Albanol A from the Root Bark of *Morus alba* L. Induces Apoptotic Cell Death in HL60 Human Leukemia Cell Line

Takashi Kikuchi,[a] Masatoshi Nihei,[a] Hisashi Nagai,[a] Hidekuni Fukushi,[a] Keiichi Tabata,[b] Takashi Suzuki,[b] and Toshihiro Akihisa*,[a]

[a] *College of Science and Technology, Nihon University; 1–8–14 Kanda Surugadai, Chiyoda-ku, Tokyo 101–8308, Japan: and* [b] *College of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan.*
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Albanol A (1), isolated from the root bark extract of *Morus alba* (mulberry), was evaluated for the cytotoxic and apoptosis-inducing activities in human leukemia (HL60) cells, and for the inhibitory activity in human DNA topoisomerases (Topo) I and II. This compound showed potent cytotoxic activity (IC_{50} 1.7 μM) on the cells, and potent inhibitory activity on topoisomerase II (IC_{50} 22.8 μM). In addition, albanol A induced early apoptosis which was detected by observing the membrane phospholipid exposure in flow cytometry. Western blot analysis showed that albanol A markedly reduced the levels of procaspases-3, 8, and 9, while being increased the levels of cleaved caspases-3, 8, and 9. The Bax/Bcl-2 ratio was significantly increased by albanol A treatment. Furthermore, albanol A induced caspase-2 activation. These results suggested that albanol A induces apoptotic cell death in HL60 *via* both the cell death receptor pathway by stimulation of death receptor, and the mitochondrial pathway by Topo II inhibition through caspase-2 activation. Therefore, albanol A may be a promising lead compound for developing an effective drug for treatment of leukemia.

Key words *Morus alba*; mulberry; albanol A; leukemia; cytotoxic activity; apoptosis inducing activity

Mulberry (*Morus* spp.; Moraceae), whose leaves represent indispensable food for silkworms, has been widely cultivated in China and Japan since early times. On the other hand, the root bark of mulberry (Mori Radicis Cortex) has been used in the traditional Chinese medicine as antiphlogistic, diuretic, antitussive, expectorant, antiheadache, and antipyretic.[1] Many novel compounds, regarded biogenetically as "Diels-Alder-type adducts" of dehydroprenyl phenols and chalcone derivatives, have been isolated from several *Morus* species.[2,3] Recently, albanol A[4] (mulberrofuran G[5]) (**1**) and several other related compounds, isolated from the stem bark of *M. australis*, have been reported to show potent cytotoxic activities against four human cancer cell lines, lung (A549), stomach (BGC 823), colon (HCT-8), and ovarian (A2780) cancer cell lines, by means of the thiazolyl blue tetrazolium bromide (MTT) assay.[3] In the course of our search for potential bioactive compounds from natural sources,[6,7] we were especially interested to undertake the investigation on the mechanisms of cytotoxic action of the "Diels-Alder-type adducts". In this study, we have isolated albanol A (**1**) and mulberofuran Q (**2**) from the root bark of *M. alba*, and have evaluated their cytotoxic activity against human leukemia (HL60) and human melanoma (CRL1579) cells. In addition, we have studied the apoptosis-inducing activity of albanol A (**1**), which exhibited potent cytotoxicity against HL60 cells, and the mechanisms of apoptotic cell death in HL60.

Results and Discussion

Albanol A (**1**) and mulberofuran Q (**2**) (Fig. 1) were isolated from the bark extract of *Morus alba* L. with ordinal- and reversed-phase column chromatography and subsequent preparative HPLC. Compounds **1**[5] and **2**[8] were identified by MS and ^{1}H-NMR comparison with the literature data. On evaluation of these compounds for their cytotoxicities on HL60 and CRL1579 cell lines, compound **1** exhibited potent activity against both HL60 (IC_{50} 1.7 μM) and CRL1579 (9.8 μM) cell lines which were almost comparable with those of a reference compound, cisplatin (Table 1).

