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Pyrrolidinium-type fullerene derivative-induced apoptosis by the generation of reactive oxygen species in HL-60 cells

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The biological activities of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide), a water-soluble cationic fullerene derivative, on human promyeloleukaemia (HL-60) cells were investigated. The pyrrolidinium fullerene derivative showed cytotoxicity in HL-60 cells. The characteristics of apoptosis, such as DNA fragmentation and condensation of chromatin in HL-60 cells, were observed by exposure to the pyrrolidinium fullerene derivative. Caspase-3 and -8 were activated and cytochrome *c* was also released from mitochondria. The generation of reactive oxygen species (ROS) by the pyrrolidinium fullerene derivative was observed by DCFH-DA, a fluorescence probe for the detection of ROS. Pre-treatment with *α*-tocopherol suppressed cell death and intracellular oxidative stress caused by the pyrrolidinium fullerene derivative. The apoptotic cell death induced by the pyrrolidinium fullerene derivative was suggested to be mediated by ROS generated by the pyrrolidinium fullerene derivative.

Keywords: *Fullerene derivative, apoptosis, HL-60, reactive oxygen species, caspase*

Introduction

Fullerene (C_{60}), discovered by Kroto et al. [1] in 1985, is a new type of carbon allotrope that has been utilized for micro devices in electronic and mechanical applications.

In physiological studies, the biological effects of water-soluble fullerene derivatives with several hydrophilic groups are noteworthy because the fullerene itself is water-insoluble. Water-soluble fullerene derivatives are known to possess various biological and pharmacological properties, which include antioxidant activity, inhibition of human immunodeficiency virus (HIV) protease and DNA photocleavage [2–6]. We have also demonstrated that pyrrolidinium fullerene derivative 1 (Figure 1) has anti-proliferative and anti-bacterial activity [7], malonic acid fullerene derivative 2 (Figure 1) has excellent antioxidant activity [8] and proline-modified fullerene derivative 3 (Figure 1) has an inhibition activity of HIV-reverse transcriptase (RT) [9]. In contrast, Sayes et al. [10] have reported that a water-soluble fullerene colloid (nano-C_{60}) generated intracellular reactive oxygen species (ROS) and, subsequently, induced cell death through the production of lipid peroxidation.

Cell death can be divided into apoptotic and necrotic ones. It is known that many existing anticancer agents induce apoptosis, i.e. programmed cell death without inflammation around the dying cells. Yamawaki and Iwai [11] reported that hydroxyl fullerene $C_{60}(OH)_{24}$ did not seem to induce apoptosis but caused the accumulation of polyubiquitylated proteins and facilitated autophagic cell death in human umbilical vein endothelial cells (HUVECs). Furthermore, Isakovic et al. [12] reported nano-C_{60}-induced necrosis following the generation of ROS in mouse fibrosarcoma (L929), rat glioma (C6) and human

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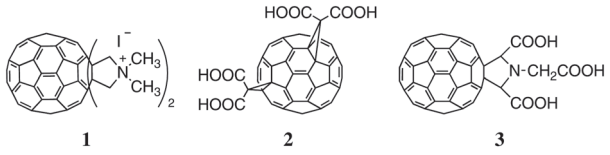

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Figure 1. Structure of fullerene derivatives.

glioma (U251) cell lines, but $C_{60}(OH)_n$-induced apoptosis through the caspase-dependent and the ROS-independent pathways. The cytotoxicity of $C_{60}(OH)_n$ is unclear and the results differ from one research group to another.

We have reported that the anti-proliferative activities of cationic fullerene derivatives, C_{60}-bis(*N, N*-dimethylpyrrolidinium iodide) 1, were comparable to those of cisplatin using a panel of 36 human cancer cell lines and suggested that the mechanism of the anti-proliferative activity of this derivative may be different from that of well-defined anti-cancer agents [7]. We have also demonstrated that C_{60} derivative 1 generated hydrogen peroxide (H_2O_2) without photo-irradiation in the presence of *Escherichia coli* (*E. coli*) inner-membrane and NADH and inhibited *E. coli* growth and respiratory chain activity [13]. We concluded that fullerene derivative 1 was reduced by NADH and that the reduced form reacted with dioxygen to form a superoxide, which is disproportionated to H_2O_2. However, the biochemical pathway leading to the anti-proliferative effects of C_{60} derivative 1 in mammalian cells remained unclear.

In this study, we demonstrated that pyrrolidinium C_{60} derivative 1 induces mitochondrion- and caspase-dependent apoptosis through the generation of ROS in human promyeloleukaemia (HL-60) cells.

