

Anti-proliferative and apoptosis-inducible activity of labdane and abietane diterpenoids from the pulp of *Torreya nucifera* in HeLa cells

SHI-PING CHEN^{1*}, MEI DONG^{1,3*}, KAZUKO KITA^{1*}, QIN-WEN SHI³, BING CONG³, WEN-ZHI GUO¹, SHIGERU SUGAYA¹, KATSUO SUGITA² and NOBUO SUZUKI¹

¹Department of Environmental Biochemistry, Graduate School of Medicine, Chiba University, Chiba 260-8670; ²Department of Clinical Medicine, Faculty Education, Chiba University, Chiba 263-8522, Japan; ³Department of Forensic Medicine, Hebei Medical University, 050017 Hebei, P.R. China

Received February 22, 2010; Accepted May 7, 2010

DOI: 10.3892/mmr_00000315

Abstract. Two abietane and one labdane type diterpenoids were isolated from the methanol extracts of *Torreya nucifera* pulp and investigated for their ability to inhibit the growth of human cancer cells. Among the three compounds, the labdane compound kayadiol was found to have the most effective inhibitory effect against a wide variety of human cancer cells. Using the MTT assay, kayadiol was determined to have an IC₅₀ (50% inhibition concentration) of 30 μ M in HeLa cells, and also to exhibit anti-proliferative effects towards six other human cancer cell lines, with IC₅₀ values of 30-50 μ M. Kayadiol treatment of HeLa cells resulted in a dose-dependent generation of apoptotic events, including DNA laddering (≤ 100 μ M). Moreover, kayadiol-treated HeLa cells showed activation of caspases-3 and -9, as well as an increase in the depolarization of mitochondrial membrane potential and the Bax/Bcl-2 ratio. These results indicate that a mitochondria-related apoptotic pathway is involved in the kayadiol-induced death of HeLa cells. Kayadiol is therefore a promising novel anti-proliferative agent and merits further investigation.

Introduction

Plants are known to contain chemical compounds that inhibit the proliferation of cancer-derived cells *in vitro*. Numerous attempts have been made to isolate anti-cancer drugs from plants. For example, the diterpene paclitaxel (Taxol®) is a

well-known anti-proliferative agent isolated from *Taxus brevifolia* (1,2). Various research has been conducted on other diterpenoid species, with the aim of identifying more effective agents for the treatment of cancer (3). We recently isolated a diterpenoid compound, sarcodonin G, from *Sarcodon scabrosus*, and demonstrated its anti-proliferative activity on human cancer cells (4).

Torreya nucifera is an evergreen coniferous tree that is commonly found in areas of Japan, Korea and China (5). The seeds of this tree exhibit significant insecticidal activity (6), while the fruit is widely used in folk medicine for the treatment of tapeworm infestation (in Korea) and for the induction of abortion (in Japan) (7). To date, analyses of the cell growth inhibition activities of *Torreya nucifera* have been focused on extracts from its leaves and wood (8), and a number of sesquiterpenoids isolated from the wood oil of the tree were reported to have an inhibitory effect on cell growth (9). However, there have been no reports on diterpenoids isolated from the pulp of the tree, although the pulp may contain numerous intriguing chemical compounds.

In the present study, two abietane diterpenoids, 18-hydroxy-ferruginol (8, 11, 13-abietatriene-12, 18-diol) (10) and hinokiol (8, 11, 13-abietatriene-3, 12-diol) (11), and a labdane diterpenoid, kayadiol [8(17), 13-labdien-15,18-diol] (Fig. 1) (12,13), were isolated from the pulp of *Torreya nucifera*. There is little available information on the biological activity of these isolated chemical compounds; thus, we investigated their inhibitory activity on the growth of cancer cells. Among the three compounds, kayadiol was found to exert the greatest growth inhibition in a wide variety of human cancer cells.

Materials and methods

Agents. Labdane and abietane diterpenoids were isolated from the air-dried pulp of *Torreya nucifera* according to previously reported methods (14). Briefly, air-dried pulp (1.3 kg) was extracted with 10 l methanol at room temperature. The combined organic extracts were evaporated under reduced pressure. Water (1 l) and then hexane (1 l) were added and the mixture was well agitated, then lipids in the upper hexane

Correspondence to: Dr Nobuo Suzuki or Dr Kazuko Kita, Department of Environmental Biochemistry, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Japan
E-mail: nobuo@faculty.chiba-u.jp or kita@faculty.chiba-u.jp

