Differentiation-Inducing Effect of Magnolialide, a 1 β -Hydroxyeudesmanolide Isolated from *Cichorium intybus*, on Human Leukemia Cells

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Cichorium intybus contains two 1 β -hydroxyeudesmanolides, magnolialide and artesin, together with several constituents. Magnolialide inhibits the growth of several tumor cell lines and appears to induce differentiation of human leukemia HL-60 and U-937 cells to monocyte/macrophage-like cells. Another 1 β -hydroxyeudesmanolide, artesin, and other constituents were inactive. The content of magnolialide was shown to be highest in the leaves of Inje cultivar among the cultivars investigated in this study.

Key words Magnolialide; 1 β -hydroxyeudesmanolide; growth-inhibitory activity; differentiation; leukemia cells; *Cichorium intybus*

The processed roots of *Cichorium intybus* (Chicory) have been used as a substitute for coffee in European countries. This herbaceous plant was not native to Korea, but was introduced from abroad and is now cultivated in alpine areas of Korea. It has been reported that crude extracts of this plant exhibit some biological effects, such as anti-inflammatory and anti-hepatotoxic activity, perhaps due to their free radical scavenging activity.^{1–3} On the other hand, various kinds of triterpenoids, such as triterpene alcohols, triterpene acetates and triterpene esters, and phytosterols have been isolated from this plant,⁴ and some sesquiterpene lactones had been reported to have a cytotoxic effect on L-5178 leukemia cells.⁵ In this study, we tried to isolate the cytotoxic principles from a number of varieties of *Cichorium* spp. in Korea, and found that a 1 β -hydroxyeudesmanolide, magnolialide,⁶ exhibited growth-inhibitory activity without cell death and induced differentiation of human leukemia cells to monocyte/macrophage-like cells.

MATERIALS AND METHODS

Plant Material The roots of *Cichorium intybus* were collected in September, at the National Alpine Agricultural Experiment Station, RDA, Pyongchang, Korea, and the plant was identified by Dr. K. O. Yoo (National Alpine Agricultural Experiment Station of Korea). A voucher specimen was deposited there. Plant material for quantitative analysis of the cytotoxic principles was collected in several areas of Kangwon Province.

Extraction and Isolation Dried roots (1.5 kg) of *Cichorium intybus* were pulverized and extracted three times with MeOH under reflux. The MeOH extract was filtered and evaporated under reduced pressure to give a viscous residue (390 g). This material was suspended in H₂O and then partitioned with CHCl₃, EtOAc and *n*-BuOH to give CHCl₃ (245 g), EtOAc (46 g) and *n*-BuOH (30 g) extracts, respectively. Part of the CHCl₃ extract (20 g) was subjected to column chromatography on silicagel (Merck, Art No. 7734).

The column was eluted with a gradient of *n*-hexane–EtOAc (10:1→10:3) to give 8 separate fractions. Fraction 1 (37 mg) was found to be a mixture of palmitates of germanicol and taraxasterol, fraction 2 (95 mg) was a mixture of the acetates of germanicol and taraxasterol, and fraction 4 (83 mg) was a mixture of germanicol and taraxasterol following GC-MS analysis and comparison with authentic samples. Fraction 5 was found to consist of β -sitosterol, campesterol and stigmasterol by NMR and GC-MS analyses. Fraction 8 was also found to be a mixture of oleanolic and ursolic acids by co-TLC, HPLC and NMR analysis.^{7,8} Following gas liquid chromatography, fraction 7 (60 mg) was found to contain two major components with retention times at 5.18 min and 5.41 min [column: OV-1 (15 m×0.53 mm×2.65 μ m capillary; oven temp. 40 °C (4 min, 10 °C/min)—220 °C (final temp.); injection temp. 300 °C; detection temp. 300 °C; carrier: 20 ml/min (helium)]. In addition, magnolialide⁶ (15 mg) and artesin⁹ (26 mg) were isolated by reversed phase column chromatography [YMC gel ODS-A, 5×50 cm (YMC), elution: MeOH/H₂O (2:5, v/v)] and by preparative HPLC [CAPCELL PAK C-18, 4×250 μ m (Shiseido), elution: MeOH/H₂O (2:3, v/v)] and their chemical structures were confirmed by IR, MS, ¹H-NMR and ¹³C-NMR (Fig. 1).