Materials and methods

Materials

C_{60} derivative 1, the mixture of the main regio isomers of C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide, was synthesized as described previously [13]. Aprotinin, leupeptin, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), hoechst 33258 and an RPMI 1640 medium were purchased from Sigma Inc. (St. Louis, MO); propidium iodide (PI) was from Calbiochem (La Jolla, CA). Anti-caspase 3, caspase 8 and cytochrome *c* antibodies were purchased from Pharmingen Becton Dickinson (San Diego, CA) and anti-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the highest purity commercially available. C_{60} derivative 1 was dissolved in dimethylsulphoxide (DMSO) and stored as a 10 mM stock solution. It was used after dilution of the stock solution with DMSO. DMSO at concentrations lower than 0.5% had no effect on cell growth. Pure water was freshly prepared with a Millipore Milli-Q Labo (Bedford, MA).

Cell culture

Human myeloblastic leukaemia HL-60 cells were grown in the RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere in a 5% CO_2 incubator.

Determination of cell penetration

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well (2 mL) and treated with C_{60} derivative 1 (30 μM) for 24 h. Cells were collected and washed with PBS and the cell pellets were solubilized by Triton X-100 (10 μL) for 1 h. After the addition of DMSO (1 mL) and centrifugation (1000 rpm for 10 min), the amount of incorporated C_{60} derivative 1 was determined by absorbance at 317 nm.

Cytotoxicity assay

Cells were seeded in 6-well plates at a density of 1×10^6 cells/well and then treated with C_{60} derivative 1 for 24 h. Cell viability was determined using trypan blue dye exclusion. The data were expressed as a percentage of living control cells.

Cell cycle analysis

Apoptotic cells were confirmed as well by the appearance of a hypodiploid cell (sub-G_1 peak) fraction 0 in the cell cycle analysis. For the cell cycle analysis, ethanol-fixed cells were stained with propidium iodide (25 μg/mL) in the presence of DNase-free RNase A (200 μg/mL) and then analysed using the fluorescence-activated cell sorter (FACS) Calibur with a CELLQuest program (Becton Dickinson, Mountain View, CA).

DNA fragmentation assay

Following exposure to C_{60} derivative 1 for various durations, cells were washed with PBS and lysed with 200 μL of a 0.5% SDS-PBS solution. Two microlitres of protease K (10 mg/mL) was added and then incubated for 3 h at 65°C. The supernatant was extracted by phenol/chloroform/isoamyl alcohol and DNA was precipitated with ethanol at −20°C overnight. The DNA pellet was dissolved in a 50 μL TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and 1 μL of DNase-free RNase A (10 mg/mL) was added. After incubation at room temperature for 20 min, DNA was separated by agarose gel (1.5%) electrophoresis and visualized under UV illumination after staining with SYBR™ Green I.

Condensation of nuclear chromatin

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well, treated with C_{60} derivative 1 and fixed in 1% glutaraldehyde for 30 min at room temperature. After washing with PBS, cells were stained with hoechst 33258 (167 μM in PBS) and observed using fluorescence microscopy.

Western blot analysis

Cells were washed with PBS and lysed in an ice-cold whole cell lysis buffer (50 mM Tris-HCl buffer (pH 8.0), 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10 mM *β*-glycerophosphate, 2.5 mM NaF, 0.1 mM Na_3VO_4, 2 μg/mL aprotinin, 2 μg/mL leupepton). After 20 min of incubation on ice, lysates were centrifuged at 15 000 rpm for 15 min at 4°C and supernatants were collected and stored at –20°C. The protein concentration was determined according to the method of Bradford [14] using bovine serum albumin as a standard. Samples were added using a Laemmli sample buffer and boiled for 10 min and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis. After being transferred to PVDF membranes, the samples were blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20 (T-TBS) for 1 h and then incubated with a primary antibody overnight at 4°C. After incubation with the secondary antibody coupled to horseradish peroxidase, detection was achieved using the enhanced chemiluminescence system (Amersham Pharmacia Biotech.). Molecular sizes were determined by the relative mobilities of pre-stained molecular weight markers.

Mitochondrial cytochrome c release

Cells were washed with PBS and lysed in an ice-cold lysis buffer (10 mM HEPES-KOH buffer (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 2 μg/mL aprotinin, 2 μg/mL leupepton). After 20 min of incubation on ice, lysates were centrifuged at 5000 rpm for 5 min at 4°C and supernatants were further centrifuged at 15 000 rpm for 15 min at 4°C to remove mitochondrial fraction. Cytochrome *c* levels in cytosolic fraction were analysed by Western blot analysis using a specific antibody as described above.

Measurement of intracellular oxidative stress

The fluorescent probe DCFH-DA was used to monitor the intracellular oxidative stress. Cells were seeded in 6-well plates at a density of 1×10^6 cells/well and 10 μM DCFH-DA was loaded. After 15 min of incubation at 37°C in the dark, the cells were washed with PBS, pre-incubated with *α*-tocopherol for 1 h and then treated with C_{60} derivative 1 for 1 h. The fluorescence intensity of DCF was monitored using FACS Calibur with the CELLQuest program.

