Taraxinic Acid, a Hydrolysate of Sesquiterpene Lactone Glycoside from the *Taraxacum coreanum* NAKAI, Induces the Differentiation of Human Acute Promyelocytic Leukemia HL-60 Cells

Jung-Hye CHOI,^a Kyung-Min SHIN,^a Na-Young KIM,^a Jung-Pyo HONG,^b Yong Sup LEE,^c Hyoungh Ja KIM,^c Hee-Juhn PARK,^d and Kyung-Tae LEE*,^a

^a College of Pharmacy, Kyung-Hee University; and ^b Dentistry, Kyung-Hee University; Dongdaemun-ku, Hoegi-Dong, Seoul 130–701, Korea; ^c Division of Life Sciences, Korea Institute of Science and Technology; P.O. Box 131, Cheongryang, Seoul 130–650, Korea; and ^d Division of Applied Plant Sciences, Sang-Ji University; Woosan-Dong, Wonju 220–702, Korea. Received June 11, 2002; accepted July 13, 2002

The present work was performed to elucidate the active moiety of a sesquiterpene lactone, taraxinic acid-1'-*O*- β -D-glucopyranoside (**1**) from *Taraxacum coreanum* NAKAI on the cytotoxicity of various cancer cells. Based on enzymatic hydrolysis and MTT assay, the active moiety should be attributed to the aglycone taraxinic acid (**1a**), rather than the glycoside (**1**). Taraxinic acid exhibited potent antiproliferative activity against human leukemia-derived HL-60. In addition, this compound was found to be a potent inducer of HL-60 cell differentiation as assessed by a nitroblue tetrazolium reduction test, esterase activity assay, phagocytic activity assay, morphology change, and expression of CD14 and CD66b surface antigens. These results suggest that taraxinic acid induces the differentiation of human leukemia cells to monocyte/macrophage lineage. Moreover, the expression level of c-myc was down-regulated during taraxinic acid-dependent HL-60 cell differentiation, whereas p21^{CIP1} and p27^{KIP1} were up-regulated. Taken together, our results suggest that taraxinic acid may have potential as a therapeutic agent in human leukemia.

Key words taraxinic acid; differentiation; c-myc; p21^{CIP1}; p27^{KIP1}; HL-60 cell

The imbalance between cell proliferation, apoptosis and differentiation leads to the development of clones of malignant cells. Based on the understanding of tumor biology in respect to the kinetics of cell populations, two new strategies, the induction of differentiation and apoptosis, have recently emerged in the fields of cancer chemoprevention and chemotherapy. Differentiation from malignant or premalignant cells into more mature or normal-like cells, as well as apoptosis in multi-step carcinogenesis, are theoretically amenable to preventive cancer intervention. Thus, a compound with differentiation effects could be a candidate for the prevention and/or treatment of cancer.^{1,2)}

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, provides a unique *in vitro* model for studying the cellular and molecular events involved in the differentiation process. Moreover, a recent approach in the treatment of leukemia includes the use of differentiation-inducing agents such as interferon,³⁾ retinoids,⁴⁾ and 1 α ,25-dihydroxyvitamin D₃.^{5,6)} Based on this, the strategy of HL-60 cell differentiation has been accepted as a valid model in detecting or screening for potential cancer chemopreventive and/or chemotherapeutic agents in preclinical evaluation.

Thus, as a part of our screening program to evaluate the chemopreventive potential effect of natural compounds, we have investigated the effect of taraxinic acid-1'-*O*- β -D-glucopyranoside isolated from *Taraxacum coreanum* NAKAI and its hydrolysate, taraxinic acid, on HL-60 growth. The whole plant of *Taraxacum coreanum* NAKAI has long been used for medicinal purposes due to its diuretic and anti-inflammatory activities.⁷⁾ Taraxinic acid, the enzymatic hydrolysate of taraxinic acid-1'-*O*- β -D-glucopyranoside (Fig. 1) isolated from *Taraxacum coreanum* NAKAI, showed significant cytotoxicity, supporting that the sesquiterpene lactone is a generally active moiety. The cytotoxic effect of taraxinic acid was

assessed by its differentiation-inducing activity against HL-60 cells by various biochemical examinations in the present study. Furthermore, we demonstrated that the expression of p21^{CIP1} and p27^{KIP1} cyclin dependent kinase inhibitor (CDKI) were increased during 48 and 72 h, whereas c-myc oncogene expression was down-regulated during the taraxinic acid-induced differentiation of HL-60 cells. This is the first report showing the mechanism of the anti-cancer effect of taraxinic acid, which may be a candidate as a differentiation-inducing cancer chemopreventive agent.

