Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 1756–1763
Bioorganic & Medicinal Chemistry

A novel sesquiterpenoid dimer parviflorene F induces apoptosis by up-regulating the expression of TRAIL-R2 and a caspase-dependent mechanism

Takashi Ohtsuki, Mayu Tamaki, Kazuhumi Toume and Masami Ishibashi*

Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

Received 18 September 2007; revised 6 November 2007; accepted 6 November 2007
Available online 13 November 2007

Abstract—Parviflorene F (**1**), a novel sesquiterpenoid dimer isolated from *Curcuma parviflora* Wall, is a cytotoxic compound. In this study, we examined the mechanism of its cytotoxic effect in HeLa cells. Treatment with **1**enhanced the mRNA and protein expression of TRAIL-R2 (tumor necrosis factor α-related apoptosis inducing ligand receptor 2). Apoptosis was induced by **1** as revealed by the distribution of DNA and Annexin V/PI staining using flow cytometry. In addition, **1**-induced apoptosis was inhibited by human recombinant TRAIL-R2/Fc chimera protein, TRAIL-neutralizing fusion protein. Also, we found that **1** induced the activation of caspase-8, caspase-9, and caspase-3, indicating that the cytotoxic effect of **1** is correlated with apoptosis by a caspase-dependent mechanism through TRAIL-R2. In addition, **1** enhanced TRAIL-induced cell death against HeLa and TRAIL-resistant DLD1 cells. Taken together, up-regulation of TRAIL-R2 by **1** may contribute to sensitization of TRAIL-induced cell death.

1. Introduction

Cancer is a class of diseases characterized by the uncontrolled division of cells. In the USA and other developed countries, cancer is presently responsible for about 25% of all deaths.[1] Chemoprevention is regarded as one of the most promising and realistic approaches to the prevention of cancer. In fact, epidemiological studies have provided convincing evidence that natural compounds and dietary agents can modify the processes of carcinogenesis, including initiation, promotion, and progression of several types of human cancer. Among anticancer drugs derived from plants, vinblastine and vincristine have already been used clinically.[2] Therefore, natural products from plants are rich sources of agents used for the treatment of cancer. During our search for natural bioactive products in tropical plants,[3,4] we recently isolated novel sesquiterpenoid dimers, parviflorenes A–J from*Curcuma parviflora* Wall. (Zingiberaceae),[5–8] and elucidated their structures. Most of these compounds exhibited cytotoxicity against human cancer cell lines. In particular, parviflorenes A and F (**1**), obtained in abundance from this plant, were cytotoxic to all of the cell lines tested at considerably low concentrations, whereas both compounds displayed low differential cellular sensitivity. Also, COMPARE analyses showed no significant correlation with the profile of any standard anticancer drug, suggesting that both compounds have a unique mechanism of action.[7] In this study, we investigated the mechanism of the cytotoxic effect of parviflorene F (**1**) in HeLa and TRAIL-resistant DLD1 cells. Our study demonstrates that **1** enhanced the mRNA and protein expression of TRAIL-R2, and activation of caspase-8, -9, and -3. Furthermore, **1**-induced apoptosis was inhibited by human recombinant TRAIL-R2/Fc chimera protein, TRAIL-neutralizing fusion protein. The cytotoxic effect of **1** was correlated with apoptosis by a caspase-dependent mechanism through TRAIL-R2. Also, **1** enhanced TRAIL-induced cell death against HeLa and TRAIL-resistant DLD1 cells.

2. Results

Previously,[6,7] we found that parviflorene F (**1**) exhibited cytotoxicity against VCR-resistance KB (nasopharyngeal epidermal carcinoma), LnCaP (prostate cancer), and TNF-related apoptosis-inducing ligand (TRAIL)-resistant KOB (human adult T cell leukemia). In addi-

Keywords: Apoptosis; TRAIL-R2; Sesquiterpenoid dimer; Caspase.
* Corresponding author. Tel./fax: +81 43 290 2913; e-mail: mish@p.chiba-u.ac.jp

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.11.022

tion, the GI_{50} value of **1** against 39 human cancer cell lines was 2.6 μM (1.1 μg/mL), although the effect was not selective. The COMPARE analysis of **1** also showed no strong correlation (correlation index, $r < 0.5$) with standard anticancer drugs, suggesting that **1** has a unique mode of action. In this study, the decrease of viability was observed in a dose-dependent manner by treatment of parviflorene F (**1**) against HeLa cells and the IC_{50} value was determined to be 4.3 μg/mL (Fig. 1B). Moreover, a time-dependent reduction in cell survival was observed in HeLa cells (Fig. 1C).

In order to elucidate which genes are differentially expressed in relation to the cytotoxic effect of **1**, a DNA microarray assay and real time RT-PCR were carried out as follows. HeLa cells (4×10^6 cells/well) were treated with **1** at 5 μg/mL and incubated for 24 h, and mRNA, obtained from the total RNA extracted from the cultured cells, was subjected to a DNA microarray analysis using IntelliGene® Human Cancer CHIP ver.4.0 (TaKaRa), which examines 886 genes related to cancer diseases. The results obtained from the microarray analysis indicated that treatment with **1** changed the mRNA expression of seven genes (Table 1). Among them, the expression levels of five genes were increased over 1.4 times, whereas those of two genes were decreased (<0.7 times), compared to cells with no treatment. Since molecular biological information on four of the seven genes was available, the microarray assay results for the four genes (gene code: NM_003607, AF016266, NM_001554, and NM_012484) were further examined using real time quantitative PCR techniques. Three template cDNA were prepared from total RNA extracted from HeLa cells incubated after treatment with **1** at 0, 5, and 8 μg/mL. The results presented in Figure 2A show that the levels of AF016266 and NM_003607 mRNA on treatment with **1** increased in

