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Induction of Apoptosis in Human Pancreatic Carcinoma Cells by a Synthetic Bleomycin-like Ligand

Rei Suginaka,[1] Reiko Izui,[1] Jun-ichiro Inoue,[2] Yasuhiko Muraoka,[3] Ken Yamaguchi,[4] Masami Otsuka[5] and Kazuo Umezawa[1, 6]

[1]Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-0061, [2]Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-0071, [3]Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, [4]National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045 and [5]Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973

Histidine-pyridine-histidine-3 (HPH-3) is an oxygen-activating ligand based on the structure of bleomycin. HPH-3 induced the death of human pancreatic adenocarcinoma AsPC-1 cells in 24 h, causing apoptotic morphology and internucleosomal degradation of DNA. HPH-3-induced cell death was not inhibited by antioxidants such as reduced glutathione and *N*-acetylcysteine, whereas hydrogen peroxide-induced cell death was inhibited by them, indicating that hydrogen peroxide is not involved in the induction of apoptosis by HPH-3. Induction of apoptosis by HPH-3 was inhibited by zinc and copper ions, indicating that chelation with ferrous ion is responsible for induction of apoptosis, as is the case in chelation by bleomycin to cleave DNA. Bleomycin A_2 and its fragment having no DNA-binding region, glycopeptide-3, did not induce apoptosis in AsPC-1 cells. Bleomycin A_2 induced G2/M block in flow-cytometric analysis, but HPH-3 did not and instead induced an apoptotic pre-G1 peak. Thus, HPH-3 induced apoptosis in human pancreatic carcinoma cells, which is a unique characteristic among bleomycin-related compounds.

Key words: HPH-3 — Bleomycin — Apoptosis — AsPC-1 pancreatic carcinoma cells

Cell death can be classified into necrosis and apoptosis. In the process of apoptosis, the cells programmed to die are considered to be removed without the induction of inflammation in their microenvironment. In cultured cells, apoptosis is often induced by activation or inhibition of specific signaling pathways. Activation of FAS on the cell surface by anti-FAS,[1)] addition of tumor necrosis factor-α (TNF-α)[2)] or transforming growth factor-β1 (TGF-β1),[3)] inhibition of tyrosine kinase,[4, 5)] and intracellular injection of an inactive Grb 2 isoform[6)] were reported to induce apoptosis. Characteristic features of apoptosis include cell shrinkage, apoptotic body formation, internucleosomal DNA fragmentation, and early induction of cell death after the signal transduction. Based on these criteria, radiation and many anticancer agents induce apoptosis. For example, etoposide,[7)] adriamycin,[8)] and cisplatin[9)] were reported to induce apoptosis in cancer cells.

The effector phase of apoptosis may involve reactive oxygen species (ROS) and proteases such as caspases. ROS were reported to be involved in various instances of apoptosis such as that induced by TNF-α,[10)] TGF-β1,[11)] or erbstatin.[12)] Addition of H_2O_2 or menadione, which produces H_2O_2, to the culture medium also induced apoptosis in cultured cells.[13)]

Bleomycin is an anticancer agent that induces double-strand scission in DNA by oxygen activation.[14)] Although a low-molecular-weight compound, bleomycin consists of oxygen-activating and DNA-binding domains, as shown in Fig. 1A. Glycopeptide-3 (GP-3) corresponds to the portion of bleomycin having no DNA-binding region. Many physico-chemical data indicate that the β-aminoalanin-amide-pyrimidine-β-hydroxyhistidine region of bleomycin forms an iron complex to activate molecular oxygen. Based on the oxygen-activating region of bleomycin, one of us designed a new ligand system of molecules with a symmetrical structure consisting of two histidine units and a pyridine and named them histidine-pyridine-histidine compounds (HPHs).[15, 16)] Among several HPH compounds, HPH-3 (Fig. 1B) is the most effective in activating oxygen.

