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Heterocyclic organobismuth(III) induces apoptosis of human promyelocytic leukemic cells through activation of caspases and mitochondrial perturbation

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ABSTRACT

We have synthesized novel heterocyclic organobismuth compounds that have potent antibacterial properties. In this study, we examined their anticancer activity and addressed the cellular mechanisms involved. Heterocyclic organobismuth compounds showed anticancer activities in various human cancer cell lines. These compounds have particularly potent anticancer activities against leukemia cell lines. One of them, bi-chlorodibenzo [c,f][1,5] thiabismocine (compound 3), inhibited the growth of the human promyelocytic leukemia cell line HL-60 at a concentration of 0.22 μM. Low concentrations of compound 3 (0.22–0.44 μM) induced apoptosis, whereas at a higher concentration (>1.1 μM) it causes acute necrosis. During the apoptosis, caspase-3, -8, and -9 were activated but caspase-12 was not. A broad caspase inhibitor (z-VAD-fmk), and caspase-3 (z-DEVD-fmk) and caspase-9 (z-LEHD-fmk) inhibitors suppressed the compound 3-induced apoptosis, but a caspase-8 inhibitor (z-IETD-fmk) was less effective, suggesting that the caspase-8 activity only partially participates in the apoptosis. In the apoptotic cells, cytochrome *c* was released from mitochondria to cytosol and a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) was detected. Compound 3-induced apoptosis was associated with enhanced generation of intracellular reactive oxygen species (ROS). Pretreatment of the cells with *N*-acetyl-L-cysteine or catalase suppressed the apoptosis. On the other hand, buthionine sulfoximine enhanced the compound 3-induced collapse of $\Delta\Psi_m$ and apoptosis. Taken together, these results indicate that compound 3 is a potent inducer of apoptosis, triggering a caspase-3-mediated mechanism *via* the generation of ROS and release of cytochrome *c* from mitochondria, suggesting a potential mechanism for the anticancer activity of compound 3.

1. Introduction

Metals and metal compounds have been used in medicine for thousands of years. Because metal compounds exhibit anticancer activities *via* a wide range of mechanisms, they are regarded as a valuable source of novel chemotherapeutic reagents. Bismuth belongs to group V of the periodic table along with arsenic and antimony and is recognized as a low toxic metal. Traditionally, inorganic salts of bismuth have been used in medicine and veterinary practice [1,2]. Unlike inorganic salts, organobismuth compounds that contain at least one carbon atom directly bound to the bismuth centre are toxic to mammalian cells and fungal cells as well as to bacterial cells. This cytotoxicity is known to differ depending on the compound, but the mechanism of bioactivity is still unclear [2].

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Recently, anticancer activities of metals and metal compounds have been reported [3], suggesting bismuth compounds to be useful as chemotherapeutic reagents against tumors. Indeed, organobismuth compounds have been studied as antitumor drug. Klapötke et al. tested their organobismuth(III) bis (thiolates) for antiproliferative properties against Ehrich ascites tumor growing as a non-solid tumor in the peritoneal cavity of mice. Among their compounds, methylbis[4-(methylammonio) phenylthiolato] bismuthane diiodide was most active, suggesting that the molecular structure of organobismuth(III) compounds can be modified to influence their toxic effects on tumors [4,5].

Some anticancer reagents cause cell death by interfering with the processes of the cell cycle, while others cause cell death through apoptosis. Therefore, apoptosis has been recognized as an important determinant of the response of tumor cells to chemotherapeutic agents. Leukemia, one of the most threatening hematological malignant cancers today, has been found to be very sensitive to anticancer chemotherapeutic reagents which either interfere with the cell cycle or cause apoptosis. Thus, the manipulation of apoptosis can probably provide novel strategies for cancer chemoprevention or chemotherapy in general, either by selectively activating apoptosis in malignant cells or by suppressing apoptosis in normal cells. Mitochondrial production of reactive oxygen species (ROS) seems to play a role in cell death [6]. Exogenous and endogenous ROS, such as H_2O_2 and O_2^-, cause apoptosis through mitochondrial permeability transition. Some anticancer drugs induce the formation of ROS in apoptosis [7,8]. Several studies suggest that arsenic trioxide can induce apoptosis *via* a direct mitochondrial effect leading to the activation of caspases [9]. Arsenic trioxide is an effective treatment for acute promyelocytic leukemia, but arsenic is a highly toxic agent. The development of new anticancer agents with lower toxicity, higher therapeutic index, and lower capacity to induce resistant phenotypes would greatly improve chemotherapy.

We have previously reported that novel heterocyclic organobismuth(III) compounds have pronounced antibacterial activity [10]. We report here that one of these compounds, bi-chlorodibenzo [*c*,*f*][1,5] thiabismocine, exerted a dual dose-dependent effect on the human promyelocytic leukemia cell line HL-60, inhibiting cell proliferation and triggering apoptosis at a low concentration (0.44 μM) and resulting in necrosis at a high concentration (1.1 μM). The compound caused the activation of caspases-3 and -9, $\Delta\Psi m$ collapse, and the release of cytochrome *c* from mitochondria to the cytosol, suggesting that the main lethal apoptotic pathway induced by this organobismuth involves mitochondrial damage.

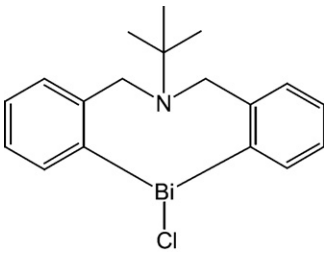
2. Materials and methods

2.1. Materials and chemicals

Catalase, *N*-acetyl-L-cysteine (NAC), 2,7-dichlorodihydrofluorescein diacetate (2′,7′-dichloroflorecin diacetate) (DCFH-DA), Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Propidium iodide (PI) and buthionine sulfoximine (BSO) were purchased from Sigma–Aldrich (St. Louis, MO), Rhodamine 123 was obtained from Molecular Probe (Eugene, OR) and the caspase inhibitors (z-VAD-fmk, z-DEVD-fmk, z-LEHD-fmk, and z-IETD-fmk), from MBL (Nagoya, Japan). Annexin V-HiLyte Fluor™ 488 was from AnaSpec International (San Jose, CA). The primary antibodies used were anti-caspase-3 (C31720) from Transduction Laboratories (Lexington, KY), anti-caspase-9 (M054-3) from MBL, anti-caspase-8 (AM46T) from Calbiochem (San Diego, CA), anti-caspase-12 (C 7611) from Sigma, anti-lamin B (NA12) from Calbiochem, anti-actin antibody (BT-560) from Biomedical Technologies (Stoughton, MA), and anti-cytochrome *c* antibody (C 5723) from Sigma. The goat anti-mouse IgG and IgM conjugated-AP antibody (AM13705) was obtained from Biosource (Camarilo, CA). The goat anti-rabbit IgG conjugated-AP antibody (4050-04) was obtained from Southern Biotechnology Associates (Birmingham, AL). The donkey anti-sheep IgG conjugated-AP (A5187) and the rabbit anti-rat IgG conjugated-AP (A6066) antibody were obtained from Sigma. All other chemicals were obtained from Sigma–Aldrich or Wako and were of the highest quality commercially available.

2.2. Cyclic organobismuth compounds

The organobismuth compounds were synthesized as previously described [10]. The compounds used in this study are *N*-*tert*-butyl-bi-chlorodibenzo[*c*,*f*][1,5]azabismocine (compound 1), bi-chlorodibenzo [*c*,*f*][1,5] thiabismocine (compound 3) bi-chlorophenothiabismin-*S*,*S*-dioxide (compound 5) (Fig. 1).



