3-*O*-(*E*)-*p*-Coumaroyl Tormentic Acid from *Eriobotrya japonica* Leaves Induces Caspase-Dependent Apoptotic Cell Death in Human Leukemia Cell Line

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Eleven triterpene acids, 1—11, isolated from the leaves of *Eriobotrya japonica*, were evaluated for inhibition of DNA topoisomerase (Topo) I and cytotoxicity against human leukemia (HL60) and melanoma cell lines (CRL1579). Among the compounds tested, four compounds, δ-oleanolic acid (4), ursolic acid (5), 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (8), and betulinic acid (10), exhibited potent Topo I inhibitory activity (IC_{50} 20.3—36.5 μM) and cytotoxicity against HL60 (EC_{50} 5.0—8.1 μM). Upon assessing the apoptosis-inducing activity in HL60 cells, compound 8 exhibited induction of apoptosis detected by the observation of DNA fragmentation and membrane phospholipid exposure in flow cytometry. Western blot analysis showed that compound 8 markedly reduced the levels of procaspases-3 and 9, while being increased the levels of cleaved caspases-3 and 9. On the other hand, compound 8 exerted almost no influence on the expression of caspase-8. In addition, compound 8 increased significantly Bax/Bcl-2 ratio and activated caspase-2. These results suggested that compound 8 induced apoptotic cell death in HL60 *via* mainly mitochondrial pathway by, at least in part, Topo I inhibition. Therefore, compound 8 may be promising lead compound for developing an effective drug for treatment of leukemia.

Key words 3-*O*-(*E*)-*p*-coumaroyl tormentic acid; triterpene acid; *Eriobotrya japonica*; cytotoxic activity; caspase-dependent apoptosis

Leaves of *Eriobotrya japonica* Lindl. (Rosaceae) have been used in folk medicine to treat various skin diseases and diabetes mellitus.[1] Various triterpenoids have been isolated from the leaves, and several of them have been reported to possess anti-inflammatory,[2] antiviral,[3] antimutagenic,[4] anti-tumor-promoting,[5] anti-oxidative,[6] and hypoglycemic properties.[7,8] In the course of our search for potential bioactive compounds from natural sources,[9,10] we have reported the isolation and identification of sixteen triterpene carboxylic acids from the methanol (MeOH) extract of the leaves of *E. japonica* and their inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and on the Epstein–Barr virus early antigen (EBV-EA) activation induced by TPA.[11] In addition, we have reported that euscaphic acid (**9**) exhibited marked antitumor-promoting activity in an *in vivo* two-stage mouse skin carcinogenesis.[11] In this paper, we report the inhibitory effect on DNA topoisomerase (Topo) I and cytotoxicity against human leukemia (HL60) and melanoma cell lines (CRL1579) for eleven triterpene acids isolated from the MeOH extract of *E. japonica* leaves in our previous study.[11] Furthermore, we report the apoptosis-inducing activity in HL60 cell line of 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**), which exhibited potent cytotoxicity against both cell lines, and the mechanisms of apoptotic cell death in HL60.

Results and Discussion

Eleven triterpene acids (Fig. 1), including four oleanane-type: oleanolic acid (**1**), maslinic acid (**2**), 3-epiasiatic acid (**3**), and δ-oleanolic acid (**4**); five ursane-type: ursolic acid (**5**), methyl ursolate (**6**), corosolic acid (**7**), 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**), and euscaphic acid (**9**); and two lupane-type: betulinic acid (**10**) and methyl betulinate (**11**), isolated from the MeOH extract of *E. japonica* leaves,[11] were evaluated with respect to their Topo I inhibitory and cytotoxic activities. On evaluation of Topo I inhibition,[12] four compounds, **4**, **5**, **8**, and **10**, exhibited potent Topo I inhibitory activity (IC_{50} 20.3—36.5 μM) (Table 1), which being almost comparable with or more inhibitory than reference camptothecin (IC_{50} 28.1 μM), a well known Topo I poison.[13] Upon evaluation of cytotoxicity, three compounds, **5**, **8**, and **10**, exhibited potent activity against both HL60 (EC_{50} 5.0—6.9 μM) and CRL1579 (EC_{50} 8.7—16.9 μM) cells (Table 1), which being almost comparable with or more inhibitory than reference cisplatin (EC_{50} HL60: 2.5 μM; CRL1579: 21.1 μM), one of the most effective and widely used anti-cancer agents. In addition, compound **4** against HL60 (EC_{50} 8.1 μM) and compound **7** against CRL1579 (EC_{50} 20.8 μM) showed potent cytotoxicity. Among these Topo I inhibitory and cytotoxic compounds, compounds **5**[14] and **10**[14] for their Topo I inhibitory activity and compounds **5**,[5,14—17] **7**,[18] **8**[19] (and its 3-*O*-(*Z*)-coumaroyl isomer),[5,19] and **10**[14,15,20—23] for their potent cytotoxicity against some tumor cell lines have been reported. Moreover, compounds **5**,[16,17] **7**,[18] and **10**[20,21,23] have been demonstrated to induce apoptotic cell death, and the mechanisms of the induction of apoptosis have been investigated.[16—18,21]