Isolation or preparation of apoptosis inducers may be useful to develop new anticancer agents, since many solid tumor cells are resistant to apoptosis caused by anticancer agents. In the present study we examined the induction of apoptosis by HPH-3 in human pancreatic carcinoma cells. We also compared its effect with that of bleomycin A_2

[6] To whom reprint requests should be addressed.

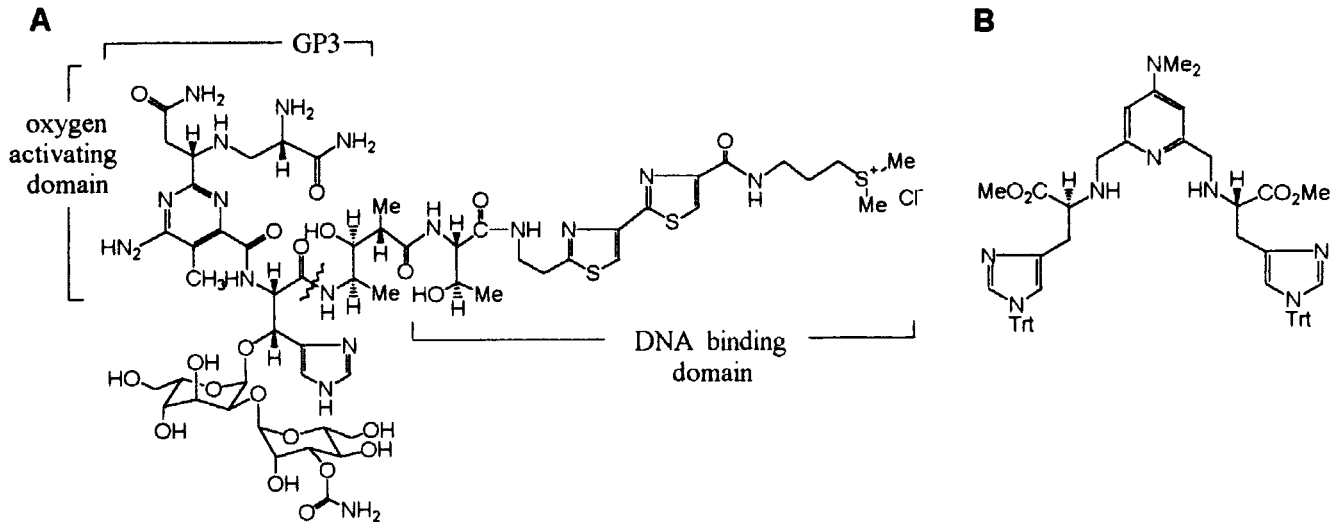

Fig. 1. The structures of bleomycin A_2 (A) and HPH-3 (B).

and with that of its fragment without the DNA-binding region, GP-3.

MATERIALS AND METHODS

Cell lines and cell culture Human pancreatic adenocarcinoma AsPC-1 cells[17] were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Bioserum, Lenexa, KS) at 37°C in a 5% CO_2 atmosphere.

Materials HPH-3 was synthesized as described before.[15] Bleomycin A_2 was obtained from the Institute of Microbial Chemistry, Tokyo. GP-3 was prepared from bleomycin A_2 (Y. Muraoka, unpublished result). HPH-3, bleomycin A_2, and GP-3 were all free of metal ions. HPH-3 was initially dissolved in dimethyl sulfoxide, whereas bleomycin A_2 and GP-3 were dissolved in H_2O.

Cell viability Cells (1×10^5) were seeded in 24-well culture plates. After 24 h, chemicals were added to the cells; and then the cells were incubated for the desired periods. Cell viability was determined by cell counting with a hemocytometer after staining with trypan blue. Surviving cells were expressed as a percentage of the total cell population. Values were expressed as means±SD of triplicate determinations.

Nuclear fragmentation Cells (2×10^5) were plated on coverslips in 12-well culture plates and treated with HPH-3 for 24 h. Then the cells were fixed for 15 min with 3% paraformaldehyde solution, and the nuclei were stained for 5 min with Hoechst 33258 dye. The cells on coverslips were examined under a fluorescence microscope.

