Isolation of Apoptosis- and Differentiation-Inducing Substances toward Human Promyelocytic Leukemia HL-60 Cells from Leaves of *Juniperus taxifolia*

Norio Muto, Tatsuru Tomokuni, Mari Haramoto, Hideki Tatemoto, Tsutomu Nakahashi, Yuka Inatomi, Hiroko Murata, and Akira Inada

1Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, 562 Nanatsuka, Shobara 727-0023, Japan
2School of Bioresources, Hiroshima Prefectural University, 562 Nanatsuka, Shobara 727-0023, Japan
3Faculty of Agriculture, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan
4Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata 573-0101, Japan

Received September 5, 2007; Accepted November 26, 2007; Online Publication, February 7, 2008

A chloroform extract of the leaves of *Juniperus taxifolia* exhibited a marked antiproliferative effect on human promyelocytic leukemia HL-60 cells at a concentration of 2.5 μg/ml. Deoxypodophyllotoxin (4) was identified in the extract as an outstanding antiproliferative compound, and five diterpenes (1–3, 5, and 6) were isolated as known compounds with weak or no cytotoxicity. These compounds were examined for their respective apoptosis- and differentiation-inducing activities toward HL-60 cells by DNA fragmentation and NBT-reducing assays, respectively. Among them, 7α-hydroxysandaracopimaric acid (6) was found to have a potent differentiation-inducing activity in a dose-dependent manner at 0.125–2 μg/ml (0.39–6.29 μM), together with apoptosis-inducing activity at concentrations of more than 2.5 μg/ml (7.86 μM). Deoxypodophyllotoxin (4) that exerted cytotoxic and apoptosis-inducing activities at 2 ng/ml (5 nM) did not induce differentiation at the same concentration, and the other diterpenes (1–3 and 5) showed no effect on cell differentiation, even at 5 μg/ml. It was thus demonstrated for the first time that 7α-hydroxysandaracopimaric acid was an effective differentiation-inducing compound toward HL-60 cells.

**Key words:** 7α-hydroxysandaracopimaric acid; deoxypodophyllotoxin; differentiation; HL-60 cell; *Juniperus taxifolia*

Apoptosis-inducing therapy is one of the most remarkable methods for cancer treatment to emerge in recent years, and most anticancer drugs that induce apoptosis utilize an active process controlled by cascades of specific cellular suicidal pathways. However, they often exert nonspecific toxicity on various types of normal cells with proliferation potency, resulting in severe adverse effects. Differentiation-inducing therapy is therefore anticipated as a novel medical treatment that could reduce such adverse effects, when compared to previous anticancer drugs.

The human promyelocytic leukemia HL-60 cell line has been used as a model system for studying cellular differentiation. HL-60 cells can be differentiated into monocyte/macrophage-like or granulocyte-like cells by various agents such as all-trans retinoic acid (ATRA), 1,25-dihydroxyvitamin D₃, anticancer drugs and natural compounds. Differentiated cells lose their abnormal proliferative and tumor-forming abilities. In fact, ATRA is used as a differentiation-inducing drug for treating human promyelocytic leukemia. Although ATRA has been assessed to be safer than most anticancer drugs, such adverse effects as leukocytosis and retinoic acid syndrome have often been observed during the course of its administration. In order to make differentiation-inducing therapy safer and more effective, it is therefore very important to search for a variety of promising compounds that possess differentiation-inducing activity.

During the course of screening promising plant materials possessing cell disorder activities toward HL-60 cells, the extract of *Juniperus taxifolia* ("shima-muro" in Japanese) was found from coniferous bioresources. Plants of the genus *Juniperus* (Cupressaceae) comprise about 60 species, most of which grow in the northern hemisphere. Among them, *J. taxifolia* Hook. et Arn. is indigenous to the Bonin islands located in the subtropical regions of Japan. Previous phytochemical studies on this plant have revealed only the composition of essential oils in the leaf. The detection of marked antiproliferative activity in the leaves of *J. taxifolia* prompted us to further investigate the constituents of this plant. We describe in this paper the isolation of
some compounds from the leaves of *J. taxifolia* and their apoptosis- and differentiation-inducing activities toward HL-60 cells.

