Bioorganic & Medicinal Chemistry 17 (2009) 6748–6754

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Death receptor 5 promoter-enhancing compounds isolated from *Catimbium speciosum* and their enhancement effect on TRAIL-induced apoptosis

Takashi Ohtsuki [a], Hiroyuki Kikuchi [a], Takashi Koyano [b], Thaworn Kowithayakorn [c], Toshiyuki Sakai [d], Masami Ishibashi [a,*]

[a] *Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*
[b] *Temko Corporation, 4-27-4 Honcho, Nakano, Tokyo 164-0012, Japan*
[c] *Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand*
[d] *Department of Molecular-Targeting Cancer Prevention, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan*

ARTICLE INFO

Article history:
Received 30 May 2009
Revised 17 July 2009
Accepted 21 July 2009
Available online 24 July 2009

Keyword:
DR5
TRAIL resistance
Cardamomin
Catimbium speciosum
Apoptosis

ABSTRACT

The TRAIL/death-receptor signaling pathway has been considered a promising target for selective cancer therapy, although some malignant tumors exhibit TRAIL resistance. We previously found that isoflavonoid enhanced TRAIL-induced apoptosis in TRAIL-resistant cells, which is achieved through up-regulation of death receptor 5 (DR5). In our screening program targeting DR5 promoter enhancement activity, activity-guided fractionations of the extract of *Catimbium speciosum* led to the isolation of six compounds. Of the isolates, cardamomin (**6**), the most potent compound, enhanced the expressions of DR5 and DR4 and decreased the Bcl-xL level in TRAIL-resistant DLD1 cells. The combination of **6** and TRAIL synergistically enhanced TRAIL-induced apoptosis against TRAIL-resistant cells upon the activation of caspase-8, 9, and 3. In addition, enhancement of apoptosis by **6** was inhibited by human recombinant DR5/Fc and DR4/Fc chimera proteins, TRAIL-neutralizing fusion proteins, indicating that **6** sensitize TRAIL-resistant cells to TRAIL through the induction of DR5 and DR4. Also, up-regulation of DR5 by **6** paralleled that of CCAAT/enhancer-binding protein-homologous protein (CHOP).

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) belongs to the tumor necrosis factor family and is distributed in a wide range of cancer cell types.[1] TRAIL binds with death receptors such as DR5 (death receptor 5 = TRAIL-R2) or DR4 (death receptor 4 = TRAIL-R1) and induces apoptosis in a various cancer cells without affecting most normal cells *in vitro* or *in vivo*.[2–6] Thus, the TRAIL/death-receptor signaling pathway has been considered a promising target for selective cancer therapy, however, it has become a problem that considerable types of cancer cells as malignant glioma and breast cancer cells are resistant to apoptosis induction by TRAIL.[7–10] It was reported that several chemotherapeutic agents and natural products, such as rottlerin,[11] withaferin A,[12] kaempferol,[13] and PS-341 (bortezomib),[14] increased the expression of DR5, and caused the sensitization of TRAIL-resistant tumor cells to TRAIL-induced apoptosis. These previous reports indicated that it was an excellent strategy to increase the number of death receptors against TRAIL-resistant tumor cells.

During our search for natural bioactive products in tropical plants, we previously identified natural products with DR5-inducing activity, such as cardinane-sesquiterpene dimer,[15] new isoflavones,[16,17] and new flavonoids.[18] In the course of extensive studies of the search for natural products with DR5-inducing activity from plants, we found that the MeOH extract of leaves of *Catimbium speciosum* (Zingiberaceae) was potently active.

C. speciosum (synonym: *Alpinia speciosa*) is species distributed in subtropical areas.[19] The rhizomes of this plant have pharmacological activities, including antiplatelet activity,[19] and its seeds have been used as an aromatic stomachic in Japan.[20] Moreover, the leaves and seeds of this plant are used for food wrapping and health maintenance, respectively, in the Okinawa area of Japan.[21] Previous chemical investigations of *C. speciosum* by other groups led to the isolation of α-pyrone,[19] diterpene,[20] sesquiterpeme,[22] flavonoid,[23] and calcone.[23,24]

Here, we describe the activity-guided isolation of *C. speciosum*, which has led to the identification of six compounds (**1**–**6**) with DR5 promoter-enhancing activity (=DR5-inducing activity). We also described that the combination of a nonapotosis-inducing dose of compound **6**, the most active compound, and TRAIL synergistically led to the induction of apoptosis in TRAIL-resistant human colon cancer (DLD1/TR) and human gastric adenocarcinoma

* Corresponding author. Tel./fax: +81 43 290 2913.
E-mail address: mish@p.chiba-u.ac.jp (M. Ishibashi).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.07.041

(AGS) cells. Moreover, we found that the mechanism of this apoptosis induction against DLD1/TR cells correlated with the up-regulation of DR4 and DR5 following the activation of caspase-8, 9, and 3. Up-regulation of DR5 by **6** paralleled that of CCAAT/enhancer-binding protein-homologous protein (CHOP).

2. Results

The MeOH extract of the leaves of *C. speciosum* showed the up-regulation of DR5 promoter activity (3.1-fold at 100 μg/mL), partitioned between hexane and 10% aqueous MeOH, and the aqueous phase was further extracted with EtOAc and *n*-BuOH to give four fractions. Among them, the most potent activity was found in the EtOAc-soluble fraction (DR5 promoter activity; EtOAc-soluble fraction: 4.8-fold at 100 μg/mL; hexane soluble fraction: 2.4-fold at 100 μg/mL; other fractions: inactive). In order to identify the active compounds responsible for the activity in the EtOAc-soluble fraction, it was fractionated by silica gel, ODS, and Sephadex LH-20 column chromatography as well as ODS-HPLC to isolate six known compounds, such as pinocenbrin (**1**),[25] naringenin (**2**),[26] 3-methoxykaempferol (**3**),[27] pinocembrin chalcone (**4**),[28,29] 5,6-dehydrokawain (**5**),[30] and cardamomin (**6**).[30] The structures of these compounds were identified by comparing their spectral data (NMR, EIMS, and $[\alpha]_D$) with those in the literature (Fig. 1).