In order to determine the content of the cytotoxic component, magnolialide, in several cultivars, samples were prepared from the leaves and roots. One gram of each plant part

Fig. 1. Structures of Magnolialide and Artesin

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was extracted with acetone (100 ml) by sonicating at 40 °C and then filtered. The extract was subjected to GC analysis and the magnolialide content was determined by comparing the peak area ratio of the sample with the authentic compound.

Growth-Inhibition Assay *In vitro* cytotoxicity testing against P-388 mouse lymphoma, L-1210 mouse lymphoma leukemia, U-937 human histiocytic lymphoma, HL-60 human promyelocytic leukemia, HepG2 human hepatoma and SNU-C5 human colon cancer cells was performed essentially according to the method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) described previously.¹⁰⁾ Cells (1×10^4) were seeded in wells containing 100 μ l RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a 96-well microtiter plate and incubated overnight. Test samples were dissolved in dimethylsulfoxide (DMSO) and were added in serial dilutions (the final DMSO concentrations in all assays did not exceed 0.1%). Twenty-four hours after seeding, 100 μ l sample solution was added, and the plates were incubated for 48 h at 37 °C. Cells were washed once before adding 50 μ l FBS-free medium containing 5 mg/ml MTT (Sigma Chemical Co., St. Louis, MO, U.S.A.). After a 4 h incubation at 37 °C, the medium was discarded and the formazan blue in the cells was dissolved in 50 μ l DMSO. The optical density was measured at 540 nm. The cell viability was also determined by the Trypan blue dye exclusion method.

Differentiation Assay (1) Nitroblue-tetrazolium (NBT) reduction testing¹¹⁾: Cells were incubated with 1.0 mg/ml NBT (Sigma) for 30 min at 37 °C, and the percentage of cells capable of reducing NBT was determined by counting the number of cells which contained precipitated formazan particles. 12-*O*-Tetradecanoylphorbol-13-acetate (Sigma) was used as a formazan stimulator. (2) Esterase activity testing: A smear preparation was chemically stained for α -naphthyl ac-

etate esterase¹²⁾ and 3-hydroxy-2-naphthoic acid *O*-toluidine (AS-D chloroacetate) esterase¹³⁾ by standard techniques.

RESULTS AND DISCUSSION

Cichorium intybus contained two 1 β -hydroxyeudesmanolides, magnolialide and artesin, together with several other constituents. In the MTT and Trypan blue dye exclusion assays against various tumor cell lines, only magnolialide inhibited cell growth but did not produce cell death (Table 1), while other compounds including artesin isolated from the roots of *Cichorium intybus* were all inactive. This suggests that the α -methylene- γ -lactone moiety, but not the α -methyl- γ -lactone moiety, plays an important role in the growth-inhibitory activity of sesquiterpene lactone, as previously reported.^{14,15)} The growth inhibition of tumor cells by magnolialide is supposed to be associated with terminal differentiation. This effect was verified by the NBT test used to evaluate the functional differentiation of either granulocytes or monocytes on U-937 and HL-60 cells. When these cells were incubated with magnolialide at concentrations of 3.5 and 7.0 μ g/ml for 4 d, approximately 25% and 50.8% of U-937 cells and 33.3% and 65.8% of HL-60 cells became stainable with NBT, respectively (Table 2). In order to confirm whether magnolialide induces U-937 and HL-60 cells to differentiate toward macrophage and granulocyte, the esterase activity was measured. When U-937 and HL-60 cells were treated with magnolialide, the α -naphthylacetate esterase activity in these cells markedly increased, but the AS-D chloroacetate esterase activity increased only slightly and this was also true for 1 α ,25-(OH)₂D₃ (5 ng/ml). Figure 2 shows that HL-60 cells differentiated to monocyte-like cells after treatment with magnolialide and 1 α ,25-(OH)₂D₃.

Finally, we examined the magnolialide content in Korean *Cichorium* cultivar. Generally, the content of magnolialide tended to be higher in the leaf than in the root of the plant and was highest in the leaf of the Inje cultivar, but was almost zero in some cultivars (Table 3).

