Induction of Apoptosis in Human Promyelocytic Leukemia Cell Line HL-60 by *C*-Benzylated Dihydrochalcones, Uvaretin, Isouvaretin and Diuvaretin

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Uvaretin, isouvaretin and diuvaretin, cytotoxic *C*-benzylated dihydrochalcones isolated from *Uvaria acuminata*, displayed growth inhibitory effects against human promyelocytic leukemia HL-60 cells. We examined the mechanism of the cytotoxicity. From the morphological study by staining with Hoechst 33258, the cells treated with *C*-benzylated dihydrochalcones exhibited significant chromosomal condensation and nuclear degradation. The cell cycle analysis showed the accumulation of cells in the G_1 phase and the appearance of a sub-G_1 peak. These results suggest apoptotic cell death. Further, from the detection of DNA fragmentation and the activation of caspase-3, the biological hallmarks of apoptosis, these *C*-benzylated dihydrochalcones appeared to induce apoptosis against HL-60 cells. The cytotoxicity of uvaretin and diuvaretin was stronger than that of isouvaretin, which suggest that the 5′-substitution of the 2-hydroxybenzyl group increased the cytotoxicity.

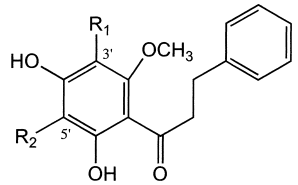
Key words *C*-benzylated dihydrochalcone; cytotoxicity against HL-60 cell; apoptosis; uvaretin; diuvaretin; isouvaretin

Uvaretin (**1**), isouvaretin (**2**) and diuvaretin (**3**) are known as *C*-benzylated dihydrochalcones peculiar to the genus *Uvaria* in the Annonaceae (Fig. 1).[1,2] Many members of this family and genus are used in folk medicine for various purposes.[1,2] It was earlier reported that dihydrochalcones of the *Uvaria* like **1**, **2** and **3** have cytotoxic activity against both PS and KB cell cultures,[3] although the mechanism of cytotoxicity was not mentioned.

Recently, we isolated six dihydrochalcones including **1**, **2** and **3** from petroleum ether extract of the root of *Uvaria acuminata*, an important folk medicine in Kenya,[4] and examined their cytotoxic activity against human promyelocytic leukemia HL-60 cells by tetrazolium-based colorimetric (WST-8) assay.[5] **1** and **3** were the main ingredients of petroleum ether extract of this plant and both exhibited significant cytotoxicity, while **2** was a minor ingredient and its activity was comparatively low. In addition, since uvangoletin, a dihydrochalcone lacking benzyl groups, was not cytotoxic, it became clear that benzyl portions had an important role in cytotoxic activity.[5]

In this paper, the mechanism of cytotoxicity of *C*-benzylated dihydrochalcones, especially **1**, **2** and **3**, was explored by investigating the apoptosis-inducing effects of those compounds in HL-60 cells.

Apoptosis is a cell suicide program that has been conserved through evolution. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue. It also plays an important role in the development of various diseases including cancer.[6,7] Recent interest has focused on apoptotic processes, and much effort has been spent in identifying the compounds that influence apoptosis from natural resources. To determine the possible mechanism of cytotoxicity that involved apoptosis, we examined the effects of *C*-benzylated dihydrochalcones, *e.g.*, **1**, **2** and **3**, on cell morphology, cell cycle progression, DNA fragmentation and activation of caspase-3.



uvaretin (**1**) : R_1 = H, R_2 = 2-hydroxybenzyl
isouvaretin (**2**) : R_1 = 2-hydroxybenzyl, R_2 = H
diuvaretin (**3**) : R_1 = R_2 = 2-hydroxybenzyl

Fig. 1. Chemical Structures of *C*-Benzylated Dihydrochalcones Isolated from the Roots of *U. acuminata*

MATERIALS AND METHODS

Materials Uvaretin (**1**), isouvaretin (**2**) and diuvaretin (**3**) were isolated from *Uvaria acuminata*. The procedures for their extraction and purification were reported previously.[5] PBS (−) was purchased from Dainippon Pharmaceutical Co., Ltd., DMSO and other reagents used were guaranteed grade.