**Materials and Methods**

*General.* Optical rotation values were measured by a Jasco-DIP-140 digital polarimeter. EI-MS data were recorded by a Jeol JMS-700T spectrometer with a direct inlet system at 30 eV. The 1H- and 13C-NMR spectra were recorded by a GE- Omega 600 (600 and 150 MHz, respectively) or a Jeol GX-400 (400 and 100 MHz, respectively) spectrometer, with CDCl3 used as a solvent and tetramethylsilane (TMS) as an internal standard. HPLC was performed with a JaiIc-908 instrument (Japan Analytical Industry Co.), using a Jaigel-ODS column (φ20 mm × 250 mm) with a differential refractometer.

*Plant material.* The leaves of *J. taxifolia* Hook. et Arn. were collected in February 2002 at Chichi-Zima in the Bonin Islands (Tokyo, Japan), and a voucher specimen (no. 160) has been deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Setsunan University.

*Extraction and isolation.* The dried and cut leaves (600 g) were successively extracted at room temperature with hexane, CHCl3, acetone, and MeOH (6 liters each, 2 times). Each solvent was evaporated off to give hexane (25.0 g), CHCl3 (23.0 g), acetone (24.1 g), and MeOH (85.5 g) extracts. The CHCl3 extract was treated with diethyl ether (300 ml) to give an ether-soluble portion (14.2 g) and an insoluble residue (8.8 g). A part (4.0 g) of the ether-soluble portion was chromatographed on silica gel (200 g), with hexane and hexane-EtOAc (4:1, 2:1, 1:1, 1:2, and 1:4) used as the eluents. Fractions B (300 mg), D (350 mg), and F (330 mg), eluted with hexane-EtOAc (2:1), were further purified by reversed phase HPLC, using MeOH-H2O (9:1), to isolate compounds I (40 mg), 2 (70 mg), and 3 (30 mg), respectively. Fraction G (550 mg), eluted with hexane-EtOAc (1:1), was purified by silica gel (33 g) column chromatography [hexane-EtOAc (1:1)] and reversed phase HPLC [MeOH-H2O (9:1)] to afford 4 (27 mg). Fraction H (320 mg), eluted with hexane-EtOAc (1:2), was separated by silica gel (25 g) column chromatography [hexane-EtOAc (1:2)] and reversed phase HPLC [MeOH-H2O (5:1)] to give compounds 5 (3.0 mg) and 6 (6.3 mg), respectively (Fig. 1).

*E-Communic acid (1):* Colorless crystals. Mp 156–158°C; 1H-NMR (CDCl3) δ: 3.98–4.05 (3H, s), 1.67 (CH, t, J = 7.2 Hz) and 1.21 (CH3, s) (Fig. 1).

*13C-NMR (100 MHz, CDCl3) δ: 38.4 (C-1), 19.9 (C-2), 39.2 (C-3), 44.11 (C-4), 56.3 (C-5), 23.2 (C-6), 40.3 (C-7), 147.6 (C-8), 56.1 (C-9), 37.8 (C-10), 25.8 (C-11), 133.2 (C-12), 141.3 (C-13), 133.6 (C-14), 109.7 (C-15), 12.8 (C-16), 107.5 (C-17), 29.0 (C-18), 183.5 (C-19), 11.8 (C-20).

*Sandaracopimicin acid (2):* Colorless crystals. Mp 162–163°C; 1H-NMR (CDCl3) δ: 3.98 (3H, s), 1.51 (CH, J = 7.2 Hz) and 1.22 (CH3, s) (Fig. 1).

*13C-NMR (100 MHz, CDCl3) δ: 28.3 (C-1), 22.6 (C-2), 132.8 (C-3), 39.4 (C-4), 25.8 (C-5), 44.1 (C-6), 127.0 (C-7), 137.2 (C-8), 44.7 (C-9), 37.1 (C-10), 25.8 (C-11), 133.3 (C-12), 131.4 (C-13), 133.5 (C-14), 109.7 (C-15), 12.8 (C-16), 107.5 (C-17), 29.0 (C-18), 183.5 (C-19), 11.8 (C-20).