Effect of α-tocopherol on C_{60} derivative 1-induced apoptosis

Cells were seeded in 6-well plates at a density of 1×10^6 cells/well and pre-incubated with *α*-tocopherol for 3 h. Then the cells were treated with 30 μM C_{60} derivative 1 for 24 h. Cell viability was determined using trypan blue dye exclusion. DNA fragmentation assay and Western blot analysis were also performed as described above.

Statistical analysis

All data are presented as mean ± SD of at least three independent experiments. Statistical differences were evaluated using the Student's *t*-test at significance levels of $p < 0.05$.

Results

Penetration of C_{60} derivative 1 into HL-60 cells or membrane

Cells turned brown after a 24 h incubation with C_{60} derivative 1 (30 μM). The amount of penetrated C_{60} derivative 1 calculated from the absorbance at 317 nm was 0.42 nmol/10^6 cells. The concentration of C_{60} derivative 1 in HL-60 cells was estimated as 100 μM, which means that C_{60} derivative 1 was concentrated 3-fold.

Cytotoxicity of C_{60} derivative 1 in HL-60 cells

We investigated the effects of C_{60}-derivative 1 on cell viability. HL-60 cells were exposed to 0.05–100 μM C_{60} derivative 1 for 24 h and cell viability was measured by the trypan blue exclusion assay. Exposure of HL-60 cells to various concentrations of C_{60} derivative 1 resulted in a dose-dependent decrease in cell viability (Figure 2). IC_{50} of C_{60} derivative 1 was ~ 10 μM. In particular, all cells died after a 24 h exposure to 50 μM C_{60} derivative 1, while ~ 25% of the cells exposed to etoposide, an excellent anti-tumour agent, were still alive.

C_{60} derivative 1 induced apoptosis in HL-60 cells

To investigate the mechanism of C_{60} derivative 1-induced cell death in HL-60 cells, we evaluated whether or not exposure of the cells to C_{60} derivative 1 resulted in apoptotic death. The morphological assessment of apoptosis in HL-60 cells was analysed using DNA fragmentation and condensation of nuclear chromatin. These phenomena are important hallmarks

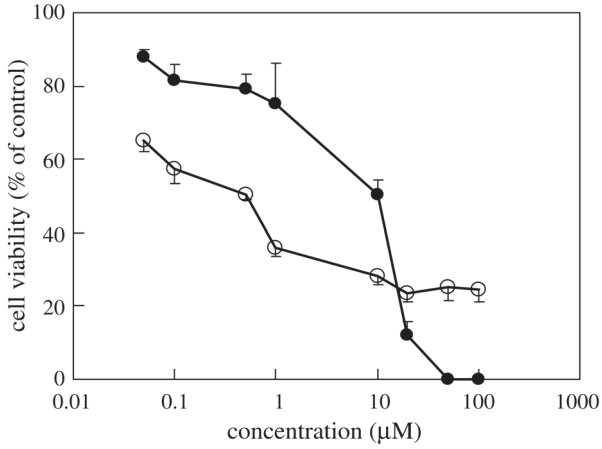

Figure 2. Cytotoxicity of C_{60} derivative **1** in HL-60 cells. Cells were treated with various concentrations of C_{60} derivative 1 for 24 h and were stained with trypan blue dye. •: C_{60} derivative 1, : etoposide (n = 3).

for apoptosis. As shown in Figure 3, we observed the appearance of a characteristic DNA ladder during 24–48 h of exposure to C_{60} derivative 1 (30 μM). Furthermore, the condensation of nuclear chromatin was apparent after 24 h of treatment with C_{60} derivative 1 (30 μM) (Figure 4). The rate of condensed cells was 27 and 33% after 24 and 48 h, respectively. It was lower than almost 100% in the case of etoposide (10 μM).

Cell cycle analysis was used to quantitatively estimate C_{60} derivative 1-induced apoptotic cell death. C_{60} derivative 1 (30 μM) led to apoptosis, as indicated by the appearance of the sub-G_1 phase (Figure 5). These results were consistent with the morphological