On the basis of the results in Table 1, the following conclusions can be drawn about the structure–activity relationship of the compounds:

(i) Among the oleanane-type triterpene acids (**1**—**4**), hydroxylation at C-2 (**2**, **3**) decreases the Topo I inhibitory and cytotoxic activities against HL60 cells. On the contrary to this, hydroxylation at C-2 enhances cytotoxic activity against

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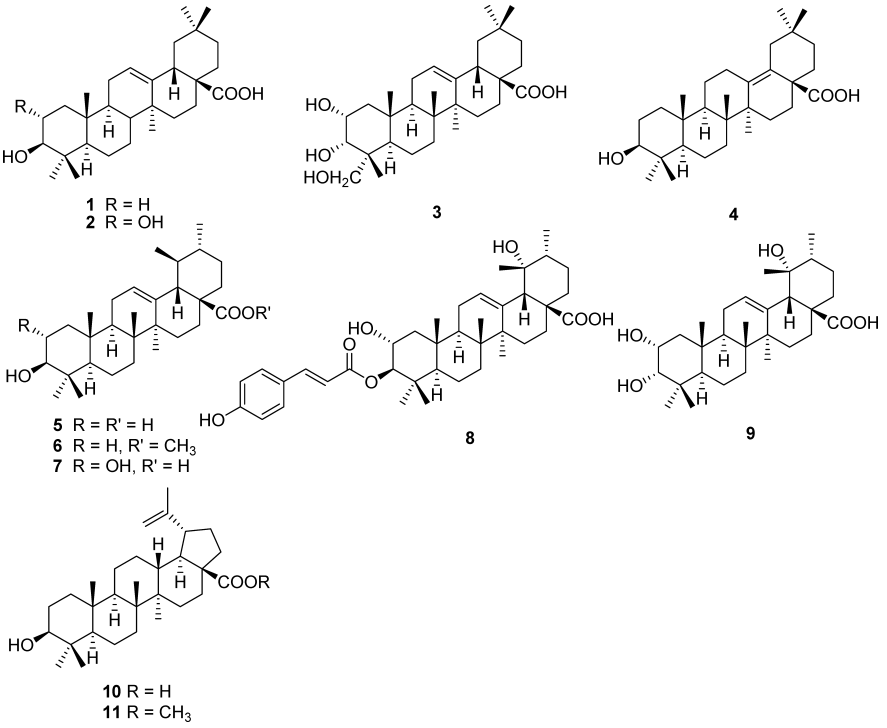

Fig. 1. Chemical Structures of Eleven Triterpene Acids (**1**—**11**)

Table 1. DNA Topoisomerase I Inhibitory Activity (IC_{50}) and Cytotoxicity (EC_{50}) of Triterpene Acids **1**—**11** from the Leaves of *Eriobotrya japonica*

Compound	IC_{50} (μM)[a]	EC_{50} (μM)[a]	
		HL60[b]	CRL 1579[b]
Oleanane-type			
1 Oleanolic acid	64.3	21.9	>100
2 Maslinic acid	80.6	63.7	68.7
3 3-Epiasiatic acid	>100	62.6	98.4
4 δ-Oleanolic acid	33.0	8.1	>100
Ursane-type			
5 Ursolic acid	26.3	5.0	8.7
6 Methyl ursolate	>100	14.1	47.3
7 Corosolic acid	>100	46.4	20.8
8 3-*O*-(*E*)-*p*-Coumaroyl tormentic acid	20.3	6.9	15.1
9 Euscaphic acid	>100	>100	66.8
Lupane-type			
10 Betulinic acid	36.5	6.4	16.9
11 Methyl betulinate	>100	56.7	75.9
Refernce compound			
Camptothecin	28.1		
Cisplatin		2.5	21.1

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

CRL1579 cells.