DNA fragmentation Cells (5×10^5) were seeded in 60-mm culture dishes. After 24 h, chemicals were added and the cells were incubated for a further 24 h, after which they were collected and lysed in 400 μl of lysis buffer (50 m*M* Tris, 10 m*M* EDTA, 0.5% sodium *N*-lauroyl sarcosinate; pH 8.0) containing 100 μg/ml of proteinase K. The mixture was incubated at 37°C overnight. After addition of 300 μl of TE buffer (10 m*M* Tris, 10 m*M* EDTA; pH 7.5), the whole was centrifuged at 15,000*g* for 20 min, and the precipitate was removed. After sequential extractions with 800 μl of phenol (twice) and chloroform (once), DNA was precipitated with ethanol. Precipitated DNA was resuspended in 40 μl of TE buffer containing 1 μg/ml of RNase. The DNA samples were subjected to electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Flow-cytometric analysis Cells (2×10^6) were seeded in 10-cm culture dishes. After 24 h, chemicals were added to the cells, and the cultures were incubated for the desired periods. The cells were subsequently collected and fixed for 1 h with 70% ethanol, and their DNA was stained for 30 min with 0.1 mg/ml propidium iodide solution. Finally, the cells were analyzed with a flow cytometer (Epics Elite, Coulter, Hialeah, FL).

RESULTS

Induction of cell death and inhibition of growth by HPH-3 HPH-3 reduced the viability of human pancreatic carcinoma AsPC-1 cells in 24 h at 10–30 μg/ml, when viability was assayed by trypan blue dye exclusion, as shown in Fig. 2A. It also inhibited the proliferation of the

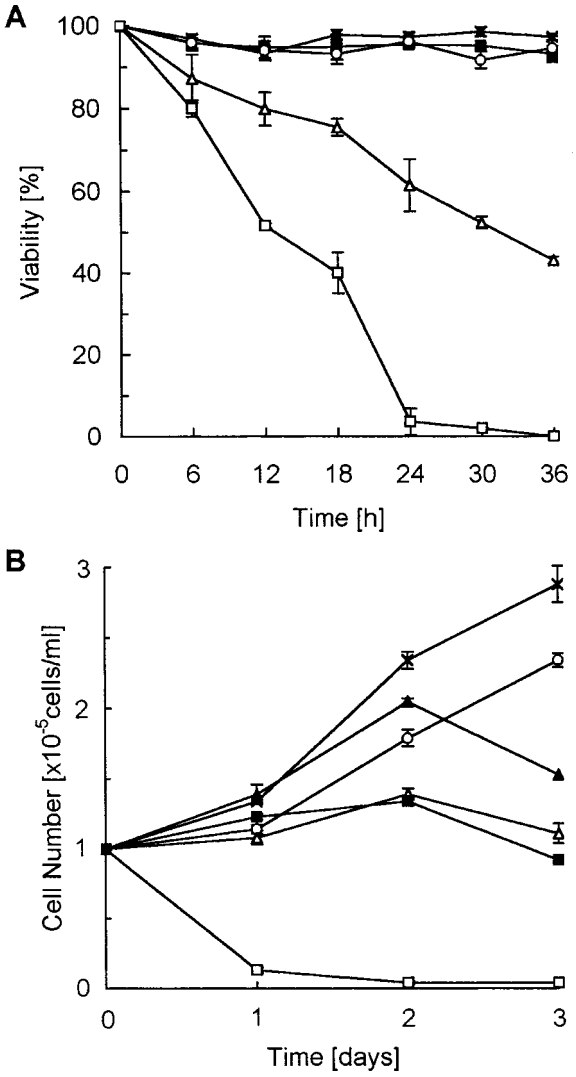

Fig. 2. Induction of cell death (A) and inhibition of growth (B) of AsPC-1 cells by HPH-3. (A) Cells were treated with 0 (×), 3 (○), 10 (△) or 30 (□) μg/ml of HPH-3 or 30 μg/ml of bleomycin A_2 (■) for the indicated periods. Surviving cells determined by the trypan blue dye exclusion assay were expressed as a percentage of the total cell population. Values are means±SD of triplicate determinations. (B) Cells were incubated with 0 (×), 1 (○), 3 (△) or 10 (□) μg/ml of HPH-3, or with 3 (▲) or 10 (■) μg/ml of bleomycin A_2 for the indicated periods. Values are means±SD of triplicate determinations.