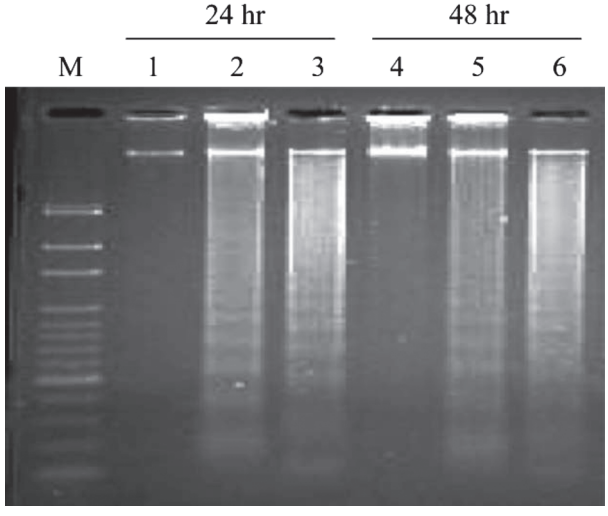

Figure 3. DNA fragmentation in C_{60} derivative 1-treated cells. Cells were treated with C_{60} derivative 1 (30 μM) or etoposide (10 μM) for 24–48 h. Isolated DNA was run on a 1.5% agarose gel and visualized by SYBR™ Green I staining. Lane 1, 4: control, lane 2, 5: C_{60} derivative 1, lane 3, 6: etoposide, lane M: marker (100 bp).

ones. After a 48 h exposure to 30 μM C_{60} derivative 1, apoptosis was induced in ~ 21% of the cells that was comparable to 16% in the case of etoposide.

Activation of caspase cascade and release of cytochrome c by C_{60} derivative 1

We examined the effects of C_{60} derivative 1 on the caspase-3 and -8 activities in HL-60 cells. Caspases play a critical role in the induction of apoptosis and are divided into initiator caspases, such as caspase-8 and -9, and executioner caspases, such as caspase-3, -6 and -7, according to their function and sequence of activation [15]. Western blot analysis showed that pro-caspase 3 was cleaved after 24 h of treatment with C_{60} derivative 1 (Figure 6A). Simultaneously, caspase-8 was also cleaved after 24 h. Caspase-8 is reported as an early marker of the apoptotic pathway and its activation is dependent on the stimulation of death receptors, such as TNF and Fas receptors [16,17]. These results indicated that the death receptor pathway and the caspase cascade were involved in C_{60} derivative 1-induced apoptosis in HL-60 cells.

The mitochondrial pathway of apoptosis is characterized by the release of the cytochrome *c*, the apoptogenic molecule, from mitochondria to the cytosolic fraction. Cytochrome *c* release into the cytosol occurred at nearly the same time as the activation of caspase 3 (Figure 6B). These results suggested that the cytotoxic activity of C_{60} derivative 1 in HL-60 cells results mainly from the induction of the apoptosis machinery, which is dependent on the activation of the caspase cascade via the mitochondrial pathway.

Generation of intracellular ROS by C_{60} derivative 1 in HL-60 cells

We have already shown that C_{60} derivative 1 inhibited the growth of *E. coli* and the inhibition was suggested to be caused by the generation of ROS by C_{60} derivative 1 at the inner membrane of *E. coli*. To demonstrate that oxidative stress is involved in C_{60} derivative 1-induced cell death, we evaluated the effects of C_{60} derivative 1 on ROS generation in HL-60 cells. The intracellular oxidative stress was measured using a non-fluorescent compound, DCFH-DA. DCFH-DA is taken up by cells and then undergoes deacetylation by esterases to 2′,7′-dichlorofluorescin (DCFH), which is not fluorescent. Oxidation of DCFH within cells leads to fluorescent 2′,7′-dichlorofluorescein (DCF), which can be easily visualized [18]. This technique is becoming popular as an index of intracellular ROS production. As shown in Figure 7, enhanced generation of ROS was observed in HL-60 cells treated with 30 μM C_{60} derivative 1 for 1 h. We used α-tocopherol, an excellent antioxidant, to examine the role of ROS production in C_{60} derivative-mediated cytotoxicity. Pre-treatment with *α*-tocopherol

