RESEARCH ARTICLE

Isoangustone A present in hexane/ethanol extract of *Glycyrrhiza uralensis* induces apoptosis in DU145 human prostate cancer cells *via* the activation of DR4 and intrinsic apoptosis pathway

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Glycyrrhiza uralensis (licorice) is one of the most frequently prescribed ingredients in Oriental medicine, and licorice extract has been shown to exert anti-carcinogenic effects. However, its use as a cancer chemopreventive agent is rather limited, due to the fact that its principal component, glycyrrhizin, is known to induce hypertension. This study determined the effects of a hexane/ethanol extract of *G. uralensis* (HEGU), which contains undetectable amounts of glycyrrhizin, on the apoptosis of androgen-insensitive DU145 cells. HEGU induced apoptosis and increased the levels of cleaved caspase-9, caspase-7, caspase-3 and poly (ADP-ribose) polymerase (PARP). HEGU also induced mitochondrial membrane depolarization and cytochrome *c* release to the cytosol. HEGU increased the levels of Fas, death receptor 4 (DR4), cleaved caspase-8, Mcl-1S, and truncated Bid proteins. A caspase-8 inhibitor suppressed HEGU-induced apoptosis. An active fraction of HEGU was separated *via* column chromatography and the structure of the active compound isoangustone A was identified *via* ^{1}H-NMR and ^{13}C-NMR. Isoangustone A increased apoptotic cells, the cleavage of PARP and caspases, and the levels of DR4 and Mcl-1S. Transfection with DR4 small interfering RNA attenuated HEGU- and isoangustone A-induced apoptosis. These results demonstrate that the activation of DR4 contributes to HEGU- and isoangustone A-induced apoptosis of DU145 cells.

Keywords:
Bcl-2 family proteins / Caspases / Death receptor / Glycyrrhizin / Licorice

1 Introduction

In recent years, the anti-cancer effects of a variety of bioactive food components have become the focus of a great deal of attention in cancer research, owing primarily to their potential cancer-preventive properties. Prostate cancer is the sixth most common cancer worldwide, the third most common cancer in men, and the second leading cause of cancer-related deaths in men in the Western world [1, 2]. As the incidence of prostate cancer increases as men age and

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Abbreviations: 7-AAD, 7-aminoactiomycin D; **DR,** death receptor; **FBS,** fetal bovine serum; **HEGU,** hexane/ethanol extract of *Glycyrrhiza uralensis*; **GA,** glycyrrhetic acid; **GL,** glycyrrhizin; **MTT,** 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PARP,** poly (ADP-ribose) polymerase; **siRNA,** small interfering RNA; **TRAIL,** tumor necrosis factor-related apoptosis-including ligand; **tBid,** truncated Bid

because prostate cancer grows slowly, the identification of bioactive compounds with the ability to suppress prostate cancer growth promises to be an effective strategy for the prevention of this disease.

Glycyrrhiza uralensis (licorice) has been extensively used for more than 4000 years as a sweetening and flavoring agent in candies, foods, tobacco products, and toothpastes. It is also contained in almost all Oriental medical herbal prescriptions. Furthermore, several complementary/alternative medicines for cancer treatment contain licorice, because licorice and its derivatives have been demonstrated to exert anti-carcinogenic effects [3, 4]. Glycyrrhizin (GL), a triterpene compound, is normally considered to be the principal biologically active component of licorice (reviewed in [3, 5]). GL is converted by human intestinal bacteria to its aglycone, glycyrrhetic acid (GA) [6], and GA has been demonstrated to inhibit the proliferation of prostate cancer cells [7] and induce apoptosis in hepatoma, promyelotic leukemia, and stomach cancer cells [8]. However, the chronic consumption of large quantities of licorice containing GL results in undesirable mineralocorticoid excess, hypertension, and hypokalemia [9, 10]. This hypertension is caused by a reduction in the activities of 11β-hydroxysteroid dehydrogenase type 2. GA is a well-known inhibitor of 11β-hydroxysteroid dehydrogenase type 2 [11, 12].

Apoptosis, or programmed cell death, is a physiological process essential to normal development and the maintenance of tissue homeostasis. Excessive apoptosis can lead to devastating degenerative diseases of the nervous system and heart, whereas a lack of cell death can result in the development of autoimmune diseases or cancer (reviewed in [13]). As the evasion of apoptosis is one of the hallmarks of cancer [14], bioactive compounds with the ability to induce apoptosis in cancer cells may potentially be utilized as cancer chemopreventive agents. There are two major apoptosis pathways – intrinsic (mitochondria-dependent apoptotic pathway) and extrinsic (death receptor (DR)-dependent apoptotic pathway) apoptotic signaling – in apoptotic stimuli-responsive cells [15]. The mitochondria-dependent apoptotic pathway is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins [16]. The pro-apoptotic Bcl-2 proteins, including Bax, Bok, and Bak (multidomain proteins) and the BH3-only subfamily, which includes Bid, Bim, Bad, and PUMA, induce the permeabilization of the mitochondrial outer membrane. As a result, cytochrome *c* is released into the cytosol, resulting in the activation of the caspase cascade and the induction of apoptotic cell death [17]. The other apoptotic pathway, the DR-dependent apoptotic pathway, is mediated by plasma membrane DRs, including tumor necrosis factor, Fas, and tumor necrosis factor-related apoptosis-including ligand (TRAIL) receptors, and caspase-8 is a major initiator caspase in this pathway, which in turn activates effector caspases including caspase-3 [18]. Caspase-8 can also activate the mitochondria-dependent apoptotic pathway *via* the cleavage of Bid. Upon cleavage, truncated Bid (tBid) migrates to the mitochondria where it induces the permeabilization of mitochondrial membranes, the release of cytochrome *c*, and the activation of caspase-9, a process that ultimately results in cell shrinkage and nuclear condensation [19, 20].

In a previous work, we prepared the hexane/ethanol extract of *G. uralensis* (HEGU), which lacks GL, and have demonstrated that HEGU suppresses doxorubicin-induced apoptosis in H9c2 rat cardiac myoblasts. Additionally, we have demonstrated that HEGU reduces the viable cell numbers of HT-29 human colon, MDA-MB-231 human breast, and DU145 human prostate cancer cells, among which DU145 cells were the most sensitive to HEGU [21]. As these results suggest that HEGU may be a promising anti-carcinogenic agent, this study attempted to ascertain whether HEGU induces apoptosis in DU145 human prostate cancer cells. Additionally, we separated out an active compound present in HEGU and determined its structure.