cells at slightly lower concentrations, as shown in Fig. 2B. As expected, HPH-3 lowered the cell number when added at 10 μg/ml. On the other hand, bleomycin A_2 did not lower the viability even at 30 μg/ml (Fig. 2A), but it inhibited the growth at 3–10 μg/ml (Fig. 2B).

Induction of apoptosis by HPH-3 HPH-3 induced AsPC-1 cell shrinkage and apoptotic body formation in 24

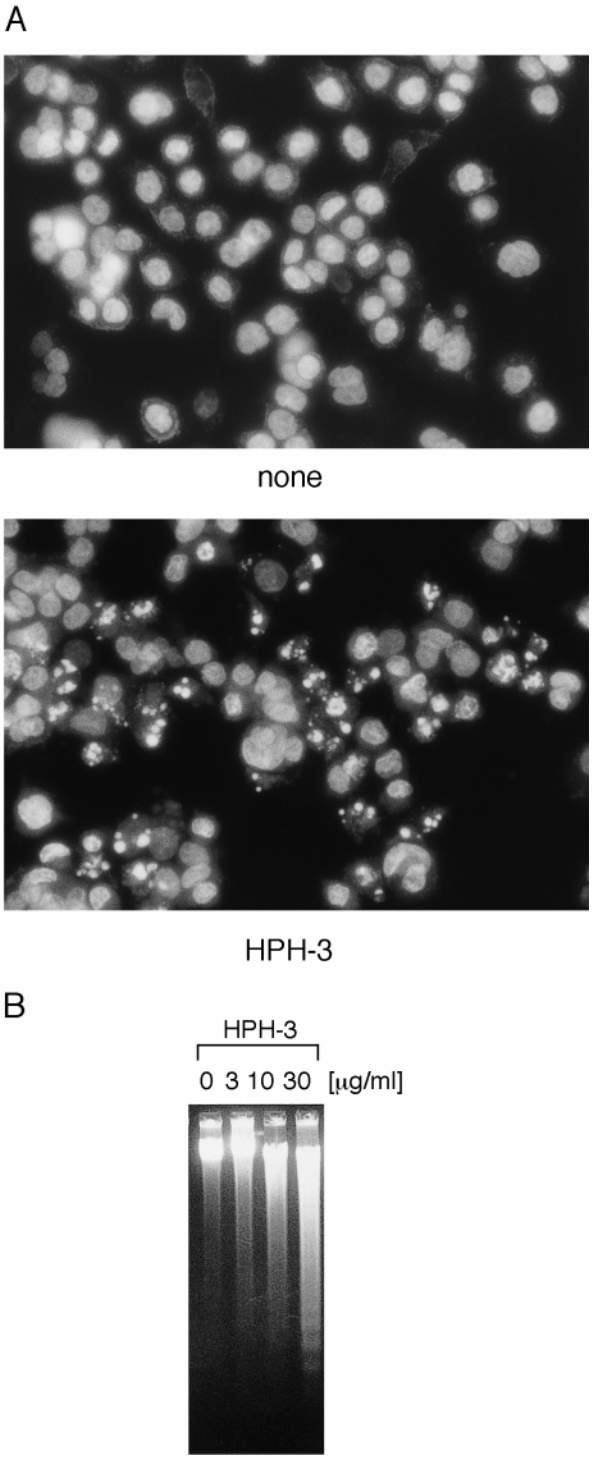

Fig. 3. Induction of apoptosis in AsPC-1 cells by HPH-3. (A) Induction of nuclear fragmentation in AsPC-1 cells by HPH-3. Nuclear fragmentation after 24 h of treatment with 30 μg/ml of HPH-3 was detected by staining with Hoechst 33258. (B) Induction of DNA fragmentation in AsPC-1 cells by HPH-3. The DNA was extracted from cells treated with HPH-3 for 24 h and electrophoresed on an agarose gel.