(ii) Among the ursane-type triterpene acids (**5**—**9**), hydroxylation at C-2 (**7**, **9**) decreases the Topo I inhibitory and cytotoxic activities against both HL60 and CRL1579 cells. However, esterification at C-3 with a *p*-coumaroyl group (**8**) enhances these activities of C-2 hydroxylated triterpene acids (**7**—**9**).

(iii) Methyl esterification of the carboxyl group at C-28 decreases the Topo I inhibitory and cytotoxic activities against both HL60 and CRL1579 cells (**5** *vs.* **6**, and **10** *vs.* **11**).

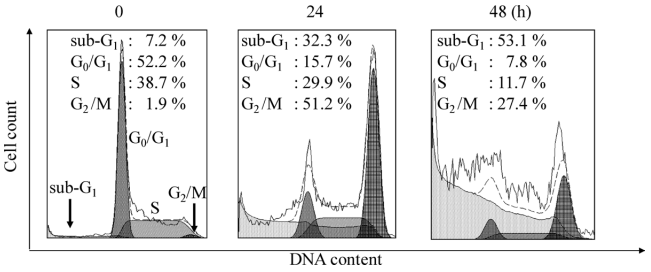

Fig. 2. Flow Cytometric Analysis of Cell Cycle Distribution

The HL60 cells were treated with 40 μM 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**) for 24 and 48 h. Three independent experiments were carried out, and all gave similar results.

(iv) HL60 cells were more sensitive than CRL1579 cells for all of the triterpene acids tested with two exceptions. Thus, the cytotoxic activity of two triterpene acids, **7** and **9**, were more potent against CRL1579 cells than against HL60 cells.

Since the nature of cytotoxicity of compound **8** remained unexplored, we undertook, in this study, flow cytometric analysis of **8** in HL60 cells with 4′,6-diamidino-2-phenylindol (DAPI) for cell cycle analysis, and annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for detection of early apoptotic cells. On treatment with **8**, the cells generated sub-G_1 peak after 24 h (32.3% *vs.* 7.2% for 0 h) and 48 h (53.1%) of incubation in cell cycle analysis (Fig. 2). Sub-G_1 peak means fragmentated DNA, which is known to one of the features of apoptosis.[24] G_2/M cell cycle arrest also was detected after 24 h and 48 h (51.2% for 24 h and 27.4% for 48 h *vs.* 1.9% for 0 h). Furthermore, we detected the early apoptotic cells with annexin V-FITC and PI double staining. The phosphatidylserine externalization to cellular membrane is one of the earliest markers of apoptotic cell death.[25] An-

