Parthenolide enhances sensitivity of colorectal cancer cells to TRAIL by inducing death receptor 5 and promotes TRAIL-induced apoptosis

SE-LIM KIM[1,2], YU-CHUAN LIU[1,2], YOUNG RAN PARK[1,2], SEUNG YOUNG SEO[1,2], SEONG HUN KIM[1,2], IN HEE KIM[1,2], SEUNG OK LEE[1,2], SOO TEIK LEE[1,2], DAE-GHON KIM[1,2] and SANG-WOOK KIM[1,2]

[1]Department of Internal Medicine, [2]Research Institute of Clinical Medicine, Biomedical Research Institute, Chonbuk National University Hospital, Chonbuk National University Medical School, Jeonju, Republic of Korea

Received October 1, 2014; Accepted November 26, 2014

DOI: 10.3892/ijo.2014.2795

Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent. Recombinant human TRAIL has been evaluated in clinical trials, however, various malignant tumors are resistant to TRAIL. Parthenolide (PT) has recently been demonstrated as a highly effective anticancer agent and has been suggested to be used for combination therapy with other anticancer agents. In this study, we investigate the molecular mechanisms by which PT sensitizes colorectal cancer (CRC) cells to TRAIL-induced apoptosis. HT-29 (TRAIL-resistant) and HCT116 (TRAIL-sensitive) cells were treated with PT and/or TRAIL. The results demonstrated that combined treatment induced apoptosis which was determined using MTT, cell cycle analysis, Annexin V assay and Hoechst 33258 staining. Interestingly, we confirmed that HCT116 cells have much higher death receptor (DR) 5 than HT-29 cells and PT upregulates DR5 protein level and surface expression in both cell lines. Apoptosis through the mitochondrial pathway was confirmed by detecting regulation of Bcl-2 family members, p53 cytochrome C release, and caspase cascades. These results suggest that PT sensitizes TRAIL-induced apoptosis via upregulation of DR5 and mitochondria-dependent pathway. Combination treatment using PT and TRAIL may offer an effective strategy to overcome TRAIL resistance of certain CRC cells.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) superfamily and known to induce apoptosis of cancer cells without significant toxicity toward normal cells (1-3). The TRAIL induces apoptosis through an extrinsic pathway by binding to the death receptors (DR) 4 and DR5. Activation of DR4 or DR5 recruits Fas-associated death domain (FADD) protein and procaspase-8, thereby forming a death-inducing signaling complex (DISC), which leads to activation of the caspase cascade (1,4). Caspase activation can be suppressed by inhibitors of apoptosis protein (IAP) family members, as well as by anti-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins (5). Application of TRAIL to cancer treatment is currently under intensive clinical evaluation. Especially, human recombinant TRAIL or high-affinity agonist monoclonal antibodies against DRs are now in phase I/II clinical trials (6-9). However, recent studies have shown that many types of cancer cells are resistant to the apoptotic effects of TRAIL including colorectal cancer (CRC) cells (10). TRAIL resistance in CRC cells can occur at several steps in signaling cascade such as deficient receptor redistribution to the membrane, mutation of caspase-8, cellular fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory protein (cFLIP) expression, Bax deficiency, or through X-linked inhibitor of apoptosis protein (XIAP) expression have been reported (11-15). Therefore, overcoming TRAIL resistance is a major challenge for the development of effective TRAIL-based therapeutic strategies.

Parthenolide (PT), a natural product, has been used for the treatment of fever and inflammatory disease. It is well known to inhibit interleukin-1 (IL-1) and tumor necrosis factor-α-mediated nuclear factor-κB (NF-κB) activation, which is responsible for its inflammatory activity (16-18). For over two decades, it is known that anticancer property of PT is through induction of apoptotic cell death in a number of human cancer cells (19-23). Apoptotic effect of PT is associated with inhibition of NF-κB and the activator of transcription 3 (STAT3), enhanced oxidative stress, and mitochondria-mediated apoptosis (19,20,24-26). In our previous studies, we found that PT can be a potential chemopreventive and therapeutic agent for CRC treatment by inducing apoptosis through mitochondrial dysfunction and inhibition of angiogenesis (20,27).

Correspondence to: Professor Sang-Wook Kim, Department of Internal Medicine, Chonbuk National University Hospital, 20 Geonji-ro, Deokjin-gu, Jeonju 561-712, Republic of Korea
E-mail: clickm@jbnu.ac.kr

Key words: parthenolide, TRAIL, death receptor, TRAIL resistance, apoptosis

In recent years, various investigations of combined therapy using PT were reported; PT sensitizes cancer cells to the non-steroidal anti-inflammatory drugs (NSAIDs), anticancer drug toxicity and radiation (25,28-33). Previously we have shown that combination of PT and 5-FU can overcome 5-FU resistance in human CRC cells and that intra-peritoneal injection of PT and 5-FU significantly inhibits tumor growth in the xenograft model (34). However, the effect and functional role of PT on TRAIL-induced apoptosis in CRC cells has not been reported.

In this study, we evaluated effects of combination therapy with PT and TRAIL on TRAIL-sensitive and -insensitive human CRC cells to gain insight into a potential treatment for CRC, especially TRAIL-resistant CRC. We also investigated the molecular mechanism of enhancing sensitivity of TRAIL by PT.

Materials and methods

Chemicals and reagents. PT and z-VAD-FMK were from Calbiochem (San Diego, CA, USA). TRAIL was purchased from Pepprotech (Rocky Hill, NJ, USA). Parthenolide was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) to a concentration of 100 μM and stored in the dark at -20˚C. Annexin-V-FITC and propidium iodide (PI) were purchased from Invitrogen (Eugene, OR, USA). Hoechst 33258 was from Sigma. Levels of DR4 and DR5 protein were analyzed using a respective specific antibody from ProSci Inc. (San Diego, CA, USA). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment. Human CRC cell lines, HT-29 and HCT 116 cells (American Type Culture Collection, Rockville, MD, USA) were employed as TRAIL-resistant and TRAIL-sensitive CRC cells, respectively. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U penicillin and 100 U streptomycin. For treatment of cells with PT or TRAIL or PT plus TRAIL, cells were sub-cultured in RPMI-1640 medium without FBS for 12 h. PT and TRAIL in the stock were diluted with FBS-free medium to achieve designated concentrations. Same concentration of DMSO was applied to the cells as a control.

Cell viability assay. Human CRC cells were plated at a density of $1.0x10^4$ cells per well in 96-well plates. Cells were treated with PT and/or TRAIL for 24 h, the medium was removed, and 200 μl of fresh medium plus 20 μl of 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT, 2.5 mg dissolved in 50 μl of dimethylsulfoxide, Sigma) were added to each well. After incubation for 4 h at 37˚C, the culture medium containing MTT was withdrawn and 200 μl of dimethylsulfoxide (DMSO) was added, followed by shaking until the crystals were dissolved. Viable cells were detected by measuring absorbance at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Annexin-V-fluorescein staining. After being incubated with single or dual agent for 24 h, the cells were trypsinized, collected, washed with ice-cold PBS, suspended in a 500 μl Annexin V binding buffer containing 5 μl of Annexin V-FITC, and incubated for 15 min at room temperature in the dark. The fluorescence was measured using a BD LSR flow cytometer and processed with CellQuest software for analysis.