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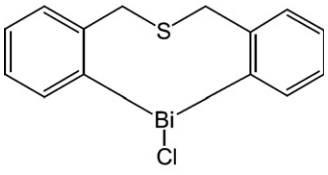

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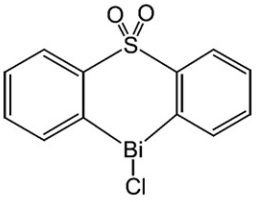

#5

Fig. 1 – Chemical structure of the cyclic organobismuth(III) compounds.

2.3. Cells and culture

HL-60, Molt-4, U937, NB-4, K562, colo201, DLD-1, (ATCC) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37 °C with 5% CO_2. MDA-MB-435S, HT1080, MG-63, MIA-paca, H226, A549, H596, HeLa and TIG cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. KATO3 cells were cultured in 40% RPMI1640 and 40% DMEM medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. SK-N-SH cells were cultured MEM-α medium supplemented with 10% fetal bovine serum.

2.4. Analysis of inhibition of cell growth

Cytotoxicity was measured using the MTT assay. The MTT assay was performed using a modified method described by Plumb et al. Briefly, the cells (5×10^4 cells/ml) were seeded in each well in 96-well plates. After 24 h, various concentrations of compound 3 were added. After 12 h, MTT was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue formed in the cells was dissolved with 100 μl DMSO. The optical density was measured at 570 nm. Cell number was measured with a Beckman-Coulter Z1 Particle Size Counter.

2.5. Cellular viability

Cellular viability was determined by the ability of the cells to exclude PI. HL-60 cells were treated with compound 3 in the absence or presence of z-VAD-fmk pretreatment and stained with 50 μg/ml PI. The vital cells were analyzed by Becton-Dickinson FACSCalibur flow cytometer.

2.6. Morphological change and determination of apoptotic cells treated with organobismuth(III) compounds

HL-60 cells were treated with compound 3 for various periods. Cells were washed with ice-cold PBS and fixed with 1% glutaraldehyde. The suspensions were then washed with PBS and stained with 0.2 mM Hoechst 33258. Cells were examined under a fluorescence light microscope when the nucleus exhibited typical apoptotic features such as chromatin condensation around the periphery and/or fragmentation.

2.7. Electrophoretic analysis of apoptotic DNA degradation

Approximately 5×10^5 cells were harvested, washed in PBS, and centrifuged again. The cell pellets were lysed in 200 μl of lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5 mg/ml RNase A, and 0.5 mg/ml proteinase K), and incubated at 37 °C for 30 min. The lysates were resuspended in 300 μl of NaI solution (6 M NaI, 10 mM Tris–HCl pH 8.0, 13 mM EDTA, 0.5% sodium N-laulylsarcosine, and 30 μg/ml glycogen), and incubated at 60 °C for 15 min. DNA was precipitated with 1 volume of 100% isopropanol, and washed with 50% isopropanol. DNA pellets were dried in air and resolved in TE. DNA was electrophoresed on a 2.5% agarose gel and visualized with ethdium bromide, and the DNA pattern was examined by ultraviolet transillumination.

2.8. Measurement of nuclear DNA fragmentation by flow cytometer

HL-60 cells were treated with compound 3, harvested and washed with PBS. The cells were fixed in ice-cold 70% ethanol, and stored at −20 °C. They were pelleted, washed with PBS, resuspended in PBS, added to PBS containing 0.5 mg/ml of RNase A, and incubated at 37 °C for 20 min. The cells were then pelleted, resuspended in PBS containing 50 μg/ml of propidium iodide, and incubated at 4 °C for 10 min in the dark. Finally, the stained cells were analyzed with a Becton-Dickinson FACSCalibur flow cytometer and the number of cells in the area corresponding to the sub-G1 population was calculated using CellQuest software.

2.9. Assessment of apoptosis by annexin-V staining

After drug treatment, 4×10^5 cells were washed in PBS, and resuspended in 500 μl of staining solution containing annexin V-HiLyte Fluor™ 488 (0.5 μg/ml) in HEPES buffer (10 mM HEPES pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). After a 15 min incubation in the dark at room temperature, the cells were washed in HEPES buffer and analyzed by Becton-Dickinson FACSCalibur flow cytometer.

2.10. Electron microscopy

The electron microscopy was performed as described previously [11]. HL-60 cells were treated with compound 3 at 0.22 μM for 12 h or 1.1 μM for 24 h. They were collected and washed twice with PBS, fixed with 0.1% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h, and rinsed twice in 0.2 M cacodylate buffer (pH 7.2). The cells were next fixed with osmium tetroxide, embedded in 2% agar in cacodylate buffer, treated with 0.5% uranyl acetate, dehydrated with an ascending series of ethanol and propylene oxide, and embedded in Epon. After staining with lead acetate, ultrathin sections were viewed at 80 kV on a JEOL100S electron microscope.

2.11. Measurement of ATP levels

Measurement of ATP levels was performed according to the method of manufacturer's instruction. Briefly, HL-60 cells were treated with various concentrations of compound 3, washed with PBS and then treated with cold 0.5% trichloroacetic acid for 15 min on ice. After centrifugation at 12,000 × *g* for 10 min at 4 °C, the supernatants obtained were neutralized with Tris–acetate buffer. The ATP content in the supernatants was determined using an ATP assay kit (LL-100-1, TOYO. B-Net Co., Tokyo). Bioluminescence was assessed on a PerkinElmer Wallac ARVO MX/Light 1420 multilabel/Luminonescence Counter).

2.12. Western blotting

The Western blotting was performed as described previously [12]. Before cell lysis, HL-60 cells were washed once in PBS. Cells were pelleted by centrifugation and resuspended in cell lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% NP-40,

1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 5 μg/ml antipain). After 5 min on ice, cells were centrifuged at 12,000 × *g* for 15 min, at 4 °C, and assayed for protein concentration. The supernatant was then added to boiling sample buffer and boiled for 10 min. Equal amounts of proteins were loaded and separated by SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to a PVDF membrane and probed by corresponding antibodies. The protein signals were detected with a BCIP/NBT reaction system.

2.13. Preparation of mitochondria-enriched and cytosolic fractions

The mitochondrial and cytosolic extracts were obtained as described elsewhere [13], with slight modifications. Briefly, HL-60 cells were incubated in ice-cold buffer A (10 mM HEPES pH 7.5, 250 mM sucrose, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 5 μg/ml antipain) containing 75 μg/ml digitonin on ice for 1 min, followed by centrifugation at 12,000 × *g* for 1 min. The supernatant was saved as the cytosolic fraction, and the pellet (mitochondrial fraction) was resuspended in the same volume of cell lysis buffer. The pellet was solubilized on ice for 5 min and centrifuged at 4 °C. The supernatant was stored as the mitochondrial fraction.

2.14. Assessment of loss of mitochondrial membrane potential

$\Delta\Psi_m$ was monitored using rhodamine 123, taken up by mitochondria in proportion to $\Delta\Psi_m$. Rhodamine 123 is a fluorescent cationic dye that accumulates in the mitochondrial matrix because of its charge and solubility in both the inner mitochondrial membrane and the matrix space [14]. For each condition, cells were incubated for 15 min at 37 °C in 1 ml of 10 μg/ml rhodamine 123. The cells were washed with PBS and subsequently analyzed using a Becton-Dickinson FACSCalibur flow cytometer and CellQuest software.

2.15. ROS determination

The production of ROS was detected using a previously described method [15], with minor modifications. Briefly, 2×10^5 cells/ml of HL-60 cells were incubated in the absence or presence of compound 3. Intracellular ROS were labeled with 10 mM DCFH-DA. Within the cells, DCFH-DA is converted to DCFH, which can be oxidized to the fluorescent compound DCF in the presence of ROS. After incubation, the cells were washed twice with PBS and analyzed with a Becton-Dickinson FACSCalibur flow cytometer.

