcinoma cells were incubated with HET (14). In addition, flow cytometric analysis showed that HET induced cell cycle arrest and apoptosis in Hep3B cells (14). These results suggested that the underlying mechanisms of the anti-tumor activity of HET involve the suppression of cell viability and induction of apoptosis.

However, high concentrations of HET in culture medium may have non-specific cytotoxic effects owing to components including saponins (e.g. saikosaponin in HET) and other detergent-like compounds. When cells are incubated with >5,000 μg/ml, necrosis has been identified to be markedly increased in ovarian cancer cells by flow cytometric analysis (15). To rule out the non-specific cytotoxic effects, the present study used a low dose of HET (50 μg/ml) that did not suppress cell viability, and assessed the synergistic effect of HET pre-treatment on the potency of cisplatin against HeLa cells. Cell survival was measured by colony survival and crystal violet assays, and the apoptotic rate was analyzed by flow cytometry. Proteins associated with cell viability and apoptosis, including phosphorylated Akt (p-Akt), p53, B-cell

lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and active caspase-3 were analyzed by immunoblotting.

Materials and methods

Reagents and drugs. HET was obtained from Tsumura Co. (Tokyo, Japan). HET is a mixture of spray-dried powder from hot water extracts obtained from the following ten medicinal plants: Ginseng radix (16.7%), Atractylodis rhizoma (16.7%), Astragali radix (16.7%), Angelicae radix (12.5%), Ziziphi fructus (8.3%), Bupleuri radix (8.3%), Glycyrrhizae radix (6.3%), Zingiberis rhizoma (2.0%), Cimicifuga rhizoma (4.2%) and Aurantii nobilis pericarpium (8.3%). HET powder was dissolved at 10 mg/ml in culture medium at 37˚C for 10 min and thoroughly mixed. The solution was passed through a 0.22-μm filter to sterilize and remove any insoluble components. Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells and culture conditions. The human cervical cancer cell line HeLa, obtained as previously described (16) was used in the present study. The cells were cultured in Eagle's Minimum Essential Medium (EMEM; Nissui, Tokyo, Japan) supplemented with 10% calf serum (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were grown at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell survival assay. The colony survival assay was used to determine the dose of HET that did not affect cell viability (17). Briefly, HeLa cells were plated in 100-mm dishes ($1x10^3$ cells/dish) in medium containing HET at various concentrations (0, 5, 25, 50, 250 and 500 μg/ml). After culturing for 14 days, the colonies were stained with 0.2% methylene blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 30% methanol (Wako Pure Chemical Industries, Ltd.), and visible colonies with a diameter greater than 3 mm (containing greater than 50 cells) were counted by eye.

The sensitivity of cells to cisplatin was measured by colony survival and crystal violet assays (18). For the colony survival assay, cells were plated in 100-mm dishes ($1x10^3$ cells/dish) in medium with or without 50 μg/ml HET. After culturing for 24 h, the medium was changed to serum-free medium containing the indicated concentrations of cisplatin (0, 0.31, 0.63, 1.3, 2.5 or 5.0 μM). After incubation in the cisplatin-containing medium for 2 h, the HET-pre-treated cells (cells treated with cisplatin following HET pre-treatment) or HET-untreated cells (cells treated with cisplatin without HET pre-treatment) were cultured in fresh medium for 14 days followed by colony counting.

For the crystal violet assay, cells were plated in 60-mm dishes ($5x10^5$ cells/dish) in medium with or without 50 μg/ml HET and incubated for 24 h. Cells were harvested, plated onto 96-well plates ($4x10^3$ cells/well) containing cisplatin at the indicated concentrations (0, 0.39, 0.78, 1.6 or 3.1 μM), and cultured for 2 days. After culture, viable cells were stained with crystal violet (Wako Pure Chemical Industries, Ltd.) followed by measurement of absorbance at 595 nm. Cell viability was calculated from the absorbance using an Emax Precision Microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and expressed as the percentage of surviving cells with regard to the group treated without cisplatin.