*Contributed equally

Key words: diterpenoid, kayadiol, human cancer cells, anti-proliferative effect, apoptosis

phase were removed. This step was repeated three times. The aqueous layer was salted out and extracted with methylene chloride, then the combined methylene chloride extract was dried with anhydrous sodium sulfate, filtered and evaporated, yielding a translucent yellow extract. A portion of the methylene chloride extract (31 g) was subjected to normal phase silica gel column chromatography (300–400 mesh; Qingdao Haiyang Chemical Group Corp., Qingdao, P.R. China). Successive stepwise elution with a Petro-EtOAc gradient (10:7–10:2) yielded 40 fractions (fractions 1–40). An aliquot of each fraction was applied to analytical thin layer liquid chromatography (TLC) using pre-coated silica gel F254 glass-backed plates (Qingdao Haiyang Chemical Group Corp.) without activation. The plates were dipped in a solution of 10% (v/v) H_2SO_4 in ethanol, followed by heating on a hot plate for 5 min. The spots of chemical compounds contained in the fraction were visualized under ultraviolet (UV) light (254 and 365 nm). The resulting pattern of each fraction from TLC was used to pool the fractions. Each fraction was further purified by high performance liquid chromatography (HPLC) using a Partisil ODS-2 preparative column (9.4x250 mm) (Whatman International Ltd., Maidstone, UK) eluted with a 50-min linear gradient of acetonitrile (25–100%) in water at a flow rate of 3 ml/min. The ODS-2 column was equipped with a Waters Delta Prep 3000 instrument coupled to a UV 2487 dual λ absorbance detector (Waters Corporation, Milford, MA, USA). Fractions 15 and 29 generated 18-hydroxyferuginol [retention time (Rt) = 29.693 min] and kayadiol (Rt = 33.751 min), respectively. Fractions 37–40 were combined and purified by preparative TLC and HPLC and generated hinokiol (Rt = 31.430 min).

The structures of the labdane and abietane diterpenoid compounds were elucidated on the basis of spectral data (Fig. 1). Each of the isolated compounds was dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industry, Osaka, Japan). The characteristics of the isolated chemical compounds have been reported elsewhere (15). Unless specifically stated, the other reagents were purchased from Wako Pure Chemical Industry.

Cells and culture conditions. The following human cell lines were used: HeLa (a cervical cancer cell line) (16), U251-SP (a glioma cell line) (17), HAC-2 (an ovarian carcinoma cell line) (17), T-Tn (a esophageal carcinoma cell line) (18), T-98 (a glioma cell line) (18), HEC-1 (an endometrial adenocarcinoma cell line) (18) and HLE (a hepatoma cell line) (18). Cells were cultured in Eagle's minimal essential medium (EMEM) (Gibco Brl, Grand Island, NY, USA) containing 10% (v/v) calf serum (Intergen, NY, USA) and antibiotics (100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin G) (Meiji Seika, Tokyo, Japan) at 37°C in a humidified atmosphere containing 5% CO_2 .

Measurement of cell viability. Cell viability was determined by the MTT assay as previously described (19). Briefly, logarithmically proliferating cells were plated onto 96-well plates (Asahi Glass, Tokyo, Japan) (5×10^3 cells/well) with medium containing the test compounds at the indicated doses, followed by culture for 2 days. The activity of mitochondrial succinic dehydrogenase was measured by further incubation of the cells with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2-

,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) for 4 h, followed by measurement of absorbance at 570 nm with a reference wavelength of 655 nm. Cell survival was calculated from the absorbance and presented as a percentage of the surviving cells.

Measurement of cell-cycle phase distribution. Cell-cycle phase distributions were measured as previously described (20). Briefly, cells were cultured in 60-mm dishes with the test compounds (up to 100 μM) for 24 h followed by trypsinization, then washed three times with phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. After fixation, the cells were suspended in staining solution [50 $\mu\text{g}/\text{ml}$ propidium iodide (PI), 4 mM sodium citrate, 0.5 mg/ml RNase A and 0.1% Triton X-100] for 10 min on ice. NaCl was then added to the solution to a final concentration of 0.15 M. The stained cell samples were analyzed using a FACScan instrument (Becton Dickinson, NJ, USA). The percentage of cells undergoing apoptosis was measured by calculating sub-G1 fractions using CellQuest software (Becton Dickinson). Cell-cycle phase distribution measurements were repeated in three different cultures.