Cell cycle and sub-G1 analysis. Cell cycle and sub-G1 distribution were determined by staining of DNA with propidium iodide (PI; Sigma-Aldrich) (Ex/Em = 488 nm/617 nm). PI is a fluorescent biomolecule that can be used to stain DNA. In brief, $1x10^6$ cells were incubated with single or dual agents for 24 h. Cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight. Cells were washed again with PBS and then incubated with PI (10 μg/ml) with simultaneous treatment of RNase at 37˚C for 1 h. The percentage of cells in different phases of the cell cycle or having sub-G1 DNA content was measured with a BD LSR flow cytometer and analyzed using CellQuest software.

Hoechst 33258 staining. Apoptotic feature of cancer cells was assessed by determining DNA condensation using Hoechst 33258. The cells were treated with single or dual agents for 24 h and then stained with Hoechst 33258 (1 μg/ml) at 37˚C for 10 min. Nuclear morphology was examined under a Confocal Laser Scanning Microscope (Carl Zeiss, Germany) to identify cells undergoing apoptosis.

Quantification of death receptor expression on cell surface. In order to quantify the cell surface expression of death receptors, DR4 and DR5, cells were harvested by trypsinization, washed in PBS and incubated for 30 min a 4˚C with phycoerythrin (PE)-conjugated monoclonal anti-human DR4 and DR5 antibody (eBioscences, San Diego, CA, USA). Non-immune mouse IgG was used as the negative control. The fluorescence was measured using a BD LSR flow cytometer and processed with CellQuest software for analysis.

Cell extraction and western blotting. After being incubated with single or dual agent for 24 h, the cells were collected, washed twice with PBS, and then lysed for 30 min on ice in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM EDTA, 1% Triton X-100, 0.5% SDS and protease inhibitor cocktail). The protein concentration in cell lysates was measured by using Protein Quantification kit from Bio-Rad. Total 50 μg proteins were loaded onto an SDS-PAGE gel. After transferring and blocking, the membrane was probed with various antibodies (anti-DR4, anti-DR5, anti-Bcl2, anti-Bax, anti-cytochrome C, anti-p53, anti-caspase-3, anti-caspase-8, anti-caspase-9 and anti-actin antibody). The binding of antibody to antigen was detected by using enhanced ECL prime (Amersham, UK), captured, and analyzed by the Las-3000 luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

Statistical analysis. The data are presented as the mean ± standard error (SE) of at least three independent experiments done in duplicate. Representative blots are shown. The data were entered into the Microsoft Excel 5.0, and SPSS software was used to perform the two-tailed t-tests or the analysis of the variance, where appropriate. P-value <0.05 was considered significant.

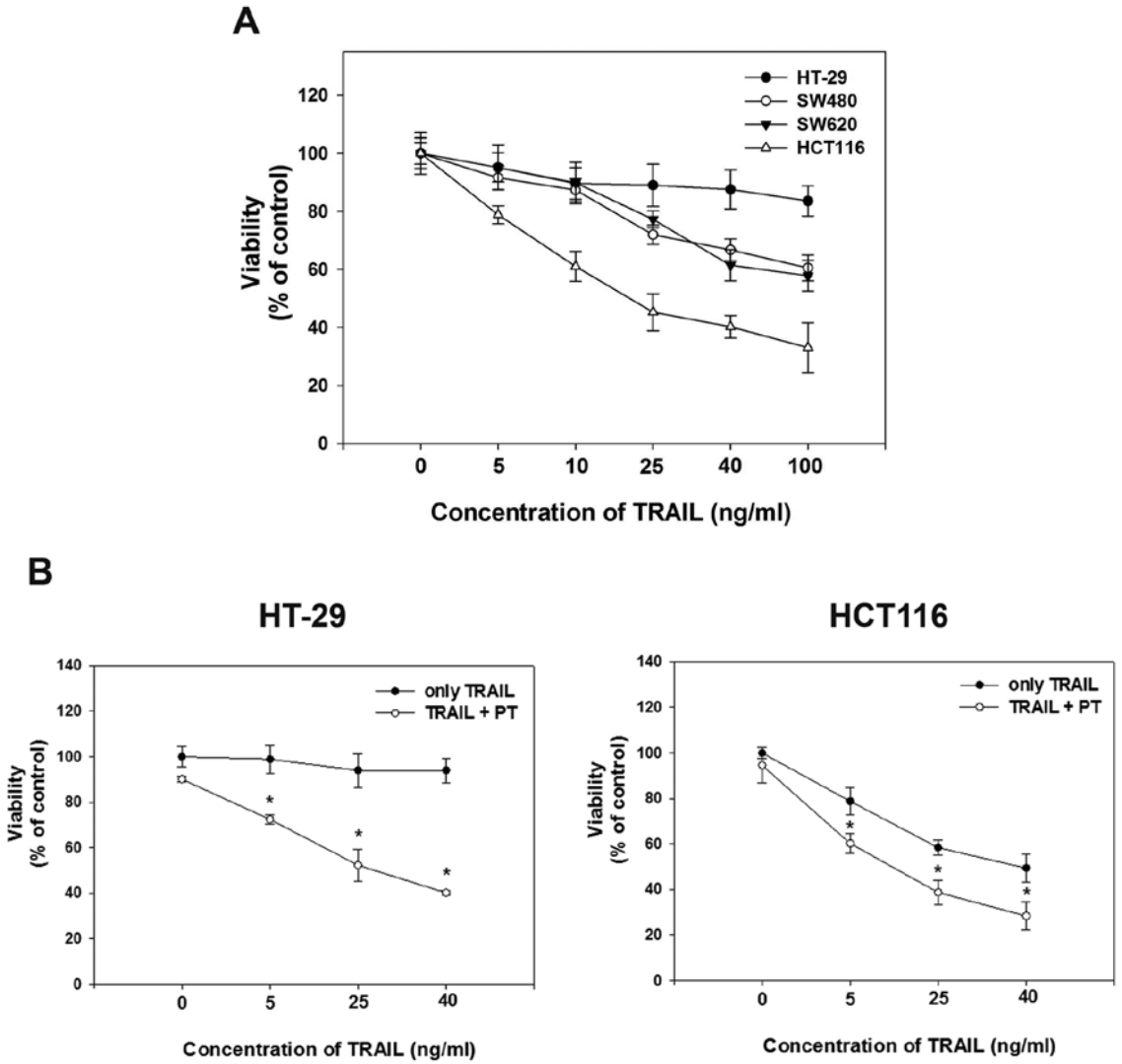

Figure 1. Inhibitory effect induced by combination of PT and TRAIL on cell growth. (A) HT-29, SW620, SW480 and HCT116 cells were treated with various concentration of TRAIL for 24 h. Then, cells were analyzed for viability by MTT assay. Data represent the mean values ± SE of three independent experiments. (B) HT-29 and HCT116 cells were treated with PT (10 μM) plus various concentrations of TRAIL for 24 h. The data represent the mean ± SE of three independent experiments. *P<0.05, versus TRAIL only.