Compound **1** was then evaluated for its inducing activity of early apoptosis on HL60 cells. Exposure of the membrane phospholipid, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death.[9] Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface. Propidium iodide (PI) does not enter whole cells with intact membranes and was used to differentiate between early apoptotic (Annexin V possitive, PI negative), late apoptotic (Annexin V, PI double positive), or necrotic cell death (Annexin V negative, PI positive). The proportion of early apoptotic HL60 cells (lower right) was significantly increased after 6 h of incubation with **1** (30 μM)

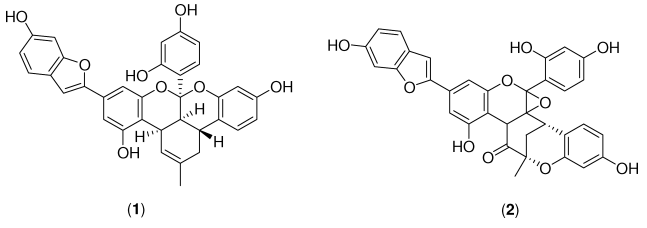

Fig. 1. Chemical Structures of Albanol A (**1**) and Mulberrofurane Q (**2**)

Table 1. Cytotoxic Activity of Compounds against Human Leukemia (HL60) and Human Melanoma (CRL1579) Cell Lines

Compound	IC_{50} (μM)[a]	
	HL60	CRL1579
1	1.7	9.8
2	37.6	83.9
Cisplatin[b]	1.9	21.1

Cells were treated with compounds (1×10^{-4}—1×10^{-6}) for 48 h, and cell viability was analyzed by the MTT assay. *a*) IC_{50} based on triplicate three points. *b*) Reference compound.

* To whom correspondence should be addressed. e-mail: akihisa@chem.cst.nihon-u.ac.jp

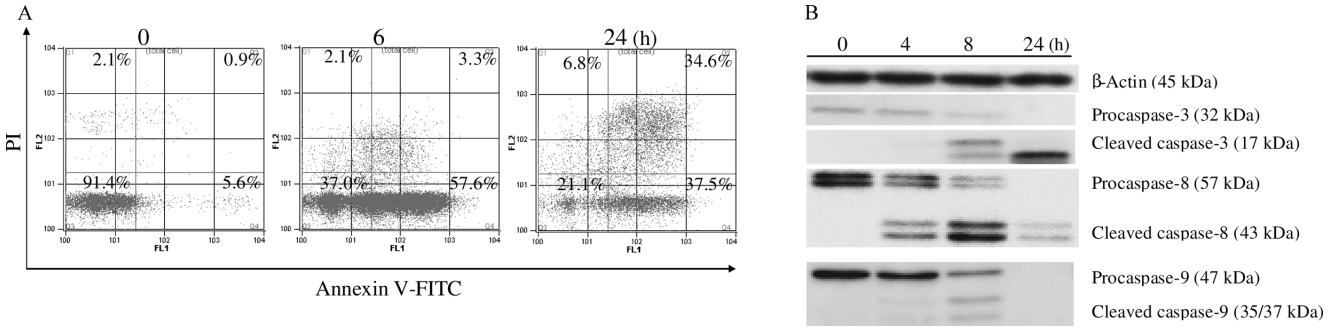

Fig. 2. Albanol A (**1**) Induced Apoptosis against HL60

The HL60 cells were treated with 30 μM albanol A for the indicated times. (A) Detection of early apoptotic cells by Annexin V–PI double staining. HL 60 cells were cultured with 30 μM albanol A for 6 h and 24 h. (B) Western blot analysis of caspases-3, 8, and 9 in HL60 cells treated with albanol A. The results are from one representative experiment among three runs which showed the similar patterns with one another.