Flow cytometric analysis of the apoptotic cell population in sub-G_1 phase. Cells that were pre-treated with and without HET (50 μg/ml) for 24 h were then treated with cisplatin (0, 15 and 30 μM) at the indicated concentrations for 24 h. The HET-pre-treated or -untreated cells were harvested and the cell population in sub-G_1 phase (apoptotic fraction) was analyzed with Guava Cell Cycle Reagent (EMD Millipore, Billerica, CA, USA) and an Accuri C6 cytometer (Tomy Digital Biology, Encyclopedia Circle, Fremont, CA, USA). The data were analyzed with FlowJo software, version 7.6 (FlowJo, LLC, Ashland, OT, Canada) as described previously (19).

Immunoblotting. Apoptosis-associated molecules were analyzed by immunoblotting (19). Cells were cultured in medium in the presence or absence of 50 μg/ml HET for 24 h and then treated with cisplatin at indicated concentrations for 24 h. Next, whole-cell lysates in an SDS sampling buffer (62.5 mM Tris-Cl, pH 6.8, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) were prepared from the HET-pre-treated or -untreated cells. Briefly, cells were harvested with phosphate-buffered saline, cell number was counted, then whole cells were dissolved directly with the SDS sampling buffer. The whole-cell lysates from approximately $2x10^4$ cells were applied to each lane of SDS-PAGE. Electrophoresis and blotting were performed with constant 15 mA and 250 mA current using a Tris-glycine buffer system, for 2 h and 3 h, respectively. All reagents for the western blotting were obtained from Wako Pure Chemical Industries, Ltd. The active form of caspase-3 protein was detected using rabbit anti-cleaved caspase-3 antibody (5A1E; Cell Signaling Technology, Inc., Beverly, MA, USA; 1:500 dilution). Bcl-2, Bax, p-Akt and p53 proteins were detected using mouse anti-Bcl-2 monoclonal antibody (sc-7832; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,000 dilution), mouse anti-Bax monoclonal antibody (sc-7480; Santa Cruz Biotechnology; 1:1,000 dilution), rabbit anti-p-Akt antibody (193H12; Cell Signaling Technology, Inc.; 1:1,000 dilution), and mouse anti-p53 monoclonal antibody (sc-126; Santa Cruz Biotechnology; 1:1,000 dilution), respectively. The secondary antibodies used were as follows: Horseradish peroxidase (HRP)-linked anti-rabbit antibody (NA934V; GE Healthcare Life Sciences, Chalfont, UK; 1:4,000 dilution) and HRP-linked anti-mouse antibody (NA931V; GE Healthcare Life Sciences; 1:4,000 dilution). The protein signals were visualized by the Enhanced Chemiluminescence system reaction (GE Healthcare Life Sciences). Protein levels of actin were also analyzed using mouse anti-actin antibody (C4; ICN Biomedicals, Costa Mesa, CA, USA; 1:10,000 dilution) as a loading control. The intensities of the protein signals were quantified using Multi Gauge 2.2 image analysis software (Fuji Foto Film, Tokyo, Japan) and expressed as a relative value to that of actin. Immunoblotting analysis was performed three times using cell samples prepared twice independently.

Statistical analysis. P-values were calculated to compare the HET-pre-treated cells and the HET-untreated cells by Student's t-test with Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA). Values are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

HET sensitizes HeLa cells to cisplatin-induced cytotoxicity. The present study first examined the effect of HET alone on HeLa cell viability. No growth suppression by HET was observed at concentrations of up to 500 μg/ml (Fig. 1).

The present study therefore adopted 50 μg/ml HET to exclude any cytotoxic effects. Next, the sensitivity of HeLa cells to cisplatin after pre-treatment in medium containing 50 μg/ml of HET for 24 h was assessed. The colony survival assay indicated that the HET-pre-treated cells had a significantly higher sensitivity to cisplatin-induced cell death than the HET-untreated cells (Fig. 2A).