Results

TRAIL sensitivity of human colorectal cancer cells and effect of PT on TRAIL-induced cell death. A panel of 4 human colorectal cancer cell lines (HT-29, SW480, SW620 and HCT116) were screened for TRAIL sensitivity by determining viability at various concentrations (0, 5, 10, 25, 50 or 100 ng/ml) for 24 h using the MTT assay. Of the 4 cell lines, 3 showed inhibition of viability in a dose-dependent manner to TRAIL treatment (Fig. 1A). Especially, HCT116 sensitively responded to TRAIL, showing ~70% of growth inhibition at 100 ng/ml concentration. On the contrary, treatment with TRAIL induced marginal cell death in HT-29 cells. These results indicate that HT-29 cells are TRAIL-resistant and HCT116 cells are TRAIL-sensitive.

To determine the synergistic effect of PT on TRAIL-induced cell death, HT-29 and HCT116 cells were incubated in the absence or presence of PT (10 μM) and TRAIL (with 5, 25 and 40 ng/ml) for 24 h. Co-treatment of PT with TRAIL significantly increased death of not only HCT116 cells but also HT-29 cells, suggesting that PT may sensitize HT-29 cells to TRAIL (Fig. 1B).

Effect of PT on TRAIL-induced apoptosis. To ascertain the above observations, Annexin V analysis was performed using FACScan. We found that treatment of HT-29 cells with PT and TRAIL alone induced 15.29 and 8.6% apoptosis in HT-29 cells, respectively, and 14.77 and 22.01% in HCT116 cells, respectively. In agreement with cell growth inhibition, treatment with TRAIL plus PT dramatically increased the Annexin V-positive cells (41.86%) by 5-fold than treatment with TRAIL, indicating that PT promotes TRAIL-induced apoptosis in HT-29 cells. Similar combination effect on apoptotic cell death was found in HCT116 cells (Fig. 2A).

We also evaluated cell cycle modifications induced by PT and TRAIL on TRAIL-resistant and -sensitive cells. After 24-h incubation with PT or PT plus TRAIL, cells were analyzed by PI staining using flow cytometric analysis (FCM). Treatment with PT and/or TRAIL resulted in the presence of a sub-G1 population, suggestive of apoptotic cell death. Similarly, FCM

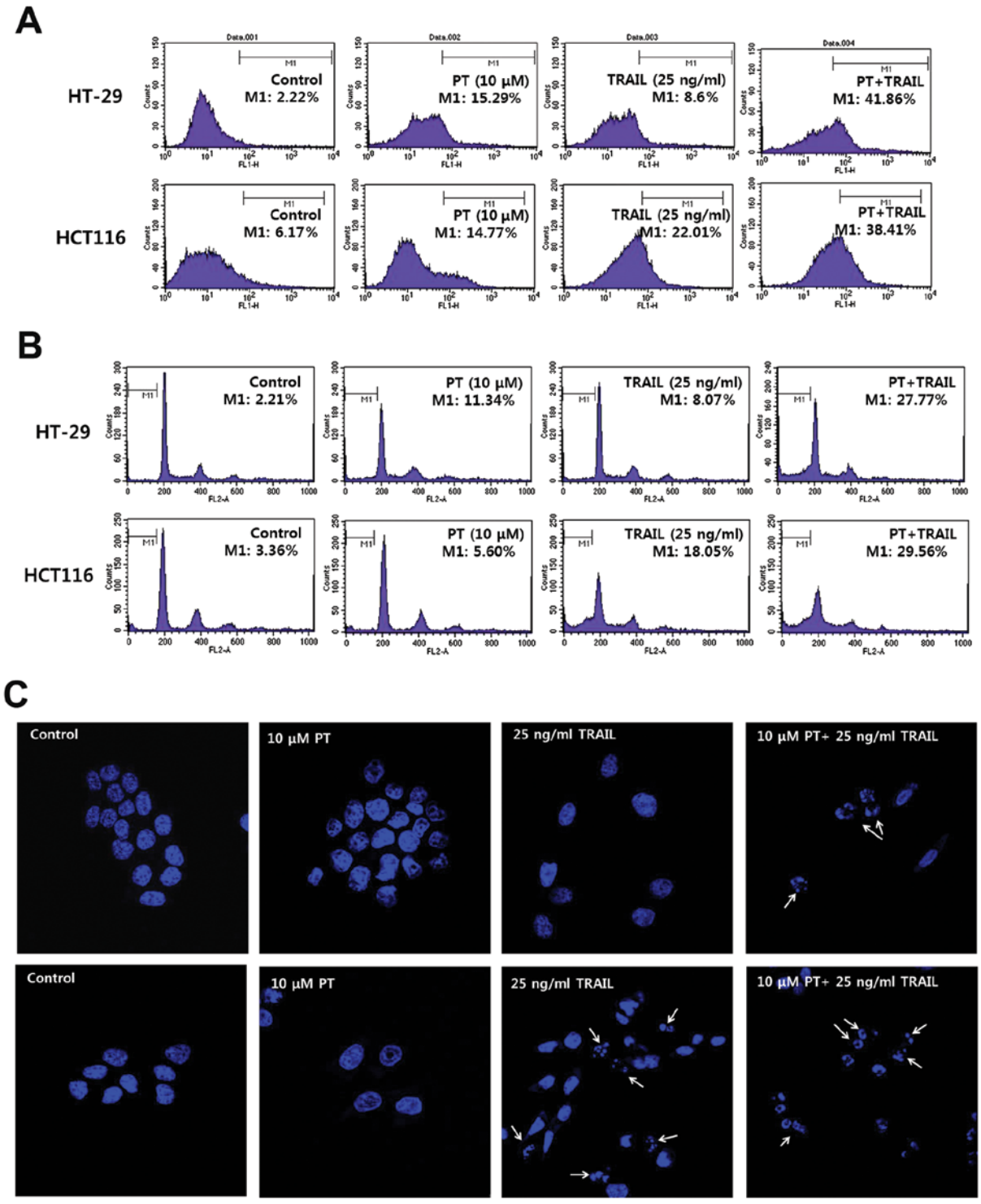

Figure 2. Apoptotic effect induced by combination treatment. (A) Apoptotic cell death induced by combination treatment. After treatment with TRAIL and/or PT for 24 h, cells were harvested and stained with Annexin V-FITC. Results from Annexin V assay showing apoptosis induced by combination treatment. (B) Cell cycle modification induced by combination treatment. After PT treatment and/or TRAIL for 24 h, cells were harvested and stained with PI. The percentage of sub-G1 population is shown in each histogram, and total number of events analyzed for each condition was 10,000. (C) DNA condensation and fragmentation induced by combination treatment. DNA condensation through the apoptotic cell death was determined using Hoechst 33258 (1 μg/ml). Apoptotic nuclei stained with Hoechst 33258 show intense fluorescence corresponding to chromatin condensation (arrowhead) and fragmentation.

revealed that PT significantly enhanced TRAIL-induced apoptosis >3-fold in HT-29 cells (8.07 versus 27.77%). In addition, higher sub-G1 arrested cells were detected.