Cell Cultures Human promyelocytic leukemia HL-60 cells were purchased from Dainippon Pharmaceutical Co., Ltd., and maintained in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 2 mM L-glutamine (Sigma) at 37 °C in a humidified atmosphere containing 5% CO_2. The doubling time of cells was approximately 24 h.

Assay for Cytotoxicity (WST-8 Assay)[8] The test compounds were dissolved in DMSO at 50 mM and stored at −20 °C. These stock solutions were further diluted with medium from 50 to 0.6 μM prior to use. The final concentrations of DMSO in the culture medium were less than 0.1%. 0.1% DMSO treated cells were used as a control for all experiments.

Cytotoxic effects of test compounds on HL-60 cells were detected by WST-8 assay. HL-60 cells were plated at 5×10^3 cells/90 μl medium/well into 96 well plates. After overnight growth, cells were treated with various concentrations of **1**, **2** and **3** for 48 h. Following incubation with *C*-benzylated dihy-

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drochalcones, the cell viability was assayed with a Cell Counting Kit-8 (Dojindo Molecular Technologies). Ten microliters of WST-8 solution (5 mM) was added to each well and then incubated for 3 h. The relative viablity of cells was determined by measuring the absorbance at 450 nm (reference at 650 nm) with a micro plate reader Anthos Lucy 2 (Aloka Co., Ltd.,).

Nuclear Staining with Hoechst 33258 HL-60 cells were plated at 4×10^5 cells/2 ml medium/well into each of 6 well plates. After overnight growth, cells were treated with **1**, **2** and **3** (**1**, **3**; 10, 20 μM, **2**; 20, 50 μM) for 24 or 48 h. The final concentration of DMSO in the culture medium was 0.1%. The cells of each well were harvested, washed once with PBS (−) and fixed with 100 μl of 1% glutaraldehyde in PBS (−). After 30 min, the cells were washed with PBS (−) and stained with 1 mM Hoechst 33258 (Nacalai Tesque) in PBS (−), then were observed by a fluorescent microscope (OLYMPUS IX70).

Cell Cycle Analysis[9] HL-60 cells were plated at 4×10^5 cells/2 ml medium/well into each of 6 well plates. After overnight growth, cells were treated with **1**, **2** and **3** (**1**, **3**; 5, 10, 20 μM, **2**; 10, 20, 50 μM) for 6, 12 and 24 h. The cells of each well were harvested, washed once with PBS (−) and fixed with 70% ethanol at 4 °C. After 1 h, 1ml of RNaseA (500 μg/ml) (Nacalai Tesque) was added to the cell and incubated at 37 °C for 30 min. Cells then were stained with 0.5 μg/ml propidium iodide (Sigma) on ice for 20 min, analyzed with a flow cytometer (FACSCalibur™, Becton Dickinson) and cell cycle distribution was analyzed using software attached to FACSCalibur (ModFit LT™, Becton Dickinson).

DNA Fragmentation[10] HL-60 cells were plated at 4×10^5 cells/2 ml medium/well into each of 6 well plates. After overnight growth, cells were treated with **1**, **2** and **3** (**1**, **3**; 5, 10, 20 μM, **2**; 10, 20, 50 μM) for 24 and 48 h. The cells of each well were harvested and washed once with PBS (−). DNA of the cells was extracted by a Master Pure™ DNA Purification kit (Epicentre Technologies). Extracted DNA was dissolved in Tris–EDTA buffer. Electrophoresis was carried out with Tris–Acetate–EDTA as the running buffer on 2% agarose gel containing 0.5 μg/ml ethidium bromide. DNA in the gel was visualized and photographed under UV light.

Caspase-3 Activity HL-60 cells were plated at 4×10^5 cells/2 ml medium/well into each of 6 well plates. After overnight growth, cells were treated with **1**, **2** and **3** (**1**, **3**; 5, 10, 20 μM, **2**; 10, 20, 50 μM) for 24 h. Caspase-3 activity was measured with ApoProbe-3, a caspase-3 fluorescent assay kit (BioDynamics Laboratory). Briefly, the cells of each well were harvested and centrifuged. Cell pellets were suspended in 50 μl lysis buffer, and incubated on ice for 10 min. After centrifugation, the supernatant was transferred to a new micro tube. To the 40 μl supernatant, 40 μl reaction buffer containing dithiothreitol (10 mM) and 2 μl of substrate (2.5 mM *N*-acetyl-Asp-Glu-Val-Asp-AMC) were added and incubated for 30 min. The release of AMC (7-amino-4-methylcoumarin) was measured with a fluorescence spectrophotometer (excitation at 355 nm, emission at 460 nm, Fluoromark™, Bio-Rad Laboratories). The amount of protein in each cell extract was measured using a protein assay kit (Proteostein Protein Quantification Kit Wide Range, Dojin Laboratory Inc.).

