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Death receptor 5 targeting activity-guided isolation of isoflavones from *Millettia brandisiana* and *Ardisia colorata* and evaluation of ability to induce TRAIL-mediated apoptosis

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ABSTRACT

Death receptor 5 (DR5) is an apoptosis-inducing membrane receptor for TNF-related apoptosis-inducing ligand (TRAIL). On screening for compounds that enhance DR5 expression using a luciferase assay with DLD-1/*Sac*I, we previously identified 4′-demethyltoxicarol isoflavone (**1**) isolated from the leaves of *Millettia brandisiana*. In this study, we revealed that **1** sensitized TRAIL-resistant human gastric adenocarcinoma (AGS) cells to TRAIL-induced apoptosis by up-regulating the expression of DR5. **1** induced DR5 expression at both the mRNA and protein level. A human recombinant DR5/Fc chimera remarkably inhibited **1**-induced apoptosis. These results suggest that the enhancement of DR5 expression by **1** was critical to the cell death. Furthermore, a MeOH extract of the bark of *Ardisia colorata* markedly enhanced DR5 activity in this screening system. Bioassay-guided fractionation of *A. colorata* led to the isolation and identification of a new isoflavone, coloratanin A (**3**), together with ten known compounds. The chemical structure of the new compound was elucidated on the basis of a spectroscopic analysis.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is a promising agent for anticancer therapy, since it triggers apoptosis in a variety of cancer cells but not in many normal cells.[1–5] TRAIL is known to bind to death receptors such as DR5 (death receptor 5 = TRAIL-R2) and DR4 (death receptor 4 = TRAIL-R1) resulting in the activation of caspase-signaling pathways leading to apoptosis.[6] However, recent studies have showed that some cancer cells have intrinsic or acquired resistance to apoptosis induced by TRAIL, which poses a potential restriction to its use in treatment.[7,8] Therefore, for the clinical use of TRAIL in cancer therapy, it is extremely important to overcome TRAIL-resistance. TRAIL-resistance has been attributed to a loss of TRAIL receptors, the upregulation of TRAIL decoy receptors, the enhanced expression of cellular FLICE-like inhibitory protein (cFLIP) and cellular inhibitor of apoptosis protein (cIAP), or changes in the expression of Bcl-2 family proteins.[7] Recently, it was reported that TRAIL-resistant cancer cells can be sensitized by combined treatment with TRAIL and chemotherapeutic drugs or natural products such as PS-341 (bortezomib),[9] tunicamycin,[10] curcumin,[11] sodium butyrate,[12] and silibinin.[13] In many cases, overcoming the TRAIL-resistance involved an up-regulation of the expression of death receptors, especially DR5. Therefore, compounds that enhance DR5 expression are considered a potential new tool with which to abrogate TRAIL-resistance. During our search for bioactive natural products, we have examined MeOH extracts of medicinal plants collected in Thailand for substances that activate the DR5 promoter. We previously reported 11 isoflavonoids isolated from the leaves of *M. brandisiana* Kurz and found that 4′-demethyltoxicarol isoflavone (**1**) and brandisianin D (**2**) had a strong effect on expression. In addition, we suggested that these compounds might overcome TRAIL-resistance by up-regulating DR5 expression.[14] In this paper, we show that **1** sensitized TRAIL-resistant human gastric adenocarcinoma (AGS) cells by up-regulating the expression of DR5.

Furthermore, we have found that a MeOH extract of the bark of *Ardisia colorata* Roxb. (Myrsinaceae) was remarkably active in this screening system. *A. colorata* is a large shrub used as a herbal medicine for liver disease, cough and diarrhea in Thailand. Previous chemical investigations of the bark and fruits of this plant by other groups led to the isolation of rapanone, ilexol and alkylphenols.[15–17] Here, we show that the bioassay-guided fractionation of *A.*