The isolated compounds (**1**–**6**) were evaluated for DR5 promoter activity by the luciferase assay using DLD-1/*Sac*I cells. As shown in Figure 2, compound **6** was found to be the most active and caused 4.1-fold enhancement of DR5 promoter activity at 18.5 μM, which showing greater potency than luteolin at 17.4 μM, used as a positive control.[31,32] On the other hand, compounds **1** and **5** showed moderate activity of over 2.5-fold enhancement at 39.0 and 43.9 μM, respectively. Also, the IC_{50} value of **6** against DLD-1/*Sac*I cells was determined following a 24 h exposure to 41.8 μM in cytotoxicity study.

In order to evaluate the effect of compound **6** against DLD1/TR cells, we next investigated changes in the expression of the death receptor pathway-related gene using Western blotting and real-time quantitative PCR analysis. As shown in Figure 3A, we found that treatment against DLD1/TR cells with **6** for 24 h significantly increased the protein level of DR5 in a dose-dependent manner. Real-time RT-PCR analysis also revealed that **6** enhanced the gene expression of DR5 by 2.0 and 4.1 times at concentrations of 9.3 and 18.5 μM, respectively (Fig. 3B). Moreover, the gene expression of DR4 with **6** treatment increased in a dose-dependent manner, compared with the control (Fig. 3C). Recently, the

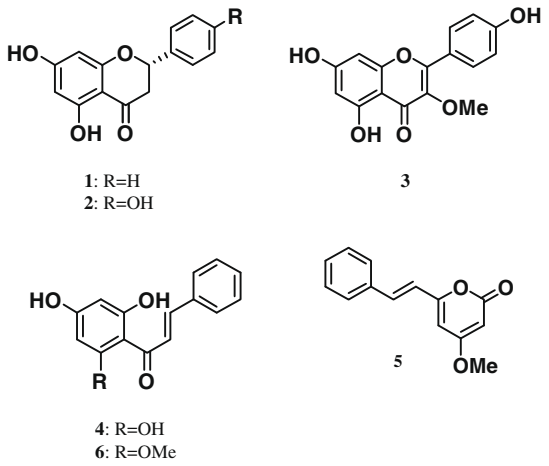

Figure 1. Structures of compounds **1**–**6**.

expression of cellular FLICE-like inhibitory protein (c-FLIP) has been implicated as an inhibitor of apoptosis mediated by death receptors,[33–36] therefore, we evaluated the protein expression level of c-FLIP in **6**-treated cells. However, we observed no change in the protein level of c-FLIP (Fig. 3A). We also checked changes in the expression of BAK (pro-apoptotic member of the Bcl-2 family) and Bcl-xL (anti-apoptotic member of the Bcl-2 family), the key protein in the intrinsic pathway, using Western blot analysis since recent data indicated the existence of considerable cross-talk between the extrinsic and intrinsic death signal pathway.[37] As shown in Figure 3A and D, treatment of DLD1/TR cells with **6** at concentrations of 9.3 and 18.5 μM for 24 h decreased mRNA and protein levels of Bcl-xL in a dose-dependent manner, while BAK expression was unaffected by treatment with **6** (Fig. 3A). These results suggested that combine treatment of TRAIL-resistant cells with **6** enhanced TRAIL-induced apoptosis through both extrinsic and intrinsic pathways by up-regulation of DR5 and/or DR4.

We next investigated the enhancement effects of TRAIL-induced apoptosis of **6** against DLD1/TR and TRAIL-resistant human gastric adenocarcinoma (AGS) cells by treatment with **6**, TRAIL, or their combined treatment.

First, the occurrence of apoptosis was determined by flow cytometric analysis of sub-G1, which is considered a marker of apoptotic DNA fragmentation, using propidium iodide (PI) staining. As shown in Figure 4A, for treatment with 100 ng/mL TRAIL for 24 h of DLD1/TR cells, sub-G1 increased only 22.0 ± 4.0%, which was similar to the result with **6** alone (15.5 ± 1.0%). On the other hand, treating cells with 100 ng/mL TRAIL and 9.3 and 18.5 μM **6** increased sub-G1 to 41.9 ± 2.5% and 55.8 ± 1.4%, respectively. In AGS cells, combined treatment of the compounds and TRAIL caused greater increase in sub-G1 than with **6** or TRAIL alone (Fig. 4B).

To further evaluate the enhancement effects of TRAIL-induced apoptosis, both cells were also stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V, a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface, and PI in order to detect apoptosis, specificity. As shown in Figure 4C, in combined treatment with 9.3 μM **6** and 100 ng/mL TRAIL, the proportion of early apoptotic cells (lower right) and late apoptotic cells (upper right) increased 14.1 ± 0.9% versus 5.7 ± 0.8% for the control and 14.3 ± 0.8% versus 7.4 ± 0.5% for the control, respectively, against DLD1/TR cells. Also, in combined treatment with 18.5 μM **6** and 100 ng/mL TRAIL, the proportion of early apoptotic cells (26.7 ± 1.6% vs 5.7 ± 0.8% for the control) and late apoptotic cells (upper right) (20.5 ± 0.9% vs 7.4 ± 0.5%) significantly increased. Similarly, in AGS cells, combined treatment resulted in a significant increase of the proportion of apoptotic cells, indicating that **6** enhanced TRAIL-induced apoptosis against TRAIL-resistant DLD1/TR and AGS cells.