DNA fragmentation analysis. Apoptotic DNA fragments were detected as previously described (21). Briefly, cells treated with vehicle and the test compounds (up to 100 μM) were cultured for 48 h and then lysed for 3 h at 37°C in a solution containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% SDS and 100 $\mu\text{g}/\text{ml}$ proteinase K. Following the addition of 1/10 volume of 3 M sodium acetate, nucleotides were extracted with phenol/chloroform, and then with chloroform alone. High molecular weight DNA was precipitated by the addition of 7/10 volume of 2-propanol followed by centrifugation at 15,000 rpm for 5 sec at room temperature. Low molecular weight DNA was recovered from the supernatant by centrifugation after incubation overnight at -20°C, then re-suspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 50 $\mu\text{g}/\text{ml}$ of DNase-free RNase A, and finally incubated for 3 h at 37°C. Samples were applied to 1.5% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide and electrophoresed in 90 mM Tris-borate (pH 8.0) and 2 mM EDTA at 100 V for 3 h. DNA ladders were photographed under UV illumination.

Immunoblotting analysis. Immunoblotting analysis was carried out as previously described (20). After treatment with the test compounds for the indicated periods, cells were solubilized in 1X SDS sampling buffer. The whole cell lysates were separated by electrophoresis on 12% (w/v) SDS-PAGE. Active forms of caspase-3 and -9 proteins were detected using rabbit anti-cleaved caspases-3 antibody (2305-PC-020; Trevigen, MD, USA) (1:800 dilution) and mouse anti-human caspase-9 antibody (MAB8301; R&D, Minneapolis, MN, USA) (1:1,000 dilution), respectively. The latter antibody detects non-cleaved and cleaved caspase-9. Bax and Bcl-2 proteins were detected using mouse anti-Bax monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:300 dilution) and mouse anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology) (1:300 dilution), respectively. The antigen-antibody complexes were detected by anti-rabbit IgG (NA934V; GE, Healthcare, UK) and anti-mouse IgG (NA931V; Amersham Biosciences, Buckinghamshire, UK), followed by

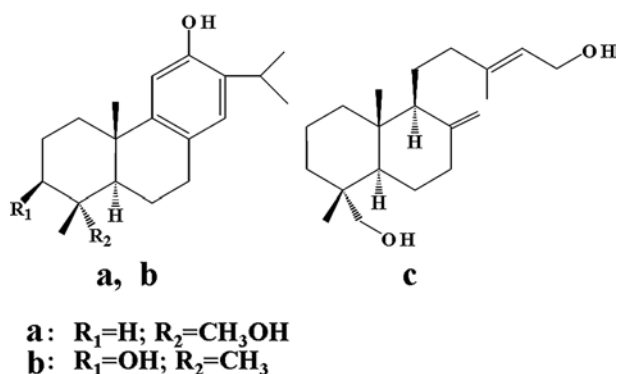


Figure 1. Chemical structures of (a) 18-hydroxyferruginol, (b) hinokiol and (c) kayadiol.

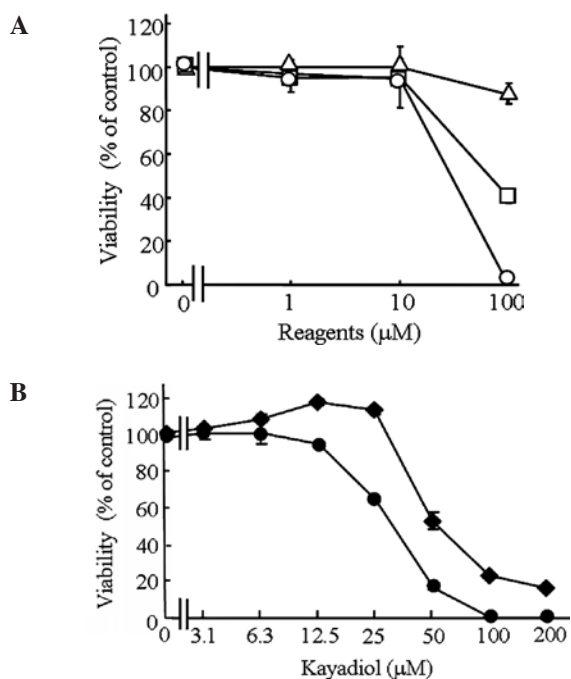


Figure 2. Cell viability as determined by the MTT assay. (A) HeLa cells were treated for 2 days with three diterpenoid compounds: 18-hydroxyferruginol (\square), hinokiol (Δ) or kayadiol (\circ). (B) HeLa (\bullet) and HLE (\blacklozenge) cells were treated for 2 days with kayadiol at the indicated doses. Data are presented as the means \pm SD of three independent experiments.

visualization using the ECL system (GE). Amounts of actin were also analyzed by mouse anti-actin antibody (Illkirch, France) (1:20,000 dilution) as the loading control. The intensities of the protein signals were quantified using Multi Gauge ver. 2.2 image analyzing software (Fuji Foto Film, Tokyo, Japan) and expressed as the relative value to that of actin.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured using a fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) (Cell Technology Inc., Mountain View, CA, USA) according to the manufacturer's instructions (22). As the membrane is hyperpolarized, JC-1 accumulates as aggregates in the mitochondria and stains red. When the membrane potential is depolarized, JC-1 exists as a monomeric form in the cytosol and stains green.