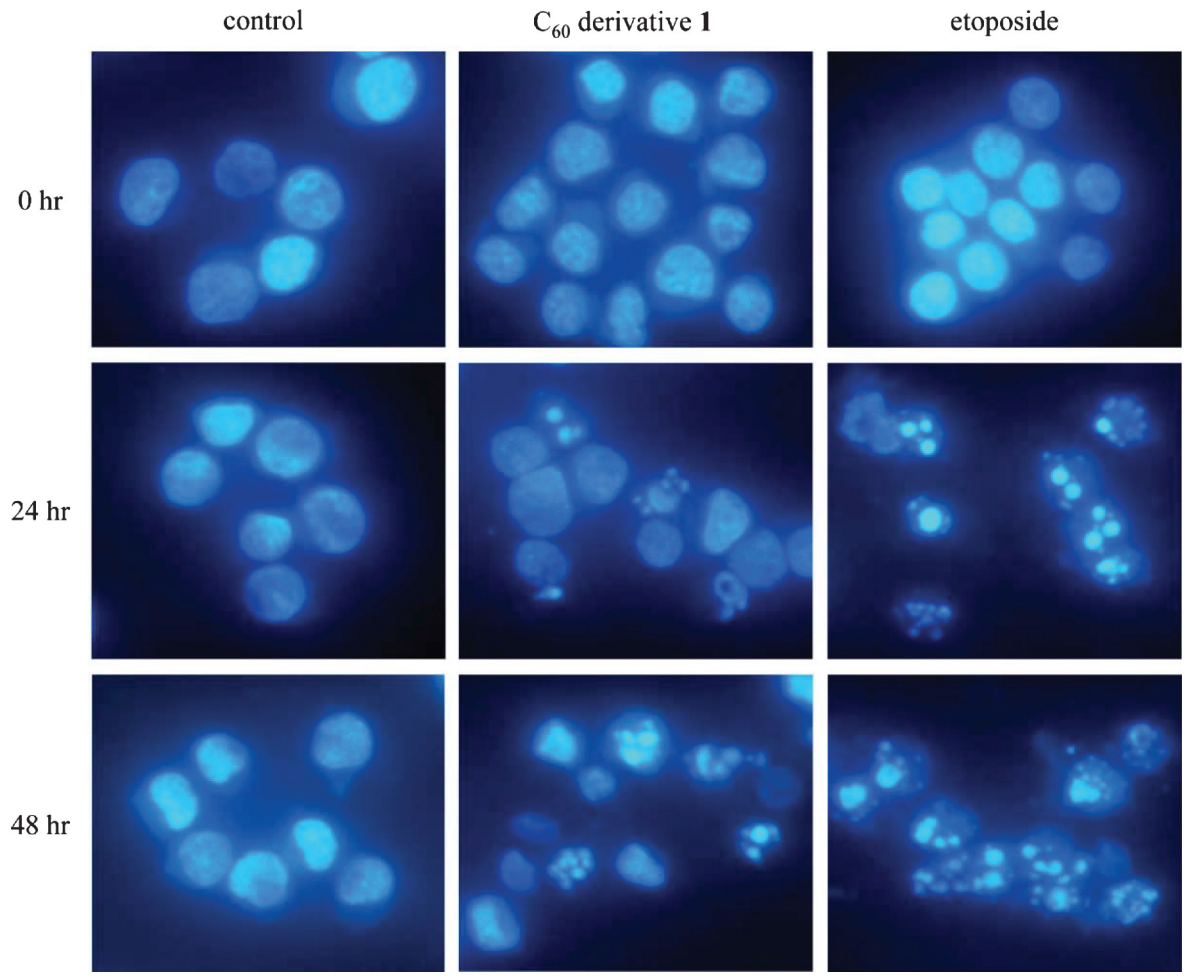

Figure 4. Morphological analysis of nuclear chromatin condensation after exposure of HL-60 cells to C_{60} derivative 1 or etoposide. Cells were treated with C_{60} derivative 1 (30 μM) or etoposide (10 μM) and were stained with Hoechst 33258.

significantly suppressed C_{60} derivative 1-induced intracellular oxidative stress in a dose-dependent manner (Figure 7). At 300 μM *α*-tocopherol, intracellular oxidative stress was almost completely suppressed to control levels. Pre-treatment with *α*-tocopherol also reduced C_{60} derivative 1-induced cell death (Figure 8, but not even 300 μM *α*-tocopherol could completely prevent cell death. The cells exposed to *α*-tocopherol did not exhibit any significant alterations in cell viability. *α*-Tocopherol also prevented DNA fragmentation and cleavage of procaspase 3 by C_{60} derivative 1 (Figures 9) . These results suggest that the cationic C_{60} derivative 1 induced apoptotic cell death in HL-60 cells, at least in part, mediated by ROS.

Discussion

If the biological activities of C_{60} derivative 1 are contemplated, it is necessary to consider the incorporation of it into human cancer cells. C_{60} derivative 1 caused pellets of HL-60 cells to turn brown and these colours did not fade after three washes with PBS. C_{60} derivative 1 was suggested to be concentrated 3-fold in HL-60 cells. Foley et al. [19] reported that the water-soluble C_{60} derivative [$C_{61}(COOH)_2$] is able to cross the cell membrane and preferentially binds to the mitochondria. Thus, C_{60} derivative 1 is expected to be on the surface of cells or to be ingested into the cells.