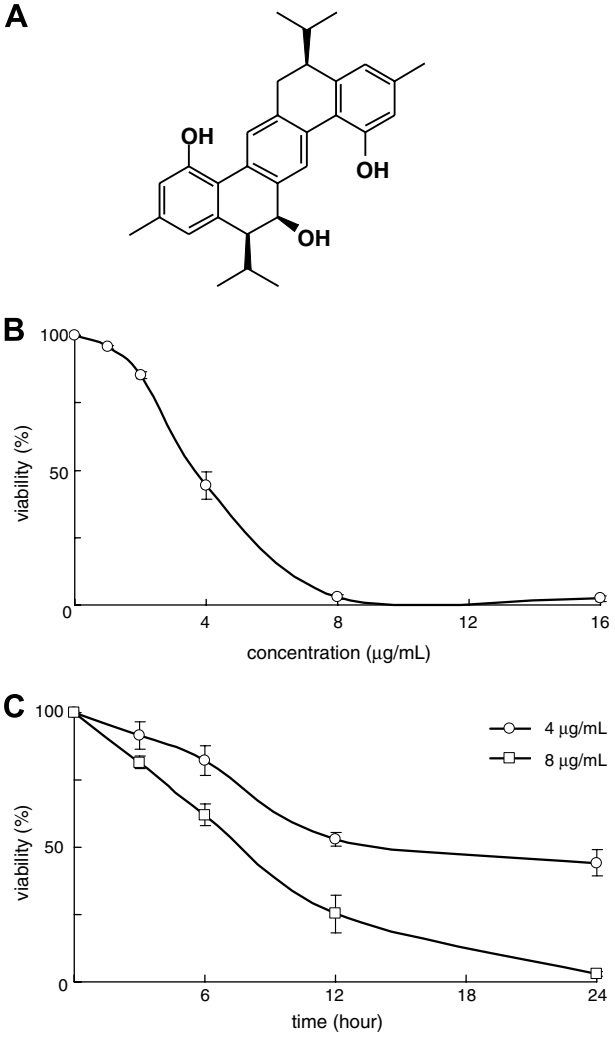


Figure 1. Effect of parviflorene F (**1**) on cell survival in HeLa cells. (A) Structure of **1** (B) Viability of HeLa cells treated with **1** for 24 h. (C) Viability of HeLa cells treated with **1** (4 and 8 μg/mL) for various interval. The error bars represent standard deviation of triplicate samples.

Table 1. The genes differentially expressed on treatment with parviflorene F (**1**)

Gene code	Protein	Ratio[a]
ID 27017[b]	Unknown	2.52
ID 27288[b]	Unknown	1.94
ID 27032[b]	Unknown	1.73
NM_003607	MRCKα[c]	1.71
AF016266	TRAIL-R2[d]	1.44
NM_001554	Cyr61[e]	0.61
NM_012484	RHAMM[f]	0.68

[a] Ratio of cells treated with **1** over control (cells not treated with **1**).
[b] TaKaRa clones.
[c] Myotonic dystrophy kinase-related Cdc42-binding kinase.
[d] TNF (tumor necrosis factor) α-related apoptosis inducing ligand receptor 2.
[e] Cystein rich 61 (angiogenic factor).
[f] Receptor for hyaluronan mediated motility.

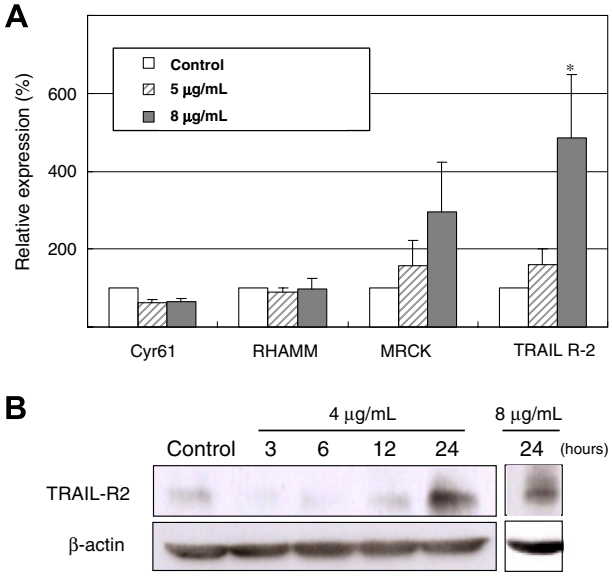
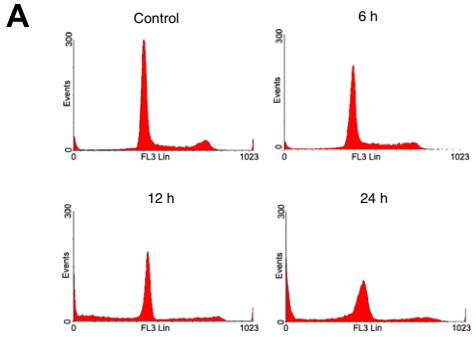