To determine whether the apoptosis enhanced by **6** was mediated via a caspase cascade, we first measured the activation of caspase-3 by a luminescent assay. As shown in Figure 5A, combine treatment with **6** and TRAIL caused marked activation of caspase-3 in a dose-dependent manner. Also, Western blot analysis of treated cells was performed to assess the activation of caspase-8, which was characterized by the decrease of precursor protein, procaspase-8, in the extrinsic apoptotic pathway. As shown in Figure 5B, the protein level of procaspase-8 was significantly decreased in DLD1/TR cells exposed to combined treatment, especially 18.5 μM **6**. Similarly, flow cytometric analysis of sub-G1 with a specific caspase-8 inhibitor (z-IETD-fmk) also revealed that the addition of 25 μM z-IETD-fmk efficiently suppressed apoptosis enhanced by combined treatment with **6** and TRAIL since the decreased sub-G1 was observed compared to combined treatment

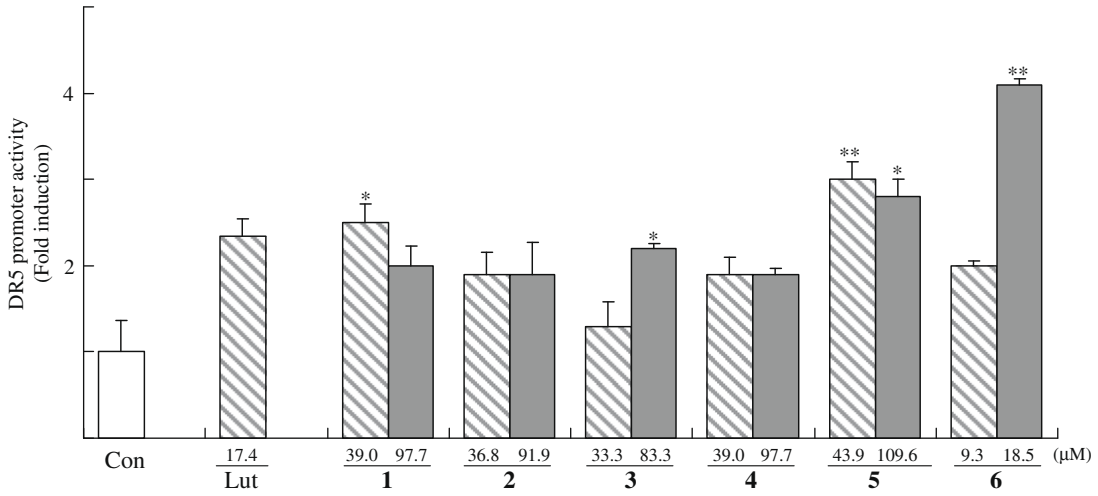

Figure 2. Activation of DR5 promoter activity by compounds **1**–**6**. Bar represents the means ± SD of three independent experiments. Significant differences in the activation of DR5 promoter activity were shown at p <0.05 (*), p <0.01 (**), compared with the control (Con). Luteolin (Lut) was used as the positive control.

without an inhibitor (Fig. 6). Furthermore, we evaluated the activation of caspase-9, which plays a crucial role in the initiation phase of the intrinsic apoptosis pathway, using flow cytometric analysis with a specific caspase-9 inhibitor (z-LEHD-fmk). As shown in Figure 6, treatment of cells with a caspase-9 inhibitor significantly suppressed apoptosis enhanced by combined treatment. These results indicated that TRAIL-induced apoptosis enhanced by **6** involved the activation of caspase-8, 9, and 3.

From the above results, it was suggested that up-regulation of DR5 and DR4 expression played a significant role in apoptosis induced by combined treatment of TRAIL-resistant cells with **6** and TRAIL. Accordingly, to clarify the functional role of DR5 and/or DR4 up-regulation, we investigated using recombinant human DR5/Fc and DR4/Fc chimera proteins, which have a dominant-negative effect by competing with endogenous DR5 and DR4, respectively. As shown in Figure 6, the addition of 9.3 μM DR5/Fc chimera significantly blocked the apoptosis induced by combined treatment. DR4/Fc chimera protein also suppressed apoptosis. These results collectively indicated that up-regulation of both death receptors is important for enhancement of TRAIL-induced apoptosis by **6**.

In previous studies, it was reported that CHOP had been considered to play an important role in regulating DR5 expression, and a CHOP binding site was identified in DR5 promoter.[38–40] To understand the mechanism of increased DR5 expression by **6**, we next analyzed the expression of CHOP. As shown in Figure 7, it was found that the expression of CHOP mRNA increased in a dose-dependent manner with **6** treatment of DLD1/TR cells, indicating that the increase of DR5 expression by **6** mediated the induction of CHOP.

3. Discussion

In the present study, activity-guided separation from *C. speciosum* led to the isolation of six compounds (**1**–**6**) with DR5 promoter-enhancing activity. Among them, cardamomin (**6**) was the most potent compound in enhancing DR5 promoter activity. We also found that treatment of DLD1/TR cells with **6** significantly increased not only the expression of DR5 (mRNA and protein level) but also that of DR4 (mRNA level) by Western blotting and real-time RT-PCR analyses (Fig. 3A–C). It is generally recognized that both the extrinsic and intrinsic apoptotic pathways for apoptosis, are regulated by multiple proteins, such as c-FLIP, and the Bcl-2 family, such as Bax, Bcl-2, and Bcl-xL.[41] Therefore, we focused on c-FLIP, BAK, and Bcl-xL in this study and our results showed that **6** alone decreased mRNA and protein levels of Bcl-xL against DLD1/TR cells (Fig. 3A and D). Decrease of Bcl-xL suggested the inhibition of NF-κB, since **6** have been reported to possess a NF-κB inhibitory activity[42] and thus inhibition of NF-κB correlated with inhibition of Bcl-xL.[43] Previously,[11–14] it was reported that several chemotherapeutic drugs and natural products induced the expression of DR4 and/or DR5. The activation of death 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.[44,45] In fact, combined treatment with **6** and TRAIL showed synergistic enhancement of TRAIL-induced apoptosis against DLD1/TR and AGS cells in this study (Fig. 4C). Caspase activation plays crucial roles in both the extrinsic and intrinsic apoptotic pathways.[46] Our results indicated that combine treatment induced an increase in caspase-3 activity in a dose-dependent manner (Fig. 5A). Also, Western blot and flow cytometric analyses showed that the initiation of apoptotic signaling by treatment with **6** led to the activation of caspase-8 (Figs. 5B and 6). Similarly, apoptosis by combined treatment significantly prevented by caspase-9 inhibitor (z-LEHD-fmk), suggesting that it occurred through both extrinsic and intrinsic pathways (Fig. 6). Our study further demonstrates that combine treatment of DLD1 cells with **6** enhanced TRAIL-induced apoptosis by up-regulating DR5 and DR4. This is evidenced by the prevention of apoptosis by combined treatment using human recombinant DR5/Fc, and DR4/Fc chimera protein (Fig. 6). Recently, the induction mechanism of DR5 expression was correlated to CHOP, a transcriptional factor known to regulate DR5 expression via binding to its promoter region.[38–40] Our results showed that CHOP expression was induced by **6** at the mRNA level in DLD1/TR cells. Thus, we concluded that **6** induced CHOP-dependent DR5 up-regulation. On the other hand, **6** have been reported to have the potent inhibition of NF-κB activation as mentioned above.[42] Also, a recent study has been indicated that inhibition of NF-κB resulted in inhibition of Yin Yang 1, a negative regulator of DR5, leading to the up-regulation of DR5.[47,48] From these reports, DR5 up-regulation by **6** may be correlated with the inhibition of NF-κB and Yin Yang 1.