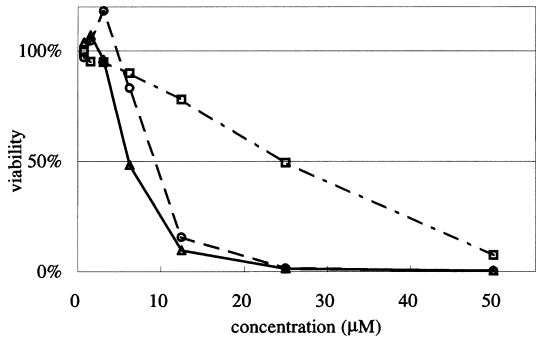

Fig. 2. Growth Inhibitory Effects of *C*-Benzylated Dihydrochalcones against HL-60 Cells

Cytotoxicity was determined by WST-8 assay. ○: uvaretin (**1**); □: isouvaretin (**2**); △: diuvaretin (**3**).

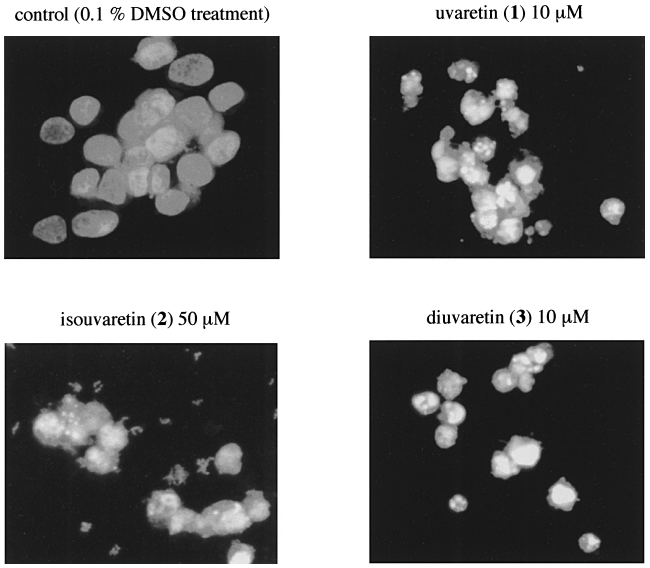

Fig. 3. Condensation of Nuclear Chromatin by *C*-Benzylated Dihydrochalcones

The cells were treated with test compounds for 24 h, followed by fixation and staining with Hoechst 33258.

RESULTS

Cytotoxic Activity Compounds **1**, **2** and **3** showed a dose-dependent inhibition on HL-60 cells (Fig. 2). The IC_{50} values of **1**, **2** and **3** against HL-60 cells were 9.3, 24.7 and 6.1 μM, respectively. **1** and **3** showed a strong cytotoxicity, while **2** showed a moderate cytotoxicity.

Nuclear Staining with Hoechst 33258 A fluorescent microscope (Fig. 3) showed that cells treated with **1** (10, 20 μM), **3** (10, 20 μM) and **2** (50 μM) clearly exhibited significant morphological changes, *i.e.*, chromosomal condensation and nuclear degradation, which is indicative of apoptotic cell death. No morphological change was observed in the cells treated with 20 μM of **2** (data not shown). The number of cells that cause these morphological changes increased with increasing concentration.