2.16. Statistical analysis

Statistical analyses were performed with Excel 2003 using Student's *t*-test.

3. Results

3.1. Anticancer activity of heterocyclic organobismuth compounds on human leukemia cell lines

Using MTT assay, we determined the effect of heterocyclic organobismuth compounds on human tumor cell lines. Fifty percent of growth inhibition (IC_{50}) was measured in these cells treated with heterocyclic organobismuth compounds at concentrations ranging from 0.01 to 10 μM. As shown in

Table 1 – Cytotoxic activities of heterocyclic organobismuth compounds against different tumor cell lines

Human tumor type	Cell line	IC_{50}[a] (μM)		
		#1	#3	#5
Leukemia	Molt-4	0.059 ± 0.009	0.036 ± 0.005	0.202 ± 0.013
	U937	0.072 ± 0.003	0.057 ± 0.005	0.295 ± 0.017
	HL-60	0.095 ± 0.064	0.151 ± 0.015	1.126 ± 0.503
	NB4	0.104 ± 0.009	0.054 ± 0.005	0.249 ± 0.022
	K562	0.688 ± 0.161	0.823 ± 0.300	0.953 ± 0.305
Brest cancer	MDA-MB-435S	0.255 ± 0.046	0.453 ± 0.045	1.398 ± 0.252
Colon cancer	Colo201	0.262 ± 0.084	0.595 ± 0.203	1.839 ± 0.343
	DLD-1	1.286 ± 0.954	1.437 ± 0.865	3.018 ± 1.547
Neuroblastoma	SK-N-SH	0.327 ± 0.060	0.975 ± 0.262	1.269 ± 0.089
Pancreatic cancer	MIA paca	0.675 ± 0.099	1.202 ± 0.157	2.328 ± 0.158
Lung cancer	H226	0.613 ± 0.202	1.298 ± 0.044	2.895 ± 0.171
	A549	1.146 ± 0.144	2.677 ± 0.997	4.292 ± 2.036
	H596	0.993 ± 0.189	2.827 ± 0.796	4.202 ± 0.784
Osteosarcoma	MG-63	2.084 ± 1.014	1.988 ± 0.665	2.337 ± 0.618
Fibrosarcoma	HT1080	2.472 ± 0.588	3.256 ± 0.241	3.869 ± 0.921
Gastric cancer	KATO3	4.702 ± 1.198	3.716 ± 0.968	5.141 ± 1.022
Cervical cancer	Hela	4.266 ± 1.897	4.780 ± 0.421	4.848 ± 0.657
Normal human cell fibroblasts	TIG	2.420 ± 0.142	2.140 ± 0.277	4.166 ± 0.116

[a] IC_{50} values were determined from MTT assays after incubation with test compounds for 12 h.

Table 1, compounds 1, 3, and 5 represented IC_{50} in the range of 0.059–4.7, 0.036–4.8 and 0.20–5.1 μM, respectively. The sensitivity of the leukemia cell lines to these compounds was higher than those of solid tumor lines. As well as antimicrobial activities [10], the eight-membered-ring compounds, compounds 1 and 3, showed higher antitumor activity than six-membered ones, e.g. compound 5, against leukemia cell lines. Normal human fibroblasts, TIG cells, were also tested. Concentrations of these compounds required for IC_{50} of the TIG cells were much higher than those of leukemia cell lines.

3.2. *The heterocyclic organobismuth(III) compounds inhibit the cell growth of cancer cells and induce either apoptosis or necrosis depending on its final concentration*

A concentration of 0.22 μM of compound 3 was high enough to inhibit cell growth (Fig. 2A). Next, we examined whether the inhibition of cell growth by compound 3 could be caused by apoptotic cell death. HL-60 cells were treated with various concentrations of compound 3 for the times indicated. Compound 3-induced apoptosis was determined by Hoechst 33258 staining of cells with nuclear condensation and fragmentation (Fig. 2B). Compound 1 also induced apoptotic cell death in HL-60 cells at much lower concentration than compound 3, but compound 5 induced apoptosis at maximum 30% (data not shown). As shown in Fig. 2C, the apoptosis increased in a time-dependent manner when the cells were exposed to 0.22 or 0.44 μM compound 3. However, the cells exposed to 1.1 μM exhibited no morphological changes representative of apoptosis.

Nucleosomal DNA fragmentation (DNA ladder formation) is one of the hallmarks of apoptosis and is executed by DNase activated by apoptotic stimuli. DNA-gel electrophoresis showed that HL-60 cells treated with 0.22–0.44 μM compound 3 displayed DNA fragmentation in multiples of 180–200 bp, but at 1.1–2.2 μM compound 3 did not induce the fragmentation (Fig. 3A). Flow cytometric analysis of DNA content also showed that the percentage of cells with a sub-G1 DNA content increased along with the concentration of compound 3 until 0.44 μM (Fig. 3B). However, this increase was suppressed at a concentration higher than 1.1 μM (Fig. 3B). As shown in Fig. 3C, the cytofluorometric analysis using annexin V-HiLyte Fluor™ 488, which stains phosphatidylserine residues, also showed that the percentage of annexin V-positive cells increased among compound 3-treated HL-60 cells at 0.22 or 0.44 μM, but this increase was suppressed at a concentration higher than 1.1 μM.

It was concluded that low concentrations of compound 3 induced apoptosis, but high concentrations (more than 1.1 μM) did not. The MTT assay of cells treated with compound 3 for 12 h at concentrations higher than 1.1 μM indicated cell viability to be decreased to <10% (data not shown). Thus, a high concentration of compound 3 was expected to promote a cell death without apoptosis. As shown in Fig. 4, transmission electron microscopy of cells exposed to 1.1 μM compound 3 showed representative necrotic cell features in which the cytoplasm became swollen and the plasma membrane disintegrated (Fig. 4B), whereas cells exposed to 0.22 μM compound 3 had the condensed chromatin and fragmented nuclei (Fig. 4A).