MATERIALS AND METHODS

Materials P388 mouse leukemia, L-1210 mouse leukemia, SNU-C5 human colon cancer, HL-60 human promyelocytic leukemia, U-937 human histocytic lymphoma and HepG2 human hepatoma cell lines were obtained from the Korean Cell Line Bank (KCLB). RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY, U.S.A.). β -Glycosidase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), nitrobluetetrazolium (NBT), 1 α ,25(OH)₂D₃, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), α -naphthyl acetate esterase kit and 3-hydroxy-2-

Fig. 1. Chemical Structure of Taraxinic Acid-1'-*O*- β -D-glucopyranoside (**1**) and Taraxinic Acid (**1a**)

* To whom correspondence should be addressed. e-mail: ktleee@khu.ac.kr

naphthoic acid *o*-toluidine (naphthol AS-D chloroacetate) esterase kit were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CD14 and CD66b were obtained from Pharmingen (San Diego, CA, U.S.A.). Anti-human c-myc, p21^{CIP1} and p27^{KIP1} mouse monoclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Plant Material The whole plant of *Taraxacum coreanum* NAKAI was collected at Namyangju, Kyonggi, Korea, in May 2000. Voucher specimens (576-1F) have been deposited in the Natural Products Chemistry Laboratory at the Korea Institute of Science & Technology (KIST).

Extraction, Fractionation and Identification The dried whole plant of *Taraxacum coreanum* (850 g) was finely cut and extracted three times with MeOH at room temperature to afford a dark-brown residue by the removal of solvent under reduced pressure. The methanol extract was suspended in water and then successively partitioned with CH₂Cl₂, EtOAc, and *n*-BuOH, respectively. The EtOAc extract was evaporated under reduced pressure to yield 6.0 g of a residue. This residue was divided into four fractions (Fr. E1–E4) by column chromatography on silica gel using the following solvent mixtures: CH₂Cl₂:MeOH:H₂O (100:10:0.5→40:10:1→70:30:3). Fraction E2 (109.0 mg) was purified by Sephadex LH-20, eluted with MeOH and further purified by silica gel column elution with CH₂Cl₂:MeOH:H₂O (100:20:1.5) to give compound **1**.

Taraxinic Acid-1'-*O*- β -D-glucopyranoside (**1**): mp 85–90 °C; IR ν_{max} (KBr) cm⁻¹: 3400 (OH), 1755 (lactone C=O), 1725 (C=O), 1630 (C=C) cm⁻¹; the spectral data were identical with those reported in the literature.⁸⁾

Enzyme Hydrolysis of (1) β -Glycosidase (3.4 mg) was added to a solution of sesquiterpene lactone (**1**, 36.4 mg) in 5 ml of NaOAc buffer (pH 5.2), and the solution was left at 37 °C for 2 d. The precipitate was collected by filtration and extracted with CHCl₃. The combined organic solvent was evaporated to dryness and the residue was purified by preparative TLC coated with silica gel to give taraxinic acid (**1a**).

Cell Culture P-388, L-1210, U-937, HL-60, SNU-C5 and HepG2 cell lines were grown at 37 °C in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μ g/ml) in a humidified atmosphere of 95% air 5% CO₂, and were then seeded in plates after 3 passages.

MTT assay for Cell Viability The *in vitro* tests against P-388, L-1210, SNU-C4, HL-60, MA-104 and U-937 cells were performed essentially according to the method described previously.⁹⁾ Cells (2×10^5) were seeded in each well containing 100 μ l of RPMI medium supplemented with 10% FBS in a 96-well microtiter plate and incubated overnight. Compounds **1** and **1a** were dissolved in dimethylsulfoxide (DMSO), and the DMSO concentrations in all assays did not exceed 0.1%. Twenty-four hours after seeding, 100 μ l new media or compound **1** or **1a** was added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μ l FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μ l DMSO. The optical density was measured at 540 nm. The concentration required to reduce absorbance by 50% (IC₅₀) in comparison to control cells was determined.⁹⁾