Cell Cycle Analysis The results of cell cycle analysis are shown in Fig. 4. The cells treated with **1** (≧10 μM, 12 and 24 h, Fig. 4B-2, 3) and **3** (≧10 μM, 12 and 24 h, Fig. 4D-2, 3) induced an accumulation of cells in the G_1 phase. Furthermore, a sub-G_1 peak, in which DNA content was less than

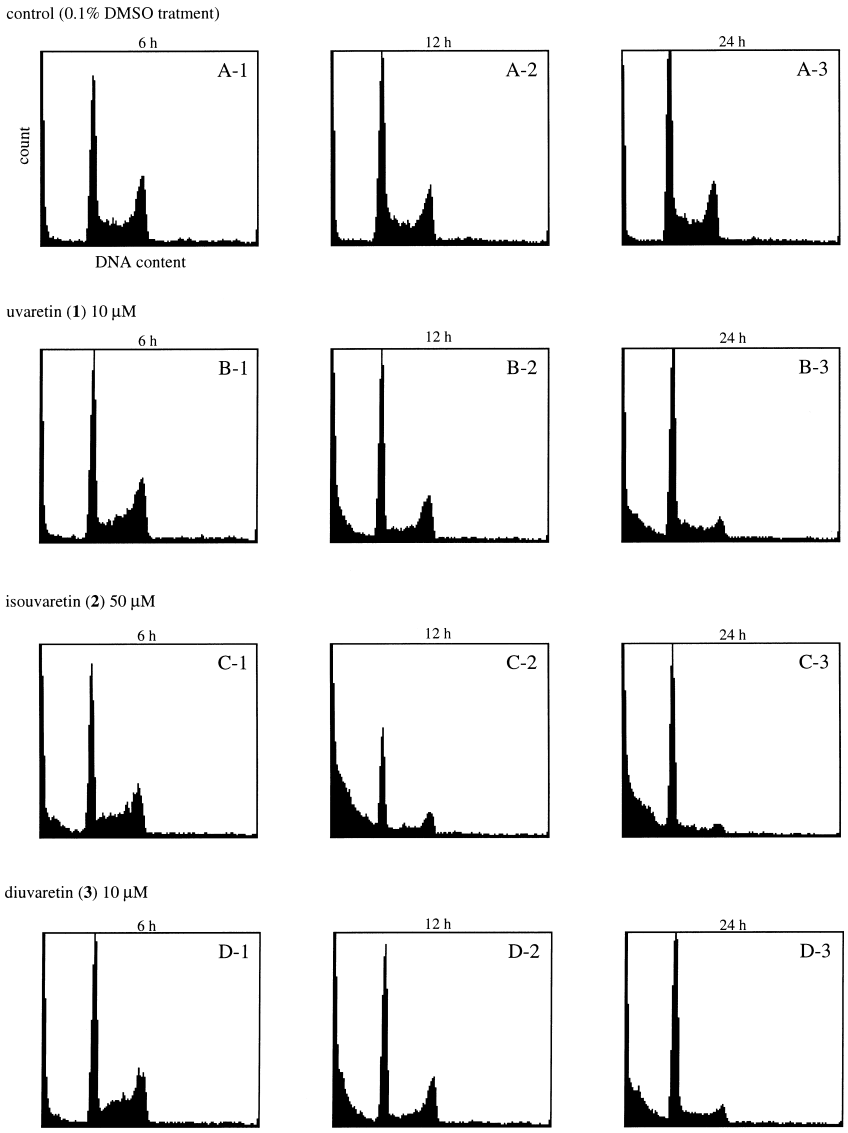

Fig. 4. The Cell Cycle of HL-60 Cells Treated with *C*-Benzylated Dihydrochalcones

DNA content of HL-60 cells was analyzed by flow cytometry. Control cells (A) clearly show G_1 phase (the first peak) and G_2/M phase (the second peak) of cell cycle.

the G_1 peak and usually considered apoptotic cells,[11] was detected by treatment with **1** (≧10 μM, 12 and 24 h, Fig. 4B-2, 3) and **3** (≧10 μM, 12 and 24 h, Fig. 4D-2, 3). No change of cell cycle was observed by treatment with **1** or **3** at 5 μM (6—24 h, data not shown). In the cells treated with **2** at 50 μM for 24 h (Fig. 4C-3), we observed an accumulation of cells in the G_1 phase. A sub-G_1 peak appeared when cells were treated with **2** at 50 μM for 12 and 24 h (Fig. 4C-2, 3); no change of cell cycle was observed by treatment with **2** at 10 and 20 μM (6—24 h, data not shown).

DNA Fragmentation DNA fragmentation is a characteristic feature of apoptosis.[12,13] DNA fragmentation was assessed by electrophoresis on agarose gel. As shown in Fig. 5, the cells treated with **1** and **3** at more than 10 μM showed a typical ladder pattern of DNA fragmentation, while those treated with **2** showed a ladder pattern at higher concentration (>20 μM).