The characteristics of apoptosis, such as DNA fragmentation and the condensation of chromatin and hypodiploid cells, were observed upon exposure to C_{60} derivative 1 for 24 h (Figures 3–5). Simultaneously, caspase-3 and -8 were activated and cytochrome *c* was released from mitochondria to the cytosol (Figure 6). In Figure 2, C_{60} derivative 1 at 30 μM induced more than 80% cell death and C_{60} derivative 1 is more toxic than etoposide. However, in a different assay for the DNA condensation (Figure 4), it seems that etoposide (10 μM) induces more dramatic DNA condensation than that of C_{60} derivative 1 (30 μM). The caspase cleavage and cytochrome *c* assay also suggest that etoposide is a stronger apoptotic inducer than C_{60} derivative 1. One possibility to explain the inconsistency is that C_{60} derivative 1 may induce non-apoptotic cell death in addition to apoptotic cell death.

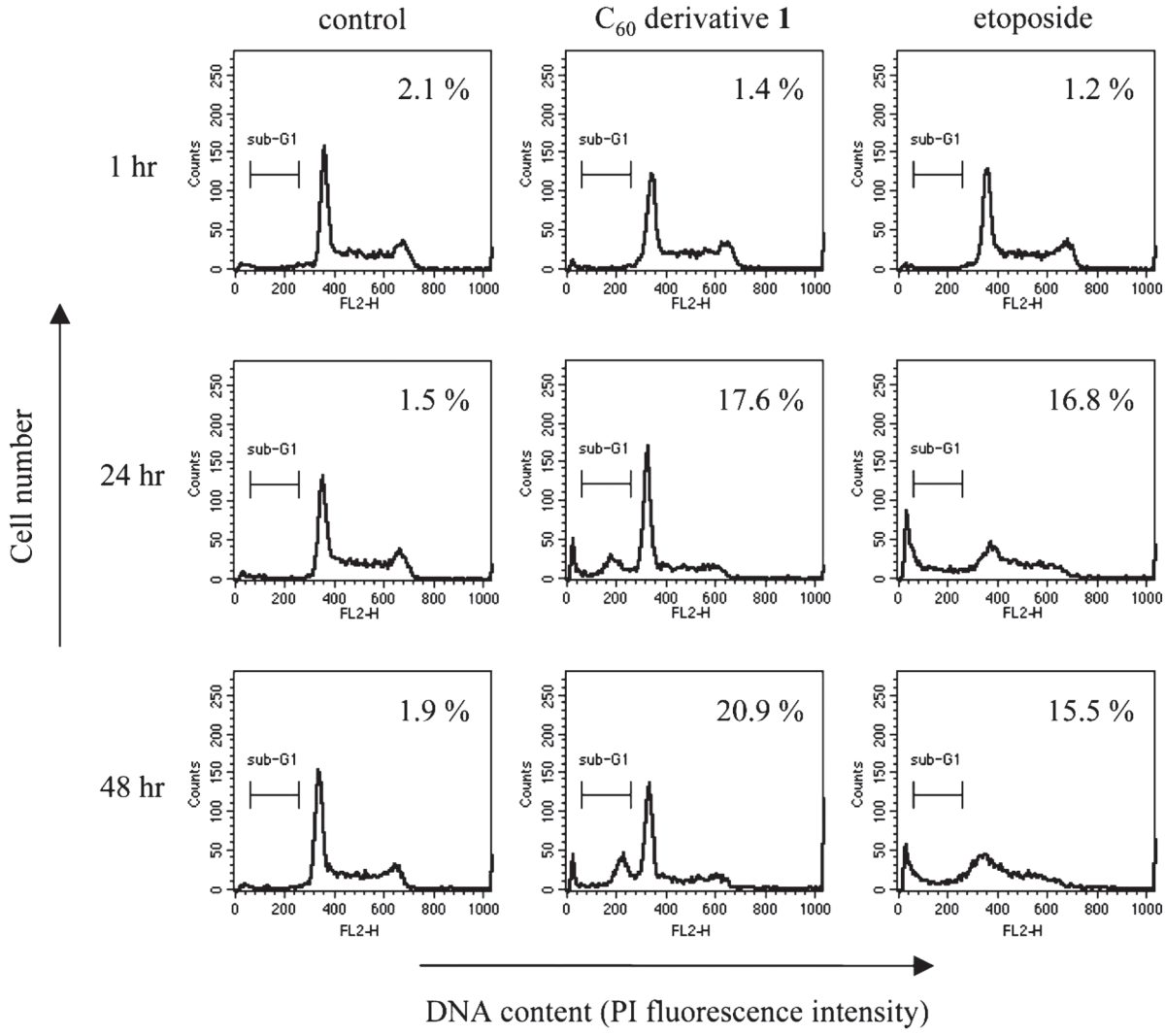

Figure 5. Cell cycle analysis in C_{60} derivative 1 or etoposide-treated cells. Cells were treated with C_{60} derivative 1 (30 μM) or etoposide (10 μM) for 1–48 h and were stained with propidium iodide (PI). Hypodiploid cells (sub-G_1 peak) consisted of mainly apoptosis.