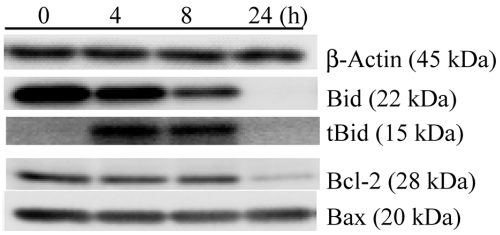

Fig. 3. Effect of Albanol A (**1**) on Bid, tBid, Bcl-2, and Bax Proteins in HL60

The HL60 cells were treated with 30 mM albanol A for the indicated times. The results are from one representative experiment among three runs which showed the similar patterns with one another.

(57.6% *vs.* 5.6%) compared with 0 h of incubation (Fig. 2A). After treatment with **1** for 24 h, early apoptotic cells decreased (37.5% *vs.* 57.6% for 6 h) and late apoptotic cells increased when compared with 6 h of incubation (34.6% *vs.* 3.3% for 6 h). In addition, a small number of necrotic cells also were detected. These results suggested that the most of cytotoxicity of **1** to HL60 cells is due to inducing apoptotic cell death.

Caspases are known to mediate the apoptotic pathway.[10,11] In order to clarify the mechanism by which compound **1** induces apoptotic cell death, activation of caspases-3, 8, and 9 was evaluated by Western blot analysis. After treatment of HL60 cells with **1** (30 μM), the level of procaspases-3, 8, and 9 gradually diminished in a time dependent manner, and cleaved caspases-3, 8, and 9 were detected (Fig. 2B). These results indicated that compound **1**-induced cell death occurs through activation of caspases-3, 8, and 9.

Next, we investigated the effect of compound **1** on Bax, Bcl-2, Bid, and truncated Bid (tBid). The proapoptotic proteins Bax and Bid, and the anti-apoptotic mitochondrial protein Bcl-2 are important regulators of cytochrome *c* release from mitochondria.[12,13] Expression of these proteins was examined by Western blot analysis. Treatment of compound **1** (30 μM) for 24 h in HL60 cells decreased the level of Bcl-2 whereas unchanged the level of Bax (Fig. 3). In addition, the level of Bid decreased in a time dependent manner, and tBid was detected after 4 and 8 h of treatment. It was reported that caspase-8 could truncate Bid, and tBid could activate the mitochondrial pathway.[14] The Bax/Bcl-2 ratio is one of the indices of the intrinsic mechanism of apoptosis in mitochondria. Since compound **1** increased this ratio in HL60 cells, it seems that compound **1**-induced apoptosis involved Bax/Bcl-2 signal transduction.[15] Compound **1** is, therefore, suggested to induce apoptosis in HL60 cells by involving mitochondrial signal transduction pathway.

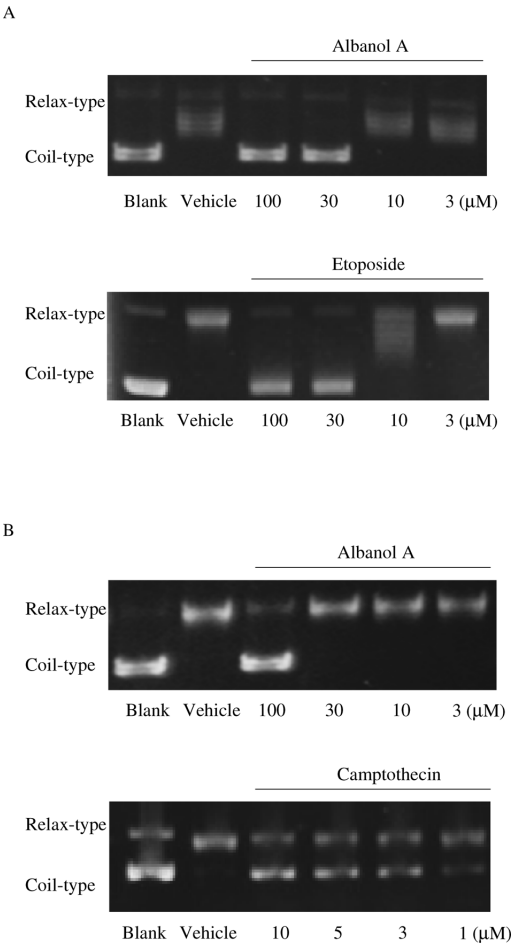

Fig. 4. DNA Topoisomerase (Topo) Inhibbitory Activity of Albanol A (**1**) and Positive Controls

(A) Topo II inhibitory activity of albanol A. Albanol A showed comparable activity to that of etoposide (n=3). (B) Topo I inhibitory activity of albanol A. Albanol A exhibited almost no activity (n=3).