Caspase-3 Activity The caspase family of proteases plays key roles in promoting the degradative changes of DNA that are associated with apoptosis. Moreover, one of

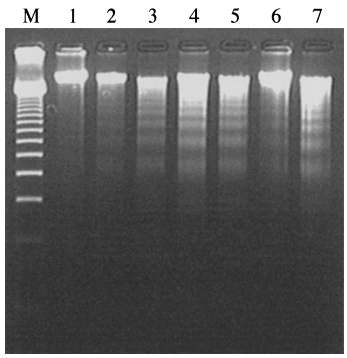

Fig. 5. DNA Fragmentation in HL-60 Cells Treated with *C*-Benzylated Dihydrochalcones

DNA was electrophoresed on 2% agarose gel and stained with ethidium bromide. M: 123-base pair marker; lane 1: control (0.1% DMSO treatment groups); lanes 2, 3: uvaretin (**1**) 10 and 20 μM treatment groups; lanes 4, 5: diuvaretin (**3**) 10 and 20 μM treatment groups; lanes 6, 7: isouvaretin (**2**) 20 and 50 μM treatment groups.

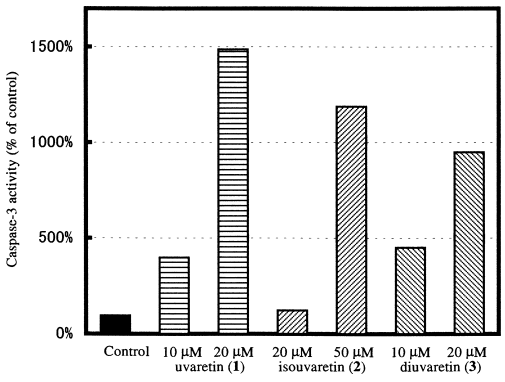

Fig. 6. Increase of Caspase-3 Activity by *C*-Benzylated Dihydrochalcones

HL-60 cells were treated with *C*-benzylated dihydrochalcones for 24 h. Caspase-3 activity was measured using a fluorometric assay kit.

these caspases, caspase-3, appears to be essential for apoptosis,[14,15] and thus, the caspase-3 activity of HL-60 cells treated with **1**, **2** and **3** was examined. Caspase-3 activity of the cells was increased about ten-fold with treatment of **1** and **3** at 20 μM compared to control cells (0.1% DMSO) (Fig. 6). An increase of caspase-3 was observed with a lesser concentration of **1** and **3** (10 μM). In the cells treated with **2**, however, caspase-3 activity was not increased by the addition of 20 μM, but was increased ten-fold by the addition of 50 μM.

DISCUSSION

Cytotoxicity of *C*-benzylated dihydrochalcones against HL-60 cells was detected by WST-8 assay. The cytotoxicity is **1**≒**3**>**2**. **1**, **2** and **3** are different at position of 2-hydroxybenzyl group in a structure. Since the cytotoxicities of **1** and **3** were higher than **2**, it is important for the cytotoxicity to substitute for the 2-hydroxybenzyl group at the 5′-C position.

We investigated whether the cytotoxic effect of these *C*-benzylated dihydrochalcones was mediated by an apoptotic mechanism. From the observations of cells stained with Hoechst 33258 (Fig. 3), those treated with *C*-benzylated dihydrochalcones exhibited significant chromosomal condensation and nuclear degradation. The results of the cell cycle analysis indicated that *C*-benzylated dihydrochalcones induced an accumulation of G_1 phase on HL-60 cells. Based on the appearance of the sub-G_1 peak, it was suggested that *C*-benzylated dihydrochalcones arrested the cell cycle at G_1 phase and caused the apoptosis.

We next examined the DNA fragmentation and the caspase-3 activity, the biological hallmarks of apoptosis. These factors were caused by these *C*-benzylated dihydrochalcones, which confirmed the apoptotic cell death. Consequently, *C*-benzylated dihydrochalcones peculiar to the genus *Uvaria* were found to induce the apoptosis, and then showed the cytotoxicity.

Acknowledgments We are grateful to Prof. Francis D. Juma and Mr. Simon G. Mathenge, University of Nairobi, for the collection of *Uvaria acuminata* at Kenya.

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