2 Materials and methods

2.1 Materials

The reagents used were as follows. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 7-aminoactiomycin D (7-AAD), biobenzimide H33258 (Hoechst 33258), proteinase K, and 5,5′-6,6′-tetraethyl-imidacarbocyanine iodide (JC-1) (Sigma, St. Louis, MO, USA); horseradish peroxidase-conjugated anti-rabbit, and anti-mouse IgG (Amersham, Arlington Heights, IL, USA); Z-IETD-FMK, antibodies against heat shock protein 60, Bcl-2, Mcl-1, Bax, Fas, and Fas ligand, control small interfering RNA (siRNA), and DR4 siRNA (Santa Cruz, Santa Cruz, CA, USA); antibodies against Bad, Bid, Bik, Bcl-xL, caspase-8, cleaved caspase-9, caspase-7, caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling, Beverly, MA, USA); DR4 antibody (Imgenex, San Diego, CA, USA); phycoerythrin-conjugated annexin V and TRAIL antibody (BD Pharmingen, Franklin Lake, NJ, USA); DR5 antibody (Prosci, Flint Place, CA, USA); Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Mannheim, Germany); Nucleofector kit L solution box (Amaxa, Gaithersburg, MD, USA). If not otherwise specified, all other materials were purchased from Sigma.

2.2 Preparation of extract, fractionation and identification of active compounds

The HEGU was prepared as described previously [21]. The HEGU (4.0 g) was subjected to flash column chromatography with silica gel (30 × 500 mm glass column, 100 g, 70–230 mesh, Merck, Germany) and eluted *via* gradient systems of *n*-hexane-ethyl acetate (10:0–5:5) to yield eleven parts (1–11). The ability of each fraction to reduce DU145 cell viability was evaluated using MTT assays as described previously [22], and part 10 (0.6 g), which evidenced the highest level of activity, was further purified *via* recrystalli-

zation to yield compound 1 (76 mg isoangustone A). The structural identification of the compound was conducted *via* ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX 400 (400 MHz) spectrometer, using tetramethylsilane as an internal standard.

2.3 Cell culture

DU145 cells were maintained in DMEM/F12 containing 100 mL/L fetal bovine serum (FBS), 100 000 U/L penicillin and 100 mg/L streptomycin. The medium was changed every 2 days. In order to determine the effects of HEGU and isoangustone A, we plated the cells in multiwell plates with DMEM/F12 containing 100 mL/L FBS. Prior to HEGU treatment, the cell monolayers were rinsed and serum-deprived for 24 h with DMEM/F12 containing 10 mL/L charcoal-stripped FBS (serum deprivation medium). After serum deprivation, the medium was replaced with fresh serum deprivation medium, with or without various concentrations (2.5, 5, or 7.5 μg/mL) of HEGU or isoangustone A. Viable cell numbers were estimated *via* MTT assay. The extracts were dissolved in DMSO and all cells were treated with DMSO at a final concentration of 1 mL/L.

2.4 Hoechst 33258 staining

In an effort to determine whether HEGU induces apoptosis in DU145 cells, the cells were plated in 4-well chamber slides at 50 000 cells/slide, serum-deprived, and treated with various concentrations of HEGU for 18 h. The cells were then fixed *via* the addition of 40 g/L PBS-buffered formaldehyde for 20 min, and then stained for 30 min with 10 mg/L Hoechst 33258. The cells were immediately washed in PBS and examined *via* fluorescent microscopy. Apoptotic cells were defined on the basis of changes in their nuclear morphology, including chromatin condensation and fragmentation.

2.5 Fluorescence-activated cell sorting analysis

Cells were plated in 100 mm dishes at 1 000 000 cells/dish and treated for 18 h with various concentrations of HEGU. The cells were trypsinized and incubated for 15 min with phycoerythrin-conjugated annexin V and 7-AAD at room temperature in darkness. Apoptotic cells were analyzed *via* flow cytometry utilizing a FACScan system (Becton Dickinson). The data were analyzed by using ModFit V.1.2. software.

2.6 Western blot analysis

Total cell lysates were prepared as described previously [23]. The cytosolic and mitochondrial proteins were separated as previously described by Eguchi *et al.* [24]. They determined the protein contents of the total cell lysates and cytoplasmic and mitochondrial fractions using a BCA protein assay kit (Pierce, Rockford, IL, USA). Western blot analyses were conducted as described previously [23]. The relative abundance of each band was quantified using the Bio-profile Bio-1D application (Vilber-Lourmat) and the expression levels were normalized to β-actin.

2.7 Flow cytometry measurement of mitochondrial membrane potential

In an effort to measure the mitochondrial membrane potential, the dual-emission potential-sensitive probe JC-1 was utilized. In the presence of high mitochondrial transmembrane potential, JC-1 forms J-aggregates that emit red fluorescence, whereas the JC-1 monomeric form emits green fluorescence at low mitochondrial transmembrane potential. The ratio of red to green fluorescence of JC-1 depends solely on the membrane potential, with a reduction being considered indicative of membrane depolarization. DU145 cells treated with HEGU were harvested, loaded with 2 mg/L JC-1 at 37°C for 20 min, and analyzed using the FACScan flow cytometer (Becton Dickinson).

2.8 Cell Death Detection ELISA

Cells were plated in 12-well plates at a density of 150 000 cells/well and treated for 6 h with various concentrations of isoangustone A. The cell lysates were then assayed for the quantitative determination of mono- and oligo-nucleosomes released into the cytoplasm using a Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science) in accordance with the manufacturer's recommendations.

2.9 siRNA

Cells were transfected with DR4 siRNA or control A siRNA using the Nucleofector Device ll gene transfer system (Amaxa), in accordance with the manufacturer's recommendations. Transfected cells were plated in 100 mm dishes at a density of 1 000 000 cells/dish and treated with or without 7.5 μg/mL of HEGU or isoangustone A for 18 or 6 h, respectively.