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colorata led to the isolation of one new isoflavone, coloratanin A (**3**), together with ten known compounds, 7,4′-dihydroxy-8-methoxyisoflavone (**4**),[18] 2-hydroxyformononetin (**5**),[19] genistein (**6**),[20] derrisoflavone B (**7**),[21] derrisoflavone D (**8**),[21] formonotetin (**9**),[22] derrisoflavone A (**10**),[21] isolupalbigennin (**11**),[23] 2,3,4-trimethoxy-5-hydoxyphenyl-2,3-dihydro-7-hydroxy-4*H*-1-benzopyran (**12**)[24] and (*R*)-mucronulatol (**13**).[25] The structure of the new compound was elucidated by a spectroscopic analysis, and these isolated compounds were evaluated biologically.

2. Results and discussion

2.1. Isolation and identification

In our screening system, the MeOH extract of the bark of *A. colorata* exhibited a potent effect on the DR5 promoter (1.8-fold increase in activity at 100 μg/mL). The extract was successively partitioned between hexane, EtOAc, and *n*-BuOH, along with aqueous layers, and the DR5 promoter activity was found particularly in the EtOAc soluble fraction (3.0-fold at 100 μg/mL). The EtOAc fraction was subjected to column chromatography followed by repeated reversed-phase HPLC to yield coloratanin A (**3**) and 10 known compounds (**4**–**13**). All of these compounds were isolated for the first time from this plant.

Coloratanin A (**3**) was obtained as a pale yellow powder, and its molecular formula was determined as $C_{25}H_{28}O_6$ on the basis of HRFABMS data (*m/z* 425.1946 $[M+H]^+$, Δ *−1.8* mmu). The IR spectrum exhibited absorption bands at ν_{max} 3630 and 1650 cm^{-1} due to hydroxyl and carbonyl groups, respectively, and the UV absorption maxima at λ_{max} 269 nm were suggestive of a flavone or isoflavone. A ^1H singlet resonance at δ_H 8.01 (1H, s) and corresponding olefinic oxymethine signal at δ_C 154.5, assignable to H-2 and C-2 in the ^1H and ^{13}C NMR spectra, respectively, were characteristic of an isoflavone skeleton. In the ^1H NMR spectrum, three aromatic protons with an ABX-type aromatic spin system resonating at δ_H 7.25 (1H, d, *J* = 1.9 Hz), 7.17 (1H, dd, *J* = 1.9, 8.3 Hz), and 6.84 (1H, d, *J* = 8.3 Hz) were assigned as a 1,3,4-trisubstituted aromatic ring. The ^1H NMR spectra further revealed one singlet hydrogen (δ_H 6.37, 1H, s), one prenyl unit [δ_H 3.30, (2H, overlapped); 5.23 (1H, t-like, *J* = 7.2 Hz); 1.77 and 1.65 (each 3H, s)], and one 3-hydroxy-3-methylbutyl unit [δ_H 2.69 (2H, m); 1.77 (2H, m); 1.26 (6H, s)]. Considering the molecular formula, it could be suggested that three hydroxy groups were attached to the isoflavone's nucleus. In the HMBC spectrum of **3**, correlations were Figure 1 observed from H_2-1″ to C-5, C-6, and C-7, from H_2-1‴ to C-2′, C-3′, and C-4′, and from H-8 to C-6, C-7, C-9, and C-10, suggesting the prenyl unit to be at C-6 and the 3-hydroxy-3-methylbutyl unit to be at C-3′, respectively (Fig. 2). From these observations, the structures of coloratanin A was concluded as **3**.

2.2. The DR5 expression enhancing activity of isolated isoflavonoids

The activity of the DR5 promoter was examined using the luciferase assay in DLD-1/*Sac*I cells, and compared with that in positive control cells treated with luteolin[26,27] at 17.5 μM. As shown in Figure 3, **5** increased DR5 promoter activity 2.5-fold as compared with the control at 17.5 μM, which exhibited more potent activity than that of luteolin.