Table I. Anti-proliferative activity of kayadiol in various cancer cell lines.

Cell line	IC ₅₀ (μM)
HeLa	30.0
U251SP	32.7
HAC-2	34.4
T-Tn	38.2
HEC-1	42.2
T-98	49.4
HLE	50.3

Cells were cultured in 60-mm dishes with and without the indicated compounds at the indicated doses for 24 h followed by trypsinization, then suspended in culture medium containing JC-1 and incubated at 37°C in a 5% CO₂ incubator for 15 min. Thereafter, the cells were washed once with EMEM, and fluorescence was measured by FACSscan. The ratio of green fluorescence (FL1) intensity to red fluorescence (FL2) intensity was used to determine the extent of the depolarization of mitochondrial membrane potential.

Statistical analysis. Values are expressed as the means of two or more independent experiments, unless specifically stated otherwise, and were compared to results from a vehicle-treated (0.1% DMSO) control. Statistical analysis was performed using the Student's t-test with StatView software (ver. 4.5; Abacus Concepts, Berkeley, CA, USA) as previously described (20).

Results

Inhibition of HeLa cell viability by kayadiol. The cytotoxic potential of three labdane and abietane diterpenoid compounds (Fig. 1) was determined using the MTT assay after culture of HeLa cells in the presence of the chemical compounds (up to 100 μM) for 2 days. Hinokiol treatment resulted in only a slight decrease in cell viability at 100 μM (Fig. 2A). The inhibitory effect of 18-hydroxyferruginol treatment on cell viability was greater than that of hinokiol, while kayadiol treatment inhibited cell viability to the greatest extent (Fig. 2A). In a more precise MTT analysis, kayadiol showed an anti-proliferative effect towards HeLa and HLE cells with an IC₅₀ value of 30 and 50.3 μM , respectively (Fig. 2B and Table I). The IC₅₀ values of kayadiol in five other human cancer cell lines ranged between 30 and 50 μM (Table I). HeLa cells showed the highest sensitivity to the anti-proliferative effect of kayadiol among all the cancer cell lines tested.

Apoptotic events in kayadiol-treated HeLa cells. Cell cycle distribution in the HeLa cells was examined by flow cytometric analysis after PI staining. Cells treated with 18-hydroxyferruginol (up to 100 μM) for 24 h showed only a slight increase in the sub-G1 phase cell population (apoptotic fraction) (Fig. 3A). By contrast, a distinct dose-dependent increase in the apoptotic fraction was observed in cells treated with kayadiol (Fig. 3A). These results indicate that apoptosis