In conclusion, only magnolialide, among the constituents of *C. intybus* inhibited the growth of several tumor cell lines. In particular, this compound appeared to induce differentiation of leukemia cells to monocyte/macrophage-like cells, as did 1 α ,25-(OH)₂D₃.¹⁶⁾ Seto *et al.*⁵⁾ reported that various kinds of sesquiterpene lactones were responsible for cytotoxic activity and their structure-cytotoxicity relationships were discussed. In this study, we have shown that a 1 β -hydroxyeudesmanolide, magnolialide, has unique activity as a

Table 1. Growth-Inhibitory Activity of Magnolialide on Tumor Cells

Cell line	Origin	IC ₅₀ ^{a)} (μ g/ml)
P-388	Mouse lymphoma	22.1
L-1210	Mouse lymphatic leukemia	11.1
U-937	Human histiocytic lymphoma	9.6
HL-60	Human promyelocytic leukemia	7.2
HepG2	Human hepatoma	12.5
SNU-C5	Human colon cancer	10.4

a) IC₅₀ is defined as the concentration which resulted in a 50% decrease in cell numbers. The values represent the mean of three independent experiments.

Table 2. Induction of Differentiation Markers on U-937 and HL-60 Cells by Magnolialide and 1 α ,25-(OH)₂D₃

Cell	Compound	Concentration (μ g/ml)	NBT reduction (%)	Naphthol AS-D chloroacetate esterase activity (%)	α -Naphthyl acetate esterase activity (%)
HL-60	Magnolialide	7.0	43.5 $\pm$ 4.2 ^{a)}	16.4 $\pm$ 1.3	42.2 $\pm$ 2.5
		3.5	16.0 $\pm$ 0.9	9.2 $\pm$ 0.8	35.3 $\pm$ 1.4
	1 α ,25-(OH) ₂ D ₃	0.005	44.5 $\pm$ 2.4	6.6 $\pm$ 1.2	31.4 $\pm$ 3.3
		— ^{b)}	13.3 $\pm$ 1.3	6.9 $\pm$ 0.8	6.9 $\pm$ 0.2
U-937	Magnolialide	7.0	39.8 $\pm$ 5.5	16.4 $\pm$ 2.9	33.4 $\pm$ 2.9
		3.5	30.5 $\pm$ 3.3	9.2 $\pm$ 0.8	18.5 $\pm$ 1.4
	1 α ,25-(OH) ₂ D ₃	0.005	62.4 $\pm$ 4.6	12.4 $\pm$ 1.5	44.6 $\pm$ 5.5
		—	13.3 $\pm$ 2.1	6.9 $\pm$ 0.8	4.2 $\pm$ 0.8

a) Mean $\pm$ S.D. (n=3). b) Untreated.

Fig. 2. Morphology of HL-60 Cells

Cells were treated, with or without magnolialide or $1\alpha,25\text{-(OH)}_2\text{D}_3$, for 4 d, fixed and stained with May-Grunwald Giemsa, $\times 800$. a) un-treated control. b) treated with 5 ng/ml $1\alpha,25\text{-(OH)}_2\text{D}_3$. c) and d) treated with 3.5 $\mu\text{g/ml}$ and 7.0 $\mu\text{g/ml}$ magnolialide, respectively.

differentiation inducer. The content of magnolialide was highest in the leaf of the Inje cultivar among Korean *C. intybus* cultivars tested. It would appear that magnolialide is a potential new anti-leukemic agent likely to exhibit less adverse reactions and Korean *C. intybus* is an important source, for its production.

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Table 3. Contents of Magnolialide in Korean *Cichorium* Cultivars

Taxa	Content ($\mu\text{g/g}$)
<i>C. intybus</i> cv. Sonsino (leaf)	19.5
cv. Sonsino (root)	16.2
cv. Radice di Chiavary (leaf)	37.1
cv. Radice di Chiavary (root)	17.0
cultivar in Inje (leaf)	42.2
cultivar in Inje (root)	17.5
cv. Palla Rosa Precose (leaf)	—
cv. Palla Rosa Red (leaf)	—
cv. Variegata di Castelfranco Corma (leaf)	—
cv. Pan di Zucchero (leaf)	18.3
cv. Spadona sel. C. S. C (leaf)	—
cv. Catalogna Gigante sel. Sangria (leaf)	—
cv. Catalogna Franstagliata (leaf)	—
<i>C. endivia</i> cv. D'Inverno (leaf)	—

—, no magnolialide peak identified under the present experimental conditions.