2.3. Induction of apoptosis by combined treatment with 1 and TRAIL

We have recently shown that 4′-demethyltoxicarol isoflavone (**1**) isolated from the leaves of *M. brandisiana* had a potent effect on the DR5 promoter in our screening system.[14] Therefore in the present study, we investigated the effect of **1** on TRAIL-mediated apoptosis in AGS cells. AGS cells, human gastrointestinal tract cancer cells, are known to be resistant to TRAIL.[28,29] The cells were treated with **1** alone (40, 50, 60 μM), TRAIL alone (200 ng/mL), and a combination of **1** and TRAIL for 24 h. The cells were collected and apoptotic cells were identified by flow cytometric analysis. As shown in Figure 4A, the combination of **1** and TRAIL strongly induced apoptosis in a dose-dependent manner. In contrast, there was no change in cells treated with TRAIL or **1** alone. We estimated that the apoptosis induced by the combination of **1** and TRAIL was caused by an increase of DR5 expression. Therefore, to clarify the participation of DR5 expression in apoptosis, we used a recombinant human DR5/Fc chimera protein,[37] which has a dominant negative effect by competing with endogenous DR5. Apoptoic cells were determined using flow cytometric analysis to detect hypodiploid cell populations (Sub-G1). As shown in Figure 4B, the apoptosis caused by the combined treatment was strongly suppressed by the DR5/Fc chimera. Next, we examined caspase-8 and capsase-3/7 activity in order to investigate the mechanism behind the apoptosis. TRAIL-induced apoptosis is executed by the activation of initiator caspases such as caspase-8 and executioners such as caspase-3/7. As shown in Figure 4C, the caspase-8 and caspase-3/7 activity increased in a dose-dependent manner, suggesting that the apoptosis was caspase-dependent. Taken together, these results suggested that the apoptosis induced by the combined treatment with **1** and TRAIL is mediated via the death receptor pathway.

2.4. Effect of 1 on expression of DR5 mRNA and protein

To examine the effect of **1** on the expression of DR5 in AGS cells, we measured mRNA and protein levels by RT-PCR and Western blotting, respectively. As shown in Figure 5, treatment with **1** alone increased both the mRNA and protein levels of DR5 in a dose-dependent manner compared to the control.

2.5. Summary

TRAIL has been shown to selectively kill tumor cells. This property has made TRAIL and agonistic antibodies against its death receptors (TRAIL-R1/DR4, TRAIL-R2/DR5) some of the most promising novel biotherapeutic agents for cancer therapy.[30,31] However, recent studies have been shown that increased numbers of tumor cells are resistant to TRAIL-induced apoptosis. It was reported that the combination of TRAIL and chemotherapeutic drugs or natural products increased the expression of death receptors, especially DR5, and was implicated in the sensitization of TRAIL-resistant tumor cells to TRAIL-induced apoptosis.[32,33] Therefore, compounds which enhance DR5 expression would be useful to abrogate TRAIL-resistance.

During our search for bioactive natural products,[14,34] we have examined MeOH extracts of medicinal plants collected in Thailand for substances that activate the DR5 promoter. DLD-1/*Sac*I cells are a human colon cancer cell line stably transfected with the pDR5/*Sac*I plasmid, which doesn't contain a *p53*-binding site for the luciferase reporter assay.[35] Although it was reported that the p53 tumor-suppressor gene regulated DR5 gene expression, p53 is inactivated in most human cancers.[36] Therefore, the compounds isolated with our screening system may activate the DR5 promoter in a *p53*-independent mechanism, and be useful in the treatment of various TRAIL-resistant tumor cells with p53 mutations in combination with TRAIL.

In this study, we demonstrated that 4′-demethyltoxicarol isoflavone (**1**) isolated from the leaves of *M. brandisiana* sensitized TRAIL-resistant human gastric adenocarcinoma (AGS) cells by upregulating the expression of DR5 at both the mRNA and protein levels. These results suggest that a combination of **1** and TRAIL may be effective in the treatment of cancer.

