Goniothalamin enhances TRAIL-induced apoptosis in colorectal cancer cells through DR5 upregulation and cFLIP downregulation

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Abstract. The combination of TNF-related apoptosis-inducing ligand (TRAIL) and bioactive compound to enhance apoptosis in TRAIL-resistant cancer is one of cancer treatment strategies. TRAIL possesses the unique capacity to selectively induce apoptosis in cancer cells both in vitro and in vivo with little effect on normal cells. Recent studies have reported that there are many TRAIL-resistant cancers. Thus, bioactive compounds that enhance cytotoxicity of TRAIL would be potential candidates for cancer therapeutic application. This study evaluated the cytotoxic and apoptosis induction upon combined treatment of TRAIL and goniothalamin, the natural styril-lactone compound extracted from plant Goniothalamus spp., in LoVo cells. The results showed that a combination of goniothalamin and TRAIL enhanced caspase-dependent apoptosis induction in LoVo cells via both death receptor- and mitochondrial-mediated apoptosis pathways. In addition, goniothalamin enhanced TRAIL-induced apoptosis through increased death receptor DR5 expression and decreased anti-apoptotic regulator cFLIP. Interestingly, goniothalamin increased translocation of DR5 to cell surface and consequently contributed to the enhancement of TRAIL-induced apoptosis. In conclusion, this is the first report showing the combined treatment of goniothalamin and TRAIL was able to effectively enhance TRAIL-mediated apoptosis induction in TRAIL-refractory colorectal cancer, LoVo cells. Therefore, this study may offer a strategic cancer treatment against TRAIL-resistant cancers.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/APO2L) is a member of the tumor necrosis factor (TNF) family of cytokines and an effective inducer of apoptosis in cancer cells (1). The interaction of TRAIL with death receptors (DRs), including the death receptors TRAILR1 (also known as DR4 and TNFRSF10A) and TRAILR2 (also known as DR5, KILLER and TNFRSF10B), on the surface of cancer cells can trigger apoptotic cell death signaling through death receptor-mediated apoptosis pathway without any harmful effects to normal cells (2,3). TRAIL binding to DRs causes conformational changes in DRs, which leads to the recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8 and -10 through the cytoplasmic death domain (DD). This forms the so-called death-inducing signaling complex (DISC). Normally, the DISC fully activates caspase-8 and triggers apoptosis by directly activating the executive caspases, such as caspase-3, -6 and -7, also found in Fas type I cancer cells. However, TRAIL-mediated apoptosis can also induce the mitochondria-mediated apoptosis pathway through the implication of mitochondrial dysfunction and caspase-9 activation via the cleavage of Bid (BH3-interacting domain death agonist) protein into truncated Bid (tBid) by caspase-8. tBid is capable of inducing mitochondrial outer membrane permeabilisation (MOMP) in cells in which the ratio of pro- and anti-apoptotic Bcl-2 family members allows it to do so leading to mitochondrial dysfunction in TRAIL-treated cancer cells, also found in Fas type II cancer cells (4-7).

However, some cancer cells are resistant to TRAIL-induced apoptosis, especially some highly malignant tumors such as pancreatic cancer, melanoma, neuroblastoma, prostate cancer and colon cancer (8,9). Failure to undergo apoptosis has been implicated in the resistance of cancer cells to TRAIL surveillance and tumor development. The mechanism of resistance to TRAIL-induced apoptosis in cancer can occur at different points in the signaling pathways of TRAIL-induced apoptosis. Dysfunctions or low expression of the DRs can lead to resistance. The defects in FADD and caspase-8 can lead to TRAIL resistance. Another cause of this defect is the high expression of cellular FADD-like interleukin-1β-converting enzyme-inhibitory protein (cFILP) which correlates with TRAIL resistance in several types of cancers because it can bind to FADD and/or caspase-8 and death receptors. This interaction in turn prevents DISC formation and subsequently suppresses the activation of caspase cascade (10,11). High expression of apoptosis inhibitors have been reported to
result in TRAIL resistance in mitochondria-dependent type II cancer cells (9). Thus, developing strategies to overcome the TRAIL resistance are the topics of interest. Several observations suggest that the combination of TRAIL with effective small molecule compounds can sensitize the resistant cancer to TRAIL-induced apoptosis. Therefore, it has been assumed as strategy to potentiate the cytotoxicity of TRAIL and its therapeutic applications.