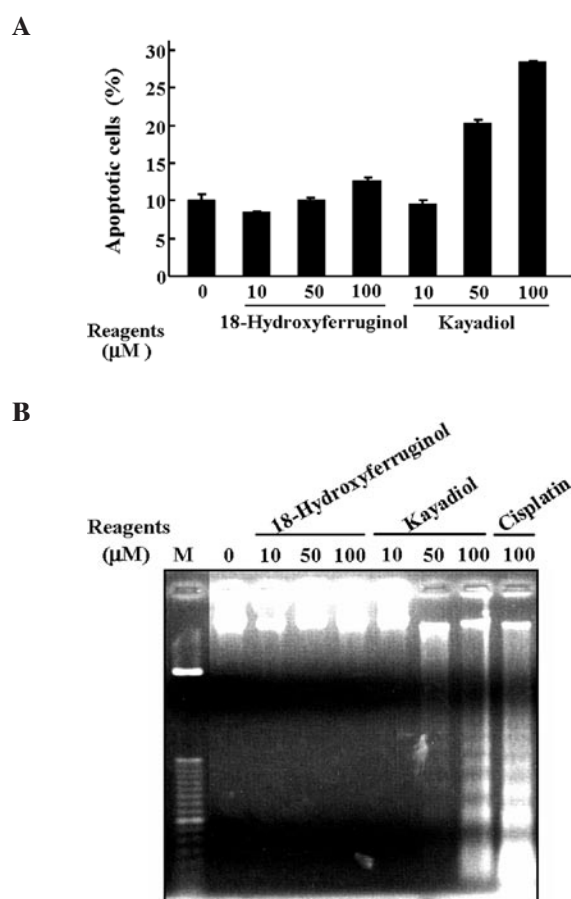


Figure 3. Apoptosis induced by kayadiol in HeLa cells. (A) Flow cytometric analysis of apoptotic population of diterpenoid compound-treated HeLa cells. HeLa cells were treated with 18-hydroxyferruginol and kayadiol for 24 h and subjected to flow cytometric analysis. At least 1×10^4 cells were counted for each condition and the fraction of sub-G1 population is presented as an apoptotic fraction. Data are presented as means \pm SD of three independent experiments. (B) DNA fragmentation in diterpenoid compound-treated HeLa cells. Cells were treated with vehicle (DMSO) at indicated concentrations of 18-hydroxyferruginol and kayadiol and cisplatin for 48 h, and then the total DNA preparation and detection of the DNA fragment were performed as described in Materials and methods. M represents molecular markers.

was induced in kayadiol-treated HeLa cells. To confirm that kayadiol induced apoptosis, the fragmentation status of genomic DNA was examined in HeLa cells treated with the compound. The results were compared to the distinct DNA fragmentation observed in HeLa cells treated with cisplatin (100 μ M) (Fig. 3B). DNA fragmentation was observed in cells treated with 50 and 100 μ M of kayadiol (Fig. 3B), but not in cells treated with 18-hydroxyferruginol (up to 100 μ M).

Involvement of mitochondria-related pathways in apoptosis in kayadiol-treated HeLa cells. To elucidate the mechanisms of kayadiol-induced apoptosis in the HeLa cells, the possible involvement of the caspases was assessed by detecting the active cleaved forms of caspases-3 and -9. The active form of caspase-3 was detected after 24 h of treatment with 50 μ M kayadiol, and was further increased in cells treated with 100 μ M kayadiol (Fig. 4A). After treatment with 50 and 100 μ M kayadiol, the amount of active caspase-9 was also increased, by \sim 3-fold in cells treated with 100 μ M kayadiol as compared to the control (Fig. 4A).