HCT116 cells treated with PT plus TRAIL were arrested at higher level than single treatment in sub-G1 stage of the cell cycle. Peaks accounting for 11.34 and 8.07% of HT-29 cells and 5.6 and 18.05% of HCT116 cells of the overall cell population were detectable in treated with PT or TRAIL, respectively. In addition, a peak accounting for 27.77% was observed in treated with combination of HT-29 and HCT116 cells, indicating that combination treatment dramatically promotes apoptosis (Fig. 2B).

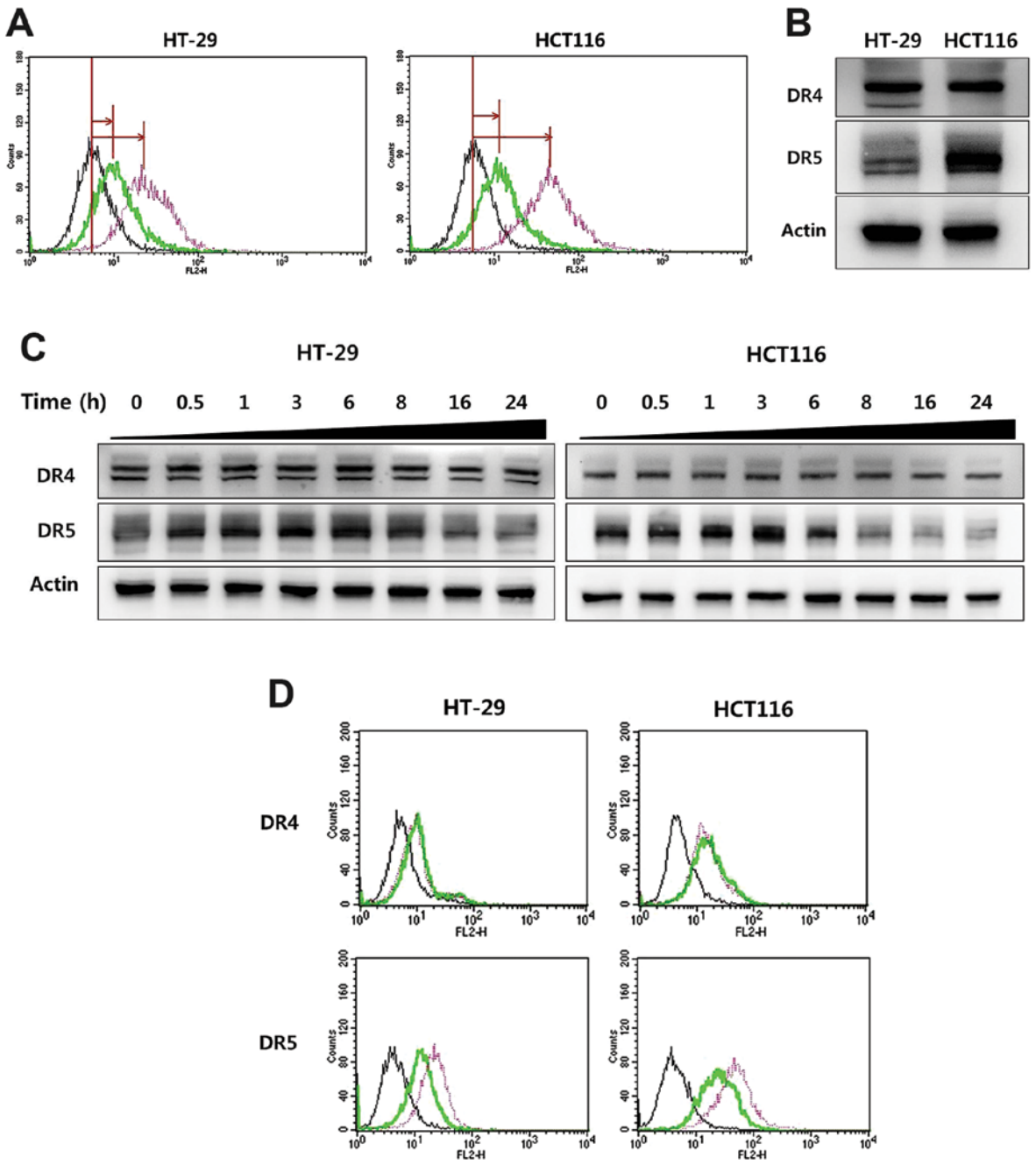

Figure 3. Expression level of DR4 and DR5 in TRAIL-resistant and -sensitive cells and effect of PT on the expression of these DRs. (A) Cell surface expression of DR4 and DR5 determined by flow cytometry. Histograms represent the mouse-IgG negative control (black), DR4 (green) and DR5 (pink) on the surface of live cell populations. (B) Expression level of DR4 and DR5 protein in each cell line. Total cell lysates were prepared and then analyzed by western blotting with DR4 or DR5 antibody. Actin was used as a loading control. (C) Effect of PT on expression of DR proteins. Cells were treated with 10 μM PT in various time-points and then equal amount of total proteins (50 μg/lane) was subjected to 12% SDS-PAGE. Expression of DR4, DR5 and actin was detected by western blotting using respective specific antibodies. (D) Flow cytometric analysis of surface expression of DRs. After treatment of 10 μM PT for 3 h, cells were harvested and incubated for 30 min at 4˚C with PE-conjugated monoclonal anti-human DR4 and DR5 antibodies and analyzed by flow cytometry. Mouse-IgG was used as the negative control. Control cells (black line) are compared with cells incubated for 3 h without (green) or with (pink) 10 μM PT.

To understand the mechanism of cell death induced by combination treatment, apoptotic nuclear morphology was evaluated after Hoechst 33258 staining. After treatment with single agent, HT-29 cells were regular in morphology and grew fully in patches and were confluent (Fig. 2C). However, treatment with PT and TRAIL together, HT-29 cells exhibited apoptotic characteristics, such as cell shrinkage, nuclear condensation and fragmentation. In HCT116 cells, treatment with TRAIL or PT plus TRAIL exhibited apoptotic nuclear morphologies while treatment with PT alone showed regular nuclear morphology.