In addition, the crystal violet assay showed that HET-pre-treated HeLa cells were more sensitive to the inhibitory effect of cisplatin on cell viability than HET-untreated HeLa cells (Fig. 2B). These findings suggested that the anti-cancer effect of cisplatin was enhanced by pre-treatment with HET.

HET- and cisplatin-induced cell death proceeds via a caspase-dependent apoptotic pathway. A previous study by our group reported that cisplatin induced apoptosis in HeLa cells and other cancer cell lines (20). Therefore, the present study examined whether the cell death induced by cisplatin and HET is induced via apoptotic pathways. Flow cytometric analysis indicated a distinct increase in the population of cells in the sub-G_1 phase (apoptotic fraction) after cisplatin treatment in the cells pre-treated with HET compared with that in the HET-untreated cells (Fig. 3). While the sub-G_1 population in the HET-untreated HeLa cells was 7.8±0.71, 10.3±3.0 and 72.2±6.7% after treatment with 0, 15 and 30 μM cisplatin, respectively, the sub-G_1 fractions were increased to 9.8±3.6, 36.8±6.4 ($P<0.005$) and 88.6±4.3% ($P<0.05$), respectively, in the HET-pre-cultured HeLa cells.

Caspase-3 is one of the effector caspases, which is cleaved and activated by initiator caspases, and executes apoptosis when activated (21). The levels of active caspase-3 increased by ~1.7-fold after incubation of HET-pre-treated HeLa cells with 20 μM cisplatin, compared with those in the HET-untreated cells (Fig. 4). These results supported that HET-pre-treatment enhanced cisplatin-induced apoptosis, which proceeded via a caspase-dependent pathway.

HET-pre-treatment enhances apoptosis signaling in HeLa cells treated with cisplatin. Bcl-2 is an anti-apoptotic protein, and Bax is a pro-apoptotic protein (22). The relative expression ratios of anti-apoptotic proteins to pro-apoptotic proteins have been reported to correlate with cellular sensitivity to the lethal effects of anti-cancer drugs (23). The Bcl-2-to-Bax ratio at 0 and 20 μM cisplatin in the HET-pre-cultured HeLa cells was decreased by 23 and 68%, respectively, compared with that in the HET-untreated cells (Fig. 5).

Next, the present study determined the cellular levels of p-Akt and p53 as candidates for upstream molecules to regulate the enhancement of the cisplatin-induced apoptosis by HET. Akt is negatively regulated by p53 (24) when apoptosis occurs. Fig. 6 shows the effect of HET on the protein levels of p-Akt and p53 in HeLa cells incubated with various concentrations of cisplatin. In the HET-untreated cells as well as in HET-treated cells, p-Akt expression was decreased at cisplatin concentrations of >20 μM, compared with that in control cells without cisplatin treatment. In addition, at almost all of the tested concentrations of cisplatin (0, 5, 10 and 20 μM), the p-Akt levels in the HET-pre-treated cells were lower than those in the HET-untreated cells. The difference between the two groups was significant at cisplatin concentrations other than 10 and 30 μM (Fig. 6A and B). Conversely, the protein levels of p53 were increased at cisplatin concentrations of >10 μM compared with

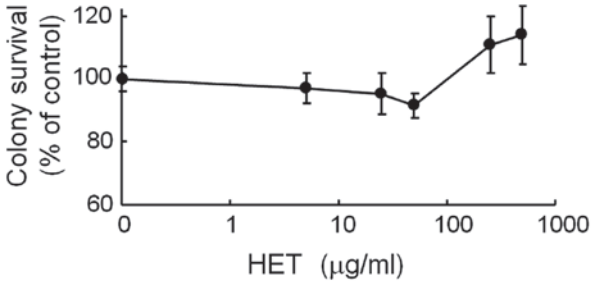

Figure 1. Effect of HET on HeLa cell viability. Cells were cultured in medium containing HET at 0, 5, 25, 50, 250 and 500 μg/ml for 14 days, followed by counting of cell colonies. Values are expressed as the mean ± standard deviation. HET, Hochu-ekki-to.