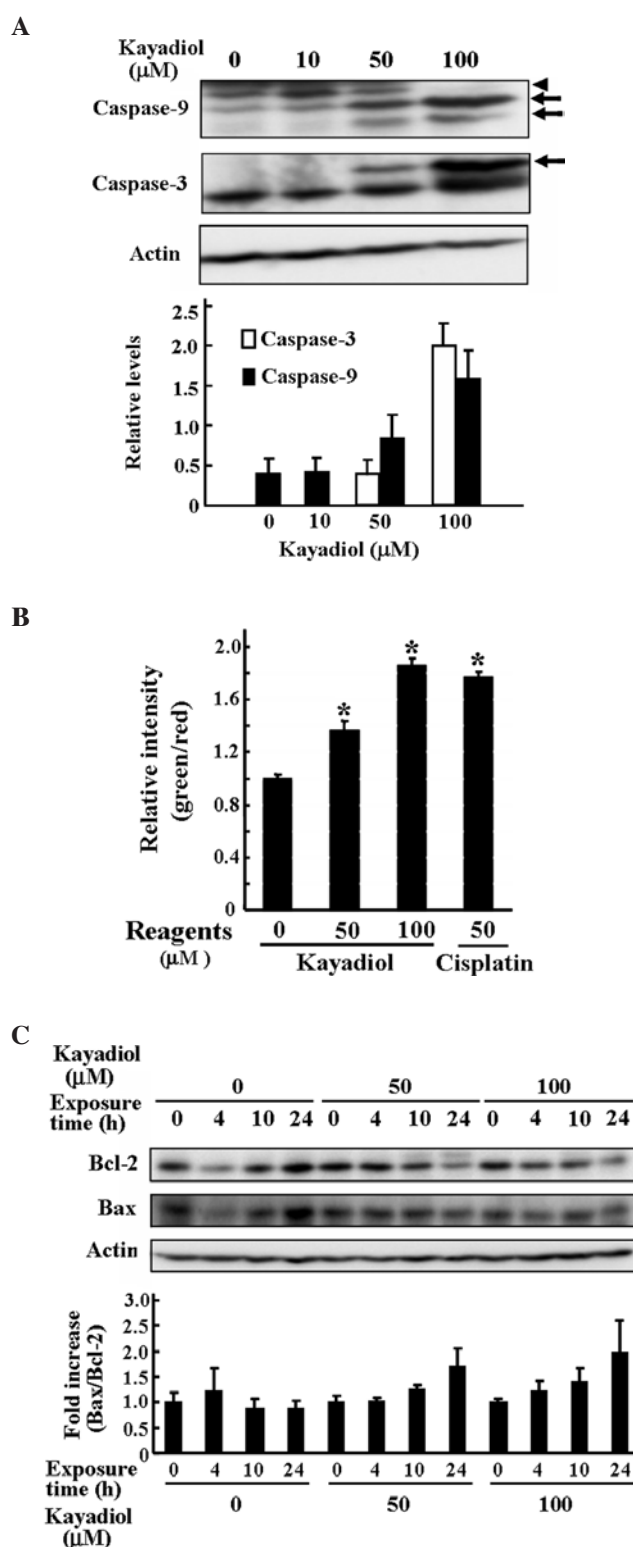


Figure 4. Apoptotic pathway in kayadiol-treated HeLa cells. Cells were treated with vehicle (DMSO) and indicated concentrations of kayadiol or cisplatin for 24 h and then the expression levels of active forms of caspases-3 and -9 were analyzed by immunoblotting (A), mitochondrial membrane potential was analyzed using JC-1 probe (B) and the expression levels of Bax to Bcl-2 protein were analyzed by immunoblotting (C), as described in Materials and methods. (A) The levels of the cleaved active forms of caspases-3 and -9, shown by an arrow, are presented as the expression levels relative to those of actin. The band of the non-cleaved form of caspase-9 is shown by an arrowhead. (B) Reduction of mitochondrial membrane potential is presented as increase in the relative intensity of green fluorescence to red fluorescence. (C) Protein levels of Bax and Bcl-2 were normalized with actin levels and the ratio of Bax to Bcl-2 are presented as relative to those of the cells without kayadiol treatment. Data are presented as means \pm SD.

The activation of caspase-9 suggested the involvement of a mitochondria-related pathway in kayadiol-induced apoptosis. Thus, mitochondrial membrane potential in HeLa cells was measured using the JC-1 fluorescent probe. After kayadiol treatment at 50 and 100 μM , a reduction in membrane potential was observed (Fig. 4B). Treatment with 100 μM kayadiol resulted in depolarization to a similar extent as treatment with 50 μM cisplatin.

Next, the protein concentrations of Bax and Bcl-2, which are involved in mitochondria-related apoptosis, were measured in HeLa cells treated with and without kayadiol. Western blot analysis showed that the expression of Bcl-2 protein decreased after kayadiol treatment (50-100 μM), while the Bax/Bcl-2 protein ratio increased in a time-dependent manner until 24 h of treatment (Fig. 4C). After 10 h of treatment with 100 μM kayadiol, the ratio was slightly increased, whereas after 24 h of treatment, it was increased by ~ 2 -fold.

Discussion

Of the three chemicals extracted from *Torreya nucifera* pulp, kayadiol exhibited the greatest inhibition of HeLa cell viability, as determined by the MTT assay (Fig. 2A). The sensitivity of HeLa cells to kayadiol was similar to their sensitivity to another diterpenoid, sarcodonin G (4). The growth inhibition of HeLa cells by kayadiol (Fig. 2) and sarcodonin G was greater than that induced by 5-FU, but weaker than that of cisplatin (4).

Chemotherapeutic anti-cancer agents, including 5-FU and cisplatin, are known to induce apoptosis (23,24). In the present study, this was demonstrated by DNA fragmentation observed in cisplatin-treated HeLa cells (Fig. 3B). Kayadiol (at concentrations in excess of 50 μM) also induced apoptotic events in HeLa cells, as evidenced by an increase in the percentage of cells in the sub-G1 fraction (Fig. 3A), DNA fragmentation (Fig. 3B) and the activation of caspase-3 (Fig. 4A), as well as of caspase-9. Caspase-9 is thought to be activated by an apoptotic signal via mitochondria-dependent mechanisms, and further cleaves and activates the other effector caspases, including caspase-3 (25,26). Here, mitochondrial membrane potential was depolarized in kayadiol-treated HeLa cells (Fig. 4B). Bax and Bcl-2 protein levels were also modulated in the kayadiol-treated HeLa cells (Fig. 4C). Bax and Bcl-2 are pro- and anti-apoptotic proteins, respectively (27). Up-regulation of the Bax/Bcl-2 ratio suggests the involvement of mitochondrial factors in caspase activation (28-30). In the present study, the Bax/Bcl-2 ratio was increased in HeLa cells treated with kayadiol (at concentrations in excess of 50 μM) (Fig. 4C).