Effect of PT on the expression of death receptor in CRC cells. In order to ascertain the mechanism by which PT sensitizes CRC cells to TRAIL, we investigated the level of expression of death receptors on the cell surface. Levels of DR4 and DR5 expression were analyzed by flow cytometry using PE conjugated anti-human DR4 and DR5 antibody (Fig. 3A).

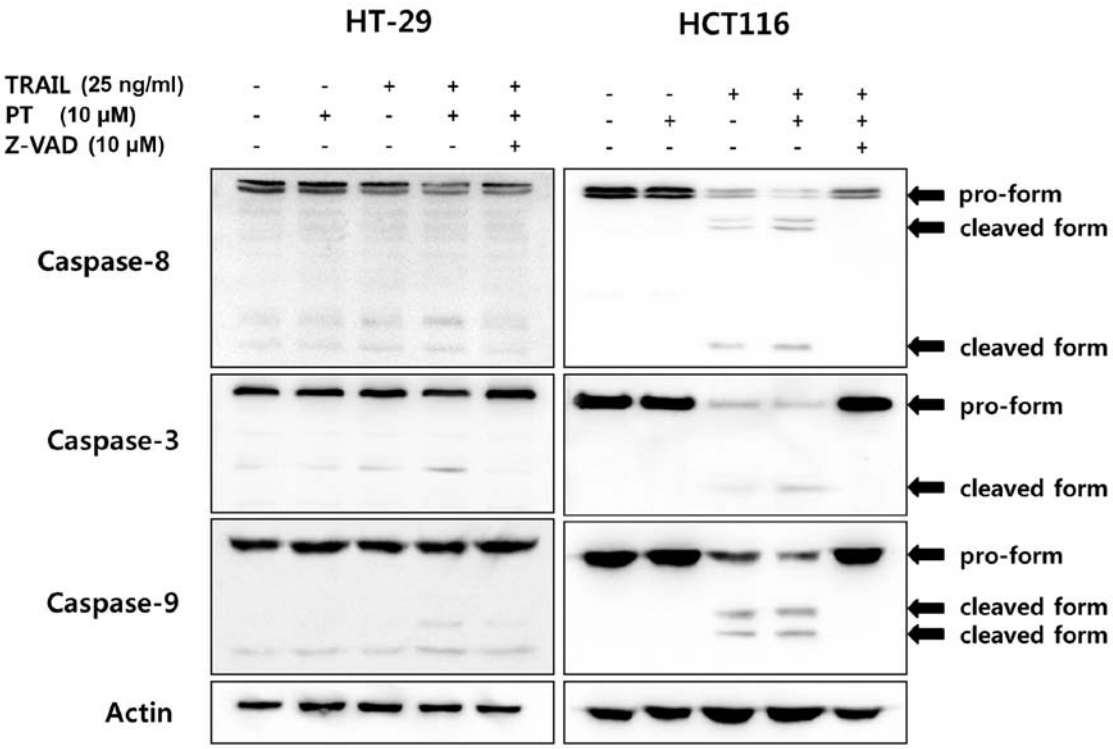

Figure 4. Activation of caspases after treatment with PT and/or TRAIL. Cell lysates were prepared after treatment with PT and/or TRAIL for 24 h and then analyzed by western blotting with caspase-3 or -8 or caspase-9 antibody. Protein levels of cleaved caspase-3, -8 and -9 were increased by combination treatment. However, activation of caspases was blocked by pretreatment with an pan-caspase inhibitor, Z-VAD-FMK.

Cell surface expression of DR4 (green line) and DR5 (pink line) was found in both of cell lines. Interestingly, the level of DR5 expression was significantly higher than the DR4 level. Moreover, the level of DR5 expression on HCT116 cells was much higher than the level on HT-29 cells. Supporting the data, western blotting results were well correlated with flow cytometry analysis (Fig. 3B).

In addition, effects of PT on DR4 and DR5 expression in HT-29 and HCT116 cells were analyzed by western blotting. Treatment with PT (10 μM) markedly changed the level of DR5 protein in both cell lines in a time-dependent manner (Fig. 3C). The PT treatment increased the level of DR5 protein up to 6 h in HT-29 cells and 3 h in HCT116 cells but the effect disappeared after that. On the contrary, the level of DR4 protein did not change in either cell line after treatment with PT.

We also investigated whether treatment with PT increases surface expression of the TRAIL receptors. In these experiments, cells treated with PT (10 μM) for 3 h were incubated for 30 min with anti-human DR4 or DR5 antibody conjugated with PE and analyzed by flow cytometry. The PT treatment did not induce surface expression of DR4, while the cell surface expression of DR5 were increased in both cell lines (Fig. 3D). Together, these observations suggest that TRAIL sensitivity is influenced by the level of DR5 expression.

PT enhances TRAIL-induced apoptosis via activation of caspase cascade. Many anticancer agents are capable of initiating caspase activation and inducing apoptotic cell death (35). The effects of PT, TRAIL or PT plus TRAIL on caspase activation in TRAIL-resistant and -sensitive cells were examined. Western blot analysis of HT-29 cells treated with PT plus TRAIL revealed that levels of cleaved caspase-3, -8 and -9 were significantly increased compared the levels in the cells treated PT or TRAIL alone (Fig. 4). Although, in HCT116 cells, TRAIL alone had effects on the activation of the caspase (-3, -8 and -9) cleavage, the combination treatment significantly increased the activation of caspases.

Western blot analysis of HCT116 cells treated with TRAIL or PT plus TRAL showed that levels of cleaved caspase-3, -8 and -9 were significantly increased compared to the levels of control and PT treated cells. In addition, the decrease in the levels of caspase-3, -8 and -9 in cells treated with PT plus TRAIL was significantly blocked by pretreatment of a general caspase inhibitor, Z-VAD-FMK (Fig. 4).

PT enhances TRAIL-induced expression of proteins involved in apotosis. To evaluate the mechanisms responsible for apoptosis by combination of TRAIL and PT, we examined the expression level of several pro-apoptosis and anti-apoptosis proteins in TRAIL-resistant and -sensitive cells, HT-29 and HCT116 cells, respectively. Western blot analysis showed that the level of Bcl-2 in both cell lines was significantly decreased by treatment with PT plus TRAIL compared to the level in the cells treated with PT or TRAIL alone. In contrast, the expression of Bax was significantly increased by treatment with PT plus TRAIL compared to the level in the cells treated with PT or TRAIL alone (Fig. 4, first and second panel).