The combined compounds synergize TRAIL-induced apoptosis in cancer through two pathways. First, to increase the death receptors DR4/DR5 expression and trigger its translocation to cell membranes thus increasing TRAIL binding resulted in extrinsic apoptosis pathway. Several chemotherapeutic agents and natural compounds, such as CDDP (12), etoposide (13), PS-341 (bortezomib) (14), tunicamycin (15), rottlerin (16), brandisianins (17), sodium butyrate (18), inostamycin (19) were reported to upregulate the death receptor expression and subsequent sensitization of TRAIL-resistant cancer cells to TRAIL-induced apoptosis. Second, resistant mechanism of TRAIL-induced apoptosis is disrupted through downregulation of cFLIP expression. Natural compounds such as kurarinone (20), icaritin (21), withanolide E (22) were reported to downregulate cFLIP expression and subsequent sensitization of TRAIL-resistant cancer cells to TRAIL-induced apoptosis. Natural compounds, such as silibinin (23), gingerol (24) and indomethacin (25) were reported to possess both mechanisms of sensitizing TRAIL-resistant cancer cells.

The LoVo colorectal cancer (CRC) cell line is derived from left supraclavicular region; stage Dukes’ C (26). The CRC is the second most and the third most common cancer in women (representing 9.2% of the total) and men (representing 10.0% of the total) worldwide, respectively (27). The CRC cell lines which resist to TRAIL-induced apoptosis remains a problem in the treatment of these cancers, thus the approaches for enhancing TRAIL-induced apoptosis are urgently required. The LoVo cell line was used as a model of TRAIL-refractory colorectal cancer cells in this study as they were reported to express significantly lower level of cell surface DR5 than the other colon cancer cell lines resulting in resistance to TRAIL treatment (8). Thus, finding the strategy to overcome the TRAIL-insensitive cancer cells is of importance.

Goniothalamin is a major bioactive styryl-lactone compound found in plant *Goniothalamus macrophyllus* (Blume) Hook. f. & Thomson, indigenous to South East Asia (28). Many reports suggested that goniothalamin showed cytotoxic activity against various cancer cell lines, such as liver, breast, and cervix (29-34). Interestingly, our preliminary studies indicated that goniothalamin could increase DR5 expression while decrease cFLIP expression in LoVo cells. These preliminary results suggested that goniothalamin has a potential use for combination with TRAIL treatment in TRAIL-resistant LoVo cells. In this study, the mechanisms to overcome the resistance to TRAIL-induced apoptosis were investigated using goniothalamin combining with TRAIL in TRAIL-resistant LoVo cells. This indicated the potential application of goniothalamin as a synergistic agent for combining with TRAIL treatment in colorectal cancer.

**Materials and methods**

**Chemical and antibodies.** Goniothalamin (IUPAC name: (2R)-2-[(E)-2-phenylethenyl]-2,3-dihydropryan-6-one) was obtained from Dr Wilawan Mahabusarakam, Faculty of Science, Prince of Songkla University, Thailand in purified powder form. The stems of *Goniothalamus macrophyllus* were collected from Songkhla province in the southern part of Thailand, in September, 2007. Identification was made by Mr. Ponlawat Pattarakulisutti, Department of Biology, Faculty of Science, Prince of Songkla University. The specimen (Uraiwan 01) has been deposited in the Herbarium of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. Recombinant TRAIL was purchased from Merck Millipore Corp. (Merck KGaA, Darmstadt, Germany). Chemicals for cell viability assay including MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Chemicals for flow cytometry analysis including Guava Cell Cycle® reagent and Guava Nexin® reagent were purchased from Merck Millipore Corp. (Merck KGaA), and PE-conjugated DR5 antibody was purchased from eBioscience, Inc. (San Diego, CA, USA). Chemical for fluorescence microscope observation Hoechst 33342 dye was purchased from Fisher Scientific, Inc. (Invitrogen™, Waltham, MA, USA). Chemical for mRNA extraction and cDNA synthesis were purchased from Qiagen N.V. (QIAzol™ lysis reagent, Venlo, LI, The Netherlands) and Thermo Fisher Scientific, Inc. (RevertAid™ First Strand cDNA Synthesis kit, Fermentas™, Waltham, MA, USA), respectively. Chemical for quantitative PCR was obtained from Thermo Fisher Scientific, Inc. (SYBR® Select Master Mix, Applied Biosystems™, Waltham, MA, USA). Antibodies (Abs) for immunoblot analysis including mouse monoclonal Abs against CHOP, and rabbit monoclonal Abs against DR5, PARP, caspase-3, caspase-8, caspase-9, Bcl2, Bax, Bid, Mcl1, and anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), and mouse monoclonal Abs against phospho-histone H2AX at Ser139 (γ-H2AX), β-actin and rabbit monoclonal Abs against cFLIP were obtained from Merck Millipore Corp. (Merck KGaA).