Compound **6** was isolated by activity-guided separation with DLD-1/*Sac*I cells, a human colon cancer cell line stably transfected with the pDR5/*Sac*I plasmid. The pDR5/*Sac*I plasmid does not contain a p53-binding site for the luciferase reporter assay.[49] Although

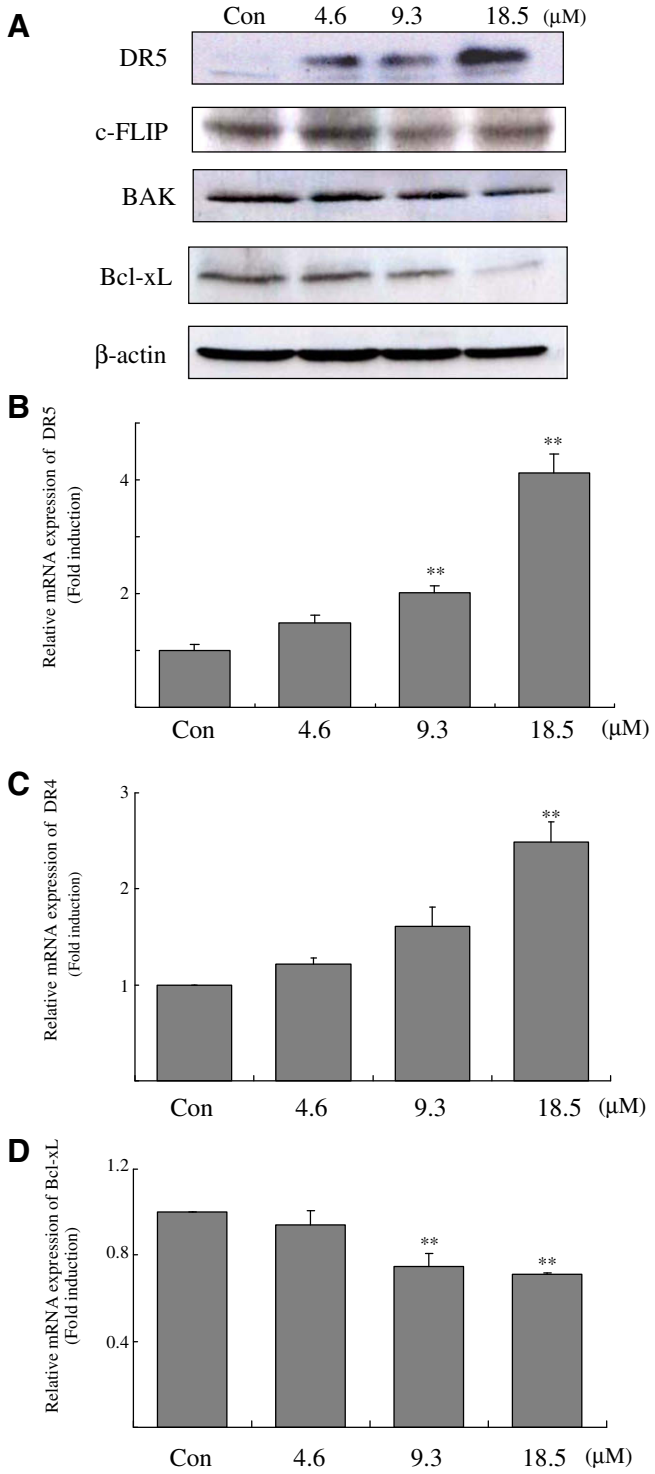

Figure 3. The effect of cardamomin (**6**) on DR5, c-FLIP, Bak, and Bcl-xL in DLD1/TR cells. (A) Western blot analysis of DR5, c-FLIP, Bak, Bcl-xL. (B)- (D) Real-time RT-PCR analysis of DR5 (B), DR4 (C), and Bcl-xL (D). Bar represents the means ± SD of three independent experiments. Significance was determined by Student's *t*-test (**, p <0.01 compared with the control).

it was reported that the p53 tumor-suppressor gene regulated DR5 gene expression, p53 was inactivated in half of all human cancers.[50] Accordingly, it was suggested that **6** induced the enhancement of DR5 promoter activity by a p53-independent mechanism. Thus, **6** might also be useful in the treatment of various TRAIL-resistant tumor cells with p53 mutations in combination with TRAIL.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured with a JASCO P-1020 polarimeter. NMR spectra were recorded on JEOL A500 and ECP600 spectrometers with a deuterated solvent, the chemical shift of which was used as an internal standard. EIMS was measured on a JEOL JMS-GC Mate.

4.2. Plant material

Rhizomes of *Catimbium speciosum* were collected in Thailand in November 1999 and were identified by T. Kowithayakorn. A voucher specimen (6-265) is maintained in our laboratory.