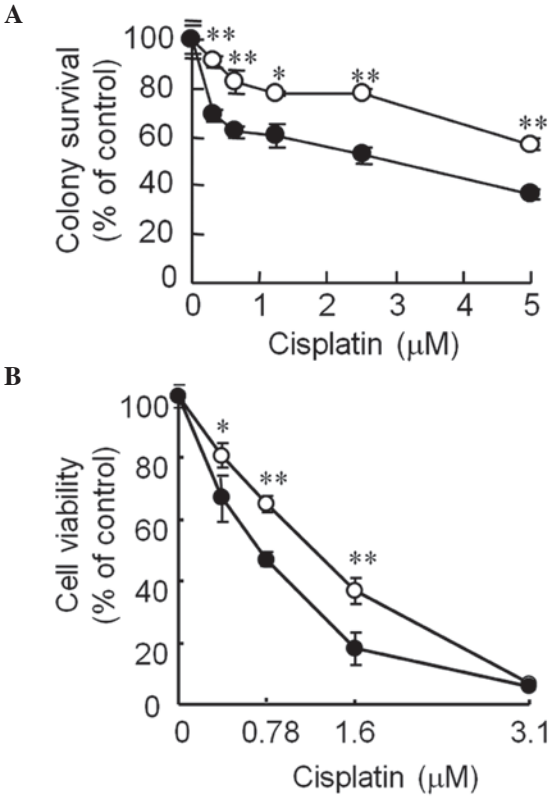

Figure 2. Effect of pre-treatment with HET on the sensitivity of HeLa cells to cisplatin-induced cell death. (A) Colony survival assay. Cells were cultured in the medium with or without 50 μg/ml HET for 24 h and then incubated with 0, 0.31, 0.63, 1.3, 2.5 or 5.0 μM cisplatin in serum-free medium for 2 h. After incubation, the cells were cultured with fresh medium for 14 days followed by colony counting. (B) Crystal violet assay. Cells were cultured in medium with or without 50 μg/ml of HET for 24 h, harvested, plated onto 96-well plates, and incubated with cisplatin at 0, 0.39, 0.78, 1.6 or 3.1 μM for 2 days. Viable cells were stained with crystal violet and cell viability was assessed. Values are expressed as the mean ± standard deviation. $^{*}P<0.05$; $^{**}P<0.005$, for HET-pre-treated cells vs. HET-untreated cells. HET, Hochu-ekki-to.

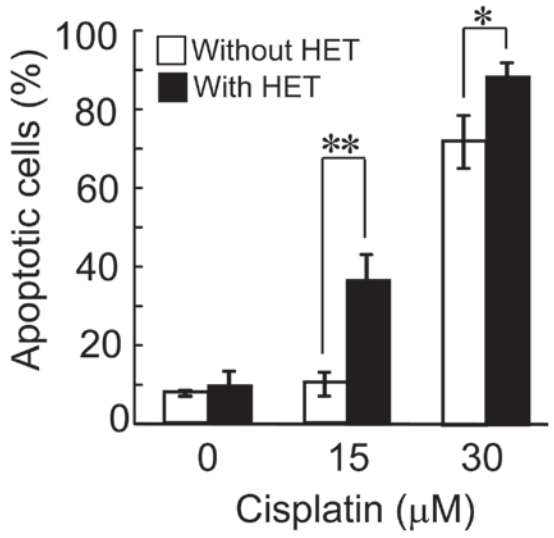

Figure 3. Flow cytometric analysis of HET- and cisplatin-treated HeLa cells. HeLa cells that were pre-treated with and without HET (50 μg/ml) for 24 h then were treated with cisplatin at the indicated concentrations for 24 . The fraction of the apoptotic cell population was then estimated by flow cytometric analysis. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05; **P<0.005 for HET pre-treated vs. HET-untreated cells. HET, Hochu-ekki to.