2.10 Statistical analysis

The results were expressed as the means ± SEM and were analyzed by ANOVA. Differences between the treatment groups were analyzed *via* Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. All statistical analyses were conducted with SAS statistical software, version 8.12.

3 Results

3.1 HEGU induces apoptosis of DU145 human prostate cancer cells

Hoechst 33258 dye staining showed that HEGU induced chromatin condensation in DU145 cells, as evidenced by an intense pycnotic bluish-white fluorescence in the nuclei of those cells (Fig. 1A). To quantify the apoptotic cells, we stained HEGU-treated DU145 cells with annexin V and 7-AAD, and then analyzed them *via* flow cytometry. Early apoptotic cell numbers were shown to have increased in a HEGU dose-dependent manner (Fig. 1B). To assess the effects of HEGU on the cleavage of caspases and PARP, the cells were treated with various concentrations of HEGU for 18 h and the total cell lysates were prepared for Western blot analysis. HEGU effected a dramatic increase in the levels of cleaved caspase-9, caspase-7, caspase-3, and PARP in DU145 cells (Fig. 1C). In addition to DU145 cells, we observed in this study that HEGU dose dependently reduced the viable cell numbers and induced apoptotic cell death in PC3 human prostate cancer cells (data not shown).

3.2 HEGU induces mitochondrial membrane depolarization and increases the levels of pro-apoptotic Bcl-2 family proteins in DU145 cells

As HEGU induced caspase-9 activation, we subsequently attempted to determine whether HEGU increases cytochrome *c* levels in the cytoplasm of DU145 cells. We noted a HEGU-

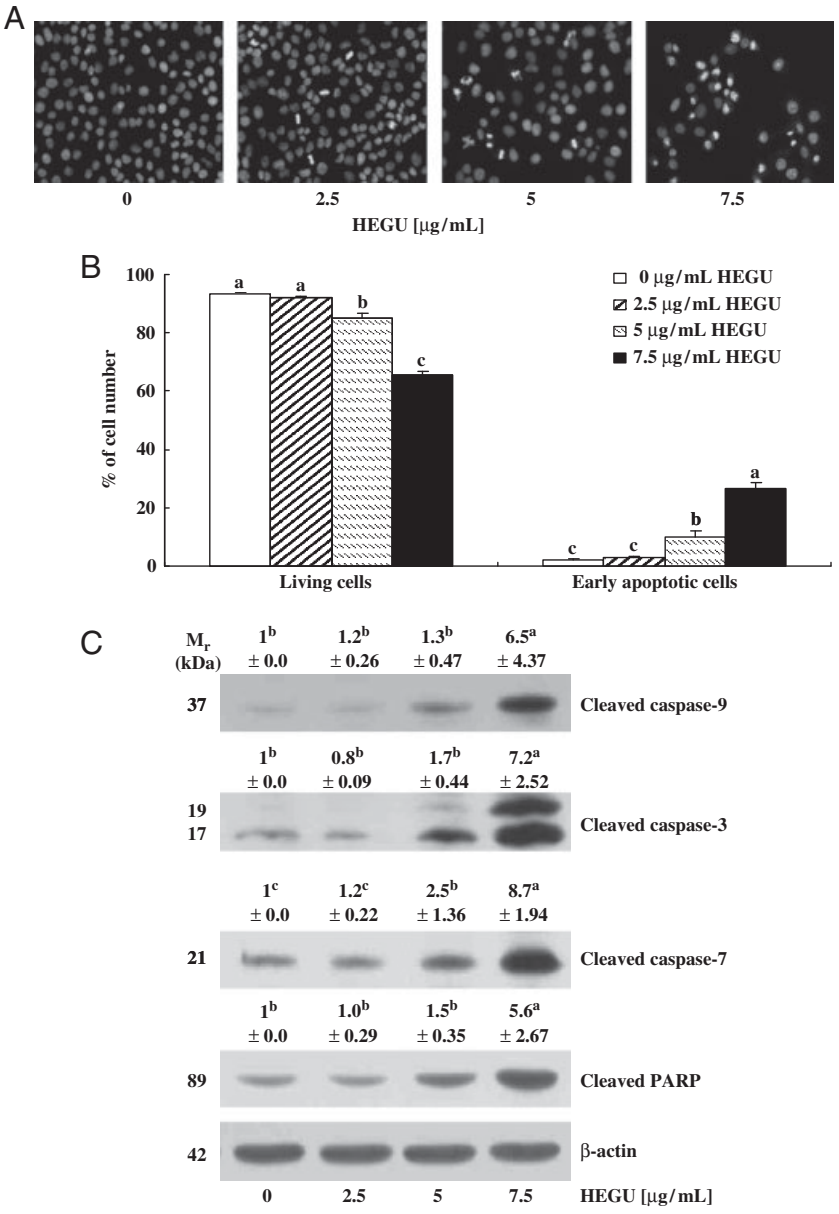

Figure 1. HEGU induces apoptosis in DU145 human prostate cancer cells. Cells were plated in chamber slides at a density of 50 000 cells/well (A) or 100 mm dishes at a density of 1 000 000 cells/dish (B and C) with DMEM/F12 supplemented with 100 mL/L of FBS. Briefly, 24 h after plating, the monolayers were serum-deprived in serum-deprivation medium (DMEM/F12 supplemented with 1% charcoal-stripped FBS). The cells were then incubated for 18 h in serum-deprivation medium with 0, 2.5, 5, or 7.5 μg/mL HEGU. (A) The cells were stained with a DNA-specific dye, Hoechst 33258. (B) Cells were stained with 7-AAD and Annexin V, then analyzed *via* flow cytometry. The numbers of living cells and early apoptotic cells were expressed as a percentage of the total cell number. Each bar represents the mean ± SEM (n=6). (C) Total cell lysates were analyzed *via* Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. Means without a common letter differ, $p<0.05$.

dependent increase in the levels of cytochrome *c* in the cytoplasm, coupled to a reduction in the mitochondrial levels of this protein (Fig. 2A). To determine whether HEGU induces the depolarization of mitochondrial membranes, the HEGU-treated cells were stained with JC-1, and the percentage of cells with green-positive and red-negative fluorescence was scored as depolarized cells. The numbers of cells with depolarized mitochondrial membranes were shown to increase in the HEGU-treated cells (Fig. 2B). Pro- and anti-apoptotic Bcl-2 family members are believed to be the core regulators of mitochondrial outer membrane permeability [25]. Western blot analysis of DU145 cell lysates showed that HEGU exerted no effect on the levels of Bcl-2, Bcl-xL Mcl-1L, Bax, or Bad proteins. HEGU induced an increase in the levels of Mcl-1S, Bik, and tBid, and a reduction in the levels of intact Bid, in a concentration-dependent fashion (Fig. 2C).