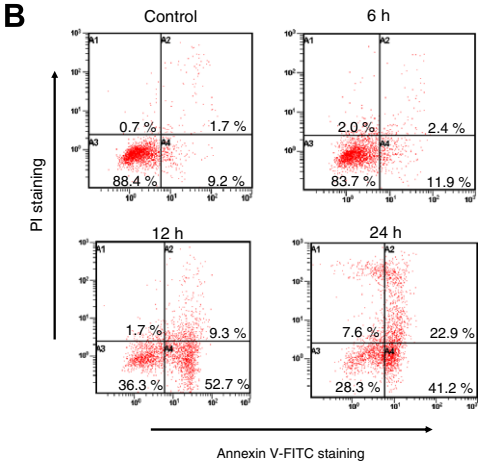
Figure 2. Expression of TRAIL-R2 in HeLa cells treated with parviflorene F (**1**). (A) Real time RT-PCR data for 4 differential genes. The bar represents means ± SD. The significance was determined by Student's *t*-test ($^*p < 0.05$ vs untreated controls) ($n = 3$). (B) Western blot analysis of TRAIL-R2 protein levels in HeLa cells treated with parviflorene F (**1**) for 3, 6, 12 (4 μg/mL), and 24 h (4 and 8 μg/mL) ($n = 3$).

dose-dependent manner compared to the control (from cells not treated with **1**). Notably, it was revealed that **1** enhanced the gene expression of AF016266 by 4.9-fold at the concentration of 8 μg/mL. The gene AF016266 codes the protein TRAIL-R2,[9] one of the death receptors involved in the signaling mechanisms inducing apoptosis. Next, we performed the Western blot analysis to determine the TRAIL-R2 protein level. As shown in Figure 2B, treatment of HeLa cells with **1** at a concentration of 8 μg/mL for 24 h significantly increased the level of TRAIL-R2, compared with the control. This result for the protein expression at 8 μg/mL was consistent with that for the mRNA expression. At 4 μg/mL, the TRAIL-R2 level was also raised in cells treated with **1** for 24 h, compared with the control. Thus, it was suggested that the cytotoxic effect of **1** is related to the induction of apoptosis.

Therefore, we next investigated whether **1** induced the apoptosis of HeLa cells. First, the occurrence of apoptosis was analyzed by flow cytometry. As shown in Figure 3A, control cells showed a normal DNA distribution in terms of fluorescent peaks. Exposure to **1** generated a prominent new peak, representing the sub-G1 peak, after 12 h (31.5% vs 3.5% for the control) and 24 h (26.1% vs 5.1%) of incubation. The HeLa cells were also stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V, a calcium-dependent phospholipid-



	Sub-G1 (%)	G1 (%)	S (%)	G2/M (%)
Control	4.4	60.8	13.3	21.3
6 h	10.9	56.1	17.3	15.6
12 h	31.5	41.6	10.3	16.6
24 h	26.1	52.1	5.5	15.3



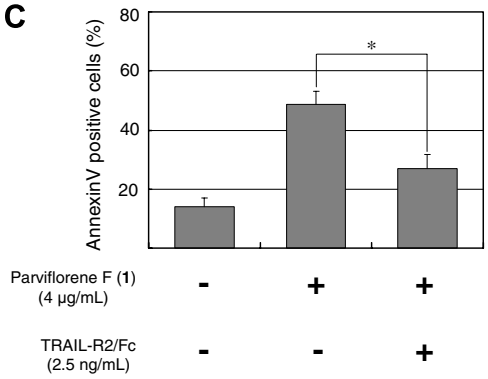


Figure 3. Induction of apoptosis by Parviflorene F (**1**) mediated through TRAIL-R2. (A) Effect of parviflorene F (**1**) on the cell cycle distribution (%) of HeLa cells. (B) Effects of parviflorene F (**1**) on the plasma membrane in HeLa cells. Exponentially growing cells were treated for the indicated period with parviflorene F at 4 μg/mL. Numbers in the respective quadrants indicate the percentage of cells present in this area. (C) TRAIL-R2/Fc chimera protein blocked the induction of apoptosis by parviflorene F (**1**). The bar represents means ± SD. The significance was determined by Student's *t*-test (*$p < 0.05$ vs parviflorene F alone) ($n = 3$).

binding protein with high affinity for phosphatidylserine expressed on the cell surface, and propidium iodide (PI) in order to characterize the early apoptosis induced by **1**. As shown in Figure 3B, the proportion of early apoptotic cells (lower right) was significantly increased (52.7% vs 9.2% for the control) after 12 h of incubation with **1** at 4 μg/mL. After 24 h of incubation, the proportion of early apoptotic cells (41.2% vs 9.2% for the control) and that of late apoptotic cells (upper right) (22.9% vs 1.7%) was significantly increased, compared with the control. We also detected a small number of necrotic cells (upper left). Taken together, these results indicated that the cytotoxic effect of **1** correlated with the induction of apoptosis.

To elucidate whether the apoptosis induced by **1** was caused by an increase of TRAIL-R2 expression, we used a recombinant human TRAIL-R2/Fc chimera protein, which has a dominant-negative effect by competing with endogenous TRAIL-R2. As shown in Figure 3C, 2.5 μg/mL of TRAIL-R2/Fc chimera protein reduced Annexin V positive cells, early and late apoptotic cells at an exposure time of 12 h, indicating that apoptosis induced by **1** was mediated through TRAIL-R2.

To determine whether the apoptosis induced by **1** was mediated via a caspase cascade, we next investigated the activation of caspase-3, a representative signaling molecule in the apoptotic pathway. The enzymatic analysis of caspase-3 activity stimulated by **1** was conducted using fluorometric detection. As shown in Figure 4A, treatment with **1** at a concentration of 4 μg/mL caused a pronounced activation of caspase-3 in a time-dependent manner. Also, a Western blot analysis of treated cells was performed to assess the activation of caspase-8. The extrinsic apoptotic pathway is characterized by the activation and cleavage of procaspase-8. As expected, cleaved caspase-8 and a decrease in procaspase-8 were detected after 6 h of incubation of the cells with **1** at 4 μg/mL, but the cleaved form was not found after 12 h (Fig. 4B).