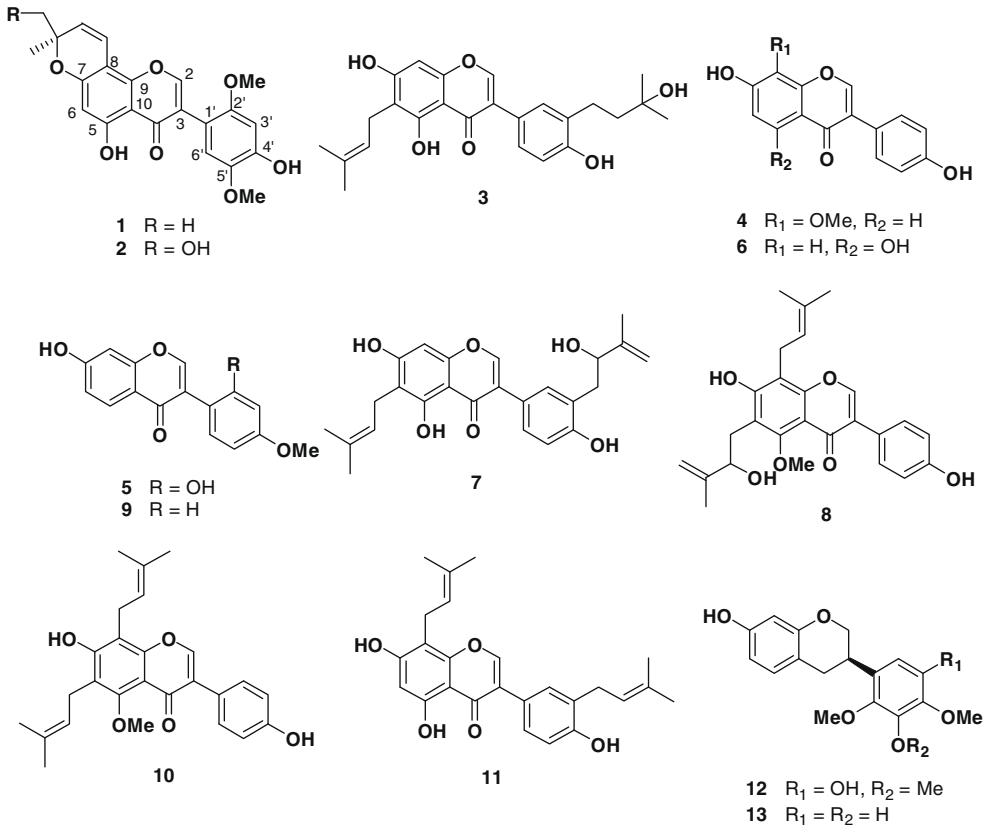

Figure 1. Structures of isoflavonoids isolated from *Millettia brandisiana* (**1** and **2**) and *Ardisia colorata* (**3**–**14**).

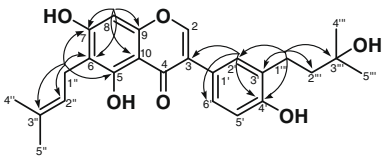

Figure 2. Key HMBC correlations observed for **3**.

3. Experiment

3.1. General experimental procedures

IR spectra were measured on a JASCO FT-IR 230 spectrophotometer. UV spectra were obtained on a Shimadzu UV mini-1240 spectrometer. The NMR spectra were recorded on JEOL JNM ECP 600 spectrometers with a deuterated solvent, the chemical shift of which was used as an internal standard. EIMS was measured on a JEOL GC-Mate spectrophotometer and high-resolution fast-atom bombardment mass spectra (HRFABMS) were recorded on a JEOL HX-110A spectrometer.

3.2. Plant material

Bark of *A. colorata* were collected in Khon Kaen, Thailand, in February 2005 and identified by T.K. A voucher specimen (6-677) is maintained at the Graduate School of Pharmaceutical Science, Chiba University.