One of the consequences following the changes of Bcl-2 family members is the dissipation of mitochondrial potential

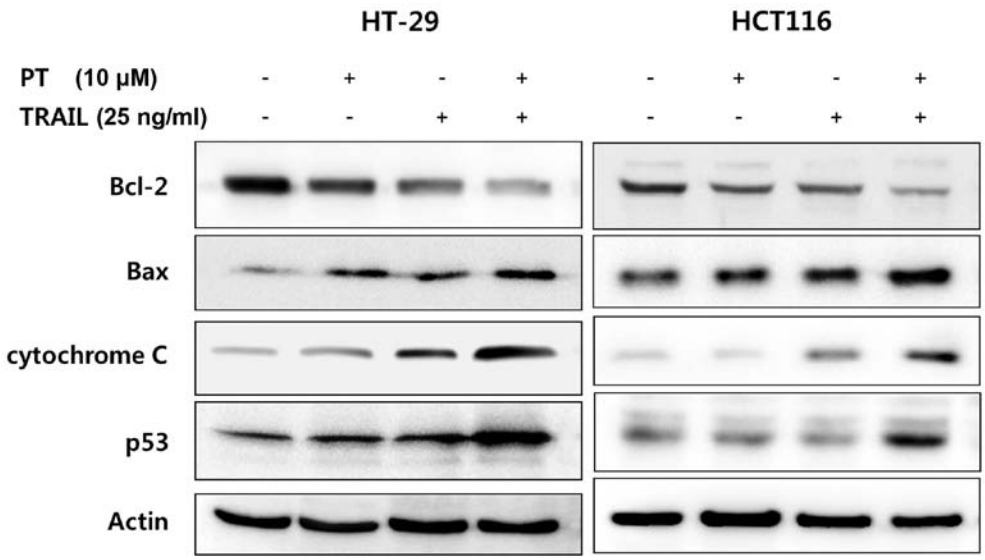

Figure 5. Effects of combination treatment on proteins involved in the apoptosis pathway. Cell lysates were prepared after treatment with PT and/or TRAIL for 24 h and analyzed by western blotting using Bcl-2, Bax, cytochrome *c*, and p53 antibody. The level of anti-apoptotic protein, Bcl-2 was significantly decreased by combination treatment with PT and TRAIL. However, pro-apoptotic protein, Bax was increased by the combination. The level of cytochrome C and p53 was increased after combination treatment. Actin was used as a loading control.

and release of the mitochondrial proapoptotic protein, cytochrome C. Treatment with PT plus TRAIL significantly increased the release of cytochrome C of both of cell lines compared to that of control and single drug treated cells (Fig. 4, third panel).

In addition, the p53 gene, which is inactivated in a majority of human cancers, has been recognized as a hallmark of apoptosis (36). The level of p53 was significantly increased by treatment with PT plus TRAIL compared to the level in cells treated with PT or TRAIL alone (Fig. 4, fourth panel). These results indicated that the apoptosis induced by the combination of TRAIL and PT may be associated with the mitochondrial pathway.

Discussion

In CRC, chemotherapy is currently used to reduce tumor recurrence and prolong survival. However, due to drug resistance, investigations of new chemotherapy strategies are required. Compared to other cancer cells, CRC cells show increased resistance to chemotherapeutic agents (37). The molecular mechanisms of drug resistance in colon cancer cells are still unknown.

Drug combination therapies play a prominent role in overcoming of drug resistance in cancer treatment. Exploration of the molecular mechanisms underlying the synergistic effects achieved by a drug combination would fuel efforts to rationally develop combination therapeutics that could significantly improve patient outcomes in cancer.

Recent studies have demonstrated that PT has anticancer activity and induces apoptosis through wide range of intracellular signals in cancer cells (22,38). We have also found that PT induces apoptosis through mitochondrial dysfunction in human CRC cells and inhibits tumor growth and angiogenesis in a CRC xenograft model (20). PT is considered to be a promising candidate as a new type of chemotherapeutic agent for cancer treatment. It would be interesting to investigate whether treatment with PT alone or in combination with an anticancer drug can overcome drug resistance. In this study, we found that combination treatment with PT and TRAIL resulted in the increase of apoptosis via caspase activation and that PT enhanced TRAIL sensitivity by upregulation of DR5 in TRAIL-resistant and -sensitive cells. These observations indicate that combination of TRAIL and PT may overcome TRAIL resistance in CRC and be an effective therapeutic strategy for patients with CRC.

Interactions between TRAIL and PT have been examined in a limited number of preclinical studies. A study in breast cancer cells has demonstrated that PT reverses resistance of breast cancer cells to TRAIL through c-Jun N-terminal kinase (JNK) activation (39). PT also sensitizes hepatocellular carcinoma cells to TRAIL through inhibition of STAT3 (28). However, the effect of PT on TRAIL-induced apoptosis remains to be understood. Therefore, in this study, we focused on the mechanism of apoptosis induced by PT, evaluating regulation of TRAIL receptors and mitochondrial apoptotic pathway using TRAIL-resistant and -sensitive human CRC cells.

Our results showed that TRAIL inhibited growth of HCT116 cells (a TRAIL-sensitive cell line) in a dose-dependent manner; however, the inhibition did not occur in TRAIL-resistant HT-29 cells. These results are very similar to the findings reported on the TRAIL sensitivity of human CRC cells (40-42). Interestingly, our results also indicated that combination of PT and TRAIL reduced cell growth and increased apoptotic cell death of not only TRAIL-sensitive cells but also TRAIL-resistant cells.

TRAIL mediates apoptotic cell death through enhancing expression of the death receptors DR4 and DR5, which are expressed on the surface of cancer cells (43,44). The

binding of TRAIL to DR4 and DR5 leads to activation of caspases, which in turn cleaves and activates executioner caspases that mediate apoptosis (45,46). Several studies have provided evidence that DR upregulation is a promising strategy for sensitizing TRAIL resistance in cancer cells (47-51). Garcinol, a polyisoprenylated benzophenone derivative, can potentiate TRAIL-induced apoptotic cell death of human CRC cell through upregulation of DR4 and DR5 (47). Quercetin, a ubiquitous bioactive plant flavonoid, enhances TRAIL-induced apoptotic cell death in prostate cancer cells via expression of DR5 (48,49). Snake venom toxin from *Vipera lebetina turanica* sensitizes cancer cells to TRAIL through upregulation of death receptors and downregulation of survival proteins (51). In the present study, our data indicated that PT markedly increased the expression of DR5 protein at early stage, while PT did not affect the expression of DR4. In particular, the effect of PT on the expression of DRs at various time-points has not been evaluated. Moreover, analysis by flow cytometry permitted to establish that PT upregulated cell surface expression of DR5 and that PT was ineffective on DR4 expression. These findings suggest that upregulation of surface expression of DR5 is the prominent event by which PT sensitizes human CRC cells to TRAIL-induced apoptosis.