To clarify whether the extrinsic pathway, the intrinsic pathway, or both pathways were activated in response to treatment with **1**, we conducted Annexin V-PI dual-staining analysis with a specific caspase-8 inhibitor (z-IETD-fmk) and a specific caspase-9 inhibitor (z-LEHD-fmk). As shown in Figure 4C, 25 μM of z-IETD-fmk efficiently prevented the **1**-induced apoptosis of HeLa cells at an exposure time of 12 h in terms of annexin V-positive staining (lower right, 2.2% vs 52.7% for parviflorene F only). In addition, incubation for 12 h with z-LEHD-fmk (20 μM) also reduced the percentage of apoptotic cells (lower right, 4.0% vs 52.7% for **1** only).

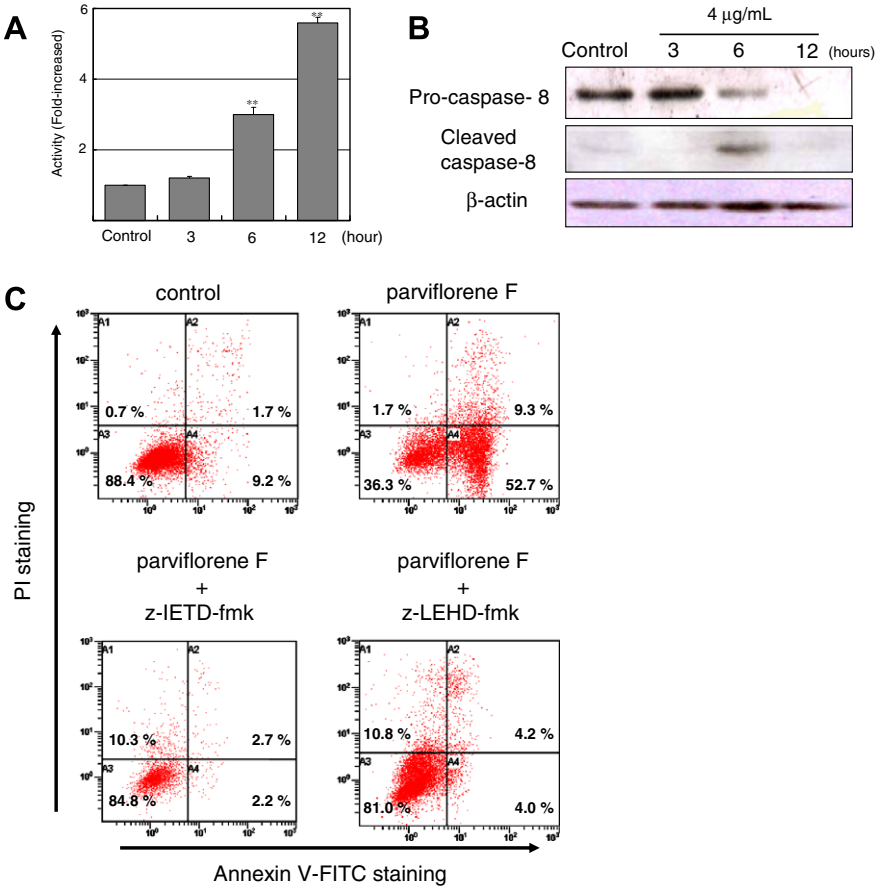


Figure 4. Treatment of parviflorene F (**1**) enhances each caspase activity. (A) Effects of parviflorene F (**1**) on caspase-3 activity. The activity in each control was defined as 1. The significance was determined by Student's *t*-test (**$p < 0.01$ vs untreated controls) ($n = 3$). (B) Western blot analysis of pro-caspase-8 (55 kDa) and the cleaved form (42 kDa) in HeLa cells treated with 4 μg/mL of parviflorene F (**1**) for 3, 6, 12 h ($n = 3$). (C) The effect of caspase-8 and -9 inhibitors on parviflorene F (**1**)-induced apoptosis ($n = 3$).

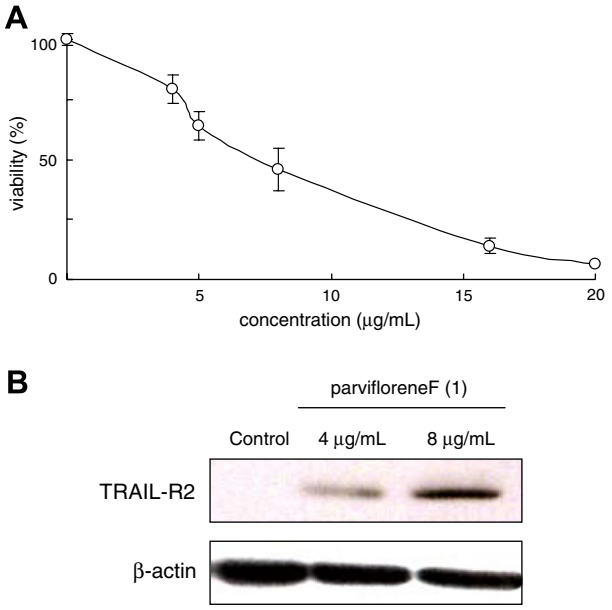


Figure 5. Effect of parviflorene F (**1**) on cell survival and expression of TRAIL-R2 in DLD1/TRAIL-R cells. (A) Viability of DLD1/TRAIL-R cells treated with **1** for 24 h ($n = 3$). (B) Western blot analysis of TRAIL-R2 protein levels in DLD1/TRAIL-R cells treated with parviflorene F (**1**) at 4 and 8 μg/mL ($n = 3$).