3.3. Extraction and isolation

The bark of *A. colorata* (470.8 g) was treated with MeOH (4 L in total) at room temperature. The MeOH extract (40.4 g) was partitioned successively with hexane (500 mL × 3), EtOAc

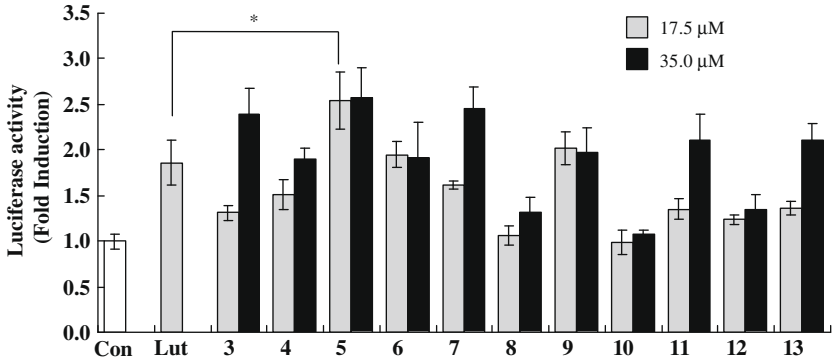

Figure 3. Activation of the DR5 promoter by the flavonids **3**–**13**, Luteolin (positive control: Lut), and DMSO (negative control: Con) in DLD-1/*Sac*I cells. All samples were tested at 17.5 and 35.0 μM. Each value represents the mean ± SD ($n = 3$). The significance was determined with Student's *t*-test (* <0.05 vs control).