3.3 A caspase-8 inhibitor attenuates HEGU-induced apoptosis

As HEGU increased the levels of tBid (Fig. 2C), we attempted to ascertain, *via* Western blotting, whether or not HEGU increases the levels of cleaved caspase-8 proteins. HEGU significantly increased the levels of cleaved caspase-8 in DU145 cells (Fig. 3A). As HEGU increased the activation of caspase-8 and Bid cleavage, we assessed whether the caspase-8 inhibitor, Z-IETD-FMK, would attenuate HEGU-induced apoptosis. DU145 cells were pretreated for 4 h with Z-IETD-FMK prior to treatment with 7.5 μg/mL HEGU. The caspase-8 inhibitor suppressed the HEGU-induced reduction in viable cell numbers and the HEGU-induced increase in apoptotic cell numbers in DU145 cells (Fig. 3B).

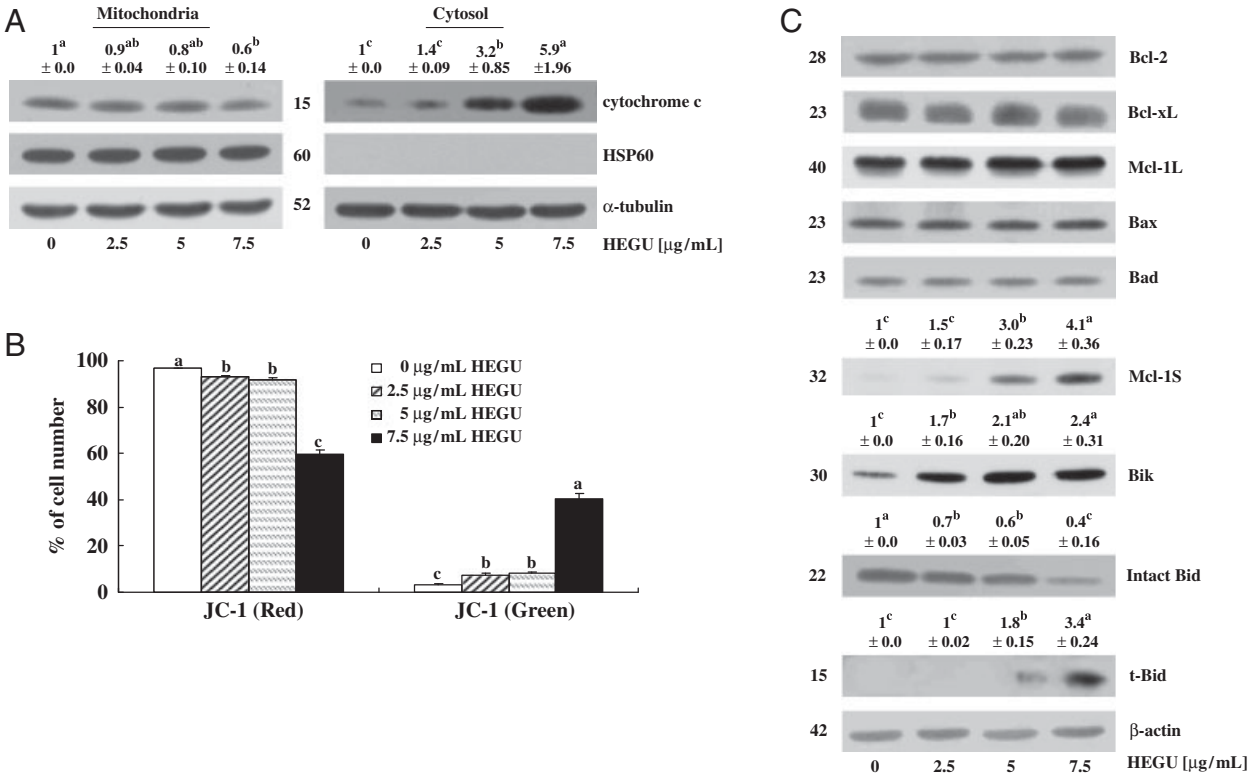

Figure 2. HEGU induces mitochondrial membrane depolarization and increases the levels of the BH3-only proteins in DU145 cells. Cells were treated with HEGU as described in Fig. 1. (A) HEGU-treated cells were subjected to subcellular fractionation. The cytosolic and mitochondrial fractions were analyzed *via* Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own α-tubulin was quantified, and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. (B) HEGU-treated cells were loaded with JC-1, and then analyzed by flow cytometry. The numbers of cells with normal polarized mitochondrial membranes (red) and cells with depolarized mitochondrial membranes (green) were expressed as a percentage of the total cell number. Each bar represents the mean ± SEM (n=6). (C) Cell lysates were subjected to Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. Means without a common letter differ, $p<0.05$.

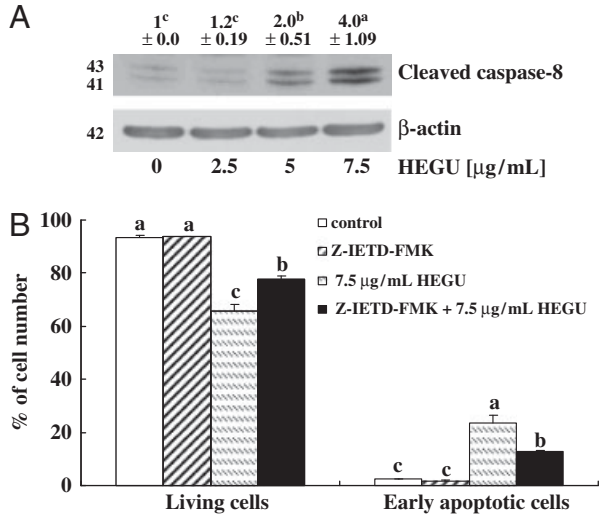

Figure 3. HEGU increases the levels of cleaved caspase-8 in DU145 cells. Cells were treated with HEGU as described in Fig. 1. (A) Cell lysates were subjected to Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. (B) Cells were cultured in the absence or presence of 20 μmol/L caspase-8 inhibitor (Z-IETD-FMK) for 4 h prior to 18 h of treatment with 7.5 μg/mL HEGU. The cells were trypsinized, loaded with 7-AAD and Annexin V, and then analyzed *via* flow cytometry. The numbers of living cells and early apoptotic cells were expressed as a percentage of the total cell number. Each bar represents the mean ± SEM (n=6). Means without a common letter differ, $p<0.05$.