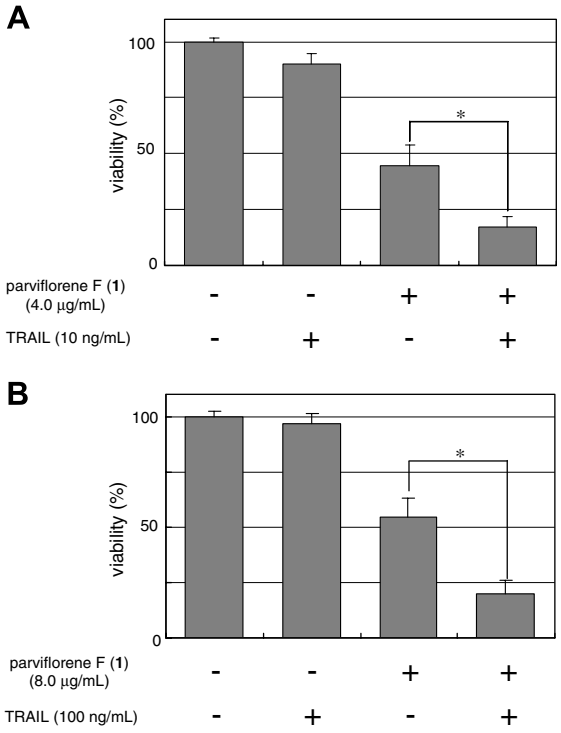


Figure 6. Enhancement of cell death by combined treatment with parviflorene F (**1**) and TRAIL against HeLa and DLD1/TRAIL-R cells. (A) Viability of HeLa cells treated with **1**(4 μg/mL) and/or TRAIL (10 ng/mL) for 24 h. (B) Viability of DLD1/TRAIL-R cells treated with **1** (8 μg/mL) and/or TRAIL (100 ng/mL) for 24 h. The bar represents means ± SD. The significance was determined by Student's *t*-test ($^*p < 0.05$ vs parviflorene F alone) ($n = 3$).

Therefore, these results indicated that **1**-induced apoptosis through both intrinsic and extrinsic apoptotic pathways.

Next we investigated the cytotoxic and TRAIL-R2 inducing effects of parviflorene F (**1**) against DLD1/TRAIL-R cells (TRAIL-resistant DLD1 cells).[10] As shown in Figure 5, parviflorene F (**1**) exhibited cytotoxicity against DLD1/TRAIL-R cells with IC_{50} value of 9.2 μg/mL. Also, treatments with 4 and 8 μg/mL of parviflorene F (**1**) up-regulated TRAIL-R2 protein in DLD1/TRAIL-R cells, suggesting that cytotoxic effect of parviflorene F (**1**) against DLD1/TRAIL-R cells was correlated with the up-regulation of TRAIL-R2.

Since correlation between parviflorene F (**1**) treatment and expression of TRAIL-R2 was suggested, we investigate the synergistic effect of parviflorene F (**1**) and TRAIL against HeLa and DLD1/TRAIL-R cells. As shown in Figure 6, treatment of parviflorene F (**1**) at 4 μg/mL showed viability rate of 44 ± 9.6% against HeLa cells and this decreased to 16 ± 2.0% with the addition of TRAIL. Similarly, combined treatment with **1** (8 μg/mL) and TRAIL significantly induced reduction of viable cells (20 ± 6.4% at combined treatment against 54 ± 8.9% at addition of **1** alone) in DLD1/TRAIL-R cells, indicating that parviflorene F (**1**) sensitized TRAIL-inducing cell death against TRAIL-sensitive and resistant cells via up-regulation of TRAIL-R2.

3. Discussion

The present study was performed to assess the mechanism responsible for the cytotoxicity of parviflorene F (**1**), a novel sesquiterpenoid dimer, in HeLa and TRAIL-resistant DLD1 cells. First, we found that **1** significantly increased mRNA and protein expression of TRAIL-R2 by conducting DNA microarray and real time RT-PCR analyses (Table 1, Fig. 2). TRAIL-R2 is a member of the TNF receptor family and is one of the receptors for TRAIL. To determine whether the cytotoxic effect of **1** was due to apoptosis through a TRAIL-R signaling pathway, we used flow cytometry. The results showed that the appearance of sub-G1 cells was also time-dependent at 4 μg/mL (Fig. 3A). As with the percentages found in the Sub-G1 peak, the proportion of annexin V-positive cells in the parviflorene F-treated cells increased in a time-dependent manner (Fig. 3B). Also, human recombinant TRAIL-R2/Fc chimera protein blocked **1**-induced apoptosis. These results indicated that the cytotoxic effect of **1** was related to the induction of apoptosis via TRAIL-R2. Apoptosis can be induced by a caspase-independent or dependent pathway, although the latter mechanism is the most common.[11] Caspase-3 is an effector caspase, which typically functions down-stream of caspase-8 and -9, and directly activates enzymes that are responsible for DNA fragmentation. Our results indicated that treatment with **1** induced an increase of caspase-3 activity in a time-dependent manner (Fig. 4A), suggesting that **1**-induced apoptosis was dependent on caspase-3. Also, the Western blot and flow cytometric analyses showed that the initiation of apoptotic signaling following treatment with **1** led to the activation of caspase-8 (Fig. 4B

and C). It was reported that caspase-8 could also initiate the intrinsic apoptotic pathway through the activation of Bid.[12,13] Both pathways lead to the activation of caspase-3 and eventual apoptotic cell death.[12] In order to confirm the role of caspase-9 in **1**-induced apoptosis, we treated HeLa cells with a combination of **1** and a specific caspase-9 inhibitor (z-LEHD-fmk). Our results showed that parviflorene F-induced apoptosis was significantly prevented by the caspase-9 inhibitor, suggesting that it occurred via both intrinsic and extrinsic pathways (Fig. 4C).