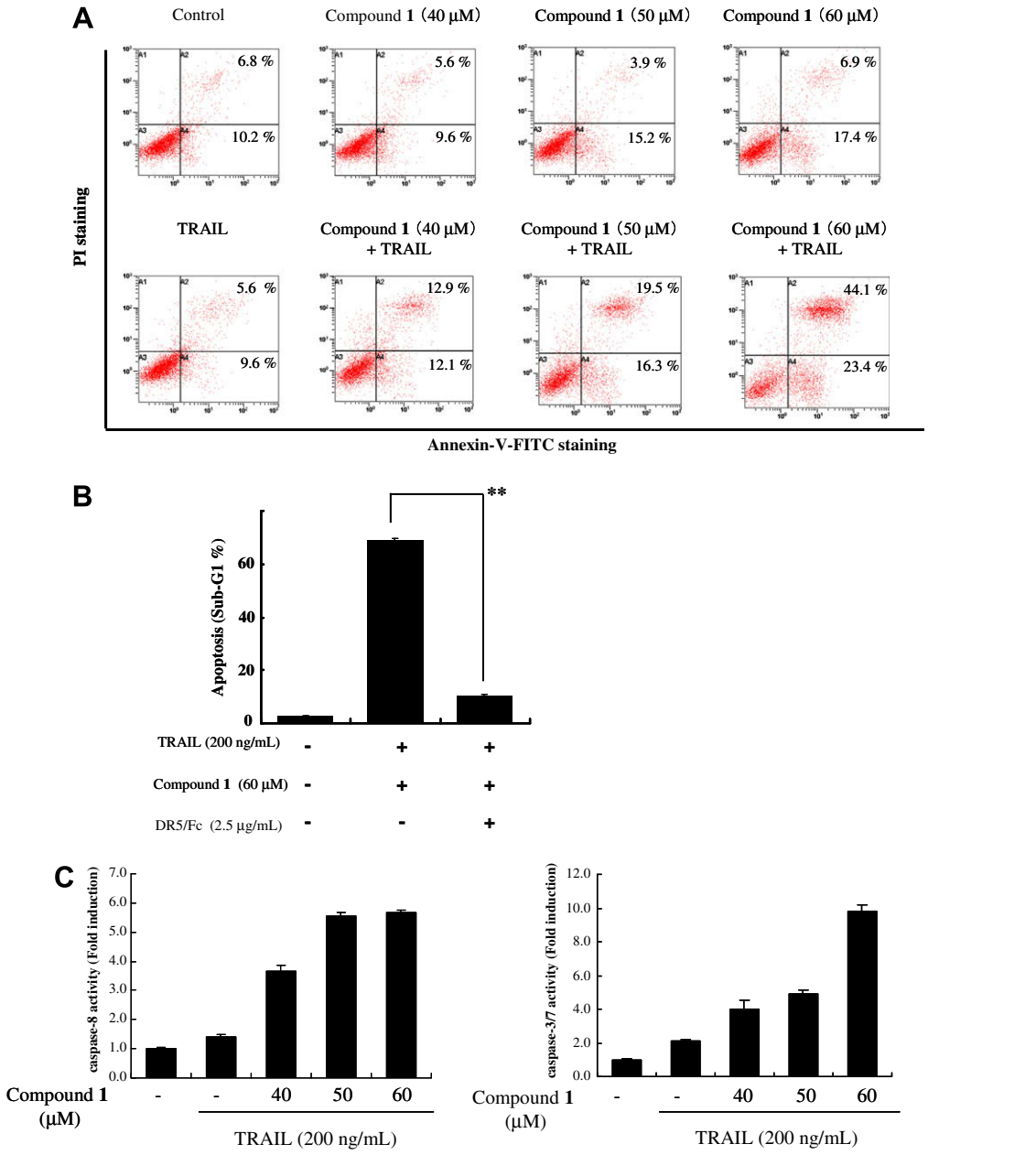

Figure 4. Induction of apoptosis by combined treatment with **1** and TRAIL in AGS cells. (A) AGS cells were treated with 40–60 μM of **1** and/or 200 ng/mL of TRAIL for 24 h. Apoptotic cells were assessed by Annexin-V-FITC and propidium iodide (PI) staining with flow cytometry. (B) DR5/Fc chimera blocked the induction of apoptosis caused by the co-treatment with **1** and TRAIL. AGS cells were treated with 60 μM of **1** and 200 ng/mL of TRAIL for 24 h with or without 2.5 μg/mL of the DR5/Fc chimeric protein. The sub-G1 populations (apoptosis) were analyzed using flow cytometry. (C) The effect of the combination of **1** and TRAIL on caspase-8 and caspase 3/7 activity. AGS cells were treated with the indicated concentrations of **1** and 200 ng/mL of TRAIL for 12 h (for caspase-8) and 24h (for caspase-3/7). The caspase activity was measured using the Caspase-Glo 8 and 3/7 assay kit. The activity in each control was defined as 1. Values represent the mean ± SD ($n = 3$). The significance was determined with Student's t-test (** <0.01 vs control).

(500 mL × 3), *n*-BuOH (500 mL × 3), and water. Since the activity was found to be concentrated in the EtOAc-soluble fraction (3.1-fold at 50 μg/mL), this fraction (6.4 g) was subjected to silica gel flash column chromatography (50 × 250 mm) with a gradient of $CHCl_3$–MeOH to yield seven fractions (1A–1F). Fractions 1C (885.1 mg) and 1D (387.1 mg) had a potent effect on DR5 expression (increase of 1.7-, and 2.6-fold, respectively, at 50 μg/mL). Fraction 1C eluted with 98% $CHCl_3$ in MeOH was subjected to ODS column chromatography (25 × 250 mm) using 50–100% MeOH in H_2O to afford thirteen fractions (2A–2M). Fraction 2A (68.8 mg), eluted with 50% MeOH, was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 250 × 10 mm; flow rate, 2.0 mL/min) with 70% MeOH to give compound **9** (4.7 mg, t_R 21 min), and three fractions (3A–3C). Compounds **12** (2.0 mg, t_R 30 min) and **13** (2.0 mg, t_R 40 min) were obtained by the separation of fraction 3B (12.1 mg) using reversed-phase HPLC (Develosil C30-UG-5, 250 × 10 mm; flow rate, 2.0 mL/min) with 67% MeOH. Fraction 2E (157.3 mg), eluted with 90% MeOH, was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18} 250 × 10 mm; flow rate, 2.0 mL/min) with 80% MeOH to give four fractions (4A–4D). Compound **10** (9.0 mg, t_R 72 min) was obtained by the separation of fraction 4C (66.4 mg) by reversed-phase HPLC (Inertsil C8-3, 250 × 4.6 mm; flow rate, 1.2 mL/min) with 65% MeOH. Fraction 2G (76.2 mg) was purified by reversed-phase HPLC (Inertsil C8-3, 250 × 4.6 mm; flow rate, 1.2 mL/min) with 70% MeOH to yield compound **11** (11.0 mg, t_R 40 min). Fraction 1D (387.1 mg) was