*Deoxydophiphyllotoxin (4):* Colorless crystals. Mp 135–137°C; 1H-NMR (CDCl3) δ: 3.98–4.05 (3H, s), 1.67 (CH, t, J = 7.2 Hz) and 1.21 (CH3, s) (Fig. 1).

*13C-NMR (100 MHz, CDCl3) δ: 38.4 (C-1), 19.9 (C-2), 39.2 (C-3), 44.11 (C-4), 56.3 (C-5), 23.2 (C-6), 40.3 (C-7), 147.6 (C-8), 56.1 (C-9), 37.8 (C-10), 25.8 (C-11), 133.2 (C-12), 141.3 (C-13), 133.6 (C-14), 109.7 (C-15), 12.8 (C-16), 107.5 (C-17), 29.0 (C-18), 183.5 (C-19), 11.8 (C-20).

*14α-Hydroxydophiphyllotoxin (5):* An amorphous solid; 1H-NMR (CDCl3) δ: 38.4 (C-1), 19.9 (C-2), 39.2 (C-3), 44.11 (C-4), 56.3 (C-5), 23.2 (C-6), 40.3 (C-7), 147.6 (C-8), 56.1 (C-9), 37.8 (C-10), 25.8 (C-11), 133.2 (C-12), 141.3 (C-13), 133.6 (C-14), 109.7 (C-15), 12.8 (C-16), 107.5 (C-17), 29.0 (C-18), 183.5 (C-19), 11.8 (C-20).
Apoptosis- and Differentiation-Inducing Substances from Juniperus taxifolia

Cell culture. The human promyelocytic leukemia cell line, HL-60, was obtained from the Riken Cell Bank (Saitama, Japan). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in RPMI1640 medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Filtron, Brooklyn, Australia).

Antiproliferative activity. The viability of HL-60 cells was determined by the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Dojin Chemicals, Kumamoto, Japan) assay. The cells (1 × 10⁶ cells/well) were seeded in a 96-well microplate and cultured for 24 h. After various concentrations of a sample had been added to the medium, the cells were cultured for an additional 24 h. All samples were dissolved in DMSO and added at a final concentration of less than 0.1% DMSO. After incubation, a 5 mM WST-1 solution containing 0.2 mM 1-methoxy-5-methylphenazium methylsulfate (Dojin Chemicals, Kumamoto, Japan) was added to each well, and the cells were incubated at 37°C for 2 h. The plates were immediately monitored at 415 nm by a microplate reader (Bio-Rad 1550). Cell viability was calculated as the percentage of live cells as compared with the untreated controls. Each value is expressed as the mean ± SD from triplicate assays.

Differentiation-inducing activity. The differentiation of HL-60 cells was assessed by the nitroblue tetrazolium (NBT) reduction assay as previously described. This assay is based on the ability of phagocytic cells, a type of differentiated leukocyte, to produce superoxide anions upon stimulation with the tissue plasminogen activator. The cells (1 × 10⁶ cells/60 mm dish) were treated with each sample at 37°C for 4 h in a dish, and then collected and washed with phosphate-buffered saline (PBS). They were incubated with 0.2 ml of solution containing 400 μg of NBT and 200 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) at 37°C for 30 min. The reaction was stopped by adding 200 μl of 1 N HCl on ice. After centrifugation, intracellular blue-black formazan deposits were extracted with 600 μl of DMSO, and the absorbance at 560 nm was measured by using a spectrophotometer (Jasco V-530).

Apoptosis-inducing activity. Apoptosis of the HL-60 cells was assessed by the DNA fragmentation assay as previously described. The cells (1 × 10⁶ cells/60 mm dish) were treated with various concentrations of a sample at 37°C for 24 h. The cells were harvested by centrifugation and washed two times with PBS. The pellet was lysed with a lysis buffer (50 mM Tris–HCl at pH 7.5, 20 mM EDTA, and 0.2% Triton X-100). The lysate was centrifuged at 2000 × g for 10 min to obtain a supernatant containing fragmented DNA. The supernatant was incubated with 2 mg/ml of RNase at 37°C for 60 min. The mixture was treated with 20 mg/ml of proteinase K at 50°C for 45 min. DNA was precipitated overnight with 50 μl of 5 M NaCl and 250 μl of isopropyl alcohol at −30°C. After recovering by centrifugation at 12,000 × g for 10 min, the DNA pellet was dried in air and suspended in a TBE buffer containing 0.25% bromophenol blue, 40% sucrose, and 1 mg/ml of ethidium bromide. The DNA samples were subjected to electrophoresis on 2% agarose gel at 50 V for 2 h. After this electrophoresis, DNA fragmentation was visualized in a UV transilluminator.