The death receptor/death ligand induced apoptosis in cancer cells.[14] In particular, TRAIL/TRAIL-receptor signaling has been implicated in the apoptosis of many tumor cell lines, but has no apoptotic effect on most normal cells.[9,15–17] So, its signaling pathway has been considered a promising target for the treatment of cancer. In fact, two approaches have been used to target TRAIL-induced apoptosis; the use of agonistic antibodies (HGS-ETR1, HGS-ETR2, and HGS-ETR2J) against TRAIL-R1 or TRAIL-R2,[18,19] and the use of a recombinant TRAIL. Both of these agents are focused on the ligand of this pathway. On the other hand, in approaches targeting the TRAIL-receptor, it was reported that several chemotherapeutic drugs and natural products, such as flavonoids,[20–22] α-tocopheryl succinate,[23] and curcumin[24], induced expression of TRAIL-R1 and/or TRAIL-R2. The activation of TRAIL receptors results in a signal transduction cascade followed by apoptosis, and is implicated in the sensitization of TRAIL-resistant tumor cells to TRAIL-induced apoptosis.[25,26] A recent study also indicated that the activation of TRAIL-R2 played a more important role than that of TRAIL-R1 in mediating TRAIL-induced cell death.[27] In fact, parviflorene F (**1**) showed cytotoxic effect in HeLa and TRAIL-resistant DLD1 cells (Figs. 1B and 5A) and synergistically enhanced TRAIL-induced cell death against both cell lines in this study (Fig. 6). Therefore, **1** might be useful in the treatment of TRAIL-resistant tumor cells alone or in combination with TRAIL.

4. Experimental

4.1. Cell cultures

HeLa cells were the same as previously described.[3,4] TRAIL-resistant DLD1 (DLD1/TRAIL-R) cells were a generous gift from Dr. Bingliang Fang, The University of Texas, MD, Anderson Cancer Center, Houston, TX, USA. HeLa cells were cultured in Iscove's Modified Dulbecco's Medium (Sigma) with 5% FBS and 4 mM L-glutamine. DLD1/TRAIL-R cells were cultured in RPMI-1640 (Wako) with 10% FBS. Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

4.2. Cytotoxicity

The procedure of assay was the same as previously described.[3,4] Briefly, HeLa (6×10^3 cells) or DLD1/TRAIL-R cells (4×10^3 cells) were treated with different concentrations of parviflorene F (**1**) and/or recombinant human TRAIL/Apo2L (Peprotech) for 24 h at 37 °C. After the medium was removed, cytotoxicity was determined by the FMCA method[28] using a fluorescence platereader.

4.3. Preparation of mRNA for DNA microarray analyses

HeLa cells (4×10^6 cells/well) were incubated for 24 h in the presence (treated) and absence (control) of parviflorene F (5 μg/mL), and total RNA was extracted using a RNeasy kit (Qiagen) according to the manufacturer's instructions. The quality and concentration of RNA was determined with a spectrophotometer (Shimadzu UV mini-1240). The extraction of mRNA from the total RNA was carried out using a TaKaRa Oligotex™-dT30 ⟨super⟩ mRNA purification kit, and the concentration was determined with a spectrophotometer (Shimadzu UV mini-1240). The extracted mRNA was concentrated using Ethachinmate (Nippon Gene), and the concentrated mRNA (0.49 μg/mL for treated sample; 0.24 μg/mL for control) was subjected to the DNA microarray analysis [IntelliGene® Human Cancer CHIP ver. 4.0 (TaKaRa)].

4.4. Real time RT-PCR analysis

The RNA samples, obtained from cultured HeLa cells treated with three concentrations (0, 5, and 8 μg/mL) of **1**, were reverse transcribed using oligo$(dT)_{20}$ primers and the ThermoScript™ RT-PCR system (Invitrogen). Template cDNA thus obtained was incubated with 2.5 μM of gene-specific primers (Fasmac) and a qPCR™ Mastermix for SYBR® Green I (Nippon Gene) in a GeneAmp® 5700 Sequence Detection System (PE Biosystems). The thermal cycling program had an initial incubation (50 °C for 2 min) and an initial denaturation (95 °C for 10 min) and then 40 cycles of denaturation (95 °C for 15 s), annealing, and extension (60 °C for 1 min). The primer sets used were as follows: NM_002046 (glyceraldehydes-3-phosphate dehydrogenase (GAPDH)), 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTGGTGACC-3′; NM_003607 (MRCKα), 5′-GGAAGCCCAAATCACAGAAA-3′ and 5′-AGGCCTGAAGATACCCTCGT-3′; AF016266 (TRAIL-R2), 5′-GAGCTAAGTCCCTGCACCAC-3′ and 5′-AATCACCGACCTTGACCATC-3′; NM_001554 (Cyr61), 5′-CCTTGTGGACAGCCAGTGTA-3′ and 5′-TGGTCTTGCTGCATTTCTTG-3′; NM_012484 (RHAMM), 5′-TGCAGCTCAGGAACAGCTAA-3′ and 5′-GCTGACAGCGGAGTTTTGAT-3′. A fluorescence signal was collected at the end of each cycle. After the reactions were terminated, the signal at each temperature from 60 to 95 °C was also collected for the dissociation curve analysis. All reactions were performed in triplicate to confirm reproducibility, and the amount of target mRNA in each sample was normalized with that of mean GAPDH, an endogenous control.