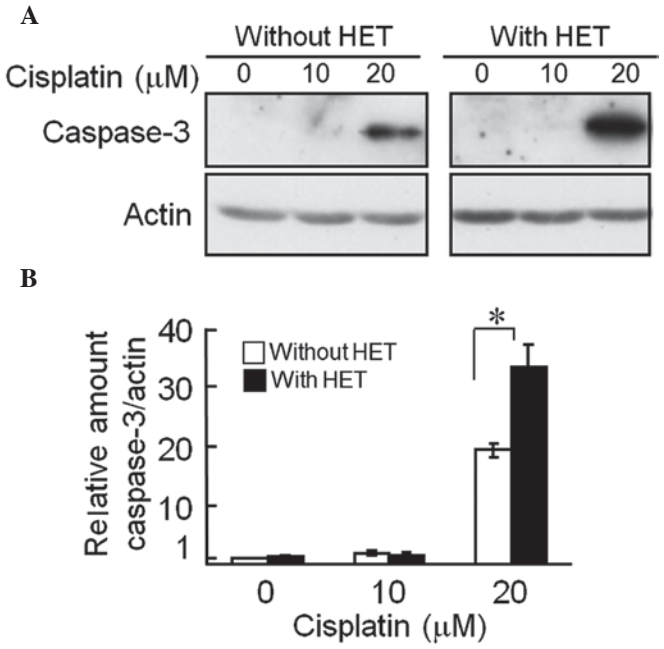

Figure 4. Effect of pre-treatment with HET on caspase-3 activation following cisplatin treatment in HeLa cells. Cells were pre-treated with or without HET (50 μg/ml) for 24 h and then incubated with cisplatin at concentrations of 0, 10 or 20 μM for 24 h. Whole-cell lysates were prepared, and the levels of the cleaved active form of caspase-3 and actin protein were analyzed by immunoblotting. (A) Western blot representative of three experiments. (B) The protein levels of active caspase-3 were normalized to actin levels and are presented as relative expression of that of the control cells without cisplatin treatment. Values are expressed as the mean ± standard deviation. *P<0.05; **P<0.005 for HET-pre-treated vs. HET-untreated cells. HET, Hochu-ekki-to.

those in the cisplatin-untreated groups. Of note, p53 levels were markedly elevated in the HET-pre-treated cells as compared with those in the HET-untreated cells (Fig. 6A and C).