3.4 Increased DR4 expression contributes to HEGU-induced apoptosis

As HEGU increased the levels of tBid and cleaved caspase-8, we subsequently assessed the effects of HEGU on the protein levels of plasma membrane DRs and their ligands. HEGU increased the levels of Fas and DR4 but had no effect on DR5. We noted small but significant increases in the levels of TRAIL in HEGU-treated cells, whereas Fas ligand was not altered by HEGU treatment (Fig. 4A). In order to elucidate clearly the function of DR4 in HEGU-induced apoptosis, DR4 expression was inhibited *via* the transfection of cells with DR4 siRNA. The Western blot analysis of total cell lysates verified that the expression of DR4 was reduced markedly in the HEGU-treated cells transfected with DR4 siRNA, and this phenomenon was correlated with reduction in the HEGU-induced cleavage of caspase-8 and caspase-3 (Fig. 4B). Additionally, HEGU-induced apoptotic cell death was suppressed significantly by DR4 siRNA (Fig. 4C).

3.5 Identification of isoangustone A as an active compound in HEGU

Among the eleven fractions eluted by gradient systems of *n*-hexane-ethyl acetate (10:0–5:5) on flash column chromatography, fraction 10 was determined to be the most effective in reducing the numbers of viable DU145 cells (Fig. 5A). The fraction 10 (0.6 g) was further purified *via* recrystallization, yielding compound 1 (76 mg yellow powder). Comparing the data with that provided in the relevant literature [26], compound 1 was identified as isoangustone A, as is shown in Fig. 5B.

3.6 Isoangustone A decreases the viability of DU145, PC3, HT-29, and MDA-MB-231 cells

The ability of isoangustone A to reduce the viability of DU145, PC3, HT-29, and MDA-MB-231 cells was determined by treating these cancer cells for 48 h with various concentrations of this compound. Isoangustone A effected a significant reduction in viable cell numbers, and induced apoptotic death in these cancer cells (Table 1).

3.7 Isoangustone A causes the cleavage of caspases and PARP and alters the levels of Mcl-1 in DU145 cells

The treatment of DU145 cells with isoangustone A for 6 h resulted in increases in the levels of cleaved caspase-9, caspase-7, caspase-3, and PARP (Fig. 6A). Additionally, isoangustone A effected a significant reduction in the levels of the anti-apoptotic Mcl-1L protein, and increased the levels of the pro-apoptotic Mcl-1S protein (Fig. 6B).

3.8 Isoangustone A induces apoptosis *via* a DR4-mediated pathway in DU145 cells

As HEGU-induced apoptosis was suppressed by the caspase-8 inhibitor (Fig. 3) and DR4 siRNA (Fig. 4), we subsequently attempted to determine whether isoangustone A alters the levels of cleaved caspase-8, Fas, and DR4, *via* Western blot analyses. Isoangustone A increased the levels of Fas, DR4, and cleaved caspase-8 in DU145 cells (Fig. 7A). The transfection of cells with DR4 siRNA significantly suppressed isoangustone A-induced increases in the levels of DR4, cleaved caspase-8, and cleaved caspase-3 (Fig. 7B). The inhibition of DR4 expression by transfection with DR4 siRNA resulted in a significant attenuation of the isoangustone A-induced increases in apoptotic cell death (Fig. 7C).

4 Discussion

Approximately 80–90% of prostate cancer is dependent on androgen at initial diagnosis. Androgen ablation therapy is