4.5. Western blot analysis

HeLa cells treated and untreated with parviflorene F were washed with ice-cold PBS, and then lysed in lysis

buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 0.1 mM NaF, and 1% protease inhibitor cocktail (Nakarai tesque Inc.). The lysate was centrifuged for 10 min at 4 °C, and supernatants were stored at −80 °C until used. Equal amounts of protein were separated by 12.5% SDS–PAGE, and were transferred electrophoretically onto a Immun-blot PVDF membrane (Bio-Rad). After blocking, the membrane was incubated at room temperature with rabbit polyclonal anti-DR5 (Sigma), rabbit polyclonal anti-caspase-8 (Stressgen), or mouse monoclonal anti-β-actin (Sigma) as primary antibody. β-Actin was used as an internal control. After being washed with TBST, the membrane was incubated at room temperature with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies as secondary antibody. After further washing with TBST, immunoreactive bands were detected using the Immobilon western chemiluminescent HRP substrate (Millipore). The secondary antibodies were obtained from Amersham biosciences.

4.6. Cell cycle analysis

The cell cycle analysis was performed as described previously.[3,4] Briefly, HeLa cells (5×10^5) were treated with different concentrations of parviflorene F at 37 °C for 24 h, fixed with 70% ethanol at 4 °C for 60 min, and resuspended in 100 μg/mL of RNAse and 100 μg/mL of propidium iodide to stain DNA. The percentages of cells having the sub-G_1 DNA content was analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter). A minimum of 20,000 events were collected for each sample. All experiments were performed in triplicate.

4.7. Annexin V/PI staining

Apoptosis was evaluated by staining cells with annexin V-fluorescein isothiocyanate (FITC) and PI labeling. After treatment with parviflorene F, 1×10^6 cells were collected and washed twice with cold PBS. Then the cells were resuspended in 500 μl of binding buffer (50 mM Hepes/NaOH [pH 7.4], 75 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, and 9 mM $CaCl_2$), 5 μl of annexin V-FITC (PharMingen, San Diego, CA) and 5 μl of 20 μg/ml PI were added, the cells were incubated in the dark at 37 °C for 15 min, and a flow cytometric analysis was performed with a Cytomics FC500 flow cytometer (Beckman Coulter) within 1 h. Viable cells were defined as negative for annexin V-FITC and PI staining; apoptotic cells were defined as positive for annexin V-FITC staining but negative for PI staining, whereas late apoptotic cells displayed both high annexin V and PI staining. Cells positive for both annexin V and PI staining were considered necrotic. For assaying correlation with TRAIL-R2 expression and **1**-induced apoptosis, HeLa cells were incubated for 12 h with a combination of parviflorene F and 2.5 μg/mL of human recombinant TRAIL-R2/Fc chimera protein (Alexis). For the inhibition of caspase-8 and -9 activities, HeLa cells were incubated for 12 h with a combination of parviflorene F and 25 μM of z-IETD-fmk (MBL), a specific caspase-8 inhibitor, or 20 μM of z-LEHD-fmk (MBL), a specific caspase-9 inhibitor. A minimum of 20,000 events were collected for each sample. All experiments were performed in triplicate.

4.8. Caspase-3 activity

The activity of caspase-3 in the lysate was determined using an EnzCheck caspase-3 assay kit (Invitrogen) according to the manufacturer's directions. Briefly, the cells were cultured in the presence or absence of **1** (4 μg/mL), collected, and lysed. The reaction mixture (total volume, 100 μl) comprising 50 μl of cell lysate and 50 μl of a caspase-3 substrate working solution containing z-DEVD-R110 substrate was incubated for 30 min at 37 °C and the fluorescence was measured at 485 nm excitation and 538 nm emission using a fluorescence platereader. Equal amounts of protein were used in this assay after the whole protein concentration was calibrated using a BCA protein assay kit (Pierce). All experiments were performed in triplicate.

Acknowledgments

We thank Professor Kazuki Saito and Dr. Masaaki Noji (Chiba University) for the use of the real time PCR instrument, Dr. Hiroaki Kodama (Chiba University) for the use of the flow cytometer, Dr. Bingliang Fang, The University of Texas, MD, Anderson Cancer Center for generous gift of TRAIL-resistant DLD1 cells, and Dr. Masaaki Sato for helpful discussions at the beginning of this study. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, from the Kampou Science Foundation, and from the Shorai Foundation for Science and Technology.