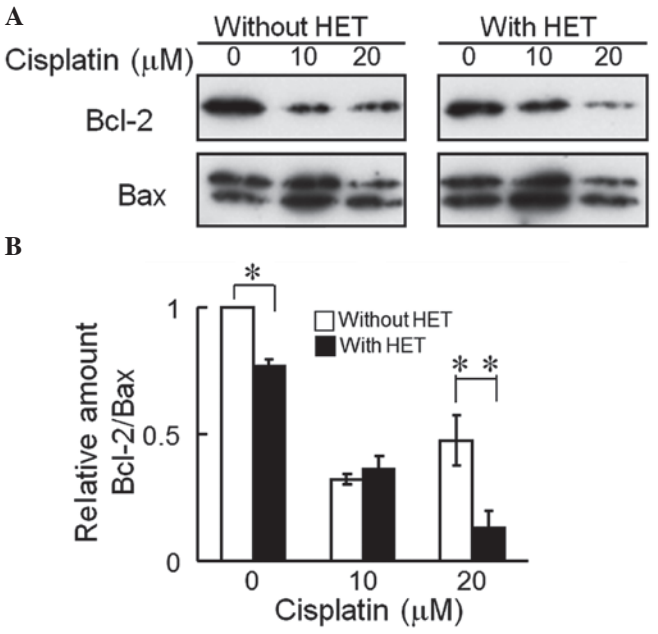

Figure 5. Effect of pre-treatment with HET on the protein levels of Bcl-2 and Bax following cisplatin treatment in HeLa cells. Cells were pre-treated with or without HET (50 μg/ml) for 24 h, and then incubated with cisplatin at concentrations of 0, 10 or 20 μM. Whole-cell lysates were prepared and the protein levels of Bcl-2, Bax and actin were analyzed by immunoblotting. (A) Representative western blots of three experiments. (B) The protein levels of Bcl-2 and Bax were normalized to actin levels, and Bcl-2/Bax ratios are presented as relative values to those in control cells without cisplatin treatment. Values are expressed as the mean ± standard deviation. *P<0.05; **P<0.005 for HET-pre-treated vs. HET-untreated cells. HET, Hochu-ekki-to; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

Discussion

Zhu *et al* (15) reported a necrotic effect of HET at concentrations greater than 5000 μg/ml in ovarian cancer cells. In the present study, no growth suppression was observed at concentrations up to 100 μg/ml, while the colony survival appeared to increase marginally (no significant difference) at >100 μg/ml (Fig. 1). Thus in the current study, 50 μg/ml HET was selected for use. The findings of the present study suggested that HET enhanced the anti-cancer effects of cisplatin by stimulation of cisplatin-induced apoptosis signaling, including upregulation of caspase-3 activation and downregulation of the Bcl-2 to Bax ratio. Furthermore, cellular levels of p-Akt were decreased, while p53 was increased in cells pre-treated with HET, leading to the enhancement of the cisplatin-induced apoptosis.

In several cancer cell lines, the proliferation is enhanced through constitutive activation of the phosphoinositide 3 kinase (PI3K)/Akt pathway through growth factor receptors including epidermal growth factor receptor (25) and platelet-derived growth factor receptor (26) as well as oncogenes including Ras and Her2/Neu (27,28). The results of the present study showed that p-Akt levels were decreased, which was in parallel with decreases in the Bcl-2 to Bax ratio (Fig. 5) in the HET-pre-treated HeLa cells compared with those in the HET-untreated cells, even without cisplatin treatment. Therefore, a mild suppression of PI3K/Akt signaling by HET at the low concentration at which it was used in the present study (50 μg/ml) may be involved in its enhancement of