4.3. Extraction and isolation

Air-dried rhizomes (379 g) were extracted with MeOH. The MeOH extract (26.2 g) was partitioned between hexane (200 mL × 3) and 10% aqueous MeOH (200 mL), and the aqueous phase was further extracted with EtOAc (200 mL × 3) and BuOH (200 mL × 3) to obtain four fractions (hexane soluble fraction, 0.24 g; EtOAc-soluble fraction, 13.7 g; BuOH soluble fraction, 5.9 g; aqueous soluble fraction, 4.9 g). Part of the EtOAc-soluble fraction (4.0 g) was subjected to silica gel PSQ100B column chromatography (48 × 300 mm) eluted with a stepwise gradient of mixtures ($CHCl_3$/MeOH: 100/0, 98/2, 95/5, 90/10, 80/20, and 0/100) to give 10 fractions (1A–1J). Fraction 1E (328 mg), eluted with $CHCl_3$/MeOH (95/5 and 90/10), was subjected to Sephadex LH-20 column chromatography (22 × 780 mm) eluted with MeOH, followed by further separation with silica gel PSQ100B column chromatography (12 × 180 mm) eluted with $CHCl_3$/MeOH (95/5) to afford compound **1** (32 mg) together with a fraction containing other compounds **1**, **2**, **3**, and **4**, which were further purified with MPLC (Ultrapack ODS-S-50A, 11 × 300 mm; eluent, 60% MeCN; UV detection, 220 nm) and HPLC (Develosil ODS UG-5, 10 × 250 mm; eluent, 45% MeCN; flow rate, 1.0 mL/min; UV detection, 220 nm) to give compound **1** (30.1 mg), **2** (2.2 mg), **3** (1.2 mg), and **4** (2.7 mg). Fraction 1C (1177 mg), eluted with $CHCl_3$/MeOH (95/5), was subjected with Silica Gel 60N column chromatography (22 × 300 mm) with eluted with hexane/EtOAc (1/1) to afford compound **5** (200 mg) together with a fraction containing compound **6**, which was further purified with Sephadex LH-20 column chromatography (10 × 480 mm) with eluted with $CHCl_3$/MeOH (3/2) to afford compound **6** (21.9 mg).

4.4. Cell cultures

AGS cells were derived from the Institute of Development, Aging and Cancer, Tohoku University; DLD1/TR cells were a generous gift from Dr. Bingliang Fang (The University of Texas M. D. Anderson Cancer Center). Both cells were cultured in RPMI-1640 medium (Wako) with 10% FBS. Cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air.

4.5. Luciferase assay to assess the enhancement of DR5 promoter activation

The assay procedure was as previously described.[16] Briefly, DLD-1/*Sac*I cells (2 × 10^5 cells per well), a human colon cancer cell line stably transfected with the DR5 promoter-luciferase reporter plasmids, pDR5/*Sac*I,[49] were treated with different concentrations of each isolated compound for 24 h at 37 °C. After medium containing the isolated compounds was removed, cells were lysed in a Cell

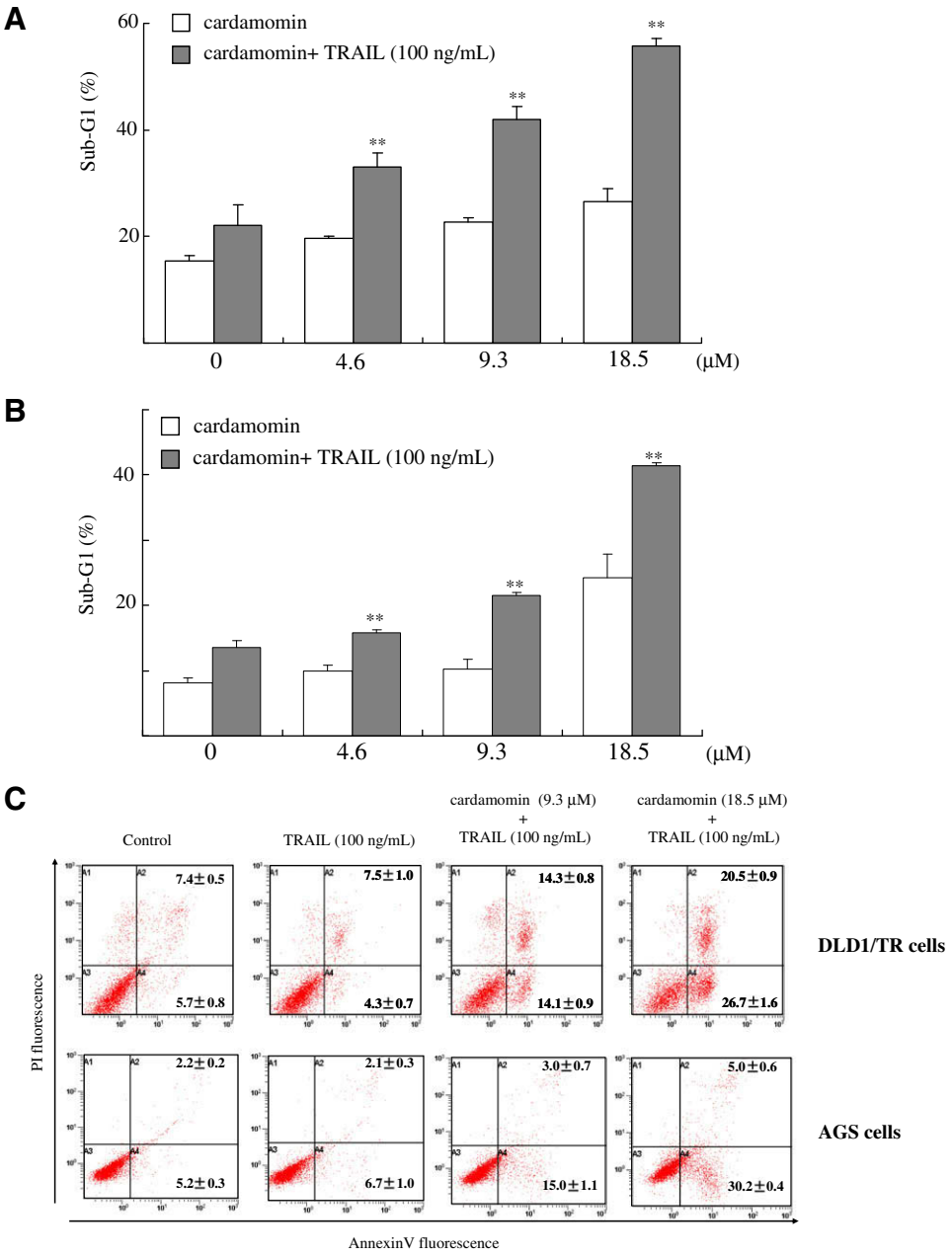

Figure 4. Cardamomin (**6**) sensitized TRAIL-induced apoptosis in DLD1/TRAIL-R and AGS cells. (A) Sub-G1 population of DLD1/TR cells treated with a combination of cardamomin (**6**) and TRAIL. (B) Sub-G1 population of AGS cells treated with a combination of cardamomin (**6**) and TRAIL. Bar represents the means ± SD of three independent experiments. Significance was determined by Student's *t*-test (**, p <0.01 compared with cardamomin alone). (C) Results of annexin V- and propidium iodide-based apoptosis analysis for combined treatment of DLD1/TR and AGS cells. Each value represents the mean ± SD of three independent experiments.