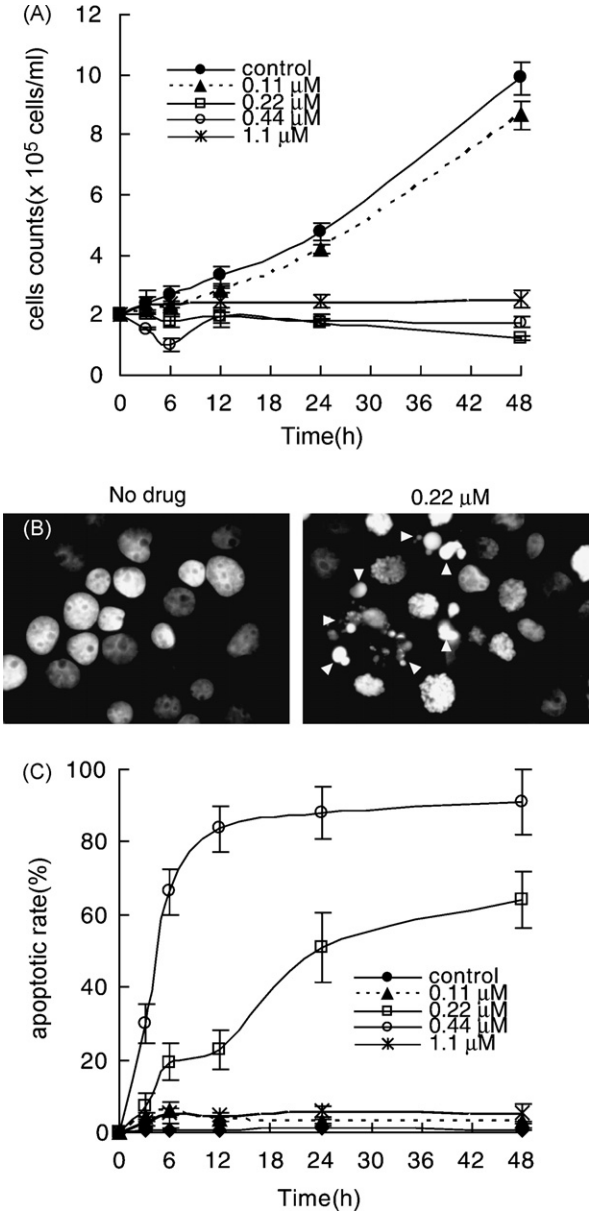

Fig. 2 – Effect of the organobismuth compound on HL-60 cells. (A) HL-60 cells were incubated with compound 3 for the indicated times. Cell numbers were measured with a Beckman-Coulter Z1 Particle Size Counter. (B) Morphological changes of HL-60 cells treated with 0.22 μM compound 3. HL-60 cells exposed to 0.22 μM compound 3 were fixed with glutaraldehyde and stained with Hoechst 33258. The cells were observed under a fluorescence microscope. Apoptotic cells showed condensed or fragmented chromatin in the nucleus (arrowhead). (C) Apoptotic rate of HL-60 cells treated with compound 3. Cells were treated with various concentrations of compound 3 for the indicated times. After treatment, cells were harvested and stained with Hoechst 33258. Apoptotic cells were examined by counting the cells with condensed and fragmented nuclei. Each point represents an average of 3 independent experiments, and standard deviation bars are shown.

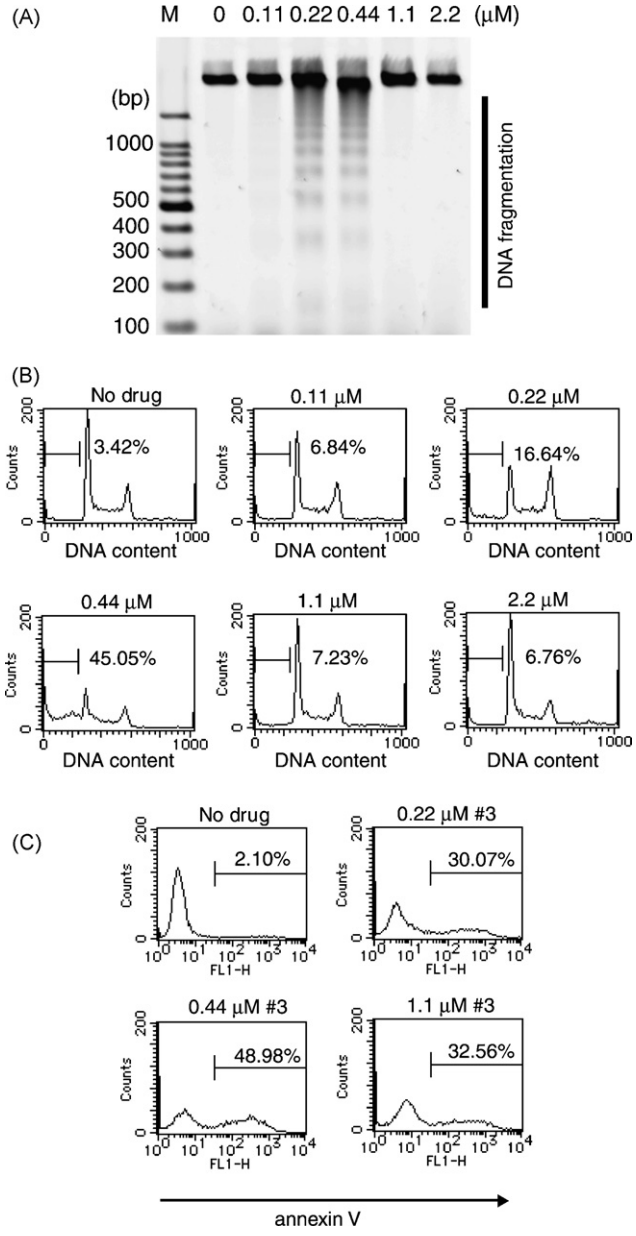

Fig. 3 – DNA fragmentation assay and cell cycle distribution. (A) DNA was extracted from HL-60 cells treated with the indicated concentrations of compound 3 for 6 h and subjected to 2.5% agarose gel electrophoresis. (B) Cell cycle distribution of cells treated with compound 3. HL-60 cells treated with the indicated concentrations of compound 3 for 6 h were harvested and fixed in 70% ethanol. After staining with propidium iodide they were analyzed using a flow cytometer. The percentage of cells in the sub-G1 population is indicated in each panel. (C) HL-60 cells were incubated with the indicated concentrations of compound 3 for 6 h, and then stained with annexin-V-HiLyte Fluor™ 488, which specifically detects exposed phosphatidyl serine residues at the cell surface. The number of annexin-V-positive cells was determined using a flow cytometer and the percentage is represented in each panel.

Apoptosis is an energy-dependent process, in such a manner that the decrease of ATP below critical levels may impede the execution of apoptosis and promote necrosis [16,17]. Then, we examined whether compound 3 decreases intracellular ATP contents. As shown in Fig. 4C, treatment with 0.22 or 0.44 μM compound 3 caused a decrease in ATP level. On the other hand, high concentrations of compound 3 (1.1 or 2.2 μM) did not decrease the ATP content (Fig. 4D). Thus intracellular ATP depletion does not implicate in compound 3-induced necrotic cell death.

3.3. *Compound 3 induces apoptosis through caspase-dependent pathways*

The caspase protease family plays a central role in the terminal, execution phase of apoptotic cell death [18–20]. It is broadly divided into two groups: initiator caspases, and executioner caspases. Caspase-3 is an executioner caspase. Caspase-8, -9, and -12, each trigger three major apoptotic cascades, the death receptor pathway, the mitochondrial pathway, and the endoplasmic reticulum pathway [21,22]. Thus, we studied whether and if so, which caspases were involved in compound 3-induced apoptosis.

To determine whether the activity of caspases is essential for the apoptosis, the effect of z-VAD-fmk, a general caspase inhibitor, was examined. As shown in Fig. 5, treatment with z-VAD-fmk before the administration of compound 3 completely prevented the appearance of the cells with apoptotic features, such as the increase in the population of cells with a sub-G1 DNA content (Fig. 5A) or nuclear condensation and fragmentation (Fig. 5B). In addition, z-VAD-fmk decreased cell death induced by 0.44 μM compound, but not by 1.1 and 2.2 μM (Fig. 5C). These results indicate that the compound 3-induced apoptotic cell death to require the activation of caspases. Moreover, caspases activation is not necessary for compound 3-induced necrotic cell death.