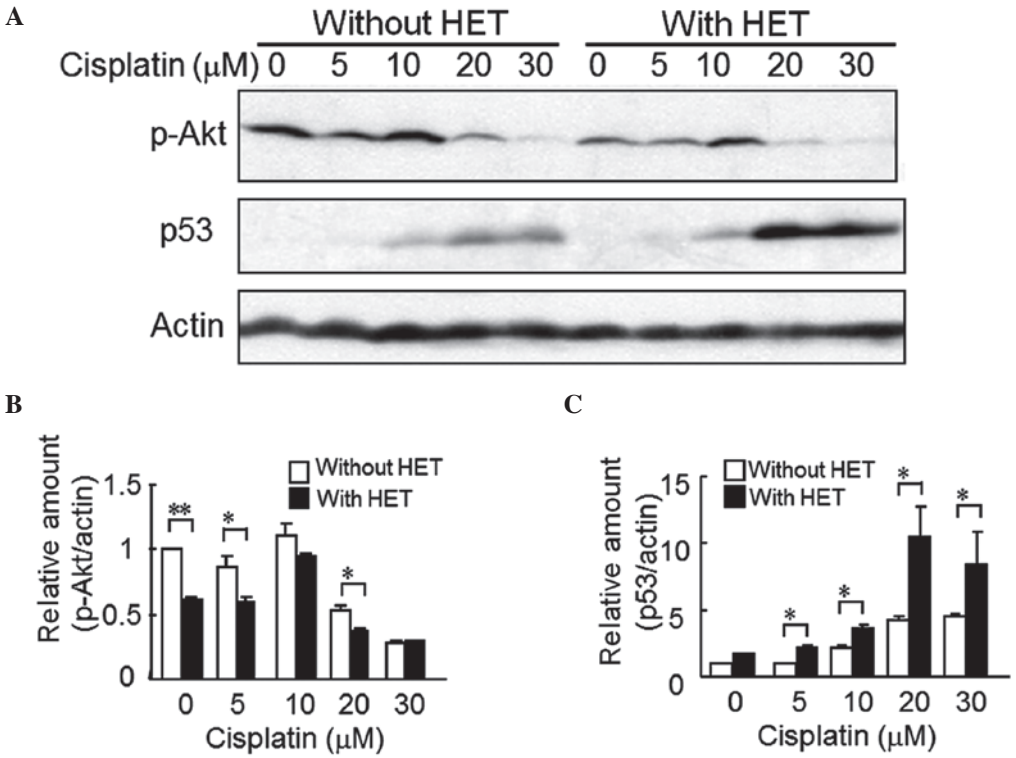

Figure 6. Effect of pre-treatment with HET on the protein levels of p-Akt and p53 following cisplatin treatment in HeLa cells. Cells were pre-treated with or without HET (50 μg/ml) for 24 h, and then incubated with cisplatin at concentrations of 0, 5, 10, 20 or 30 μM. Whole-cell lysates were prepared, and the protein levels of p-Akt, p53 and actin were analyzed by immunoblotting. (A) Representative western blots of three experiments. The protein levels of (B) p-Akt and (C) p53 were normalized to actin levels and are presented as relative ratios to those in control cells without cisplatin treatment. Values are expressed as the mean ± standard deviation. *P<0.05; **P<0.005 for HET-pre-treated vs. HET-untreated cells. HET, Hochu-ekki-to; p, phosphorylated.

anti-cancer drug-induced apoptosis, even though HET alone did not suppress cell viability.

When p-Akt (activated Akt) is outnumbered by counteracting p53, apoptosis is activated (29). Interplay between Akt and p53 determines the cell fate with regard to proliferation or apoptosis (24). Under stress conditions, such as the presence of cytotoxic cisplatin, Akt is negatively regulated by p53 (24). Furthermore, inactivation of Akt leads to suppression of Bcl-2 (30), and upregulation of p53 also suppresses the expression of Bcl-2 (31). Therefore, the downregulation of Akt and the upregulation of p53 (Fig. 6) may have contributed to the apoptosis-enhancing effect of HET.

This apoptosis-enhancing effect of HET was not observed in pancreatic cancer cell lines, including AsPC-1 and MiaPaCa-2, which carry a p53 mutation (32,33). Another study reported that the hepatoma cell line HepG2 (wild-type p53) was more sensitive to growth suppression induced by HET than HA22T (p53 mutation) (14). HeLa cells carry wild-type p53 (34). These findings suggested that the apoptosis-inducing effect of HET may be dependent on the p53 status, as wild-type p53 is required for its efficiency. In this respect, further studies are required to elucidate the underlying upstream mechanisms.

In conclusion, the present study was the first, to the best of our knowledge, to demonstrate that pre-treatment with HET at a low dose, which did not suppress cell viability, enhanced cisplatin-induced apoptosis in HeLa cells, as indicated by a distinct increase in the population of cells in the sub-G_1 phase by flow cytometry, downregulation of p-Akt and the Bcl-2 to Bax ratio, as well as stimulation of cisplatin-induced up-regulation of p53 and active caspase-3. The HET-involved augmenting effect on cisplatin-induced apoptosis may therefore be beneficial for enhancing the efficiency of anti-cancer drug treatment.

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