Determination of Growth Inhibition HL-60 cells were

grown at 37 °C in RPMI medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μ g/ml) in a humidified atmosphere of 5% CO₂. Cells were seeded at a concentration of 2×10^5 cells/ml, maintained for logarithmic growth by passaging them every 2–3 d, and incubated for 1–4 d with taraxinic acid at various concentrations. Taraxinic acid dissolved in DMSO was added to the medium in serial dilution (the final DMSO concentration in all assays did not exceed 0.1%). Cell viability was checked by the trypan blue exclusion method.⁹⁾

Differentiation Assay (1) NBT Reduction Test: The percentage of HL-60 cells capable of reducing NBT was determined by counting the number of cells which contained the precipitated formazan particles after cells had been incubated with NBT (1.0 mg/ml) at 37 °C for 30 min. TPA was used as a stimulator for the formation of formazan.

(2) Phagocytosis Test: HL-60 cells (1×10^6 cells/ml) were suspended in serum-free RPMI 1640 medium containing 0.2% latex particles (average diameter, 0.81 μ m) and incubated at 37 °C for 4 h. After incubation, the cells were washed once with phosphate-buffered saline (PBS). Cells containing more than 10 latex particles were scored as phagocytic cells.⁹⁾

(3) Esterase Activity Test: A smear preparation was chemically stained for α -naphthyl acetate esterase and naphthol AS-D chloroacetate esterase by the standard techniques.¹⁰⁾

(4) Flow Cytometry: HL-60 cells (2×10^5 cells/ml) exposed to taraxinic acid were collected and washed twice with ice-cold PBS. Cells were then incubated with direct FITC-labeled anti-CD 14 or anti-CD-66b antibody on ice for 30 min, washed twice with PBS, and antibody binding to cells was quantified using FACS flow cytometry (Becton Dickinson Co., Germany).

Western Blot Analysis Cellular proteins were extracted from control and taraxinic acid-treated HL-60 cells. The washed cell pellets were resuspended in ELB buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μ g/ml each of leupeptin and aprotinin, and incubated for 15 min at 4 °C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration was determined by Bio-Rad (CA, U.S.A.) protein assay reagent (CA, U.S.A.), as described by the manufacturer. Cellular proteins (50 μ g) from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk in PBS containing 0.02% Tween 20) at 4 °C, and then incubated for 4 h with a 1:1000 dilution of monoclonal anti-c-myc, p27^{KIP1} or p21^{CIP1} antibody. Blots were washed 2 times with PBS, and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed again three times with PBS, and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RESULTS

Cell Growth Inhibition We initiated our study by exam-

ining the cytotoxicities of taraxinic acid-1'-*O*- β -D-glucopyranoside and its aglycon, taraxinic acid, using MTT assay on various cancer cells. Taraxinic acid showed significant cytotoxicity, with IC_{50} at the concentration of 34.5–135.9 μ M, whereas its glycoside showed no effect up to 200 μ M (Table I). These results indicated that the active moiety of **1a** should be the aglycone taraxinic acid, rather than **1** with the gross structure.

The effect of taraxinic acid on the proliferation of HL-60 cells was examined first. The cell growth of HL-60 cells was inhibited in a concentration- and time-dependent manner (Fig. 2), suggesting that this chemical has an antiproliferative activity. The inhibitory effect became apparent at a concentration of 15 and 30 μ M taraxinic acid, and no cytotoxic effects were observed under the condition. Thus, these concen-

Table 1. Cytotoxic Activity of Taraxinic Acid and Taraxinic Acid-1'-*O*- β -D-glucopyranoside on Cancer Cell Growth *in Vitro*

Cell line	IC_{50}^a (μ M)		Cisplatin
	Taraxinic acid	Taraxinic acid-1'- <i>O</i> - β -D-glucopyranoside	
P-388	67.5	>200	23.1
L-1210	59.4	>200	15.4
U-937	46.5	>200	22.0
HL-60	34.5	>200	17.7
SNU-C5	115.8	>200	17.4
HepG2	135.9	>200	54.6

a) IC_{50} is defined as the concentration which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor. The values represent the mean of three independent experiments.

Fig. 2. The Effect of Taraxinic Acid on the Growth of HL-60 Cells

HL-60 cells were treated with an increasing concentration of taraxinic acid (■, control; ●, 15 μ M; ◆, 30 μ M; ▲, 60 μ M) for 4 d, and cell viability was determined by the trypan blue exclusion method. Data represent the mean and S.D. of three independent experiments.

trations were used throughout the present study.