Results

Isolation of compounds from the leaves of J. taxifolia

During the course of screening the antiproliferative activities of methanol extracts of various plants collected in Japan, the most potent activity on HL-60 cells was demonstrated by the extracts of leaves from J. taxifolia. The active compounds were separated by successively extracting the dried leaves of J. taxifolia with hexane, CHCl₃, acetone, and MeOH, and a marked antiproliferative activity at the concentration of 2.5 μg/ml was found in the CHCl₃ extract. As shown in Fig. 1, the CHCl₃ extract was further subjected to ether extraction, SiO₂ column chromatography, and reversed phase HPLC, or a combination of silica gel column chromatography and reversed phase HPLC, resulting in the isolation of the most active compound (4) and other five compounds (1–3, 5, 6). Their structures were elucidated as communic acid (1), sandaracopimaric acid (2), (15S)-15-hydroxylabd-8(17)-en-19-oic acid (3), deoxypodophyllotoxin (4), 14α-hydroxyisopimaric acid (=calliphyllin) (5), and 7α-hydroxysandaracopimaric acid (6) on the basis of spectral analyses (¹H, ¹³C-NMR, EI-MS, ¹H- and ¹³C-NMR, together with 2D NMR (COSY, NOESY, HMGC, and HMBC experiments)). Their structures were confirmed by comparison with reported spectroscopic data, their chemical structures being shown in Fig. 2.
Antiproliferative and apoptosis-inducing activities of the compounds isolated from *J. taxifolia*

The antiproliferative activity of each of these compounds toward HL-60 cells was evaluated by a WST-1 assay. Among them, 4 exhibited remarkably potent cytotoxicity toward HL-60 cells. As shown in Fig. 3A, this compound exerted cytotoxicity even at the low concentration of 2 ng/ml (5 nM). In addition, 6 also showed relatively potent cytotoxicity at concentrations of more than 2.5 μg/ml (7.86 μM), whereas the other four diterpene compounds isolated in this study had no cytotoxicity at concentrations of less than 10 μg/ml (Fig. 3B).

To characterize the cell death induced by 4 and 6, DNA fragmentation was examined by an agarose gel electrophoresis. As shown in Fig. 4, the amounts of fragmented DNA gradually increased in a concentration-dependent manner, and a ladder pattern of internucleo-
somal DNA fragmentation was apparent when the cells were treated with 4 at more than 1 ng/ml (2.5 nM) and with 6 at more than 2.5 mg/ml (7.86 μM). Differentiation-inducing activity of the compounds isolated from J. taxifolia

To verify the differentiation-inducing activity of the six compounds isolated from the leaves of J. taxifolia, an NBT reduction assay was employed to assess the functional differentiation of HL-60 cells. Among them, only 6 was found to induce differentiation at the concentration of 2 μg/ml (6.29 μM), whereas the other diterpenes (1–3 and 5) and 4 showed no effect at 5 μg/ml and 2 ng/ml (5 nM), respectively. 6 induced differentiation in HL-60 cells at 0.125–2 μg/ml (0.39–6.29 μM) in a dose-dependent manner, and the NBT-reducing activity of HL-60 cells treated with 1–2 μg/ml (3.15–6.29 μM) of 6 was comparable to that treated with 1 μM ATRA (Fig. 5). In addition, differentiation-inducing activity at the concentration of 2 μg/ml (7.86 μM) was demonstrated to increase with the time course of 1–4 d (Fig. 6). This profile was similar to that of ATRA.24)

In another experiment, we examined whether 6 must be present throughout the entire culture period of 4 d to exert its effect. Cells were separately treated with 6 at times.
2 \mu g/ml for 1, 2, 3, or 4 d, and after changing to a fresh medium, each culture was continued until a total of 4 d had elapsed. Cells treated for 1 d with \( 6 \) and then 3 d without \( 6 \) showed almost the same NBT-reducing activity as those treated for the full 4 d with \( 6 \) (data not shown). This indicates that \( 6 \) triggered signal transduction for differentiation within 24 h of the treatment and then required additional culture for more than 1 d to permit complete differentiation at 4 d. This differentiation was also confirmed by morphological changes in HL-60 cells, which were examined by Wright-Giemsa staining (data not shown).