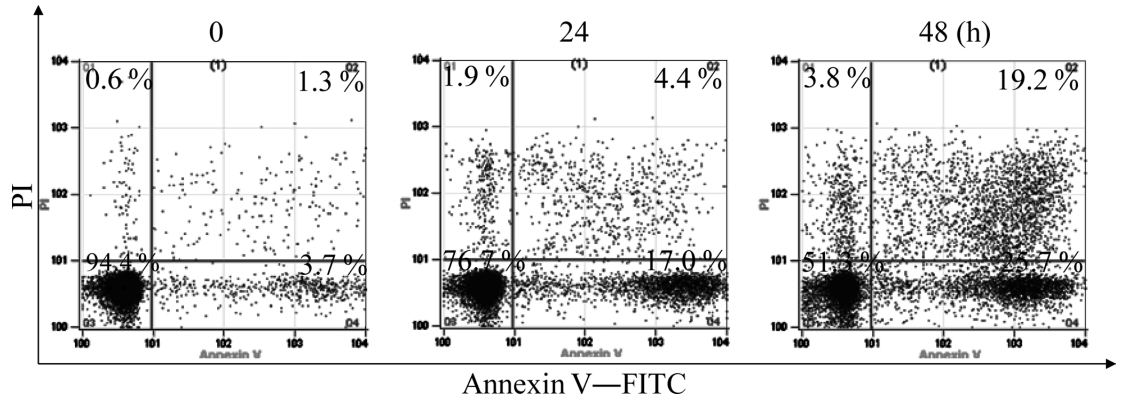

Fig. 3. Detection of Early Apoptotic Cells by Annexin V-PI Double Staining

The HL 60 cells were cultured with 40 μM 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**) for 24 and 48 h. Three independent experiments were carried out, and all gave similar results.

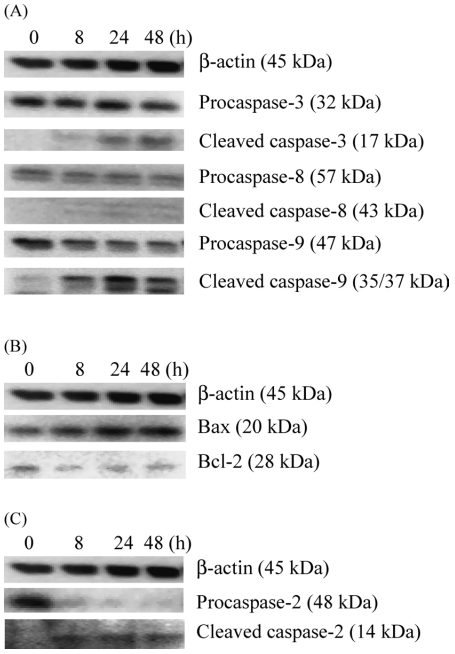

Fig. 4. Western Blot Analysis in HL60 Cells

The HL60 cells were treated with 40 μM 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**) for the indicated times. Three independent experiments were carried out, and all gave similar results. (A) Western blot analysis of caspases-3, 8, and 9 in HL60 cells treated with 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**). (B) Effect of 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**) on Bcl-2 and Bax proteins in HL60. (C) Western blot analysis of caspase-2 in HL60 cells treated with 3-*O*-(*E*)-*p*-coumaroyl tormentic acid (**8**).

nexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the surface of early apoptotic cells. Because PI, a DNA intercalating agent, does not enter whole cells with intact membranes, it is excluded from living and early apoptotic cells. They were 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 cells (lower right) was significantly increased in a time-dependent-manner after treatment with **8** (40 μM) (3.7% for 0 h *vs.* 17.0% for 24 h and 25.7% for 48 h) and late apoptotic cells (upper right) significantly increased after 48 h of incubation with **8** compared with 0 h (1.3% for 0 h *vs.* 19.2% for 48 h) (Fig. 3). Although a small number of necrotic cells (upper left) also were detected, these results showed that cytotoxic activity of **8** against HL60 is mainly due to inducing apoptotic cell death.

It is known that apoptosis is induced mainly by triggering the death receptors (extrinsic pathway) and/or mitochondria (intrinsic pathway), leading to the activation of caspases.[26,27] In the death receptor-mediated apoptosis pathway, tumor necrosis factor family activates upstream caspase-8. Activation of caspase-8 can in turn directly activate caspase-3. The mitochondrial-mediated apoptosis pathway activates procaspase-9 by releasing cytochrome *c*. Caspase-9 forms an active holoenzyme that processes and activates downstream caspase-3. In order to clarify the mechanism by which compound **8** induces apoptotic cell death, activation of caspases-3, 8, and 9 was evaluated by Western blot analysis. After treatment of HL60 cells with **8** (40 μM), the level of procaspases-3 and 9 gradually diminished in a time-dependent-manner, and cleaved caspases-3 and 9 were detected (Fig. 4A). On the other hand, compound **8** exerted almost no influence on the expression of procaspase-8 and cleaved caspase-8. These results suggested that compound **8** induces apoptotic cell death *via* mainly mithochondrial pathway through activation of caspases-3 and 9 in HL60.