Culture Lysis Reagent (Promega). The lysate was measured for 10 s as relative light units by a luminometer and DR5 promoter activity was evaluated by relative light units of the sample compared with the control (cells treated with EtOH).

4.6. Real-time RT-PCR analysis

Total RNA was extracted from DLD-1/TR cells (5×10^5 cells) with an RNeasy Mini Kit (Qiagen). cDNAs were synthesized from 500 ng total RNA using SuperScript III Platinum SYBR Green Two-Step qRT-PCR Kit (Invitrogen) as recommended by the supplier. Template cDNA thus obtained was incubated with 200 nM gene-specific primers (Fasmac) and with a SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen) in a Mx3000 QPCR System (Stratagene). The thermal cycling program had initial incubation (50 °C for 2 min) and initial denaturation (95 °C for 2 min) and then 40 cycles of denaturation (95 °C for 15 s), annealing and extension (60 °C for 30 s). The primer sets were as follows: glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-TAAAAGCAGCCCTGGTGACC-3′; DR5, 5′-GAGCTAAGTCCCTGCACCAC-3′ and 5′-AATCACCGACCTTGACCATC-3′; DR4, 5′-CCGCGGCCACACCCAGAAAGT-3′ and 5′-GTACATGGGAGGCAAGCAAACAAA-3′; Bcl-xL, 5′- TCCTTGTCTA- CGCTTTCCACG-3′ and 5′-GGTCGCATTGTGGCCTTT-3; CHOP (CCAAT/enhancer-binding protein-homologous protein), 5′-CAACTGCAGAGATG- GCAGCTGA-3′ and 5-CTGATGCTCCCAATTGTTCAT-3′. A fluorescence signal was collected at the end of each cycle. After the reactions were terminated, the signal at each temperature from

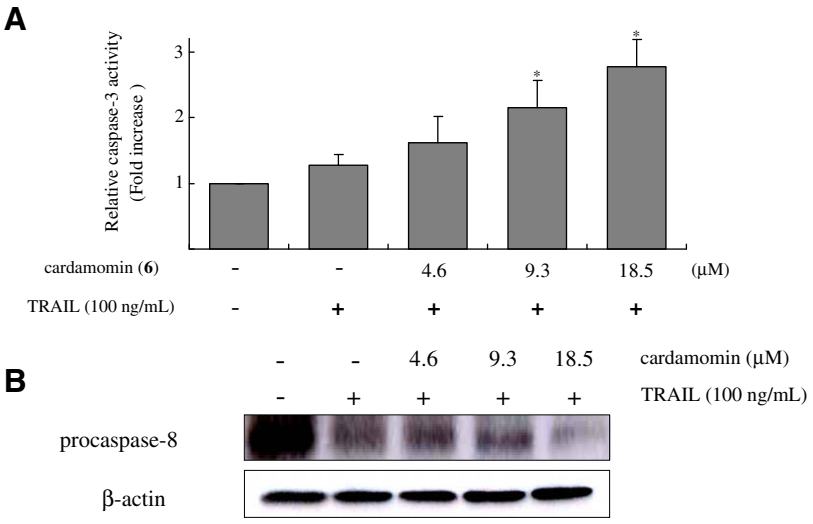

Figure 5. Combined treatment of cardamomin (**6**) and TRAIL enhances caspase activity. (A) Effects of combined treatment on caspase-3 activity. Activity in the control was defined as 1. Bar represents the mean ± SD of three independent experiments. Significance was determined by Student's *t*-test (, p <0.01 compared with the untreated control). (B) Western blot analysis of pro-caspase-8 (55 kDa) in DLD1/TR cells by combined treatment.

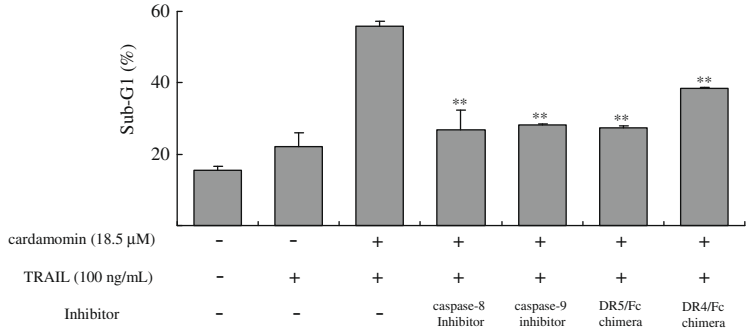

Figure 6. TRAIL-induced apoptosis enhanced the combined treatment inhibited by caspase-8 and -9 inhibitors, DR5/Fc chimera and DR4/Fc chimera proteins. Bar represents the mean ± SD of three independent experiments. Each value represents the mean ± SD of three independent experiments. Significance was determined by Student's *t*-test (, p <0.01 compared with combined treatment of cardamomin and TRAIL).

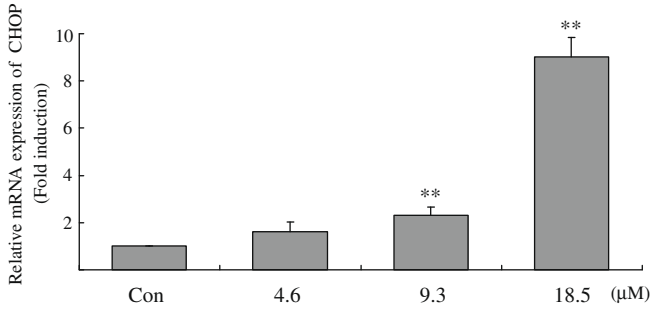

Figure 7. Expression of CHOP in DLD1/TR cells treated with cardamomin (**6**). Bar represents the mean ± SD of three independent experiments. Significance was determined by Student's *t*-test (, p <0.01 compared with untreated control).