Generally, apoptosis occurs via the mitochondrial (intrinsic) pathway (31) or the death receptor (extrinsic) pathway (32). The mitochondrial (intrinsic) pathway is controlled at the level of the mitochondrial membrane by the Bcl-2 superfamily of proteins. An increase in the expression ratio of the pro-apoptotic family of proteins vs. the anti-apoptotic family of proteins leads to the disruption of mitochondrial membrane potential and the activation of caspase-9 (25,28-30). Therefore, it is possible that kayadiol treatment (at concentrations in excess of 50 μM) induced the activation of at least the intrinsic mitochondria-related apoptotic pathway in HeLa cells.

The viability of six other human cancer cell lines was decreased by kayadiol treatment. The IC_{50} values of kayadiol in the other cell lines ranged between 30 and 50 μM . Thus, kayadiol may have a lethal effect in a wide variety of cancer cells.

From a structure-activity perspective, it would be informative to investigate which functional groups of kayadiol contribute to its substantial anti-proliferative activity. We are now isolating other diterpenoids from various plants, particularly from *Taxus mairei* (33), and searching for novel diterpenoids that have greater anti-proliferative activity in human cancer cells than kayadiol.

Acknowledgements

This work was supported in part by grants-in-aid from the Scientific Research Foundation and the Traditional Chinese Medicine Administration Bureau of Hebei (P.R. China), and by the Smoking Research Foundation, Tokyu Foundation for a Better Environment, Hamaguchi Foundation for the Advancement of Biochemistry, Kieikai Research Foundation, Goho Life Science International Foundation, Ministry of Health, Labour and Welfare for the Intractable Diseases Treatment Research Program and the Japan Society for the Promotion of Science (Japan).

References

1. Fuchs DA and Johnson RK: Cytologic evidence that taxol, an antineoplastic agent from *Taxus brevifolia*, acts as a mitotic spindle poison. *Cancer Treat Rep* 62: 1219-1222, 1978.
2. McGuire WP, Rowinsky EK, Rosenshein NB, Grumbine FC, Ettinger DS, Armstrong DK and Donehower RC: Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. *Ann Int Med* 111: 273-279, 1989.
3. Shi Q-W, Dong M, Huo C-H, Su X-H, Li C-F, Zhang X-P, Wang Y-F and Kiyota H: New 14-hydroxy-taxane and 2 α ,20-epoxy-11(15 \rightarrow 1) abeotaxane from the needles of *taxus canadensis*. *Biosci Biotechnol Biochem* 71: 1777-1780, 2007.
4. Dong M, Chen SP, Kita K, Ichimura Y, Guo WZ, Lu S, Sugaya S, Hiwasa T, Takiguchi M, Mori N, Kashima A, Morimura K, Hirota M and Suzuki N: Anti-proliferative and apoptosis-inducible activity of Sarcodonin G from *Sarcodon scabrosus* in HeLa cells. *Int J Oncol* 34: 201-207, 2009.
5. Fukushima I, Sayama Y, Kyogoku K and Murayama H: Isolation of 6-hydroxydehydro abietinol and hinokiol from the leaves of *Torreya nucifera* Sieb. et Zucc. *Agric Biol Chem* 32: 1103-1107, 1968.
6. Sakai T, Nishimura K and Hirose Y: Structure and stereochemistry of four new sesquiterpenes isolated from the wood oil of *Torreya nucifera*. *Bull Chem Soc Japan* 38: 381-387, 1965.
7. Jang YP, Kim SR and Kim YC: Neuroprotective dibenzylbutyrolactone lignans of *Torreya nucifera*. *Planta Med* 67: 470-472, 2001.
8. Sayama Y, Kyogoku K and Murayama H: New diterpenes of *Torreya nucifera*. *Agric Biol Chem* 35: 1068-1073, 1971.
9. Kariyone T and Sawaka T: Flavonoids of the leaves of Coniferae and allied plants. I. Flavonoid from the leaves of *Torreya nucifera*. *Yakugaku Zasshi* 78: 1010-1013, 1958.
10. Harrison LJ and Asakawa Y: 18-Oxoferruginol from the leaf of *Torreya nucifera*. *Phytochemistry* 26: 1211-1212, 1987.
11. Shingh M, Pal M and Sharma RP: Biological activity of the labdane diterpenes. *Planta Med* 65: 2-8, 1999.
12. Zhao Q-S, Tian J, Yue J-M, Chen S-N, Lin Z-W and Sun H-D: Diterpenoids from *Isodon flavidus*. *Phytochemistry* 48: 1025-1029, 1998.
13. Monacelli B, Pasqua G and Botta B: Abietane diterpene from callus cultures of *Taxus baccata*. *Planta Med* 68: 764-766, 2002.
14. Dong M, Zhang M-L, Wang Y-F, Zhang X-P, Li C-F, Shi Q-W, Cong B and Kiyota H: Three new taxanes with the 10-alkoxy group from the heartwood of *Taxus cuspidate*. *Biosci Biotechnol Biochem* 71: 173-179, 2007.