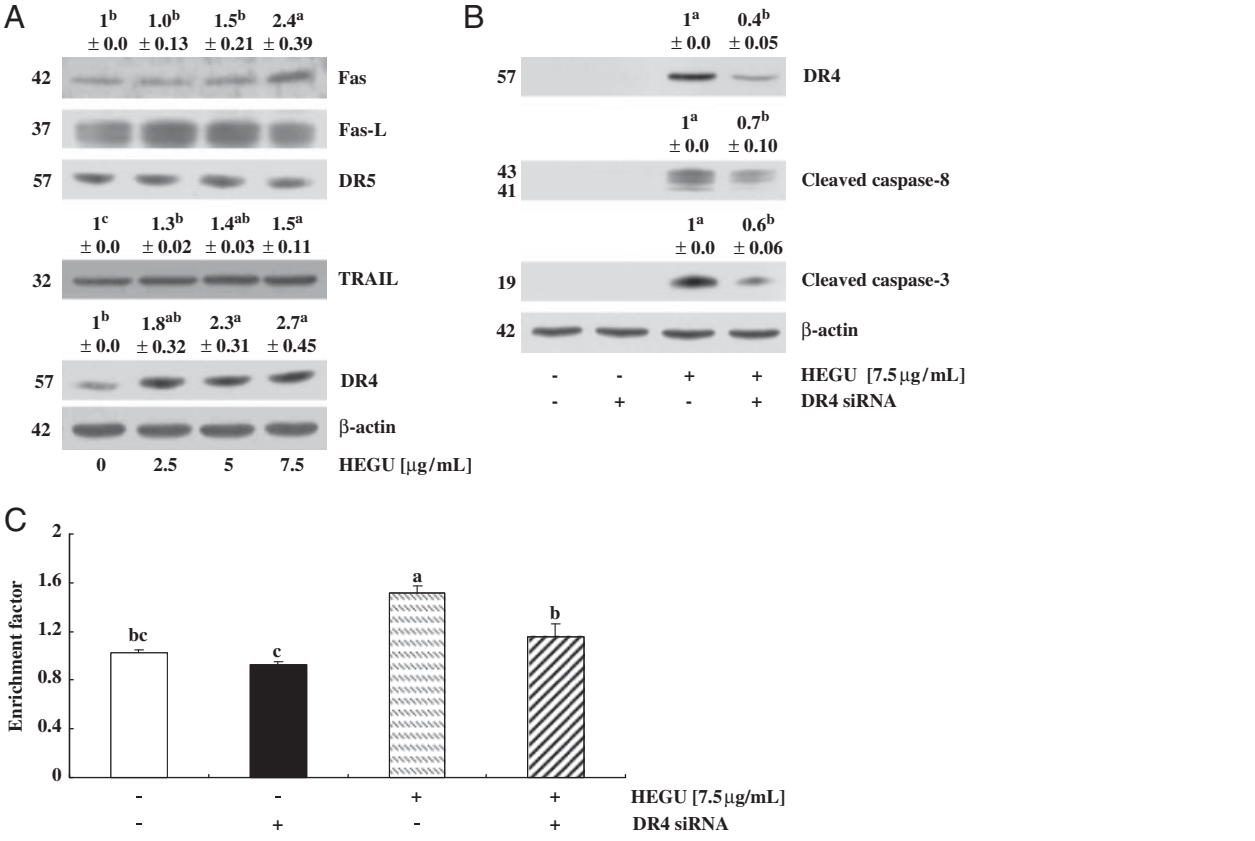

Figure 4. HEGU induces apoptosis though a DR4-mediated pathway in DU145 cells. Cells were treated with HEGU as described in Fig. 1. (A) Cell lysates were subjected to Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified, and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. (B and C) Cells were transfected with control or DR4 siRNA. The cells were plated at a density of 1 000 000 cells/dish (B) or 150 000 cells/well in 12-well plates (C) with DMEM/F12 supplemented with 10% FBS. In total, 24 h after plating, the monolayers were serum-deprived for 24 h in serum-deprivation medium. The cells were then incubated for 18 h in serum-deprivation in the presence of 0 or 7.5 μg/mL of HEGU. (B) The total cell lysates were subjected to Western blotting with the indicated antibodies. (C) Apoptotic cells were detected with Cell Death Detection ELISA^{PLUS} kit in accordance with the manufacturer's recommendations. The mono- and oligo-nucleosomes released into the cytoplasm were quantified, and the enrichment factor was calculated. Enrichment factor = absorbance of the sample/absorbance of the control (control siRNA transfected, 0 μg/mL HEGU). Each bar represents the mean ± SEM (n = 6). Means without a common letter differ, p < 0.05.

effective in the initial stages, but it ultimately fails as the cancer progresses to an androgen-independent conditions. With regard to metastatic disease, few curative therapies currently exist, and treatment generally has been palliative in nature [1, 27]. As prostate cancer is associated with old age and grows slowly, the identification of bioactive compounds with potential anti-cancer efficacy may prove an effective strategy for both preventions and interventions of prostate cancer. Licorice is a famous medicinal plant with a long history of pharmaceutical use in Oriental nations. Licorice extracts have been reported to exert a variety of beneficial effects, including anti-inflammatory [28], anti-viral [29], and anti-tumor [30] effects. However, the use of licorice extract as an anti-cancer agent has been limited, because the principal component of licorice, GL has been shown to induce severe hypertension [9, 10]. We have shown previously that HEGU lacking GL effects a reduction in the viable cell numbers of a variety of cancer cells [21]. This study was conducted in an effort to determine whether HEGU induces the apoptosis of androgen-independent DU145 prostate cancer cells, and to identify the active compound(s) present in HEGU. We found that both HEGU and its active compound isoangustone A induced the apoptosis of DU145 cells. The biological effects of isoangustone A have not, thus far, been studied extensively. Only in one study isoangustone A has been reported to exert antibacterial effects on methicillin-resistant *Staphylococcus aureus* [31]. To the best of our (admittedly limited) knowledge, this study is the first to report the anti-carcinogenic effect of isoangustone A.

The morphologic features of apoptosis include cell rounding, cell volume decrease, membrane blebbing, cytoskeletal collapase, chromatin fragmentation, and nuclear pyknosis (reviewed in [32]). In this study, we demonstrated

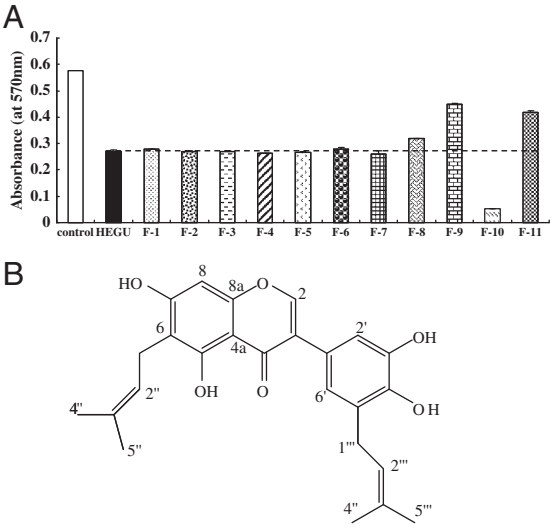

Figure 5. Isoangustone A is identified as an active compound in HEGU. (A) Cells were plated at a density of 50 000 cells/well in 24-well plates with DMEM/F12 supplemented with 10% FBS. In total, 24 h after plating, the monolayers were serum-deprived for 24 h in serum-deprivation medium. The cells were treated for 48 h with 7.5 μg/mL of the different fractions of HEGU. Cell numbers were estimated by the MTT assay. Each bar represents the mean ± SEM (n=6). (B) Structure of isoangustone A. The analytical properties of isoangustone A were as follows: $C_{25}H_{26}O_6$, MP: 191–93°C, EI-MS: m/z 422 $[M]^+$, ^{1}H-NMR (600 MHz, DMSO-d_6): 1.62 (3H, s, H-5″), 1.69 (6H, s, H-4‴, 5‴), 1.72 (3H, s, H-4″), 3.23 (4H, s, CH_2, 1″ and 1‴), 5.17 (1H, t, H-2‴), 5.28 (1H, t, H-2″), 6.44 (1H, s, H-8), 6.66 (1H, d, J = 1.2, H-6′), 6.88 (1H, s, J = 1.2, H-2′), 8.26 (1H, s, H-2), 8.36, 9.36, 10.86 (each, 1H, 3′, 4′, 7-OH), 13.28 (5-OH), ^{13}C-NMR (150 MHz, DMSO-d_6): 18.16 (C-4″, 4‴), 21.50 (C-1‴), 25.95 (C-5"), 26.01 (C-5‴), 28.75 (C-1″), 93.31 (C-8), 104.72 (C-4a), 111.42 (C-6), 114.46 (C-2′), 120.91(C-6′), 122.55 (C-3), 122.63 (C-2″), 122.99 (C-1′), 123.50 (C-2‴), 128.41 (C-5′), 131.15(C-3″), 131.37 (C-3‴), 143.58 (C-4′), 144.94 (C-3′), 154.11 (C-2), 155.75 (C-8a), 159.27 (C-5), 162.38 (C-7), 180.77 (C-4).