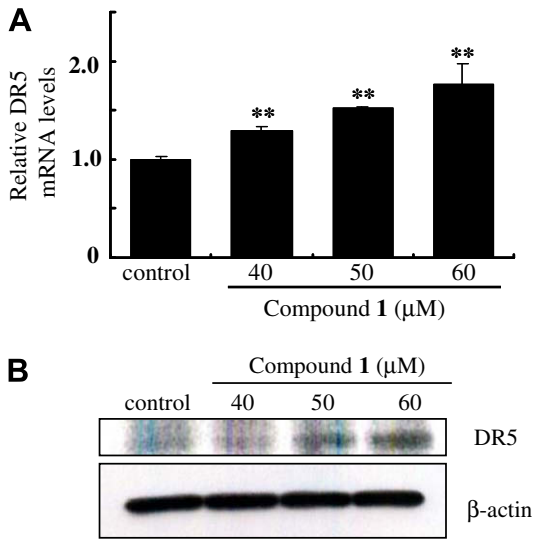

Figure 5. Effect of compound **1** on DR5 mRNA and protein in AGS cells. (A) The relative DR5 mRNA level in AGS cells. AGS cells were treated with the indicated concentrations of **1**, and the expression was determined by real-time RT-PCR. The relative quantification of the target gene expression was normalized to the mRNA expression of an endogenous reference gene, GAPDH. Values represent the mean ± SD ($n = 3$). The significance of differences was determined with Student's *t*-test (** <0.01 vs control). (B) Western blot analysis of DR5 protein levels in AGS cells after 24 h of treatment with the indicated concentrations of compound **1**.

separated into eight fractions (5A-5H) by ODS flash column chromatography (20 × 200 mm) with a step-gradient solvent system of 50–100% MeOH/H_2O. Compounds **4** (1.4 mg, t_R 32 min), **5** (3.2 mg, t_R 42 min), and **6** (3.4 mg, t_R 58 min) were obtained by the separation of fraction 5A (87.4 mg) by reversed-phase HPLC (YMC-Pack Pro C_{18}, 250 × 10 mm; flow rate, 2.0 mL/min) with 50% MeOH. Fraction 5C (12.2 mg) was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 250 × 10 mm; flow rate, 2.0 mL/min) with 75% MeOH to give compounds **3** (3.4 mg, t_R 36 min), **7** (2.4 mg, t_R 43 min), and **8** (2.5 mg, t_R 52 min).