To clearify the trigger of apoptosis induced by compound **1**, we investigated DNA Topo inhibitory activity and activation of caspase-2. Topo poisons such as camptothecin and etoposide are known to activate caspase-2 and induce apoptosis.[16,17] On investigation of Topo I and II inhibitory activ-

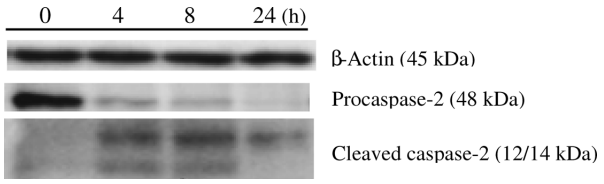

Fig. 5. Western Blot Analysis of Caspase-2 in HL60 Cells Treated with Albanol A (**1**)

The HL60 cells were treated with 30 μM albanol A for the indicated times. The results are from one representative experiment among three runs which showed the similar patterns with one another.

ity, compound **1** exhibited potent Topo II inhibitory activity (IC_{50} 22.8 μM) almost comparable with that of a positive control, etoposide (IC_{50} 34.5 μM). Compound **1** was, however, a weaker Topo I inhibitor (IC_{50} 88.4 μM) than a positive control, camptothecin (IC_{50} 1.9 μM) (Fig. 4). Activation of caspase-2 was then analyzed by Western blotting. After treatment of HL60 cells with **1** (30 μM), procaspase-2 decreased in a time dependent manner, and cleaved caspase-2 was detected after 4 h of incubation (Fig. 5). These suggested that Topo II inhibition of compound 1 is one of the triggers of apoptosis in HL60 cells.

In summary, we have revealed that albanol A (**1**) induces apoptotic cell death *via* both the death receptor and the mitochondrial pathways on HL60 cell lines. In addition, inhibition of Topo II and stimulation of the death receptor might be the triggers of apoptosis. It remains to be clarified whether these changes in apoptotic pathways might be responsible solely for apoptotic cell death of HL60 cells, since any experiment to block the apoptotic cell death, such as those with specific caspase inhibitors, has not been undertaken in this study. This is the first report for the apoptotis-inducing activity of albanol A in HL60 cells. It appears that albanol A may be a promising lead compound for developing an effective drug for treatment of leukemia.

Experimental

General The ^{1}H-NMR spectra were measured on a JEOL ECX-400 spectrometer (400 MHz) in $(CD_3)_2CO$. The high-resolution electrospray ionization (HR-ESI)-MS were recorded on an Agilent 1100 LC/MSD TOF system.

Materials The dried and pulverized root bark of *Morus alba* L. (Mori Radicis Cortex) used in this study was purchesed in 2006 from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). A voucher specimen is on file in the laboratory of the College of Science and Technology, Nihon University.

Cell Lines and Culture Conditions HL60 (human leukemia) and CRL1579 (human melanoma) cell lines, obtained from Riken Cell Bank (Tsukuba, Ibaraki), were grown in RPMI 1640 medium (Invitrogen Co., Auckland, New Zealand). The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co.) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were cultured in a 5% CO_2 humidified incubator at 37 °C.