Next, we investigated the effect of compound **8** on Bax and Bcl-2. The proapoptotic proteins Bax and Bid, and the anti-apoptotic mitochondrial protein Bcl-2 are important regulators of cytochrome *c* release from mitochondria.[28,29] Expression of these proteins was examined by Western blot analysis. Treatment of compound **8** for 24 h in HL60 cells decreased the level of Bcl-2 whereas increased the level of Bax after 8 h (Fig. 4B). The Bax/Bcl-2 ratio is one of the indices of the intrinsic mechanism of apoptosis in mitochondria.[30] Since compound **8** increased this ratio in HL60 cells, it seems that compound **8**-induced apoptosis involves Bax/Bcl-2 signal transduction in mitochondrial pathway.

Activation of caspase-2 triggers the mitochondrial apoptotic pathway,[31] and Topo poisons such as camptothecin and etoposide are known to activate caspase-2 and induce apoptosis.[32,33] To clarify the trigger of apoptosis induced by compound **8**, we investigated activation of caspase-2 by Western blotting. On treatment of HL60 cells with **8**, procaspase-2 decreased and cleaved caspase-2 expressed after 8 h (Fig. 4C). Taking into account that compound **8** exhibited potent Topo I inhibitory activity, it can be suggested that Topo I in-

hibition followed by activation of caspase-2 is one of the triggers of apoptosis in HL60 cells for compound **8**.

In summary, we have revealed that compound **8** induces apoptotic cell death in HL60 cell line *via* mainly the mitochondrial pathway by, at least in part, Topo I inhibition. This is the first report for compound **8** that this induces apoptotic cell death in HL60 cells. It appears that compound **8** as well as compounds **5**,[16,17] **7**,[18] and **10**[20,21,23] may be promising lead compounds for developing effective drugs for cancer chemotherapy, especially for leukemia. In addition to compound **8**, the 3-*O*-*p*-coumaroyl esters of other types of triterpene acids, such as oleanane- and lupane-types triterpene acids, might be worth to investigate for developing effective drugs for cancer chemotherapy. It might be worthy to note here that among the compounds exhibiting no significant cytotoxicity in this study, two compounds, **1**[17] and **2**,[34] have been demonstrated for their apoptotic cell death in some tumor cell lines, and have been discussed for their potentials to develop effective antitumor agents. Evaluation of apoptosis-induction activity of compound **4** in HL60 is now undergoing.

Experimental

Materials Eleven triterpene acids, **1**—**11**, were isolated from the MeOH exract of the leaves of loquat, *Eriobotrya japonica* Lindl. (Rosaceae).[11]

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., Carlsbad, CA, U.S.A.). The cells were cultured according to the previous method.[35,36]

DNA Topoisomerase Inhibitory Assay DNA Topo I was performed according to the previous method[12,33] with a slight modification. Briefly, calf DNA Topo I and supercoiled pBR 322 plasmid DNA were purchased from Takara Bio Inc. (Ohtsu, Japan). Reaction mixture contained 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, 0.01% bovine serum albumin (BSA), 0.5 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–ethylenediaminetetraacetic acid (EDTA)) running buffer. The agarose gel was stained with ethidium bromide (EtBr) and the DNA was visualized under UV light.

Cytotoxicity Assay Cytotoxicity assay was performed according to the previous method.[35,36]

Cell Cycle Analysis Aliquots of HL60 (4×10^5 cells) were incubated with medium containing test compounds. The cells were then washed with PBS with centrifugation. DNA was stained with Nim-DAPI (NPE Systems, FL, U.S.A.) staining solution for 10 min at room temperature. Fluorescence intensity was measured by flow cytometer (Cell Lab Quanta™ SC; Beckman Coulter K.K., Tokyo, Japan). Cell cycle distribution was analyzed using DNA Cell Analysis software (Multi Cycle AV; Phoenix Flow System, San Diego, CA, U.S.A.).

Annexin V-Propidium Iodide Double Staining Apoptosis was detected by the same way as described in our recent paper using an recombinant human (rh) Annexin V-FITC kit.[35,36]

Western Blot Analysis Western blot analysis was performed as described previously.[35,36]

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