that HEGU induced chromatin fragmentation and increased the number of annexin V-stained cells (Figs. 1A and B). Isoangustone A also effected a reduction in the viable cell numbers and induced apoptotic death in DU145, PC3, HT-29, and MDA-MB-231 cancer cells (Table 1).

In the previous study ([21]), we observed that DU145 cells were most sensitive to HEGU among the three examined cancer cell lines. However, in this study we noted that DU145 cells were most resistant to isoangustone A. There may be several active compounds other than isoangustone A in HEGU with the ability to reduce the numbers of viable cells and induce apoptosis. Additionally, there is a possibility that these various ingredients present in HEGU may exert synergistic and/or antagonistic effects. Future studies will be required to explain why prostate cancer cells are the most resistant to isoangustone A, but the most sensitive to HEGU.

Caspases are crucial players in the induction of apoptotic cell death. Caspase-9 is the only initiator caspase that has

Table 1. Isoangustone A reduces viable cell numbers and induces apoptosis in cancer cells

	Viable cell numbers (Absorbance at 570 nm)				Apoptotic cell death (Enrichment Factor)			
Isoangustone A (μg/mL)	0	2.5	5	7.5	0	2.5	5	7.5
DU145	0.804 ± 0.023^{a}	0.687 ± 0.04^{b}	0.525 ± 0.016^{c}	0.047 ± 0.001^{d}	1.0 ± 0.037^{c}	1.67 ± 0.12^{Bc}	2.57 ± 0.14^{B}	4.08 ± 0.81^{A}
PC3	1.613 ± 0.017^{a}	1.159 ± 0.02^{b}	0.943 ± 0.012^{c}	0.279 ± 0.035^{d}	1.0 ± 0.08^{d}	1.36 ± 0.07^{C}	1.71 ± 0.09^{B}	2.56 ± 0.15^{A}
HT-29	0.842 ± 0.011^{a}	0.266 ± 0.010^{b}	0.159 ± 0.011^{c}	0.123 ± 0.008^{d}	1.0 ± 0.081^{B}	1.03 ± 0.07^{B}	1.41 ± 0.24^{AB}	1.75 ± 0.10^{A}
MDA-MB-231	0.624 ± 0.009^{a}	0.581 ± 0.009^{b}	0.091 ± 0.002^{c}	0.095 ± 0.002^{c}	1.0 ± 0.067^{B}	1.13 ± 0.06^{B}	1.72 ± 0.10^{A}	1.75 ± 0.01^{A}

Cells were treated with various concentrations of isoangustone A, and the numbers of viable cells were estimated by the MTT assay. Apoptotic cell death was detected with Cell Death Detection ELISAPLUS kit. Each bar represents the mean ± SEM (n=6). Means without a common letter differ, $p < 0.05$.

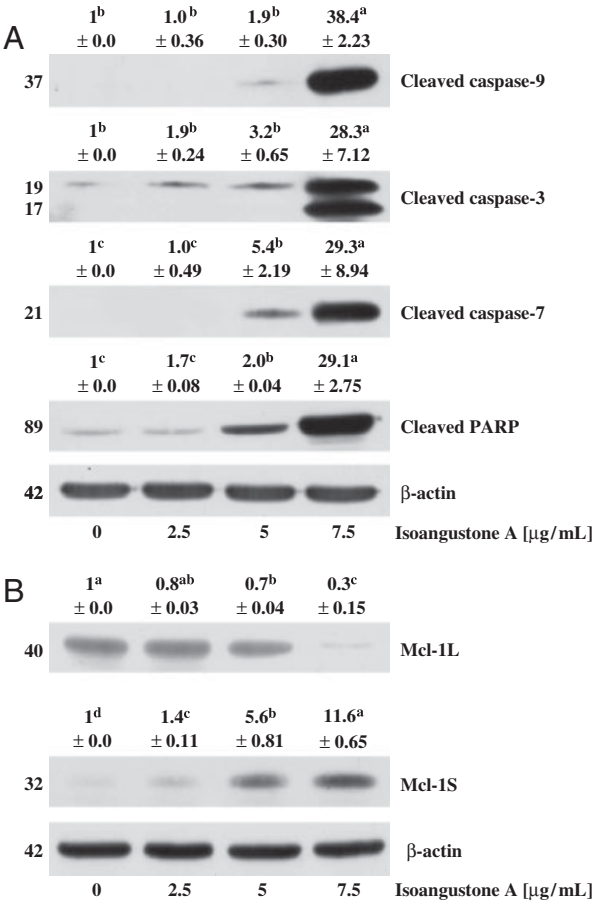

Figure 6. Isoangustone A induces cleavage of caspases and increases the levels of the Mcl-1S protein in DU145 cells. Serum-deprived cells were treated with various concentrations of isoangustone A for 6 h. Total cell lysates were analyzed *via* Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band is shown above each blot. Means without a common letter differ, $p<0.05$.

been implicated in the mitochondria-dependent pathway [33]. The complex of cytochrome *c*, Apaf-1, and procaspase-9 is a critical activator of the effector caspases [34]. In this study, HEGU treatment was shown to induce the cleavage of caspase-9, caspase-3, caspase-7, and PARP (Fig. 1C), increased the permeability of mitochondrial membranes (Fig. 2B) and increased the levels of cytochrome *c* in the cytoplasm (Fig. 2A). Isoangustone A also markedly increased the levels of cleaved caspases and PARP (Fig. 6A). These results demonstrate that HEGU and isoangustone A induce apoptosis *via* the activation of the mitochondrial apoptotic pathway and subsequent caspase activation in DU145 human prostate cancer cells.