Effect on Differentiation of HL-60 Cells After 4 d treatment, the effect of taraxinic acid on HL-60 cell differentiation was compared with that of $1\alpha,25(OH)_2D_3$, and the results are summarized in Table 1. When HL-60 cells were incubated with taraxinic acid at a concentration of 15 and 30 μ M for 4 d, approximately 28.9% and 79.7% of HL-60 cells were stained with NBT, respectively, whereas only 13.2% of the untreated cells were positive (Table 2). $1\alpha,25(OH)_2D_3$ (20 nM) gave 58.4% of NBT-reducible cells. In order to test whether taraxinic acid induces HL-60 cells to differentiate into monocyte/macrophage or granulocyte, the esterase activity was measured under identical conditions. Treatment of HL-60 cells with 15 and 30 μ M of taraxinic acid for 4 d resulted in 39.6% and 75.1% increases in α -naphthyl acetate esterase activity, respectively, but the effect of taraxinic acid on the naphthyl AS-D chloroacetate esterase activity was relatively small. Moreover, cells treated with this compound showed apparent phagocytic activity (Table 2). In addition, as shown in Fig. 3, 15 to 30 μ M taraxinic acid significantly increased the expression of membrane antigen CD14, whereas it did not show any influence on the expression of CD66b. These results indicated that taraxinic acid induced HL-60 cells to undergo monocyte/macrophage differentiation.

The Expression Level of c-myc, p27^{KIP1} and p21^{CIP1} during the Taraxinic Acid-Induced Differentiation of HL-60 Cells To assess the altered expression of the characteristic cellular growth-related proteins of HL-60 cells by taraxinic acid, we examined the expression level of c-myc, p21^{CIP1} and p27^{KIP1} using immunoblot analysis over a time frame of 0 to 4 d (Fig. 4). When HL-60 cells were incubated with taraxinic acid, the level of CDK inhibitor p21^{CIP1} became detectable after 1 d and continued to increase about 7-fold at 3 d, then decreased toward a baseline level after 4 d. The related CDK inhibitor p27^{KIP1} was also increased during 2–3 d, albeit with different kinetics; the induction of p27^{KIP1} protein level was initiated after 2 d. In contrast, c-myc protein levels decreased dramatically, about 60%, after 1 d incubation, and no protein level was detected after 2 d.

DISCUSSION

Currently, programs for the identification and testing of potential chemopreventive agents have been developed.⁽¹⁾ The preclinical laboratory work of these programs is designed to systemically test the efficacy and toxicity of chemopreventive agents in both *in vivo* and *in vitro* models.^(1,2) Among them, the *in vitro* model can be divided into

Table 2. Induction of Differentiation Markers in HL-60 Cells after Treatment with Taraxinic Acid for 4 d

Compound	Concentration (μ M)	NBT reduction (%)	Naphthyl AS-D chloroacetate esterase activity (%)	α -Naphthyl acetate esterase activity (%)	Phagocytosis (%)
Vehicle	—	13.2 $\pm$ 0.8	12.0 $\pm$ 0.8	11.0 $\pm$ 0.9	2.7 $\pm$ 0.5
Taraxinic acid	15	28.9 $\pm$ 1.1*	13.8 $\pm$ 0.9	39.6 $\pm$ 2.8*	22.4 $\pm$ 1.0*
	30	79.7 $\pm$ 2.5*	14.9 $\pm$ 1.2	75.1 $\pm$ 5.3*	36.9 $\pm$ 1.8*
Vitamin D ₃	0.02	58.4 $\pm$ 5.4*	10.5 $\pm$ 3.2	49.6 $\pm$ 4.8*	40.4 $\pm$ 3.5*

ta indicate the percentage of cells which were stainable with NBT, showed positive esterase activities or took up more than 10 latex particles, and express the mean $\pm$ S.D. of 3 experiments. * p <0.01, significantly different from the vehicle control, using Student's *t*-test.