Activation of caspases is known to occur after the cleavage of their respective precursors [23–25]. Then, to know which caspases are involved in compound 3-mediated apoptosis, the levels of each caspase precursor were examined in the compound 3-treated cells (Fig. 6). The band of procaspase-3 disappeared accompanied with the formation of a small active fragment of 20/17 kDa in the cells treated with 0.44 μM compound 3. That the cleavage of lamin B, a known substrate of caspases, to p32 occurred in parallel further confirmed the activation of caspases (Fig. 6). Next, the activation of caspase-8, -9, and -12, known as initiator caspases that undergo autoprocessing for their activation in response to apoptotic stimuli and process the precursor of effecter caspases (caspase-3, -6, and -7) was examined. The level of procaspase-8 was significantly decreased and the processed form of caspase-8 was detected in the compound 3-treated cells (Fig. 6). On the other hand, the level of procaspase-12 was only slightly decreased and the cleaved form was hard to detect (Fig. 6), a result similar to the case of etoposide-induced apoptosis that is known to be evoked *via* a mitochondrial pathway [26]. These findings suggest that caspases-8, -9, and -3 are the main caspases activated in compound 3-induced apoptosis whereas caspase-12 was not activated. Caspase-12 is known to be involved in the apoptotic process *via* ER stress

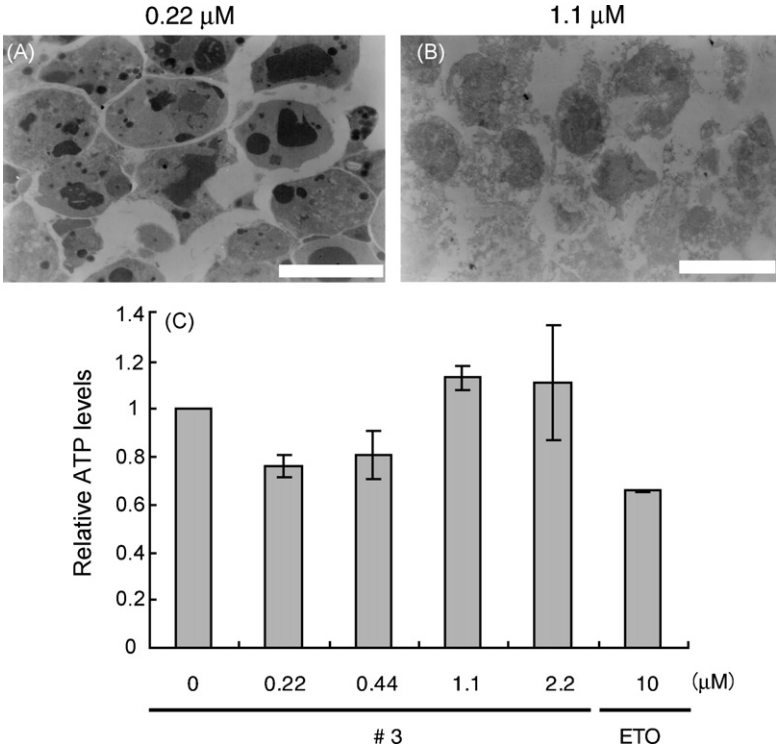

Fig. 4 – Compound 3 induced apoptotic and necrotic cell death (A) Electron microscopy of HL-60 cells treated with 0.22 μM compound 3 for 12 h. Many cells have the fragmented nuclei and condensed chromatin, features of apoptosis. (B) Electron microscopy of HL-60 cells treated with 1.1 μM compound 3 for 24 h. Necrotic cells with dissolved cytoplasm, broken organelles, and ruptured membrane were observed. *Bar* 10 μm (C) Effect of compound 3 on intracellular ATP levels. HL-60 cells were treated with compound 3 for 3 h and collected for whole cell ATP measurement as described in Section 2. Etoposide (ETO) was used as a positive control.

[23]. Therefore, it is suggested that the compound 3 induced-apoptosis occurs *via* a death receptor signaling pathway and/or mitochondrial pathway.

Then, to further clarify the members of the caspase family that are involved in compound 3-induced apoptosis, several specific caspase inhibitors were used and tested for their effect on the two typical apoptotic features induced by compound 3 treatment, namely increases in the number of cells with a sub-G1 DNA content and the cells with nuclear condensation and fragmentation. A caspase-9 inhibitor (z-LEHD-fmk) and a caspase-3 inhibitor (z-DEVD-fmk) significantly decreased the number of cells with a sub-G1 DNA content and with condensed and fragmented nuclei (Fig. 7). z-IETD-fmk, a caspase-8 inhibitor, also suppressed the increase in cells with a sub-G1 DNA content though less effectively (Fig. 7A). The assay to test the suppression of the increase in the cells with condensed and fragmented nuclei also showed z-IETD-fmk to be less effective (Fig. 7B). These results indicate that caspase-8 was not a main initiator in compound 3-induced apoptosis.

3.4. *Compound 3 induces loss of $\Delta\Psi_m$ and translocation of mitochondrial cytochrome c into the cytosol*

Clarification of the caspases participating in the compound 3-induced apoptosis suggested the main pathway of the apoptosis to be *via* a perturbation of mitochondrial functions. Recent reports provide evidence that mitochondria are intimately involved in the regulation of cell death [27]. This mitochondrial pathway results in the activation of caspase and DNA fragmentation, coupled with characteristic morphologic changes of cells. Then, we asked whether compound 3-induced apoptosis is preceded by a decrease of $\Delta\Psi_m$. As shown in Fig. 8A, compound 3 induced a loss of mitochondrial membrane potential in a time-dependent manner. Since mitochondrial swelling induced by permeability transition is known to cause the outer membrane to rupture following the release of cytochrome *c* from mitochondria, release into the cytosol was examined. The cytosolic cytochrome *c* level was increased at 6 h after compound 3-treatment (Fig. 8B).

3.5. *ROS signaling and intracellular GSH are important for compound 3-induced apoptosis*

Production of ROS is a common feature of cells treated with anticancer drugs and can result in modifications of proteins and lipids. Since compound 3 induced a loss of mitochondrial membrane potential, we then examined ROS production in compound 3-treated cells. DCFH-DA was used to detect endogeneously generated ROS. As shown in Fig. 9, the intracellular level of ROS was increased in both apoptotic and necrotic cells.