Many anticancer agents are capable of initiating activation of caspase cascade and inducing apoptotic cell death (52). Caspase-3 and -9, terminal factor of apoptosis, exist as an inactive precursor in cytoplasm, which is activated during apoptosis and takes part in apoptosis induced by multiple factors. Moreover, caspase-8 activation is the initial step in the TRAIL-mediated caspase activation cascade, and lack of caspase-8 expression has been reported to cause TRAIL resistance (53). To understand the mechanism by which PT and TRAIL induce apoptosis, we examined its effect on the activation and cleavage of these caspases. The results showed that the levels of cleaved form of caspase-3, -8 and -9 were increased by combination treatment with PT and TRAIL especially in TRAIL-resistant cells, decreasing the levels of pro-forms. The cleavage of caspases was prevented by pretreatment with the pancaspase inhibitor Z-VAD-FMK. Our findings suggest that PT and TRAIL-induced apoptosis is mediated by enhancing the apoptotic sensitivity to TRAIL via caspase-dependent pathway.

Previous studies have reported that the Bcl-2 family members play an important role in PT action in CRC. The mitochondrial pathway is regulated by the Bcl-2 family, which is divided into two groups, the anti-apoptotic members (Bcl-2 and Bcl-xl) and pro-apoptotic members (Bax, BAD, BAK and Bid) (54,55). Level of Bcl-2 is often enhanced in tumors, with a possible substitution of Bcl-2 by Bcl-xL in the most aggressive tumors (56,57). Mutation in Bax has been reported in some colon cancers, the majority of which have a defect in DNA mismatch repair which is readily detected by mutations in repetitive sequences (58). Therefore, the regulation of Bcl-2 and Bax expression has an important role in chemotherapy, and it could be a measure of chemotherapeutic effect. In this study, the results showed that expression level of Bcl-2 in HCT116 and HT-29 cells treated with PT plus TRAIL was decreased while the level of Bax was increased. These results demonstrate that resistance of TRAIL CRC cells (HCT116 cells) is overcome by combining with PT and that the combination treatment-induced apoptosis is under the control of the mitochondrial pathway.

The tumor suppressor gene p53 is involved in G1 growth arrest by inducing the cyclin-dependent kinase inhibitor p21 and also in apoptosis through transactivation of the pro-apoptotic Bax gene in response to DNA-damage (58,59). The p53 gene, which is inactivated in a majority of human cancers, has been proposed as an accurate indicator of response of CRC to anticancer drug (36). In the present study, that results showed that the level of p53 was enhanced by combination treatment with PT and TRAIL in TRAIL-resistant and -sensitive cells.

In conclusion, we investigated effects of combination of PT and TRAIL on cell growth and apoptotic cell death using TRAIL-resistant and -sensitive human CRC cells. Treatment with PT dramatically increased the surface expression of DR5 protein in both cell types. Moreover, combination of PT and TRAIL upregulated expression of proteins involved in the mitochondrial apoptotic pathway and increased caspase activation. Taken together, these results suggest that PT sensitizes CRC cells resistant to TRAIL, therefore, we believe that combined treatment with PT and TRAIL could represent a new therapeutic strategy for CRC treatment.

Acknowledgements

This study was supported by Fund of Chonbuk National University Hospital Research Institute of Clinical Medicine. The authors thank Professor Mie-Jae Im (Department of Internal Medicine, Research Center for Pulmonary Disorders, Chonbuk National University Medical School) for kindly proofreading and contribution.

References

1. Johnstone RW, Frew AJ and Smyth MJ: The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer 8: 782-798, 2008.
2. Koschny R, Walczak H and Ganten TM: The promise of TRAIL - potential and risks of a novel anticancer therapy. J Mol Med (Berl) 85: 923-935, 2007.
3. Walczak H, Miller RE, Ariail K, *et al*: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5: 157-163, 1999.
4. Gonzalvez F and Ashkenazi A: New insights into apoptosis signaling by Apo2L/TRAIL. Oncogene 29: 4752-4765, 2010.
5. Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9: 459-470, 2002.
6. Ashkenazi A, Holland P and Eckhardt SG: Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 26: 3621-3630, 2008.
7. Pan Y, Xu R, Peach M, *et al*: Evaluation of pharmacodynamic biomarkers in a Phase 1a trial of dulanermin (rhApo2L/TRAIL) in patients with advanced tumours. Br J Cancer 105: 1830-1838, 2011.
8. Wiezorek J, Holland P and Graves J: Death receptor agonists as a targeted therapy for cancer. Clin Cancer Res 16: 1701-1708, 2010.
9. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A and Ford HL: On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. Oncogene 32: 1341-1350, 2013.
10. Zhang L and Fang B: Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther 12: 228-237, 2005.