**Cell culture.** Human colorectal cancer, LoVo cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It was maintained in RPMI-1640 medium (Gibco Life Technologies, Carlad, CA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Science, Little Chalfont, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (GE Healthcare Life Science, Inc., Little Chalfont, UK) at 37°C in a humidified 5% CO₂ atmosphere and used for assays during exponential phase of growth.

**Cell viability assay.** Cells/well (5x10³) were seeded in a 96-well plate. After adherence, culture medium containing 10 and 100 ng/ml of TRAIL alone and in combination with different goniothalamin concentrations 5, 15, 25 and 50 µM were incubated for 24 h at 37°C with 5% CO₂. The control group was treated with 0.5% DMSO. Cytotoxicity of gonio-
thalamin was determined by cell proliferation analysis using MTT assay as described by Denizot and Lang (35). Briefly, after the indicated treatment, 0.5 mg/ml of MTT solution dissolved in culture medium was added and the cells were incubated for 2 h at 37˚C with 5% CO2 in the incubator; the MTT solution was removed and 100 µl of DMSO was added to dissolve the formazan crystals, a product of cell respiration as for viable cells, and the absorbance at 540 nm was quantified on Epoch™ Microplate Spectrophotometer and analyzed by Gen5™ Data Analysis software (BioTek, CA, USA).

**Chromatin condensation.** Cells/well (8x10⁴) were seeded in a 12-well plate. After adherence, culture medium containing 10 and 100 ng/ml of TRAIL alone and in combination with 15 µM goniothalamin were incubated for 24 h at 37˚C with 5% CO2. The control group was treated with 0.5% DMSO. Chromatin condensation, a character of apoptosis, was detected by cell staining with a fluorescent dye Hoechst 33342 modified from Oberhammer et al (36). After treatment, the fixed cells were washed and then stained with chromatin staining solution (5 µg/ml of Hoechst 33342) for 15 min. After staining, the stained cell were washed and then the plates were observed using a fluorescence microscope IX73 model (Olympus, Tokyo, Japan) with U-MWU2 mirror units for ultraviolet excitation.

**Cell cycle determination.** Cells/well (2x10⁵) were seeded in each 6-well plate. After adherence, culture medium containing 10 and 100 ng/ml of TRAIL alone and in combination with 15 µM goniothalamin were incubated for 24 h at 37˚C with 5% CO2. The control group was treated with 0.5% DMSO. After treatment, the whole cells were collected and stained according to the manufacturer's instructions (Guava Cell Cycle® reagent from Merck Millipore Corp.; Merck KGaA). The stained cells were then sorted and analyzed for DNA content by a Guava easyCyte™ flow cytometer and GuavaSoft™ software (Merck Millipore Corp.; Merck KGaA), respectively.

**Cell surface phosphatidyl-serine determination.** Cells/well (2x10⁵) were seeded in a 6-well plate. After adherence, culture medium containing 10 and 100 ng/ml of TRAIL alone and in combination with 15 µM goniothalamin were incubated for 24 h at 37˚C with 5% CO2. The control group was treated with 0.5% DMSO. After treatment, whole cells were collected and stained according to the manufacturer's instructions (Guava Nexin® reagent from Merck Millipore Corp.; Merck KGaA). The stained cells were then sorted and analyzed for cell surface phosphatidyl-serine content by a Guava easyCyte™ flow cytometer and GuavaSoft™ software (Merck Millipore Corp.; Merck KGaA), respectively.

**Cell surface DR5 determination.** Cells/well (2x10⁵) were seeded in a 6-well plate. After adherence, culture medium containing different goniothalamin concentrations of 1, 5, 15 and 25 µM was incubated for 24 h at 37˚C with 5% CO2. The control group was treated with 0.5% DMSO. After treatment, the whole cells were collected and resuspended in PBS buffer containing PE-conjugated DR5 antibody, then incubated in the dark for 1 h at room temperature. The cells were washed and resuspended in PBS solution then sorted and analyzed for cell surface DR5 by a Guava easyCyte™ flow cytometer and GuavaSoft software (Merck Millipore Corp.; Merck KGaA), respectively.