Extraction and Isolation The dried and pulverized root bark of *M. alba* (4 kg) was extracted successively with *n*-hexane (reflux, 3 h, ×2) and MeOH (reflux, 3 h, ×3) to yield *n*-hexane (909 g) and MeOH (620 g) extracts. A portion of the MeOH extract (562 g) was suspended in water and partitioned successively with EtOAc and *n*-BuOH to afford EtOAc (41 g), *n*-BuOH (116 g), and H_2O (356 g) fractions. The EtOAc-soluble fraction was subjected to silica gel (1.2 kg; Silica gel 60, 220—400 mesh, Merck) column chromatography. Step gradient elution with *n*-hexane–EtOAc–MeOH (9 : 1 : 0→0 : 1 : 0→0 : 1 : 9) gave 22 fractions, E1—E22. Fraction E12 (640 mg) from the eluate of *n*-hexane–EtOAc–MeOH (1 : 1 : 0) was passed through octadecyl silica (ODS) (Chromatorex-ODS, 100—200 mesh; Fuji Silysia Chemical, Ltd., Aichi, Japan) column with MeOH–H_2O (1 : 1→1 : 0) which gave six sub-fractions, E12-1—E12-6. Fraction E12-2 (74 mg) eluted with MeOH–H_2O (3 : 2) was subjected to reversed-phase preparative HPLC on an ODS column (Pegasil ODS-II 5 μm column, 25 cm×10 mm i.d.; Senshu Scientific Co., Ltd., Tokyo, Japan) eluted with MeCN–H_2O–AcOH (55 : 45 : 1; flow rate 3.0 ml/min) to yield albanol A (**1**, mulberrofuran G; 13.1 mg; retention time 16.8 min)[5] and mulberofuran Q (**2**; 1.8 mg; 10.3 min).[8]

Albanol A (**1**): HR-ESI-MS: *m/z* 562.1713 (Calcd for $C_{34}H_{27}O_8$ $[M+H]^+$: 562.1705). The ^{1}H-NMR data were indistinguishable from those of the corresponding compound reported in the literature.[5]

Mulberofuran Q (**2**): HR-ESI-MS: *m/z* 591.1257 (Calcd for $C_{34}H_{23}O_{10}$ $[M+H]^+$: 591.1296). The ^{1}H-NMR data were indistinguishable from those of the corresponding compound reported in the literature.[8]

Cytotoxicity Assay Cytotoxicity assay was performed according to the previous method.[18] Briefly, HL60 (human leukemia) and CRL1579 (human melanoma) cell lines (each 3×10^3 cells/well) were treated with compounds for 48 h, and then thiazolyl blue tetrazolium bromide (MTT) solution was added to the well. After incubation for 3 h, the generated blue formazan was solubilized with 0.04 M HCl in isopropanol. The absorbances at 570 nm (top) and 630 nm (bottom) were measured with a microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan).

Annexin V–Propidium Iodide Double Staining Apoptosis was detected using an rh Annexin V/FITC kit (Bender MedSystems®; Cosmo Bio Co., Ltd., Tokyo, Japan). HL60 cells (4×10^5 cells/well) were exposed to compound (final concentration: 30 μM). To prepare the cell sample for flow cytometry, cells were washed with annexin-binding buffer and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 10 min. The cell samples were analyzed by flow cytometer (Cell Lab Quanta™ SC; Beckman Coulter K.K., Tokyo, Japan) using the ranges of FL1 and FL2 for annexin V-FITC and PI, respectively.

Western Blot Analysis Western blot analysis was performed according to the previous method.[18] Briefly, HL60 cells (1×10^6 cells/well) lysates containing 20 μg of total protein were separated by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated with the anti-caspase-3 and anti-caspase-8 (Sigma-Aldrich Japan Co.), and anti-cleaved caspase-3, anti-caspase-9, anti-Bax, anti-Bcl-2, anti-Bid, and anti-β-actin primary antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) at 4 °C overnight. The blots were then deteced with enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare, Buckinghamshire, U.K.).