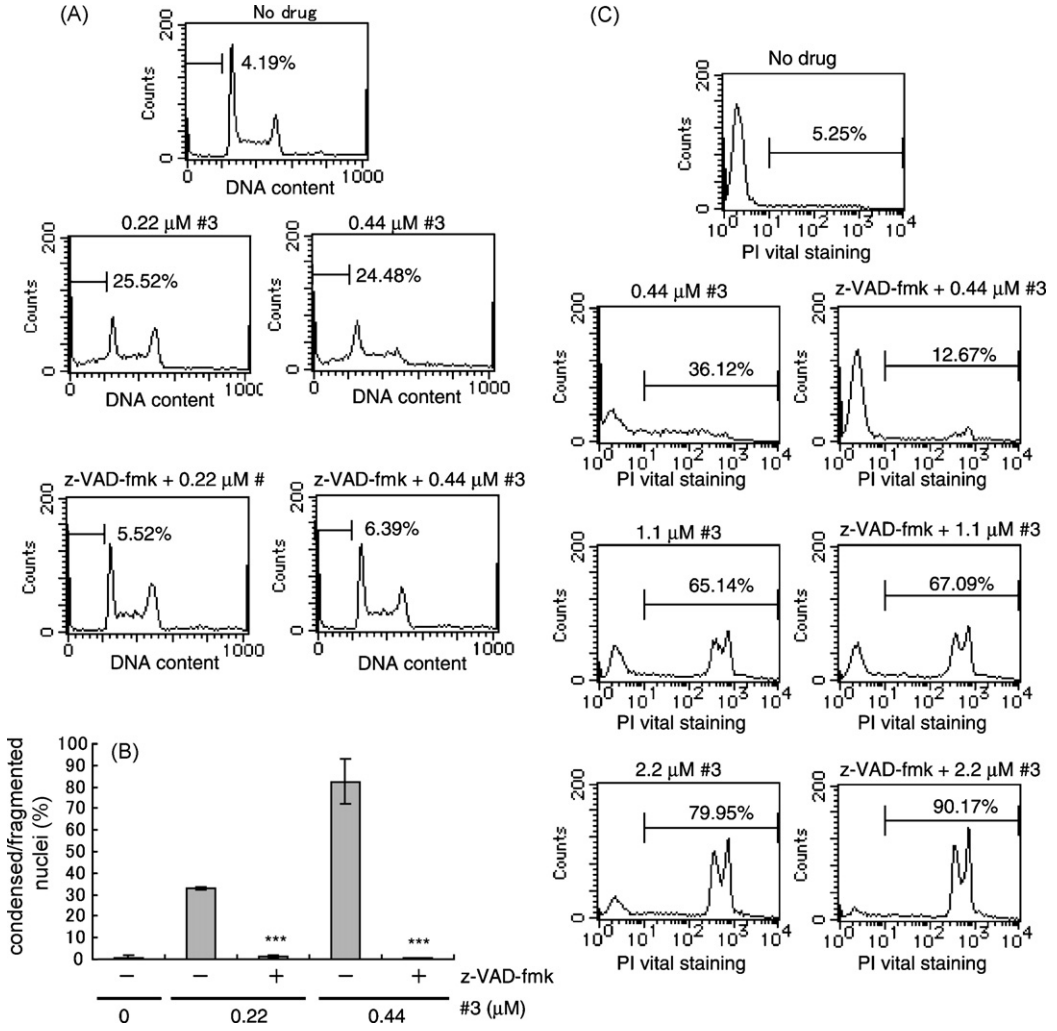

Fig. 5 – Effect of the pan-caspase inhibitor z-VAD-fmk on compound 3-induced cell death. HL-60 cells were pretreated with or without z-VAD-fmk at 40 μM for 1 h, then treated with compound 3 for 6 h. (A) Apoptotic cells with a sub-G1 DNA content were analyzed by flow cytometry. The percentage of cells in the sub-G1 population is shown in each panel. (B) Apoptotic cells were measured using Hoechst 33258 staining as described in Material and Methods. Values are the mean for 3 independent experiments, each performed in triplicate, and standard deviation bars are shown. Significant difference at ***$P < 0.001$, from compound 3-treated cells. (C) Cellular viability was determined by the ability of the cells to exclude PI. HL-60 cells were treated with compound 3 in absence or presence of z-VAD-fmk pretreatment and stained with 50 μg/ml PI. The number of PI-positive cells was determined using a flow cytometer and the percentage is represented in each panel.

To know the relation between the increase in ROS and compound 3-induced apoptosis, the effect of two antioxidants, NAC and catalase, was tested. NAC is an aminothiol and synthetic precursor of intracellular cysteine and GSH and known as a general antioxidant, which increases the intracellular levels of GSH and scavenges the ROS. As shown in Fig. 10A, both catalase and NAC decreased compound 3-induced apoptosis. In particular, NAC markedly reduced the apoptosis, in spite that it had no effect by itself. On the other hand, BSO is known to decrease intracellular GSH levels by inhibiting γ-glutamylcysteine synthase activity. Thus, BSO is expected to enhance the compound 3-induced apoptosis. In fact, BSO did enhance the apoptosis (Fig. 10B) in parallel with an increase in the collapse of $\Delta\Psi_m$ (Fig. 10C). These results indicated that the compound 3-induced apoptosis occurred *via* the generation of ROS.

4. Discussion

4.1. *Heterocyclic organobismuth compound induces cell death in HL-60 cells*

In this report, we showed that heterocyclic organobismuth compounds have antiproliferative and cytotoxic effects on human leukemia cells through apoptosis and necrosis. The heterocyclic organobismuth compound 3 is a potent and rapid inducer of apoptosis in the human leukemic cell line, HL-60, causing internucleosomal DNA degradation, nuclear

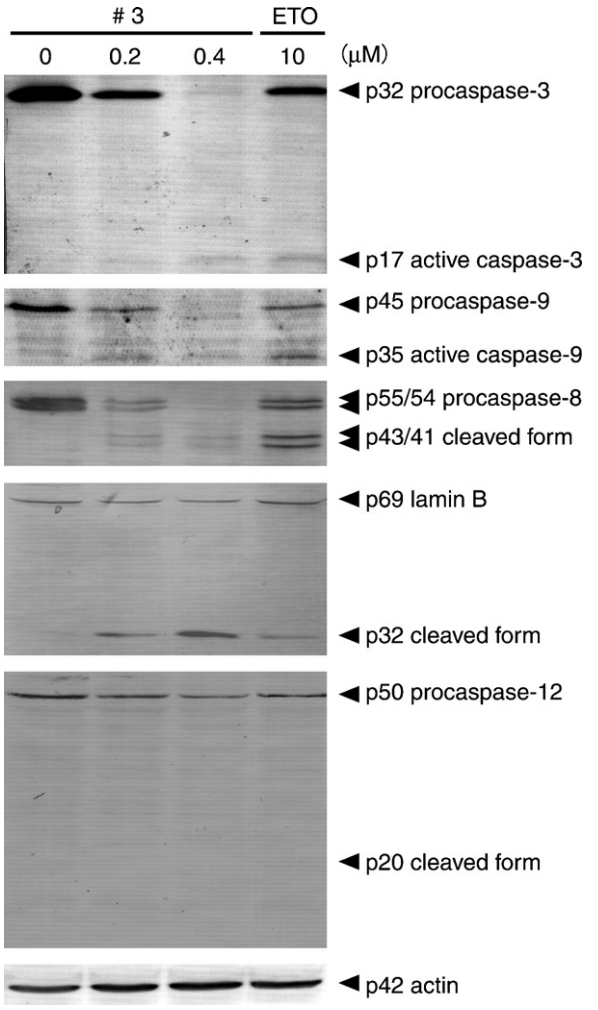

Fig. 6 – Activation of caspases in compound 3-treated HL-60 cells. Cell extracts were prepared from HL-60 cells following treatment with or without compound 3. Protein was resolved using SDS-PAGE, transferred onto PVDF membrane, and probed with each of the antibodies. Etoposide (ETO) is a positive control for the activation of caspases in HL-60 cells. Western blot analyses for cleavage of caspases-3, -9, -8, and -12 and lamin B were performed to show the activation of caspases.

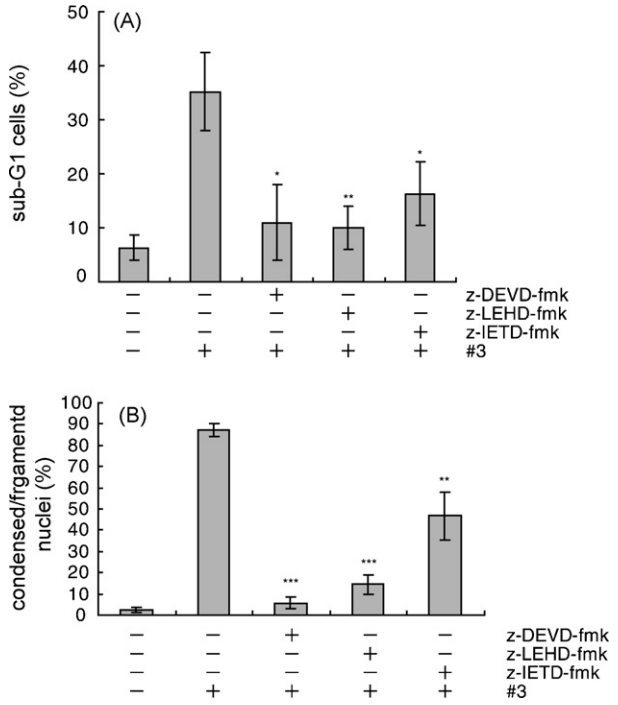

Fig. 7 – Effect of the caspase-3 inhibitor (z-DEVD-fmk), caspase-9 inhibitor (z-LEHD-fmk), and caspase-8 inhibitor (z-IETD-fmk) on the compound 3-induced apoptosis. HL-60 cells were pretreated with or without a caspase inhibitor (100 μM) for 2 h, then treated with 0.44 μM compound 3 for 6 h. Apoptotic cells were measured using flow cytometry (A) and Hoechst 33258 staining (B) as described in Section 2. Values are the mean for 3 independent experiments, each performed in triplicate, and standard deviation bars are shown. Significant difference at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, respectively, from compound 3-treated cells.