Fig. 3. FACS Analysis of Expression of CD66b and CD14 Antigens in HL-60 Cells by Taraxinic Acid

HL-60 cells (2×10^5 cells/ml) exposed to various concentration of taraxinic acid for 4 d were collected and washed twice with ice-cold PBS. Cells were then incubated with direct immunofluorescent staining using fluorescein-isothiocyanate conjugated mouse antihuman CD66b (Pharmingen) or CD14 (Pharmingen) antibody on ice for 30 min, washed twice with PBS, and antibody binding to cells was quantified using FACS flow cytometry.

three categories: inhibition of cell proliferation, blockage of transformation, and induction of differentiation. Many new agents that can selectively induce the differentiation of pre-malignant and malignant cells into more mature cells have been identified in *in vitro* and *in vivo* models. The present study has demonstrated that taraxinic acid exerts a potent differentiation-inducing activity on promyelocytic leukemia HL-60 cells. This effect of taraxinic acid was confirmed with a NBT reduction test, esterase activity assay, phagocytosis and expression of cell surface antigens. Furthermore, p21^{CIP1} and p27^{KIP1} expression was up-regulated by taraxinic acid, whereas c-myc proteins were down-regulated. Taken together with the effects of differentiation on HL-60 cells, our results suggest that taraxinic acid could be used as a potent chemoprevention agent in HL-60 human promyelocytic leukemia cells. However, it remains unclear whether taraxinic acid effectively induces the elimination of promalignant or malignant cells *via* differentiation *in vivo*.

Cell differentiation is regulated in a cell cycle-dependent manner. For example, the differentiation of hematopoietic

Fig. 4. Time-dependent Analysis of p21^{CIP1}, p27^{KIP1} and c-Myc Protein during Taraxinic Acid-Induced Differentiation of HL-60 Cells

HL-60 cells were incubated at 37 °C with 30 μM taraxinic acid for the indicated periods. Total protein (50 μg/lane) after lysis was subjected to electrophoresis on 12% SDS-PAGE gel and blotted with human p27^{KIP1}, p21^{CIP1} and c-myc antibody. Similar results were obtained from three separate experiments.

cells is associated with a loss of cell cycling capacity, and the cells become arrested in the G0/G1 phase of the cell cycle.¹²⁾ Although the mechanism of differentiation induction by taraxinic acid is not clear, it appears that p21^{CIP1} and p27^{KIP1} play an important role in modulation of the cell differentiation activity of the compound. Inhibition of the G1/S transition induces growth arrest and monocyte differentiation of both HL-60 and U937 cells, which is mediated by a block of cell cycle progression at the G1 phase.^{13,14)} The p21^{CIP1} protein is a cyclin-dependent kinase (CDK) inhibitor that is one of the regulators of cell cycle progression in the G1/S transition. Exposure of HL-60 cells to differentiation-inducing agents such as 1,25(OH)₂D₃ and TPA causes transient overexpression of p21^{CIP1}.¹⁴⁾ Another report has suggested that p27 protein is also a strong candidate as a cell cycle regulator that blocks the entry into the S phase of 1,25(OH)₂D₃-treated HL-60 cells.¹⁵⁾ Collectively, these findings indicate that p21^{CIP1} and p27^{KIP1} induction occurs through a G1 arrest mechanism, and they raise the possibility that differentiation-associated stimuli may be closely associated with the terminal differentiation of myeloid leukemic cells. In this regard, it is noteworthy that p21^{CIP1} and p27^{KIP1} protein levels continued to increase, peaking at 2–3 d and declining by 4 d of taraxinic acid treatment.

The c-myc gene product is a nuclear protein and has been implicated in the control of normal cell growth as well as transformation, but its exact function is unknown. A decrease in c-myc mRNA has been demonstrated *in vitro* during

chemically induced differentiation of various cell lines.¹⁶⁾ This decline was gradual or even biphasic, as seen during the differentiation of the leukemia cell lines.¹⁷⁾ The inverse relationship between p21^{CIP1} and p27^{KIP1} and c-myc expression after a lower concentration of taraxinic acid treatment was anticipated, given the established role of c-myc in S-phase progression,¹⁸⁾ as well as in promotion of apoptosis.¹⁹⁾ This finding indicates a cooperative interaction between c-myc down-regulation and p21^{CIP1} induction in the maturation process. Such a notion is consistent with the results of a recent report demonstrating that c-myc can act as a negative regulator of p21^{CIP1} expression.²⁰⁾

In summary, our results suggest that the induction of HL-60 cell maturation by taraxinic acid may have potential as a therapeutic approach for the treatment of leukemia, although the doses are probably high for chemoprevention. Furthermore, taraxinic acid-induced differentiation accompanying the increase of p21^{CIP1} and p27^{KIP1} and decrease of c-myc expression suggest that the targeting of p21^{CIP1} and p27^{KIP1} and c-myc regulation may have importance in the treatment of cancer.

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