3.3.1. Coloratanin A (3)

Pale yellow powder; IR (ATR) ν_{max} 3630, 2980, and 1650 cm^{-1}; UV (MeOH) λ_{max} (log ε) 269 (4.3) and 215 (4.5) nm; ^1H NMR (600 MHz, CD_3OD) δ_H 8.01 (1H, s, H-2), 7.25 (1H, d, J = 1.9 Hz, H-1′), 7.17 (1H, dd, J = 1.9, 8.3 Hz, H-6′), 6.84 (1H, *d*, J = 8.3, H-5′), 6.37 (1H, s, H-8), 5.23 (1H, t-like, J = 7.2 Hz, H-2″), 3.30 (2H, overlapped, H_2-1″), 2.69 (2H, m, H_2-1‴), 1.77 (2H, m, H_2-2‴), 1.77 (3H, s, H_3-4″), 1.65 (3H, s, H_3-5″), and 1.26 (6H, s, H_3-4‴ and H_3-5‴); ^{13}C NMR (125 MHz, CD_3OD) δ_c 182.4 (C-4), 163.7 (C-7), 160.5 (C-5), 157.6 (C-9), 156.6 (C-4′), 154.5 (C-2), 132.1 (C-3″), 131.8 (C-2′), 130.5 (C-3′), 128.7 (C-6′), 124.8 (C-3), 123.5 (C-1′), 123.4 (C-2″), 115.8 (C-5′), 113.1 (C-6), 106.1 (C-10), 93.9 (C-8), 71.6 (C-3‴), 44.9 (C-2‴), 29.1 (C-4‴ and C-5‴), 26.0 (C-5″), 26.3 (C-1‴), 22.3 (C-1″), and 17.9 (C-4″); EIMS *m/z* 424 $[M]^+$ (27), 406 (41), 389 (18), 363 (57), 351 (71), 300 (85), 143 (30), and 130 (100); HRFABMS *m/z* 425.1946 $[M+H]^+$ (calcd for $C_{25}H_{29}O_6$, 425.1964).

3.4. Cell cultures

The human gastric adenocarcinoma AGS cell line and DLD-1/*Sac*I cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS).

3.5. Assessment of DR5 promoter activity

Activity to enhance death receptor 5 expression was assayed as described in our previous report.[14] Briefly, DLD-1/*Sac*I cells were seeded in a 24-well culture plate (2×10^5 cells per well) in 1 mL of RPMI medium containing 10% FBS. The cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h, then treated with the test samples (100 μg/mL). After 24 h of incubation, the cells were rinsed with PBS and 100 μL of 1 × lysis reagent (Promega) added to each well. Forty microliters of cell lysate was transferred to a 96-well white bottom plate. Then, 200 μL of luciferase assay substrate (Promega) was added to each well, and luminescence was measured in a Luminoskan Ascent Luminometer (Thermo). The enhancement of DR5 promoter activity was evaluated based on light intensity, relative to that of the control (cells treated with DMSO). Luteolin,[26] which is known to activate the DR5 promoter, was used as a positive control at a concentration of 17.5 μM.

3.6. Analysis of apoptosis

Apoptoic cells were evaluated by annexin V-FITC and propidium iodide (PI) double staining. Briefly, AGS cells were treated with 40, 50, 60 μM of compound **1** and/or 200 ng/mL of TRAIL for 24 h. They were collected and washed twice with cold PBS solutions. Then the cells were resuspended in 500 μL of binding buffer, 5 μg/mL of annexin-V-FITC (Annexin-V-FLUOS Staining kid, Roche), and 5 μL of 20 μg/mL of propidium iodide, and mixed and incubated for 15 min at room temperature in the dark. The flow cytometric analysis was performed with a Cytomics FC500 flow cytometer (Beckman Coulter) within 1 h.

3.7. Caspase 8 and caspase 3/7 activity

The measurement of caspase-8 and caspase-3/7 activity was performed using Caspase-Glo 8 Assay and Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions. Briefly, the cells were treated with 40, 50, 60 μM of compound **1** and/or 200 ng/mL of TRAIL for 12 h (for caspase-8) and 24 h (for caspase-3/7), collected, and lysed. The cell lysates were incubated with a luminogenic substrate, z-DEVD-amino-luciferin (for caspase 3/7) or z-LETD-amino-luciferin (for caspase 8), at room temperature for 1 h and the luminescence was monitored using a luminometer.

3.8. RNA extraction and real time RT-PCR analysis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to protocols provided by the manufacturer. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a RT-PCR kit according to the manufacturer's instructions, and carried out in a Mx3000 QPCR System (stratagene), with a SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). The PCR primer pair for GAPDH and DR5 was described in our previous paper.[37]

3.9. Western blot analysis

AGS cells were lysed as previously described,[37] and 20 μL of protein lysate was resolved by 12.5% SDS-PAGE. The antibodies used were rabbit polyclonal anti-DR5 (Sigma) and mouse monoclonal anti-β-actin (Sigma). β-Actin was used as an internal control.

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