condensation/fragmentation, and DNA laddering. Another heavy metal, arsenic trioxide, has been known to induce apoptosis in a relatively wide spectrum of tumors [28]. Although the sensitivity of different cell types to arsenic trioxide varies to a great extent, the arsenic concentration needed to induce apoptosis in tumor cells is 0.5–2 μM [29]. The percentage of viable HL-60 cells is reported to be about 60% after treatment with 5 μM arsenic trioxide for 24 h [30]. The concentration of arsenic trioxide needed to exhibit antitumor activity is one order of magnitude or more above the concentration of the organobismuth compound reported in this study in HL-60 cells. Thus, the heterocyclic organobismuth compound 3 is an extremely potent and rapid inducer of apoptosis in human leukemic cells.

Apoptosis plays a central role in the maintenance of homeostasis, and there is an endogenous mechanism by which its induction is controlled. The development of tumors involves cell proliferation and impaired apoptosis. Therefore, apoptosis is the key mechanism of chemotherapeutic agents. There are several characteristic cellular and biochemical hallmarks of apoptotic cell death, including oligonucleosomal fragmentation, nuclear condensation, and DNA laddering. Here we report evidence that the heterocyclic organobismuth compound 3 is an inducer of apoptosis in human leukemic cells, leading to internucleosomal DNA degradation, nucleus condensation/fragmentation and DNA laddering. These results suggest that this compound would make a novel anticancer drug. Higher concentrations of compound 3 (>1.1 μM) induced necrosis. Apoptosis and necrosis are two distinct modes of cell death with respective morphological characteristics. Intracellular ATP levels have been implicated as a determinant of the cell's decision to die by apoptosis or necrosis [17,31]. The present study showed that intracellular ATP levels in HL-60 cells treated with low concentrations of compound 3 for 3 h were decreased, but not with high concentrations. Thus intracellular ATP is not involved in necrosis-induction by compound 3. In the previous

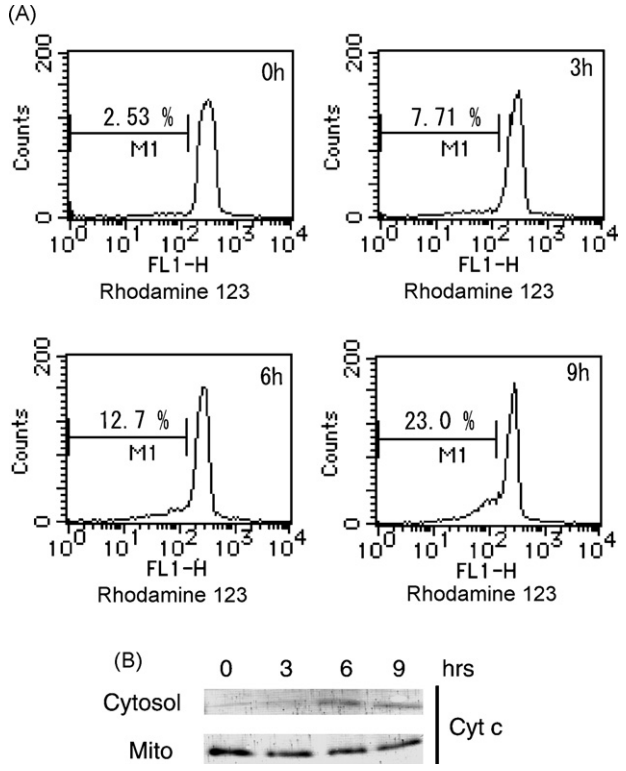

Fig. 8 – Effect of compound 3 on mitochondrial membrane potential Effects of compound 3 on mitochondrial membrane potential in HL-60 cells and the release of cytochrome c into the cytosol were examined. (A) Cells were treated with 0.22 μM compound 3 for indicated times. Then, cells were stained with Rhodamine 123 and fluorescence intensity was measured by flow cytometry. (B) Western blot analysis of cytochrome c expressed in the mitochondrial fraction (Mito) and cytosolic fraction (Cytosol) obtained from HL-60 cells treated with compound 3 at the indicated time.

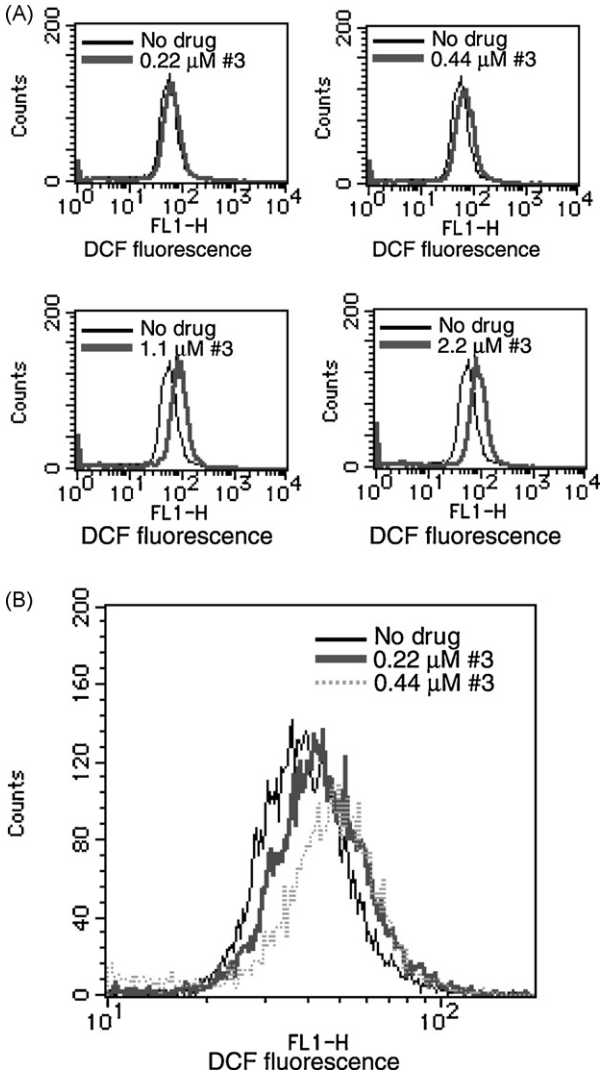

Fig. 9 – Effect of compound 3 on intracellular concentration of ROS HL-60 cells were treated with the indicated concentrations of compound 3 for 1 h (A) or 3 h (B). The intracellular ROS concentration was measured by flow cytometry using an oxidation-sensitive fluorescent probe, DCFH-DA, which is oxidized to DCF in the presence of ROS.

study, while cisplatin induced apoptosis with decrease in intracellular ATP level, BSO plus cisplatin induced necrotic cell death without ATP depletion in U937 cells [32]. Thus, necrosis by compound 3 may be accompanied by GSH depletion. The present study showed that compound 3 induced the production of ROS in a concentration-dependent manner. Since it has been known that higher concentrations of ROS can induce necrosis, whereas lower concentrations can induce apoptosis in the same cell type [33], a switch of cell death mode from apoptosis to necrosis during the treatment with compound 3 might be dependent on the concentration of ROS in HL-60 cells. In fact, we have observed that NAC reduced the concentration of ROS and suppressed apoptosis and necrosis in parallel. In addition, pretreatment of NAC switched back the mode of death from necrosis to apoptosis.