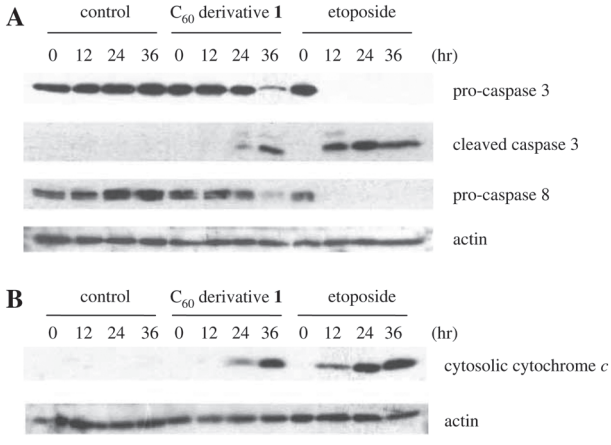

Figure 6. Activation of caspase -3, -8 and mitochondrial cytochrome *c* release in C_{60} derivative 1-treated cells. Cells were treated with C_{60} derivative 1 (30 μM) or etoposide (10 μM) for 0–36 h and whole cell extracts (A) and cytosolic fractions (B) were isolated. Caspase-3, -8 and cytochrome *c* were analysed by Western blotting using specific antibodies. Actin was included as an internal loading control.

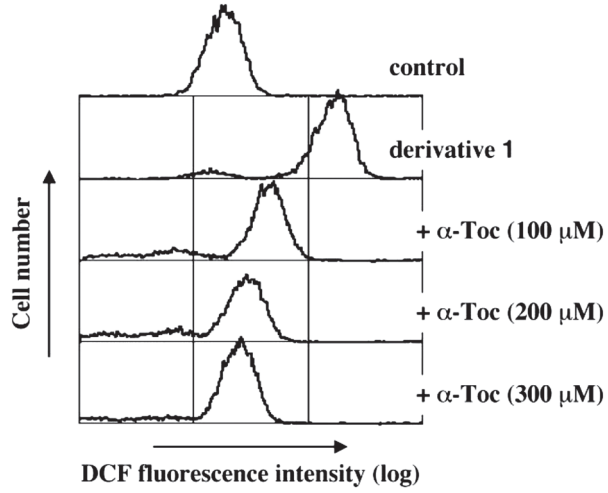

Figure 7. Effects of *α*-tocopherol on intracellular oxidative stress in HL-60 cells treated with C_{60} derivative 1. Cells were pre-incubated with *α*-tocopherol (*α*-Toc) for 1 h before exposure to C_{60} derivative 1 (30 μM) for 1 h. Oxidative stress was measured by DCFH-DA fluorescence probes.

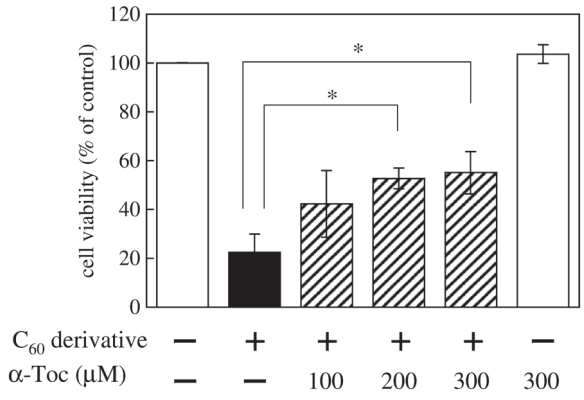

Figure 8. Effects of *α*-tocopherol on C_{60} derivative 1-induced cell death. Cells were pre-incubated with *α*-tocopherol (*α*-Toc) for 3 h before exposure to C_{60} derivative 1 (30 μM) for 24 h. $^{*}p < 0.01$ (student's *t*-test, $n = 3$).

Pre-treatment with *α*-tocopherol, an effective antioxidant, significantly suppressed C_{60} derivative 1-induced cell death, intracellular oxidative stress, DNA fragmentation and activation of caspase 3 (Figures 7–10). These results suggest that C_{60} derivative 1 is able to induce apoptosis involving the generation of ROS in the human cancer cell line. ROS are important regulators of apoptosis in the upstream/downstream signalling pathway. Mitochondria are primary organelles involved in the generation of ROS. We have reported that C_{60} derivative 1 generated H_2O_2, one of the ROS, in the *E. coli* inner membrane with NADH [13]. We have also shown by a cyclic voltammogram that the reduced form of C_{60} derivative 1 reacts with dioxygen. However, DCFH-DA, which we used as a fluorescence agent for the detection of ROS, is sensitive to the hydroxyl radical and alkoxyl radical [20], but does not react with H_2O_2 directly. It seems more likely that H_2O_2, which is produced from the reaction of the C_{60} derivative with dioxygen, was reduced through a process such