**mRNA expression analysis.** Cells/well (8x10⁴) were seeded in a 12-well plate. After adherence, culture medium containing 15 µM goniothalamin was incubated for 24 h at 37˚C with 5% CO2. The control group was treated with 0.5% DMSO. Analysis of mRNA expression was performed using the two step quantitative reverse transcriptase (RT)-PCR. After treatment, the whole cells were collected and RNA was extracted by using QIAzol lysis reagent (Qiagen N.V.) and cDNA synthesis by reverse transcription according RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific) with 2 µg of total RNA of each sample. In quantitative PCR step, it was performed with SYBR Select Master Mix (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers used for amplification were: DR5 (forward 5'-CACCGTAGTGGTACCCAAACACTGCT-3' and reverse 5'-TACGGCTGCAACTGTGACTC-3'), CHOP (forward 5'-GGCCATGAAGGAAAGACAAC-3' and reverse 5'-TACGGCTGCAACTGCAAC-3'), caspase-3 (forward 5'-ATCCGTCATACCGAAG-3' and reverse 5'-TCAGGCAATAGCAGG-3'), caspase-8 (forward 5'-ATGGGCAAGCAAGG-3' and reverse 5'-TCAGGACATGCGATGGCGG-3'), GAPDH (forward 5'-AGGTGCACTCTGTCGGCAGA-3' and reverse 5'-ATGGTTCGGACGCAGAATG-3'). The PCR amplification was analyzed by CFX96 Touch™ Real-Time PCR Detection system with CFX Manager™ software (Bio-Rad Laboratories, Inc., CA, USA). All steps were performed according to the manufacturer's instructions.

**Protein expression analysis by immunoblotting.** Cells/well (2x10⁴) were seeded in a 6-well plate. After adherence, the cells were treated with appropriate condition. After treatment, the cells were lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na2HPO4, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF). The extracted proteins were separated on 8-15% acrylamid gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore Corp., Merck KGaA). Then, the membranes were blocked with 5% skimmed-milk in TBS-Tween buffer for 1 h at room temperature and incubated with mouse monoclonal Abs against CHOP, γ-H2AX, and rabbit monoclonal Abs against DR5, PARP, caspase-3, caspase-8, caspase-9, Bcl2, Bax, Bid, cFLIP, β-actin overnight at 4˚C. Following incubation with anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the signals were developed using Immobilon™ Western chemiluminescent HRP substrate (Merck Millipore Corp., Merck KGaA, Darmstadt, Germany) and detected under Chemiluminescent Imaging system (GeneGnome gel documentation, Synoptics Ltd., Cambridge, UK).

**Statistical analysis.** To compare the data from different treatments, Student's t-test was used. All data presented
were obtained from at least three independent experiments and presented as mean ± standard deviation (SD). A p-value of 0.05 was taken as minimum basis for assigning significance.

Results

Enhanced TRAIL-induced apoptosis in LoVo cells by co-treatment with goniorthoalamin. Our preliminary study showed that LoVo cells were insensitive to goniorthoalamin treatment with high IC50 value at 65.25±1.85 µM. However, goniorthoalamin induced increased DR5 expression at lower concentration than IC50 value indicating that goniorthoalamin in combination with TRAIL may have a potential to trigger apoptosis induction via the death receptor-TRAIL mediated apoptosis pathway. Thus, we tried to investigate the mechanisms of apoptosis induction upon combination of TRAIL and goniorthoalamin in LoVo cells.

In this study, we first found that co-treatment of goniorthoalamin and TRAIL enhanced cytotoxicity induction in LoVo cells. We confirmed this cytotoxic effects and apoptosis induction using the MTT assay and Hoechst 33342 staining to assess chromatin condensation as shown in Fig. 1A and B, respectively. Treatment of LoVo cells with 10 and 100 ng/ml of TRAIL for 24 h showed >80% cell viability, while combined treatment with 15 µM goniorthoalamin resulted in enhanced cytotoxicity in LoVo cells. The increased chromatin condensation is shown in Fig. 1B upon combining treatment of 15 µM goniorthoalamin and 10 and 100 ng/ml of TRAIL as compared to a single treatment. Moreover, other apoptotic characteristics, accumulation of subG1 phase population and cell surface phosphatidyl-serine presentation, were studied using flow cytometry technique. As shown in Fig. 2A and C, a significant increased accumulation of a subG1 phase population was detected upon treatment with 15 µM goniorthoalamin and 10 ng/ml TRAIL as compared to a single treatment, but not for the combination of 15 µM goniorthoalamin and 100 ng/ml TRAIL as compared to a single 100 ng/ml TRAIL treatment. In addition, the significant increased cell surface phosphatidyl-serine presentation was detected upon combined treatment with both 10 and 100 ng/ml of TRAIL as compared to a single treatment (Fig. 2B and D). Thus, these results indicated that the combined treatment of goniorthoalamin and TRAIL enhanced cytotoxicity and apoptosis induction in LoVo cells, especially at 15 µM goniorthoalamin and 10 ng/ml TRAIL, which was selected for use in the next steps to assess apoptosis pathway.