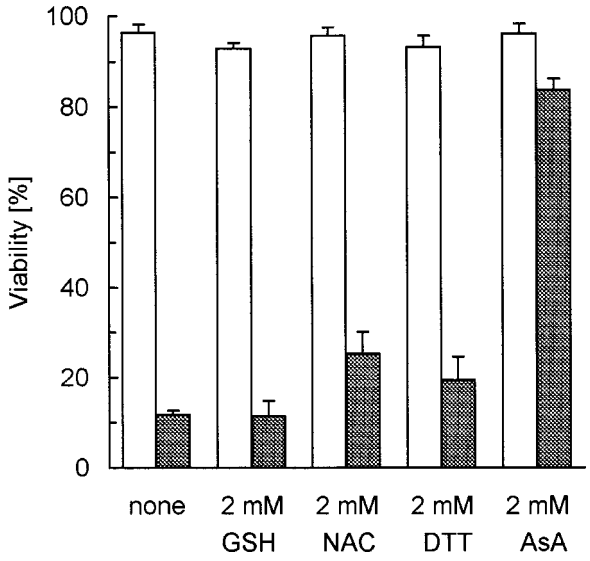

Fig. 4. Effect of antioxidants on HPH-3-induced cell death in AsPC-1 cells. After 1 h of preincubation with antioxidants, cells were treated (solid columns) or not (open columns) with 30 μg/ml of HPH-3 for 24 h. Surviving cells determined by the trypan blue dye exclusion assay are expressed as a percentage of the total cell population. Values are means±SD of triplicate determinations. GSH, reduced glutathione; NAC, *N*-acetylcysteine; DTT, dithiothreitol; AsA, ascorbic acid.

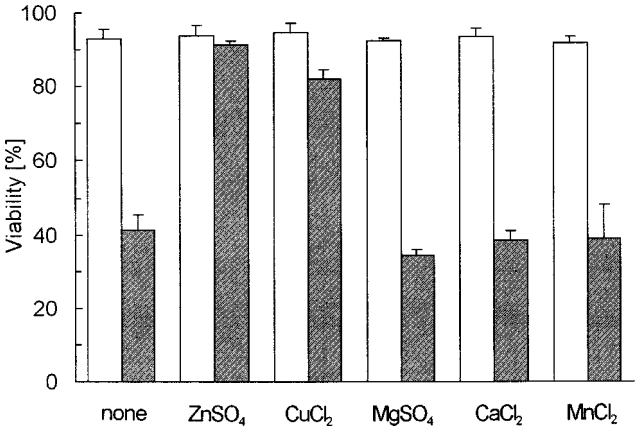

Fig. 6. Effect of metal ions on HPH-3-induced cell death in AsPC-1 cells. After 1 h of preincubation with a 30 μM concentration of the desired metal ion for 1 h, cells were treated (solid columns) or not (open columns) with 30 μM (28.2 μg/ml) of HPH-3 for 24 h. Surviving cells were determined by the trypan blue dye exclusion assay and are expressed as a percentage of the total cell population. Values are means±SD of triplicate determinations.