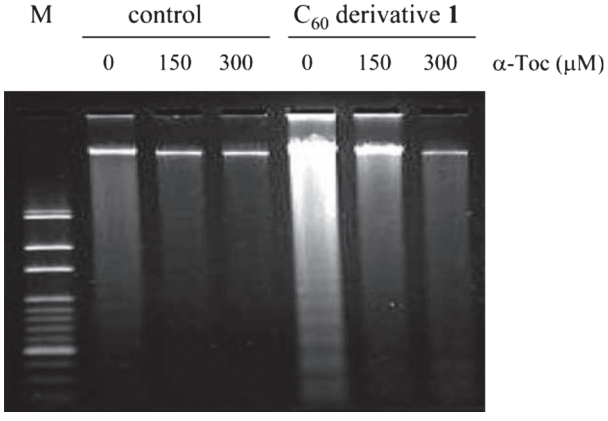

Figure 9. Effect of *α*-tocopherol on C_{60} derivative 1-induced DNA fragmentation. Cells were pre-incubated with *α*-tocopherol (*α*-Toc) for 3 h before exposure to C_{60} derivative 1 (30 μM) for 48 h. Isolated DNA was run on a 1.5% agarose gel and visualized by SYBR™ Green I staining. Lane M: marker (100 bp).

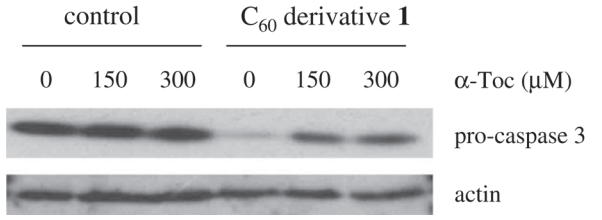

Figure 10. Effect of *α*-tocopherol on C_{60} derivative 1-induced cleavage of pro-caspase 3. Cells were pre-incubated with *α*-tocopherol (*α*-Toc) for 3 h before exposure to C_{60} derivative 1 (30 μM) for 48 h and whole cell extracts were isolated. Pro-caspase-3 was analysed by Western blotting using specific antibodies. Actin was included as an internal loading control.

as the Fenton reaction to the hydroxyl radical in the cells. The fluorescence of DCF was observed when HL-60 cells were treated with H_2O_2 without the C_{60} derivative, which indicates that H_2O_2 changes to a hydroxyl radical in HL-60 cells. Pre-treatment with *α*-tocopherol remarkably prevented the C_{60} derivative-induced cell death (Figure 8). However, *α*-tocopherol could not inhibit the cell death completely. This data suggested that part of the cell death that *α*-tocopherol could not prevent was non-apoptotic cell death. It is consistent with the difference of the effects between etoposide and C_{60} derivative 1 (Figures 2, 4 and 6).

Caspases play an important role in apoptosis. Apoptotic signals are divided into a death receptor-ligand interaction pathway and a mitochondrial pathway [21,22]. It was confirmed that caspase-8 was activated by exposure to C_{60} derivative 1 (Figure 6A). The initiator caspase-8 is activated by pro-apoptotic signals, such as death receptor stimulation. In death receptor-mediated apoptosis, two signalling pathways have been demonstrated. One is that in which activated caspase-8 directly activates other caspases, including caspase-3, without mitochondrial involvement (Type I). Another is that in which activated caspase-8 cleaves the cytosolic Bid protein, resulting in the release of cytochrome *c* from mitochondria to the cytosol (Type II) [21,22]. The formation of apoptosome (the complex of cytochrome *c*/Apaf-1/pro-caspase-9) in cytosol activates caspase-9 and then pro-caspase-3 is cleaved. Finally, caspase-3 leads to apoptosis. The release of cytochrome *c* by treatment with C_{60} derivative 1 was observed (Figure 6B). This result indicates a possibility that both the death receptor and the mitochondrial pathway are involved in C_{60} derivative 1-induced cell death. The p53 tumour suppressor protein, which is directly activated by stress, such as UV, heat, X-rays and drugs, is often involved in chemotherapy-induced apoptosis in cancer cells. However, HL-60 is a p53-deficient promyelocytic cell line [23,24]. The detailed intracellular molecular signalling pathway of C_{60} derivative-induced apoptosis should be further investigated.

In conclusion, it is evident that the cationic C_{60} derivative 1 induces apoptosis mediated by mitochondria in HL-60 cells. Apoptotic cells are removed by phagocytosis without inflammation around the dying cells; therefore, it is desired that the anti-cancer agents be able to induce apoptosis. C_{60} derivative can be a new candidate for anti-cancer agents.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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