4.2. *Caspases as essential executors of compound 3-induced apoptosis, but not necrosis*

Evidence suggests that most proapoptotic stimuli induce the activation of a family of intracellular cysteine proteases called caspases. Caspase-3 and -9 were activated in HL-60 cells exposed to compound 3 (Figs. 6 and 7). The activation of caspases represents the irreversible or execution stage of apoptosis, because caspase-mediated proteolysis is irreversible. Many proteins required for the maintenance of cell structure and function are substrates of active caspase-3. During compound 3-induced apoptosis, lamin B, a nuclear structural protein, was cleaved (Fig. 6), leading to the disruption of the nuclear architecture. Because lamin B is reported to be cleaved predominantly by caspase-6, we can assume that caspase-6 is activated in compound 3-treated HL-60 cells. Caspase-8, an initiator caspase in the death receptor-mediated signaling pathway, was also cleaved (Fig. 6), and the caspase-8 inhibitor moderately suppressed compound 3-induced apoptosis (Fig. 7). Therefore compound 3 may partially activate death receptor signaling pathway.

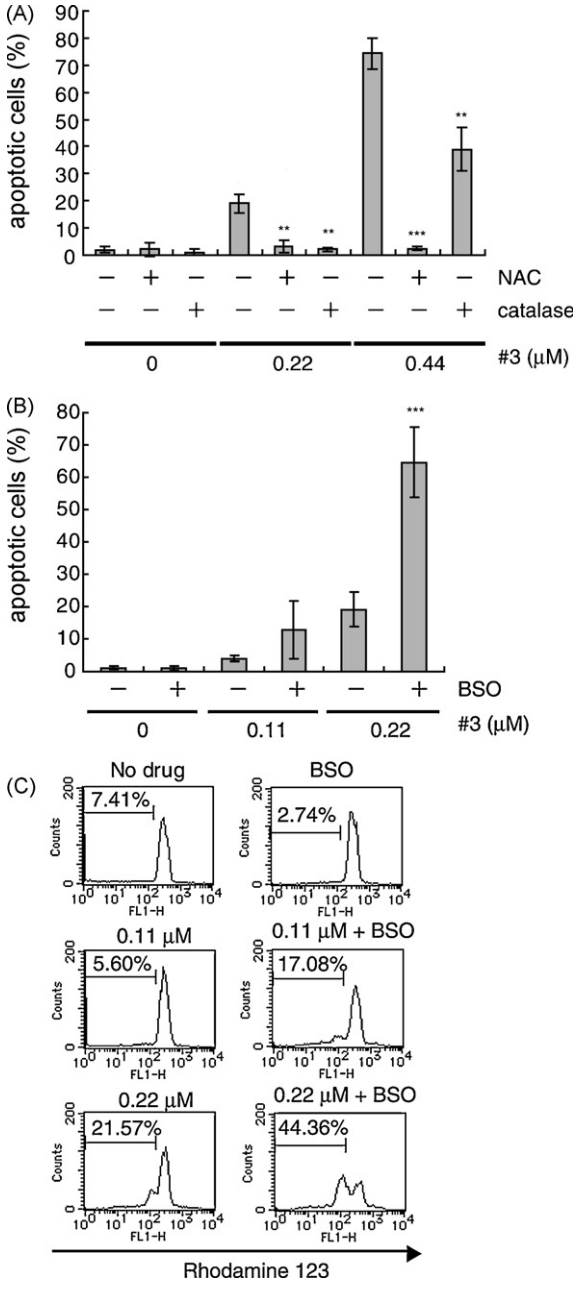

Fig. 10 – Effect of compound 3 on mitochondrial membrane potential and ROS production. (A, B) Effect of catalase, NAC, and BSO on compound 3-induced apoptosis. HL-60 cells were pretreated with or without 10^3 U/ml catalase, 10 mM NAC, or 0.2 mM BSO for 2 h, then treated with compound 3 for 6 h. Apoptotic cells were measured using Hoechst 33258 staining as described in Section 2. Values are the mean for 3 independent experiments, each performed in triplicate, and standard deviation bars are shown. Significant difference at $^{**}P < 0.01$, $^{***}P < 0.001$, respectively, from compound 3-treated cells. (C) Effect of BSO on compound 3-induced loss of mitochondrial membrane potential. HL-60 cells were pretreated with or without 0.2 mM BSO for 2 h, then treated with compound 3 for 6 h. Mitochondrial membrane potential was measured as described in Section 2.

However, as several studies have described that caspase-8 is cleaved during mitochondrial apoptosis without receptor-trigger cleavage [26,34], caspase-8's activation during compound 3-induced apoptosis may occur downstream of initiator caspases in the mitochondrial apoptotic pathway.

4.3. *Compound 3-induced mitochondrial dysfunction and ROS production*

In this study, we have demonstrated that compound 3 induced the generation of ROS and mitochondrial dysfunction (Figs. 8 and 9). Both catalase and NAC blocked ROS production and prevented compound 3-induced apoptosis. GSH's depletion by BSO facilitated the apoptosis (Fig. 10). These results indicated the generation of ROS and mitochondrial dysfunction to play an important role in compound 3-mediated apoptosis. At present, it is not obvious how compound 3 induces the production of ROS and the disruption of mitochondrial function. However, one possible explanation is that it directly or indirectly interacts with the ROS-generating system resulting in an increase in the production of O_2^-. Compound 3 may act on the mitochondrial electron transport system and subsequently cause an elevation in ROS levels similar to a pathway described elsewhere [35–37]. Alternatively, compound 3 interfered with NF-κB-related pathways [38], or altered the oxidation–reduction metabolic pathways and metabolites including the intracellular glutathione level, the xanthine/xanthine oxidase system, and/or the NADPH oxidase.

4.4. *Comparison of compound 3-induced apoptosis with arsenic trioxide*

Arsenic trioxide (As_2O_3)-induced apoptosis involves the activation of caspases [39–42]. In addition, As_2O_3 induces an increase in the level of cellular ROS, disruption of the mitochondrial membrane potential, and the release of cytochrome *c* with the consecutive activation of the caspase cascade in acute promyelocytic leukemia (APL) cells [39,43]. These actions of As_2O_3 on APL cells are similar to those of our heterocyclic organobismuth compounds. As_2O_3 can induce clinical remission in patients with APL through induction of apoptosis [44]. However, the reported chronic toxicities and carcinogenicity of arsenic trioxide has hampered its acceptance as a first-choice drug [45]. Toxicity is a serious concern for the management of human cancers by chemotherapeutic agents. We observed that mice treated with compound 3 at 0.13 mg/kg intravenously every other day for a month slightly degreased body weight. At present, we did not observed a significant damage of normal tissues, including kidney, liver, spleen, lung, heart, brain, and ovary after the treatment. In general, inorganic bismuth compounds are known as low toxic substance and have already used in medicine. In present study, we showed that the sensitivities of leukemia cell lines to heterocyclic organobismuth compounds were higher than those of solid tumor lines. Organobismuth compounds of diverse structure can be synthesized, thus heterocyclic organobismuth compounds may become to have some advantage for the treatment of APL over arsenic trioxide. In conclusion, the present study shows that heterocyclic organobismuth compounds could kill leukemia cells by inducing apoptotic and necrotic cell death. The results

should be valuable for studying the utilization of organobismuth compounds as novel anti-leukemia drugs. Moreover, organobismuth compounds may be useful for studying the mechanisms of apoptotic and necrotic actions.

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