60 to 95 °C was also collected for 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.

4.7. Western blot analysis

DLD1/TR cells were lysed as previously described,[15] and equal amounts of protein were separated by 15% (BAK and Bcl-xL), 12.5% (DR5), or 10% (c-FLIP and caspase-8) SDS–PAGE, and were transferred onto an Immun-blot PVDF membrane (Bio-Rad). After blocking, the membrane was incubated for 1 h at room temperature with rabbit polyclonal anti-DR5 (Sigma), rabbit polyclonal anti-caspase-8 (Stressgen), rabbit polyclonal anti-caspase-8, mouse monoclonal anti-c-FLIP (ALEXIS), rabbit polyclonal anti-BAK (Sigma), mouse monoclonal anti-Bcl-xL (Sigma), or mouse monoclonal anti-β-actin (Sigma) as the primary antibody. β-Actin was used as an internal control. After washing with TBST, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody as the secondary antibody (Amersham Biosciences) followed by detection using the Immobilon western chemiluminescent HRP substrate (Millipore).

4.8. Sub-G1 analysis

Sub-G1 analysis was performed as described previously.[15] Briefly, DLD1/TR or AGS cells (5×10^5) were treated with compound **6**, TRAIL, or a combine of **6** and TRAIL at 37 °C for 24 h, fixed with 70% ethanol at 4 °C for 60 min, and resuspended in 100 μg/mL RNAse and 100 μg/mL propidium iodide to stain DNA. The percentages of cells with the sub-G_1 DNA content were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter). For the inhibition of caspase-8 and -9 activities, DLD1/TR cells were incubated

for 24 h with a combination of 18.5 μM compound **6**, 100 ng/mL TRAIL, 25 μM z-IETD-fmk (MBL), a specific caspase-8 inhibitor, or 20 μM z-LEHD-fmk (MBL), a specific caspase-9 inhibitor. To assay the correlation with DR5 and/or DR4 expression and enhancement of TRAIL-induced apoptosis by combine treatment, DLD1/TR cells were incubated for 24 h with a combination of 18.5 μM compound **6**, 100 ng/mL TRAIL, and 2.5 μg/mL human recombinant DR5/Fc chimera or DR4/Fc chimera protein (Alexis). A minimum of 20,000 events were collected for each sample. All experiments were performed in triplicate.

4.9. Annexin V/PI staining

Apoptosis was evaluated by staining cells with annexin V-fluorescein isothiocyanate (FITC) and PI labeling as described previously.[15] Briefly, DLD1/TR or AGS cells (5×10^5) were treated with 9.3 and 18.5 μM compound **6** and/or 100 ng/mL TRAIL for 24 h, and then collected and washed twice with cold PBS solutions. The cells were resuspended in 500 μL binding buffer, 5 μg/mL annexin-V-FITC (Annexin-V-FLUOS Staining Kit, Roche), and 5 μL of 20 μg/mL propidium iodide, and mixed and incubated for 15 min at room temperature in the dark. 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.

4.10. Caspase-3 activity

Caspase-3 activity was measured using the Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions, as described previously.[17] Briefly, cells were treated with 4.6, 9.3, and 18.5 μM compound **6** and/or 100 ng/mL TRAIL for 24 h, collected, and lysed. Cell lysates were incubated with a luminogenic substrate, z-DEVD-amino-luciferin at room temperature for 1 h and luminescence was monitored using a luminometer.

Acknowledgements

We thank Dr. Bingliang Fang, The University of Texas M. D. Anderson Cancer Center, for the generous gift of TRAIL-resistant DLD1 cells. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by a Grant-in-Aid to T.O. from Takeda Science Foundation and from Mitsubishi Chemical Corporation Fund.