11. Jin Z, McDonald ER III, Dicker DT and El-Deiry WS: Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. J Biol Chem 279: 35829-35839, 2004.
12. Kim HS, Lee JW, Soung YH, *et al*: Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology 125: 708-715, 2003.
13. Hernandez A, Wang QD, Schwartz SA and Evers BM: Sensitization of human colon cancer cells to TRAIL-mediated apoptosis. J Gastrointest Surg 5: 56-65, 2001.
14. Burns TF and El-Deiry WS: Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma cell line SW480 using a genetic approach. J Biol Chem 276: 37879-37886, 2001.
15. Cummins JM, Kohli M, Rago C, Kinzler KW, Vogelstein B and Bunz F: X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res 64: 3006-3008, 2004.
16. Murphy JJ, Heptinstall S and Mitchell JR: Randomised double-blind placebo-controlled trial of feverfew in migraine prevention. Lancet 2: 189-192, 1988.
17. Hehner SP, Heinrich M, Bork PM, *et al*: Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. J Biol Chem 273: 1288-1297, 1998.
18. Lyss G, Knorre A, Schmidt TJ, Pahl HL and Merfort I: The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. J Biol Chem 273: 33508-33516, 1998.
19. Zhang S, Ong CN and Shen HM: Involvement of proapoptotic Bcl-2 family members in parthenolide-induced mitochondrial dysfunction and apoptosis. Cancer Lett 211: 175-188, 2004.
20. Kim SL, Trang KT, Kim SH, *et al*: Parthenolide suppresses tumor growth in a xenograft model of colorectal cancer cells by inducing mitochondrial dysfunction and apoptosis. Int J Oncol 41: 1547-1553, 2012.
21. Wen J, You KR, Lee SY, Song CH and Kim DG: Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. J Biol Chem 277: 38954-38964, 2002.
22. Zhang S, Ong CN and Shen HM: Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells. Cancer Lett 208: 143-153, 2004.
23. Pajak B, Gajkowska B and Orzechowski A: Molecular basis of parthenolide-dependent proapoptotic activity in cancer cells. Folia Histochem Cytobiol 46: 129-135, 2008.
24. Dai Y, Guzman ML, Chen S, *et al*: The NF (Nuclear factor)-kappaB inhibitor parthenolide interacts with histone deacetylase inhibitors to induce MKK7/JNK1-dependent apoptosis in human acute myeloid leukaemia cells. Br J Haematol 151: 70-83, 2010.
25. Sun Y, St Clair DK, Xu Y, Crooks PA and St Clair WH: A NADPH oxidase-dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells. Cancer Res 70: 2880-2890, 2010.
26. Sobota R, Szwed M, Kasza A, Bugno M and Kordula T: Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochem Biophys Res Commun 267: 329-333, 2000.
27. Kim SL, Lee ST, Trang KT, *et al*: Parthenolide exerts inhibitory effects on angiogenesis through the downregulation of VEGF/VEGFRs in colorectal cancer. Int J Mol Med 33: 1261-1267, 2014.
28. Carlisi D, D'Anneo A, Angileri L, *et al*: Parthenolide sensitizes hepatocellular carcinoma cells to TRAIL by inducing the expression of death receptors through inhibition of STAT3 activation. J Cell Physiol 226: 1632-1641, 2011.
29. Fang LJ, Shao XT, Wang S, Lu GH, Xu T and Zhou JY: Sesquiterpene lactone parthenolide markedly enhances sensitivity of human A549 cells to low-dose oxaliplatin via inhibition of NF-kappaB activation and induction of apoptosis. Planta Med 76: 258-264, 2010.
30. Gao ZW, Zhang DL and Guo CB: Paclitaxel efficacy is increased by parthenolide via nuclear factor-kappaB pathways in in vitro and in vivo human non-small cell lung cancer models. Curr Cancer Drug Targets 10: 705-715, 2010.
31. Yun BR, Lee MJ, Kim JH, Kim IH, Yu GR and Kim DG: Enhancement of parthenolide-induced apoptosis by a PKC-alpha inhibition through heme oxygenase-1 blockage in cholangiocarcinoma cells. Exp Mol Med 42: 787-797, 2010.
32. Lee CS, Kim YJ, Lee SA, Myung SC and Kim W: Combined effect of Hsp90 inhibitor geldanamycin and parthenolide via reactive oxygen species-mediated apoptotic process on epithelial ovarian cancer cells. Basic Clin Pharmacol Toxicol 111: 173-181, 2012.
33. Yip-Schneider MT, Nakshatri H, Sweeney CJ, Marshall MS, Wiebke EA and Schmidt CM: Parthenolide and sulindac cooperate to mediate growth suppression and inhibit the nuclear factor-kappa B pathway in pancreatic carcinoma cells. Mol Cancer Ther 4: 587-594, 2005.
34. Kim SL, Kim SH, Trang KT, *et al*: Synergistic antitumor effect of 5-fluorouracil in combination with parthenolide in human colorectal cancer. Cancer Lett 335: 479-486, 2013.
35. Fulda S and Debatin KM: Death receptor signaling in cancer therapy. Curr Med Chem Anticancer Agents 3: 253-262, 2003.
36. Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61: 759-767, 1990.
37. Bates RC, Edwards NS, Burns GF and Fisher DE: A CD44 survival pathway triggers chemoresistance via lyn kinase and phosphoinositide 3-kinase/Akt in colon carcinoma cells. Cancer Res 61: 5275-5283, 2001.
38. Woynarowski JM and Konopa J: Inhibition of DNA biosynthesis in HeLa cells by cytotoxic and antitumor sesquiterpene lactones. Mol Pharmacol 19: 97-102, 1981.
39. Nakshatri H, Rice SE and Bhat-Nakshatri P: Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. Oncogene 23: 7330-7344, 2004.
40. Vasilevskaya IA and O'Dwyer PJ: 17-Allylamino-17-demethoxy-geldanamycin overcomes TRAIL resistance in colon cancer cell lines. Biochem Pharmacol 70: 580-589, 2005.
41. Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K and Johnston PG: Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. Mol Cancer Ther 4: 2026-2036, 2005.
42. Saturno G, Valenti M, De Haven Brandon A, *et al*: Combining trail with PI3 kinase or HSP90 inhibitors enhances apoptosis in colorectal cancer cells via suppression of survival signaling. Oncotarget 4: 1185-1198, 2013.
43. Ashkenazi A and Herbst RS: To kill a tumor cell: the potential of proapoptotic receptor agonists. J Clin Invest 118: 1979-1990, 2008.
44. Eberle J, Fecker LF, Forschner T, Ulrich C, Rowert-Huber J and Stockfleth E: Apoptosis pathways as promising targets for skin cancer therapy. Br J Dermatol 156 (Suppl 3): 18-24, 2007.
45. Pennarun B, Meijer A, de Vries EG, Kleibeuker JH, Kruyt F and de Jong S: Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. Biochim Biophys Acta 1805: 123-140, 2010.
46. Jung YH, Heo J, Lee YJ, Kwon TK and Kim YH: Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. Life Sci 86: 351-357, 2010.
47. Prasad S, Ravindran J, Sung B, Pandey MK and Aggarwal BB: Garcinol potentiates TRAIL-induced apoptosis through modulation of death receptors and antiapoptotic proteins. Mol Cancer Ther 9: 856-868, 2010.
48. Kim YH, Lee DH, Jeong JH, Guo ZS and Lee YJ: Quercetin augments TRAIL-induced apoptotic death: involvement of the ERK signal transduction pathway. Biochem Pharmacol 75: 1946-1958, 2008.
49. Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L and Pintzas A: Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. Mol Cancer Ther 6: 2591-2599, 2007.
50. Sung B, Park B, Yadav VR and Aggarwal BB: Celastrol, a triterpene, enhances TRAIL-induced apoptosis through the down-regulation of cell survival proteins and up-regulation of death receptors. J Biol Chem 285: 11498-11507, 2010.
51. Park MH, Jo M, Won D, Song HS, Song MJ and Hong JT: Snake venom toxin from *Vipera lebetina turanica* sensitizes cancer cells to TRAIL through ROS- and JNK-mediated upregulation of death receptors and downregulation of survival proteins. Apoptosis 17: 1316-1326, 2012.
52. Fernandes-Alnemri T, Litwack G and Alnemri ES: CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J Biol Chem 269: 30761-30764, 1994.
53. Teitz T, Wei T, Valentine MB, *et al*: Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 6: 529-535, 2000.

54. Mignotte B and Vayssiere JL: Mitochondria and apoptosis. Eur J Biochem 252: 1-15, 1998.
55. Reed JC: Double identity for proteins of the Bcl-2 family. Nature 387: 773-776, 1997.
56. De Angelis PM, Stokke T, Thorstensen L, Lothe RA and Clausen OP: Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal carcinomas, and association with p53 genotype/phenotype. Mol Pathol 51: 254-261, 1998.
57. Maurer CA, Friess H, Buhler SS, *et al*: Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. Dig Dis Sci 43: 2641-2648, 1998.
58. Rampino N, Yamamoto H, Ionov Y, *et al*: Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 275: 967-969, 1997.
59. Sionov RV and Haupt Y: The cellular response to p53: the decision between life and death. Oncogene 18: 6145-6157, 1999.