**Discussion**

Many anticancer drugs including etoposide\(^{25}\) have been reported not only to cause cell death but also to induce apoptosis and/or differentiation to various types of tumor cells. \( 4 \) was first isolated as a highly cytotoxic compound from the extract of *J. taxifolia*. \( 4 \) has been reported as a cytotoxic and antineoplastic component from *Pulsatilla koreana*\(^{26}\) and is a deoxy form of podophyllotoxin, which is industrially used for the production of etoposide, a potent anticancer drug.\(^{25}\) This compound possessed 400–500 times more potent cytotoxicity toward HL-60 cells than etoposide. Although \( 4 \) induced apoptosis toward HL-60 cells, even in a concentration as low as 2.5 nM, it did not induce differentiation at around the same concentration. This indicates that \( 4 \) and its related compounds exhibited antitumor activity through cytotoxicity and apoptosis induction.

On the contrary, \( 6 \) was isolated as a moderate antiproliferative compound from an extract of *J. taxifolia*. \( 6 \) had been previously isolated from *Juniperus communis*\(^{23}\) and *Salvia* species,\(^{27}\) but its differentiation-inducing activity has not been reported until now. In this study, only \( 6 \) was demonstrated to have the ability to induce the differentiation of HL-60 cells, whereas such other structurally related diterpenes as \( 2 \) and \( 5 \) showed no activity at the various concentrations tested. When pimaric acid obtained as an authentic compound was also tested for its differentiation-inducing activity, this compound obviously induced differentiation of HL-60 cells at concentrations of 0.125–2 \mu g/ml (0.41–6.62 \mu M), and the NBT-reducing activity after the treatment with pimaric acid was the same as that after a treatment with \( 6 \) (unpublished results). The action mechanism, the structural characteristics of the active compounds, \( 6 \) and pimaric acid, and the structure-activity relationship...
among pimaric and isopimaric acids-related compounds are not yet apparent, and further studies are needed to resolve these aspects.

Several leukemia diseases are characterized by a breakdown of myeloid cell maturation. To restore normal cell progression, differentiation therapy using differentiation-inducing agents like ATRA is expected to be a novel medical procedure. However, remission cannot be achieved in all cases, and therapy often has side effects such as drug resistance and hypercalcemia. Other approaches for the development of differentiation therapy are therefore in progress. It has been demonstrated that the combination of established differentiation inducers can exert an additive or synergistic effect on the differentiation of HL-60 cells, and might reduce adverse effects. These combinations include DMSO and 1,25-(OH)_{2}D_{3}, dithizone and 1,25-(OH)_{2}D_{3}, yomogin and 1,25-(OH)_{2}D_{3}, etoposide and 1,25-(OH)_{2}D_{3}, TPA and ATRA, and capsaicin and 1,25-(OH)_{2}D_{3}. It seems therefore worthwhile to search for additional promising agents and to clarify that pimaric acid may be candidates of differentiation therapy for some types of leukemia diseases.

In conclusion, the present study has demonstrated an antitumor effect on HL-60 cells of 7α-hydroxysandaracopimaric acid isolated from the leaves of *Juniperus taxifolia*. This compound induced cellular differentiation in a concentration range of 0.39–6.29 μM and may also cause apoptotic cell death at the higher concentrations. These results may provide a basis for the potential therapeutic application of 7α-hydroxysandaracopimaric acid and its related compounds to cancer therapy.

Acknowledgments

We are grateful to Ogasawara branch of the Tokyo Metropolitan Government in Japan for permission to collect the plant materials.

References

21) Ikeda, R., Nagao, T., Okabe, Y., Nakano, Y., Matsunaga,