References and notes

1. Wiley, S. R.; Schooley, K.; Smolak, P. J.; Din, W. S.; Huang, C. P.; Nicholl, J. K.; Sutherland, G. R.; Smith, T. D.; Rauch, C.; Smith, C. A.; Goodwin, R. G. *Immunity* **1995**, *3*, 673.
2. Ishibashi, M.; Ohtsuki, T. *Med. Res. Rev.* **2008**, *28*, 688.
3. 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.* **1997**, *16*, 5386.
4. Ashkenazi, A.; Dixit, V. M. *Curr. Opin. Cell Biol.* **1999**, *11*, 255.
5. 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.
6. Singh, T. R.; Shankar, S.; Chen, X.; Asim, M.; Srivastava, R. K. *Cancer Res.* **2003**, *63*, 5390.
7. Van Geelen, C. M.; de Varis, E. G.; de Jong, S. *Drug Resist. Updat.* **2004**, *7*, 345.
8. Kim, E. H.; Kim, S. U.; Shim, D. Y.; Choi, K. S. *Oncogene* **2004**, *23*, 446.
9. Kim, E. H.; Kim, H. S.; Kim, S. U.; Noh, E. J.; Lee, J. S.; Choi, K. S. *Oncogene* **2005**, *24*, 6877.
10. Park, C.; Moon, D. O.; Ryu, C. H.; Choi, B. T.; Lee, W. H.; Kim, G. Y.; Choi, Y. H. *Acta Pharmacol. Sin.* **2008**, *29*, 341.
11. Lim, J. H.; Park, J. W.; Choi, K. S.; Park, Y. B.; Kwon, T. K. *Carcinogenesis* **2009**, *30*, 729.
12. Lee, T. J.; Um, H. J.; Min, D. S.; Parl, J. W.; Choi, K. S.; Kwon, T. K. *Free Rad. Biol. Med.* **2009**, *46*, 1639.
13. Yoshida, T.; Konishi, M.; Horinaka, M.; Yasuda, T.; Goda, A. E.; Taniguchi, H.; Yano, K.; Wakada, M.; Sakai, T. *Biochem. Biophys. Res. Commun.* **2008**, *375*, 129.
14. Chen, S.; Hu, L.; Lonical, S.; Khuri, F. R.; Sun, S. Y. *Cancer Res.* **2007**, *67*, 4981.
15. Ohtsuki, T.; Tamaki, M.; Toume, K.; Ishibashi, M. *Bioorg. Med. Chem.* **2008**, *16*, 1756.
16. Kikuchi, H.; Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *J. Nat. Prod.* **2007**, *70*, 1910.
17. Kikuchi, H.; Ohtsuki, T.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *Bioorg. Med. Chem.* **2009**, *17*, 1181.
18. Ohtsuki, T.; Hiraka, T.; Kikuchi, H.; Koyano, T.; Kowithayakorn, T.; Sakai, T.; Ishibashi, M. *Heterocycles* **2009**, *77*, 1379.
19. Fujita, T.; Nishimatsu, H.; Kaburagi, K.; Mizutani, J. *Phytochemistry* **1994**, *36*, 23.
20. Itokawa, H.; Morita, M.; Mihashi, S. *Chem. Pharm. Bull* **1980**, *28*, 3452.
21. Masuda, T.; Mizuguchi, S.; Tanaka, T.; Iritani, K.; Takeda, Y. *J. Agric. Food Chem.* **2000**, *48*, 1479.
22. Morita, M.; Nakanishi, H.; Morita, H.; Mihashi, S.; Itokawa, H. *Chem. Pharm. Bull.* **1996**, *44*, 1603.
23. Itokawa, H.; Morita, M.; Mihashi, S. *Phytochemistry* **1981**, *11*, 2503.
24. Krishna, B. M.; Chaganty, R. B. *Phytochemistry* **1973**, *12*, 238.
25. Liu, Y.-L.; Ho, D. K.; Cassady, J. M.; Cook, V. M.; Baird, W. M. *J. Nat. Prod.* **1992**, *55*, 357.
26. Robertson, W.; Yaipakdee, P. *Phytochemistry* **2001**, *57*, 341.
27. Hayashi, K.; Komura, S.; Isaji, N.; Ohishi, N.; Yagi, K. *Chem. Pharm. Bull.* **1999**, *47*, 1521.
28. Itokawa, H.; Morita, M.; Mihashi, S. *Phytochemistry* **1981**, *20*, 2503.
29. Meyer, J. J. M.; Bremner, P. D. *Planta Med.* **1998**, *64*, 777.
30. Itokawa, H.; Morita, M.; Mihashi, S. *Phytochemistry* **1979**, *18*, 1754.
31. Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Matsui, H.; Sakai, T. *Oncogene* **2005**, *24*, 7180.
32. Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Nakanishi, R.; Nishino, H.; Sakai, T. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 833.
33. Griffith, T. S.; Chin, W. A.; Jackson, G. C.; Lynch, D. H.; Kubin, M. Z. *J. Immunol.* **1998**, *161*, 2833.
34. Leverkus, M.; Neumann, M.; Mengling, T.; Rauch, C. T.; Brocker, E. B.; Krammer, P. H.; WalkzaK, H. *Cancer Res.* **2000**, *60*, 553.
35. Kim, K.; Takimoto, R.; Dicker, D. T.; Chen, Y.; Gazitt, Y.; EI-Deiry, W. S. *Int. J. Oncol.* **2001**, *18*, 241.
36. Hao, C.; Beguinot, F.; Condorelli, G.; Trencia, A.; Van Meir, E. G.; Yong, V. W.; Parney, I. F.; Roa, W. H.; Petruk, K. C. *Cancer Res.* **2001**, *61*, 1162.
37. Belyanskaya, L. L.; Marti, T. M.; Hopkins-Donaldson, S.; Kurtz, S.; Felley-Bosco, E.; Stahel, R. A. *Molecular cancer* **2007**, *6*, 66.
38. Abdelrahim, M.; Newman, K.; Vanderlaag, K.; Samudio, I.; Safe, S. *Carcinogenesis* **2006**, *27*, 717.
39. Yamaguchi, H.; Wang, H. G. *J. Biol.Chem.* **2004**, *279*, 45495.
40. Yoshida, T.; Shiraishi, T.; Nakata, S.; Horinaka, M.; Wakada, M.; Mizutani, Y.; Miki, T.; Sakai, T. *Cancer Res.* **2005**, *65*, 5662.
41. Harada, H.; Grant, S. *Rev.Clin. Exp. Hematol.* **2003**, *7*, 117.
42. Lee, J-H.; Jung, H. S.; Giang, P. M.; Jin, X.; Lee, S.; Son, P. T.; Lee, D.; Hong, Y-S.; Lee, K.; Lee, J. J. *J. Pharmacol. Exp. Ther.* **2006**, *316*, 271.
43. Vega, M. I.; Jazirehi, A. R.; Huerta-Yepez, S.; Bonavida, B. *J. Immunol.* **2005**, *175*, 2174.
44. Zhu, H.; Guo, W.; Zhang, L.; Wu, S.; Teraishi, F.; Davis, J. J.; Dong, F.; Fang, B. *Cancer Biol. Ther.* **2005**, *4*, 781.
45. Nakata, S.; Yoshida, T.; Shiraishi, T.; Horinaka, M.; Kouhara, J.; Wakada, M.; Sakai, T. *Mol. Cancer Ther.* **2006**, *5*, 1827.
46. Kumar, S. *Cell Death Differ.* **2007**, *14*, 32.
47. Baritaki, S.; Huerta-Yepez, S.; Sakai, T.; Spandidos, D. A.; Bonavida, B. *Mol. Cancer Ther.* **2007**, *6*, 1387.
48. Baritaki, S.; Suzuki, E.; Umezawa, K.; Spandido, D. A.; Berenson, J.; Daniels, T. R.; Penichet, M. L.; Jazirehi, A. R.; Palladino, M.; Bonavida, B. *J. Immunol.* **2008**, *180*, 6199.
49. Yoshida, T.; Maeda, A.; Tani, N.; Sakai, T. *FEBS Lett.* **2001**, *507*, 381.
50. Takimoto, R.; EI-Deiry, W. *Oncogene* **2000**, *19*, 1735.