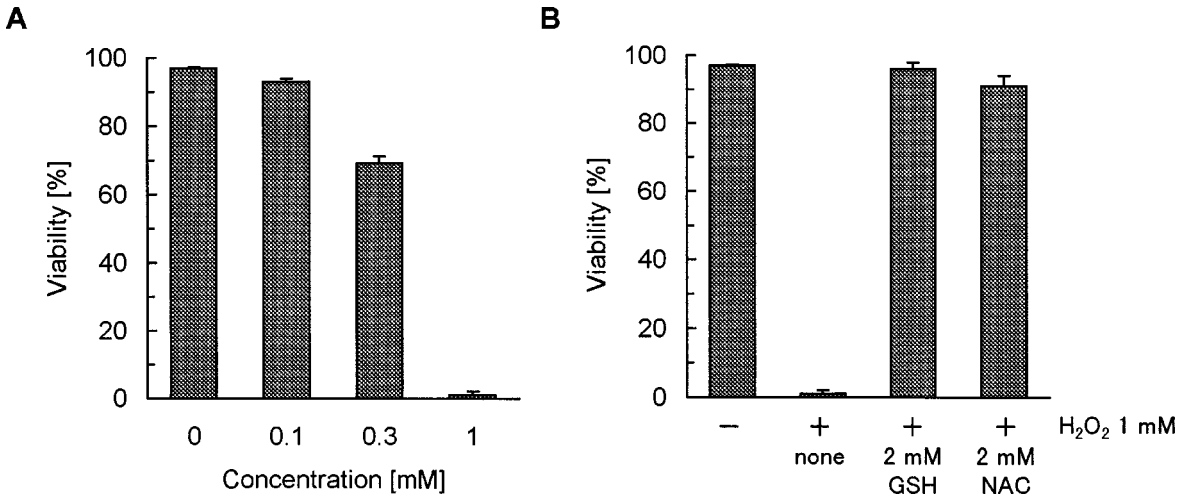

Fig. 5. Induction of cell death in AsPC-1 cells by H_2O_2. (A) Cells were treated with H_2O_2 for 24 h. (B) Effect of antioxidants on H_2O_2-induced cell death. Cells were pretreated with 2 mM reduced glutathione or *N*-acetylcysteine for 1 h, after which 1 mM H_2O_2 was added. Surviving cells were determined by the trypan blue dye exclusion assay and are expressed as a percentage of the total cell population. Values are means±SD of triplicate determinations.