The Bcl-2 family comprises anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1L), multidomain pro-apoptotic proteins (Bax and Bak), and pro-apoptotic BH3-only proteins (Bad, Bim, Bik, Bmf, and Bid), and performs pivotal functions in the regulation of mitochondrial membrane permeability [35–37]. Recently, an alternatively spliced form of Mcl-1 has been reported in humans. The short form of Mcl-1 (Mcl-1S), unlike the originally identified anti-apoptotic Mcl-1 long (Mcl-1L), is a BH3-only pro-apoptotic protein which is capable of dimerizing with the anti-apoptotic Mcl-1L, and its action is antagonized by Mcl-1L [38, 39]. In this study, we noted that HEGU effected an increase in the levels of the BH3-only proteins Mcl-1S, Bik, and tBid (Fig. 2C). Isoangustone A increased Mcl-1S levels and reduced Mcl-1L levels (Fig. 6B). These results indicate that the increase in the levels of pro-apoptotic BH3-only members of the Bcl-2 family resulted in increased mitochondrial membrane permeability in HEGU-treated cells (Fig. 2B).

The activation of plasma membrane DRs as the result of ligand binding or receptor clustering and aggregation triggers a DR-dependent apoptotic pathway. The overexpression of DR4 or DR5, which bind to TRAIL, has also been demonstrated to trigger apoptosis [40, 41]. In this study, we noted that HEGU and isoangustone A induced an increase in the levels of cleaved caspase-8, Fas, and DR4 (Figs. 3A, 4A and 7A). The caspase-8 inhibitor, Z-IETD-FMK, attenuated HEGU-induced apoptosis (Fig. 3B). Additionally, the transfection of cells with DR4 siRNA significantly attenuated the HEGU- and isoangustone A-mediated increases in apoptotic cell death and caspase-8 and caspase-3 cleavage (Figs. 4B, C and 7B and C). These results demonstrate that DR4 activation contributed to apoptosis in HEGU- and isoangustone A-treated cells, which may have been mediated by caspase-8 activation, Bid cleavage, and subsequent increases in mitochondrial membrane permeability. In addition, the activated caspase-8 may have directly activated caspase-3.

In conclusion, we have demonstrated that HEGU lacking GL induces the apoptosis of androgen-insensitive DU145 human prostate cancer cells, and isoangustone A was identified as one of the active compounds of HEGU. Isoangustone A reduces cell viability and induces the apoptosis of DU145 human cancer cells, HT-29 human colon cancer cells, and MDA-MB-231 human breast cancer cells. The data generated in this study show that HEGU and isoangustone A activate caspases *via* both the DR-dependent and the mitochondria-dependent apoptotic pathways in DU145 cells. As the evasion of programmed cell death or apoptosis is one of the hallmarks of cancer [14] and prostate cancer and hypertension increase with age, HEGU and isoangustone A deserve further evaluations of their potential as anti-carcinogenic agents.

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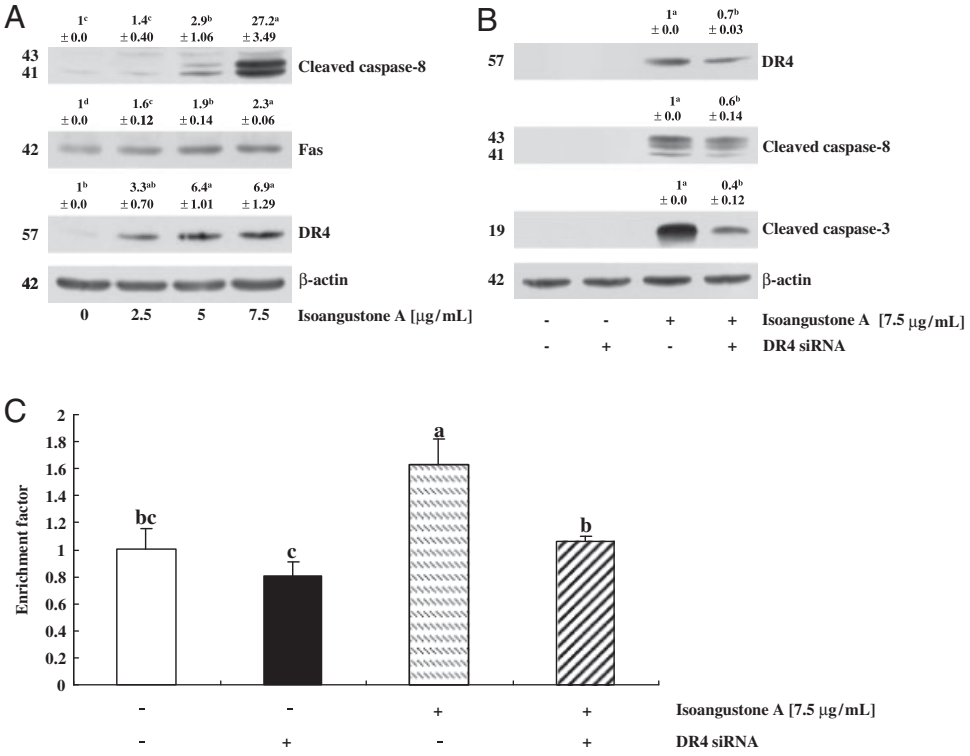

Figure 7. Isoangustone A induces DR4-dependent apoptosis in DU145 cells. (A) Cells were treated with isoangustone A as described in Fig. 6. The cell lysates were subjected to Western blotting with the indicated antibodies. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band was quantified, and the control levels were set at 1. The adjusted mean ± SEM (n=3) of each band to its own β-actin is shown above each blot. (B and C) Cells were transfected with control or DR4 siRNA, plated, and serum-deprived as described in Fig. 4. Serum-deprived cells were treated with isoangustone A for 6 h. (B) The total cell lysates were subjected to Western blotting with the indicated antibodies as described in Fig. 4B. (C) Apoptotic cells were detected with Cell Death Detection ELISA^{PLUS} kit as described in Fig. 4C. Each bar represents the mean ± SEM (n = 6). Means without a common letter differ, $p < 0.05$.

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