References and notes

1. Jemal, A.; Murray, T.; Ward, E.; Samuels, A.; Tiwari, R. C.; Ghafoor, A.; Feuer, . J.; Thun, M. J. *CA Cancer J Clin.* **2005**, *55*, 10–30.
2. Pezzuto, J. M. *Biochem. Pharmacol.* **1996**, *53*, 121–133.
3. Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Sakai, S.; Kawahara, N.; Goda, Y.; Yamaguchi, N.; Ishibashi, M. *Bioorg. Med. Chem.* **2004**, *12*, 3841–3845.
4. Ohtsuki, T.; Sato, M.; Koyano, T.; Kowithayakorn, T.; Kawahara, N.; Goda, Y.; Ishibashi, M. *Bioorg. Med. Chem.* **2006**, *14*, 659–665.
5. Takahashi, M.; Koyano, T.; Kowithayakorn, T.; Hayashi, M.; Komiyama, K.; Ishibashi, M. *Tetrahedron Lett.* **2003**, *44*, 2327–2329.
6. Toume, K.; Takahashi, M.; Koyano, T.; Kowithayakorn, T.; Yamaguchi, K.; Hayashi, M.; Komiyama, K.; Ishibashi, M. *Tetrahedron* **2004**, *60*, 10817–10824.
7. Toume, K.; Sato, M.; Koyano, T.; Kowithayakorn, T.; Yamori, T.; Ishibashi, M. *Tetrahedron* **2005**, *61*, 6700–6706.
8. Tamaki, M.; Sadhu, S. K.; Ohtsuki, T.; Toume, K.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *Heterocycles* **2007**, *72*, 649–654.
9. Walczak, H.; Degli-Esposti, M. A.; Johnson, R. S.; Smolak, P. J.; Waugh, J. Y.; Boiani, N.; Timour, M. S.;

Gerhart, M. J.; Schooley, K. A.; Smith, C. A.; Goodwin, R. G.; Rauch, C. T. *EMBO J.* **1999**, *16*, 5386–5397.
10. Zhang, L.; Gu, J.; Lin, T.; Huang, X.; Roth, J. A.; Fang, B. *Gene Ther.* **2002**, *9*, 1262–1270.
11. Serradell, M. C.; Guasconi, L.; Cervi, L.; Chiapello, L. S.; Masih, D. T. *Vet. Immunol. Immunopathol.* **2007**, *117*, 197–208.
12. Suliman, A.; Lam, A.; Datta, R.; Srivastava, R. K. *Oncogene* **2001**, *20*, 2122–2133.
13. Sinicrope, F. A.; Penington, R. C. *Mol. Cancer Ther.* **2005**, *4*, 1475–1483.
14. Ashkenazi, A.; Dixit, V. M. *Science* **1998**, *281*, 1305–1308.
15. Ashkenazi, A.; Dixit, V. M. *Curr. Opin. Cell Biol.* **1999**, *11*, 255–260.
16. Chen, X.; Thakkar, H.; Tyan, F.; Gim, S.; Robinson, H.; Lee, C.; Pandey, S. K.; Nwokorie, C.; Onwudiwe, N.; Srivastava, R. K. *Oncogene* **2001**, *20*, 6073–6083.
17. Singh, T. R.; Shankar, S.; Chen, X.; Asim, M.; Srivastava, R. K. *Cancer Res.* **2003**, *63*, 5390–5400.
18. Pukac, L.; Kanakaraj, P.; Humphreys, R.; Alderson, R.; Bloom, M.; Sung, C.; Riccobene, T.; Johnson, R.; Fiscella, M.; Mahoney, A.; Carrell, J.; Boyd, E.; Yao, X. T.; Zhang, L.; Zhong, L.; von Kerczek, A.; Shepard, L.; Vaughan, T.; Edwards, B.; Dobson, C.; Salcedo, T.; Albert, V. *Br. J. Cancer* **2005**, *92*, 1430–1441.
19. Zeng, Y.; Wu, X. X.; Fiscella, M.; Shimada, O.; Humphreys, R.; Albert, V.; Kakehi, Y. *Int. J. Oncol.* **2006**, *28*, 421–430.
20. Hasegawa, H.; Yamada, Y.; Komiyama, K.; Hayashi, M.; Ishibashi, M.; Yoshida, T.; Sakai, T.; Koyano, T.; Kam, T.-S.; Murata, K.; Sugahara, K.; Tsuruda, K.; Akamatsu, N.; Tsukasaki, K.; Masuda, M.; Takasu, N.; Kamihira, S. *Blood* **2006**, *107*, 679–688.
21. Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Sakai, T. *Mol. Cancer Ther.* **2006**, *5*, 945–951.
22. Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Sakai, T. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 833–838.
23. Tomasetti, M.; Andera, L.; Alleva, R.; Borghi, B.; Neuzil, J.; Procopio, A. *FEBS lett.* **2006**, *580*, 1925–1931.
24. Jung, E. M.; Park, J.; Choi, K. S.; Park, J.; Lee, H. I. L.; Lee, K. S.; Kwon, T. K. *Carcinogenesis* **2006**, *27*, 2008–2017.
25. Zhu, H.; Guo, W.; Zhang, L.; Wu, S.; Teraishi, F.; Davis, J. J.; Dong, F.; Fang, B. *Cancer Biol. Ther.* **2005**, *4*, 781–786.
26. Nakata, S.; Yoshida, T.; Shiraishi, T.; Horinaka, M.; Kouhara, J.; Wakada, M.; Sakai, T. *Mol. Cancer Ther.* **2006**, *5*, 1827–1835.
27. Kelley, R. F.; Totpal, K.; Lindstrom, S. H.; Mathieu, M.; Billeci, K.; DeForge, L.; Pai, R.; Hymowitz, S. G.; Ashkenazi, A. *J. Biol. Chem.* **2005**, *280*, 2205–2212.
28. Larsson, R.; Kristensen, J.; Sandberg, C.; Nygren, P. *Int. J. Cancer* **1992**, *50*, 177–185.