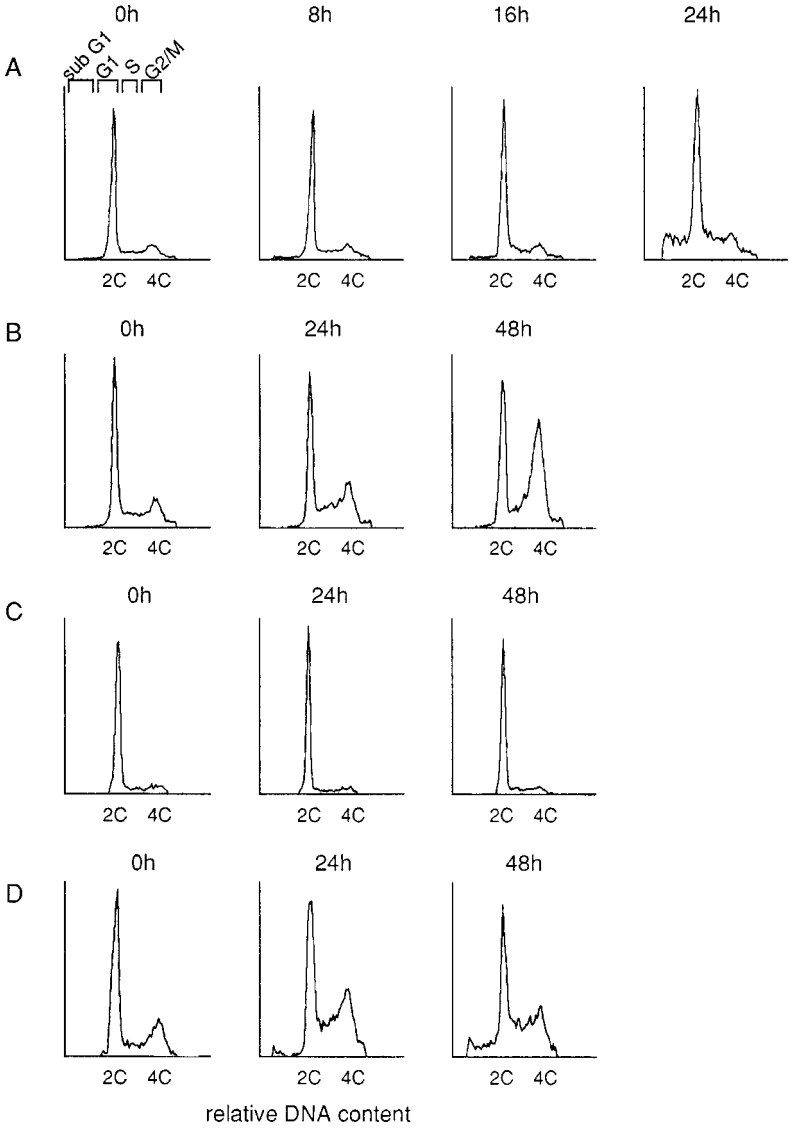

Fig. 7. DNA flow-cytometric analysis of HPH-3- (A), bleomycin A_2- (B), GP-3- (C), and H_2O_2- (D) treated AsPC-1 cells. Cells were treated with 10 μM (9.4 μg/ml) HPH-3 for 8, 16 or 24 h, or with 30 μM bleomycin A_2, 30 μM GP-3, or 1 mM H_2O_2 for 24 or 48 h. They were then stained with propidium iodate and analyzed by flow cytometry. The cells were severely damaged when treated with 10 μM HPH-3 for 48 h.

h at 30 μg/ml, as well as nuclear fragmentation detectable by Hoechst 33258 staining (Fig. 3A). It also induced internucleosomal fragmentation of DNA at 10–30 μg/ml after incubation for 24 h (Fig. 3B). Thus, the cell death induced by HPH-3 was shown to be apoptotic.

Effect of antioxidants on HPH-induced cell death Antioxidants such as reduced glutathione, *N*-acetylcysteine, dithiothreitol, and ascorbic acid were added to the cells prior to HPH-3. As shown in Fig. 4, except for ascorbate, these antioxidants did not inhibit the HPH-3-induced cell death. On the other hand, hydrogen peroxide induced cell death in AsPC-1 cells at 0.3–1 mM, as shown in Fig. 5A. The hydrogen peroxide-induced cell death was completely inhibited by reduced glutathione and *N*-acetylcysteine (Fig. 5B). Therefore, it is unlikely that hydrogen peroxide is involved in the mechanism of HPH-3-induced cell death.

Effect of metal ions on HPH-3-induced cell death In the case of bleomycin, chelate formation with a trace amount of ferrous ion (Fe^{2+}) is considered to be responsi-

ble for oxygen activation and DNA scission.[18] Bleomycin is also known to bind Cu^{2+} and Zn^{2+} more strongly than Fe^{2+}.[19] Therefore, we studied the effect of these metal ions on HPH-3-induced cell death. Each metal ion was added at the equivalent molar concentration to that of HPH-3. As shown in Fig. 6, Zn^{2+} and Cu^{2+} inhibited the reduction in viability induced by HPH-3, whereas other divalent ions such as Mg^{2+}, Ca^{2+}, and Mn^{2+} did not. Thus, metal-chelating activity is likely to be responsible for the cytotoxic effect of HPH-3.

Flow-cytometric analysis of DNA from AsPC-1 cells treated with HPH-3, bleomycin A_2 or GP-3 Neither bleomycin A_2 nor GP-3 even at 100 μM induced cell death in AsPC-1 cells. Bleomycin A_2 inhibited proliferation of the cells at 3–30 μM in 3 days, as shown in Fig. 2B; but GP-3 at 30 μM did not (data not shown). Then, we compared the effect of HPH-3 to that of related compounds, bleomycin A_2 and GP-3, on cell-cycle progression. A 24-h or 48-h treatment with HPH-3 at 10 μM (9.4 μg/ml) induced a pre-G1 peak, indicative of apoptotic cells, as shown in Fig. 7. No accumulation of G1 or G2/M cells was observed. On the other hand, bleomycin A_2 even at 30 μM did not induce pre-G1 cells, and clearly caused the accumulation of G2/M cells. GP-3 is the portion of bleomycin A_2 having no DNA-binding ability. GP-3 did not induce either pre-G1 cells or accumulation of G2/M cells. Thus, only HPH-3 induced apoptotic cell death among the 3 related compounds. Since adriamycin, cisplatin, and vinblastine did not induce early cell death in AsPC-1 cells, we used hydrogen peroxide as a positive control. It also induced pre-G1 peaks in AsPC-1 cells (Fig. 7D). Both HPH-3 and hydrogen peroxide weakly induced pre-G1 peaks in AsPC-1 cells.