DNA Topoisomerase Inhibitory Assay DNA topoisomerase I and II inhibitory assay was performed according to the previous method.[19] Briefly, human DNA Topo I and II were purchased from TopoGen (Columbus, OH, U.S.A.). Supercoiled pBR 322 plasmid DNA was purchased from Takara Bio Inc. (Ohtsu, Japan). Reaction mixture contained 10 mM Tris–HCl (pH 7.9), 0.1 mM spermidine, 5% glycerol, 0.01% bovine serum albumin (BSA), 0.25 μg DNA, the indicated compound concentrations in dimethyl sulfoxide (DMSO), and 1 U of Topo I in a final volume of 20 μl (concentration of DMSO: 2.5%). Reaction mixtures were incubated for 30 min at 37 °C. Reaction products were electrophoresed on 1% agarose gel in TAE (Tris–HCl–acetate–EDTA) running buffer. The agarose gel was stained with ethidium bromide (EtBr) and the DNA was visualized under UV light. The catalytic activity of Topo II was analyzed in the same manner as above except that the reaction mixture contained 50 mM Tris–HCl (pH 7.9), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT, 0.20 μg DNA, and 1 U human Topo II.

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References

1) Namba T., "The Encyclopedia of Wakan-Yaku (Traditional Sino–Japanese Medicines) with Color Pictures," Vol. I, revised ed., Hoikusya, Osaka, 1994, pp. 153—154.
2) Nomura T., *Yakugaku Zasshi*, **121**, 535—556 (2001).
3) Zhang Q.-J., Tang Y.-B., Chen R.-Y., Yu D.-Q., *Chem. Biodivers.*, **4**, 1533—1540 (2007).
4) Rao A. V. R., Deshpande V. H., Shastri R. K., *Tetrahedron Lett.*, **24**, 3013—3016 (1983).
5) Fukai T., Hano Y., Hirakura K., Nomura T., Uzawa J., Fukushima K., *Chem. Pharm. Bull.*, **33**, 3195—3204 (1985).
6) Akihisa T., Yasukawa K., "Biomaterials from Aquatic and Terrestrial

Organisms," ed. by Fingerman M., Nagabhushanam R., Science Publ., Enfield, 2006, pp. 63—114.
7) Akihisa T., *Oleoscience*, **7**, 445—453 (2007).
8) Hano Y., Tsubura H., Nomura T., *Heterocycles*, **24**, 1807—1813 (1986).
9) Martin S. J., Reutelingsperger C. P., McGahon A. J., Rader J. A., van Schie R. C. A. A., LaFace D. M., Green D. R., *J. Exp. Med.*, **182**, 1545—15556 (1995).
10) Salvesen G. S., Dixit V. M., *Cell*, **91**, 443—436 (1997).
11) Hornberry N. A., Lazebnik Y., *Science*, **281**, 1312—1316 (1998).
12) Kluck R. M., Bossy-Wetzel E., Green D. R., Newmeyer D. D., *Science*, **275**, 1132—1137 (1997).
13) Kluck R. M., Esposti M. D., Perkins G., Renken C., Kuwana T., Bossy-Wetzel E., Goldberg M., Allen T., Barber M. J., Green D. R., Newmeyer D. D., *J. Cell Biol.*, **147**, 809—822 (1999).
14) Luo X., Budihardjo I., Zou H., Slaughter C., Wang X., *Cell*, **94**, 481—490 (1998).
15) Oltvai Z. N., Korsmeyer S. J., *Cell*, **79**, 189—192 (1994).
16) Paquet C., Sane A.-T., Beauchemin M., Bertrand R., *Leukemia*, **19**, 784—791 (2005).
17) Lassus P., Opitz-Araya X., Lazebnik Y., *Science*, **297**, 1352—1354 (2002).
18) Tabata K., Motani K., Takayanagi N., Nishimura R., Asami S., Kimura Y., Ukiya M., Hasegawa D., Akihisa T., Suzuki T., *Biol. Pharm. Bull.*, **28**, 1404—1407 (2005).
19) Mizushina Y., Akihisa T., Ukiya M., Murakami-N. C., Kuriyama I., Takeuchi T., Sugawara F., Yoshida H., *Life Sci.*, **77**, 2127—2140 (2005).