15. Cao C-M, Dong M, Zhang M-L, Zhang X-P, Yang Y, Gu Y-C and Shi Q-W: Studies on chemical constituents of the fruit of *Torreya nucifera*. *Natural Product Research and Development* 21: 737-739, 2009.
16. Suzuki N and Fuse A: A UV-sensitive human clonal cell line, R5a, which has low repair activity. *Mutat Res* 84: 133-145, 1981.
17. Suzuki N, Oiwa Y, Sugano I, Inaba N, Sekiya S, Fukazawa I, Yoshida J, Takakubo Y, Isogai E and Saito-Ebihara M: Dipyridamole enhances an anti-proliferative effect of interferon in various types of human tumor cells. *Int J Cancer* 51: 627-633, 1992.
18. Suzuki N, Sekiya S, Sugano I, Kojima T, Yamamori H and Takakubo Y: Dipyridamole combined with tumor necrosis factor-enhances inhibition of proliferation in human tumor cell lines. *Jpn J Cancer Res* 86: 761-769, 1995.
19. Wano C, Kita K, Takahashi S, Sugaya S, Hino M, Hosoya H and Suzuki N: Protective role of HSP27 against UVC-induced cell death in human cells. *Exp Cell Res* 298: 584-592, 2004.
20. Zhai L, Kita K, Wano C, Wu Y, Sugaya S and Suzuki N: Decreased cell survival and DNA repair capacity after UVC irradiation in association with down-regulation of GRP78/BiP in human R5a cells. *Exp Cell Res* 305: 244-252, 2005.
21. Wu Y, Kita K and Suzuki N: Involvement of human heat shock protein 90 α in nicotine-induced apoptosis. *Int J Cancer* 100: 37-42, 2002.
22. Cossarizza A, Baccarani-Conti M, Kalashnikova G and Franceschi C: A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). *Biochem Biophys Res Commun* 30: 40-45, 1993.
23. Lowe SW, Ruley HE, Jacks T and Housman DE: P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74: 957-967, 1993.
24. Jakubowicz-Gil J, Rzymowska J, Paduch R and Gawron A: The effect of quercetin on pro-apoptotic activity of cisplatin in HeLa cells. *Biochem Pharmacol* 69: 1343-1350, 2005.
25. Sareen D, van Ginkel PR, Takach JC, Mohiuddin A, Darjatmoko SR, Albert DM and Polans AS: Mitochondria as the primary target of resveratrol-induced apoptosis in human retinoblastoma cells. *Invest Ophthalmol Vis Sci* 47: 3708-3716, 2006.
26. Schuler M and Green DR: Mechanisms of P53-dependent apoptosis. *Biochem Soc Trans* 29: 684-688, 2001.
27. Green DR and Kroemer G: The pathophysiology of mitochondrial cell death. *Science* 305: 626-629, 2004.
28. Luna-More S, Weil B, Bautista D, Garrido E, Florez P and Martinez C: Bcl-2 protein in normal, hyperplastic and neoplastic breast tissues: a metabolite of the putative stem-cell subpopulation of the mammary gland. *Histol Histopathol* 19: 457-463, 2004.
29. Lu C-X, Nan K-J, Nie Y-L, Hai Y-N and Min J: Delisheng, a Chinese medicinal compound, exerts anti-proliferative and pro-apoptotic effects on HepG2 cells through extrinsic and intrinsic pathways. *Mol Biol Rep*: DOI 10.1007/s11033-009-9930-7, 2009.
30. Wang H-J, Tashiro S, Onodera S and Ikejima T: Inhibition of insulin-like growth factor 1 receptor signaling enhanced silibinin-induced activation of death receptor and mitochondrial apoptotic pathways in human breast cancer MCF-7 cells. *J Pharmacol Sci* 107: 260-269, 2008.
31. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479-489, 1997.
32. Nagata S: Apoptosis by death factor. *Cell* 88: 355-365, 1997.
33. Shi Q-W, Zhao Y, Si X, Li Z-P, Yamada T and Kiyota H: 1-Deoxypaclitaxel and abeo-taxoids from the seeds of *Taxus mairei*. *J Nat Prod Res* 69: 280-283, 2006.