DISCUSSION

Although adriamycin, cisplatin, and vinblastine are known to induce apoptosis, they did not induce cell death or apoptotic morphology in AsPC-1 cells at 30 μg/ml in 24 h (manuscript in preparation). However, HPH-3 induced early cell death, internucleosomal DNA degradation, and morphological apoptosis in these cells. The HPH-3-induced cell death and DNA fragmentation were not inhibited by 100 μM caspase-1 inhibitor Ac-YVAD-CHO or by 100 μM caspase-3 inhibitor Ac-DEVD-CHO. Also, HPH-3 did not change the cellular level of Bcl-2, or that of Bax in 12 h, as judged by western blotting analysis (data not shown). Therefore, HPH-3 may act directly at the effector phase of apoptosis rather than at the induction phase by producing reactive oxygen species.

HPH-3 induced early cell death and apoptotic morphological changes not only in AsPC-1 cells, but also in mouse leukemia L1210 cells, human acute promyelocytic leukemia HL60 cells, and human epidermoid carcinoma KB cells. It was also effective in inducing apoptosis in multidrug resistant KBC-4 cells.

The structure of HPH-3 was derived from the oxygen-activating region of bleomycin.[15] It is not known what kind of reactive oxygen species is responsible for DNA scission by bleomycin. We also could not identify the type of reactive oxygen produced by HPH-3 to induce apoptosis. However, involvement of hydrogen peroxide was excluded, since reduced glutathione and *N*-acetylcysteine did not inhibit the HPH-3-induced cell death, whereas they did inhibit the hydrogen peroxide-induced cell death. Among the 4 antioxidants employed, only ascorbic acid inhibited the induction of apoptosis by HPH-3. HPH-3 did not react with ascorbic acid directly in the aqueous solution, as judged by thin-layer chromatographic analysis. Among glutathione, *N*-acetylcysteine, and ascorbic acid, only ascorbic acid was reported to be a scavenger of singlet oxygen.[20, 21] Therefore, it is possible that HPH-3 produces singlet oxygen to induce apoptosis. Ascorbic acid is a two-electron reductant, and can reduce Fe^{3+} to Fe^{2+}.[22] As in the case of bleomycin, HPH-3 is likely to chelate Fe^{2+} in the medium to form HPH-3-Fe^{2+}-O_2, which should be responsible for oxygen activation, as Fe^{2+}-O_2 is likely to be converted to Fe^{3+}-O_2^-. Thus, another possible explanation would be that ascorbate interferes with the transition from Fe^{2+} to Fe^{3+} in the chelate.

Bleomycin was reported to be a weak apoptosis inducer, and it induced apoptosis only at a high intracellular concentration in Chinese hamster ovary cells.[23] In AsPC-1 cells it did not induce apoptosis, although it caused a G2/M block (Fig. 7) and inhibited the growth. Bleomycin contains a DNA-binding region, whereas HPH-3 does not. However, the two are known to have comparable oxygen-activating ability. Therefore, we first expected that removal of the DNA-binding region from the structure of bleomycin would confer apoptosis-inducing activity upon bleomycin. However, GP-3, a fragment of bleomycin having no DNA-binding region, did not induce apoptosis at all. Thus, apoptosis-inducing activity seems to be a unique characteristic of HPH-3 among bleomycin-related compounds. HPH compounds may be useful core structures to design new potent and selective agents for induction of apoptosis in neoplastic cells.

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