Combined treatment with goniorthoalamin and TRAIL accelerate apoptosis induction by a caspase activation-dependent pathway involved with both death receptor- and mitochondrial-mediated apoptosis pathways in LoVo cells. To observe whether the combination of goniorthoalamin and TRAIL leads to activation of caspase-activated apoptosis in TRAIL-resistant LoVo cells, apoptotic-related protein was assessed by immunoblot analysis. As shown in Fig. 3, caspase-activated apoptotic mediators including PARP and caspase-3 as executive apoptosis, caspase-8 and Bid as death receptor mediated apoptosis pathway, caspase-9, Bcl2 and Bax as mitochondrial mediated apoptosis pathway were determined. The results indicated that both extrinsic and intrinsic pathway were enhanced upon combined treatment of 15 µM goniorthoalamin and 10 or 100 ng/ml of TRAIL, as confirmed by increasing the cleaved form of PARP, caspase-3, caspase-8, caspase-9, Bid, decreased antiapoptotic Bcl2 and increased proapoptotic Bax expression. Moreover, increased phosphorylation of histone
the so-called γ-H2AX, was observed upon the combined treatments, these results indicated that goniothalamin induced DNA double-strand breaks and triggered apoptosis-associated γ-H2AX accumulation, which is one of apoptotic characteristics. The results indicated that these combined treatment can induce cytotoxicity resulting in DNA double-strand break in LoVo cells.

Goniothalamin enhances TRAIL-induced apoptosis through DR5 upregulation and cFLIP downregulation.
TRAIL-stimulated death signal is initiated by the binding of TRAIL to DR5 in the subsequent caspase-8 activation. As shown in Fig. 4, immunoblot analysis showed that goniothalamin dramatically upregulated the DR5 and CHOP protein but downregulated the cFLIP and Mcl1 protein in a dose-dependent manner (Fig. 4A). Treatment with 15 μM goniothalamin at various time points showed that goniothalamin upregulated the DR5 and CHOP protein while downregulated the antiapoptotic cFLIP and Mcl1 in a time-dependent manner (Fig. 4B). These results corresponded with quantitative RT-PCR analysis (Fig. 4C) indicating that DR5 and CHOP mRNA were upregulated whereas cFLIP and cFLIPm mRNA was significantly downregulated. Moreover, the translocation of DR5 to cell surface was analyzed and the results indicated that goniothalamin increased cell surface DR5 expression in a dose-dependent manner (Fig. 4D). These results implied that DR5 upregulation induced by increased CHOP expression together with the downregulation of cFLIP and McIl contributed to enhanced TRAIL sensitization.

Discussion

TRAIL, also known as Apo-2L, is a typical member of TNF ligand family that induces apoptosis via death-receptor mediated pathway. TRAIL has potential benefits in cancer therapy because of its potent ability to be selectively toxic in cancer cells. Unlike the other death ligands such as TNF-α or FasL, the treatment of TRAIL causes less toxic in normal cells (37,38). Furthermore, the combined treatment of TRAIL and genotoxic chemotherapeutic agents synergistically inhibited cancer cell growth which are otherwise resistant or less toxicity to treatment with TRAIL or chemotherapy alone (37-39). There are several recombinant TRAIL and TRAIL-receptor agonists as an anticancer therapy that have been tested in phase I and II trials in patients with advanced cancer. Clinical studies in TRAIL-receptor agonist are being investigated using combination treatment in patients with advanced cancer stage (40). However, the single TRAIL treatment probably is not feasible since the majority of cancer cells are resistant to TRAIL. Thus, the combination treatment with TRAIL and chemotherapy is essential for use in TRAIL-resistant cancers. We also analyzed in detail that TRAIL combined treatment with cytotoxic agent goniothalamin may enhance cytotoxicity and apoptosis induction in colorectal cancer cells, indicating a potential use for cancer therapy.

In this study, we demonstrated for the first time that goniothalamin upon combined treatment with TRAIL-regulated expression of antiapoptotic- and proapoptotic-death receptor-mediated apoptotic molecules, including upregulation of DR5 and CHOP, downregulation of cFLIP and Mcl1 resulting in enhancement of the ability of TRAIL in TRAIL refractory LoVo cells. Various studies have reported the increased transcriptional activation of DR5 gene by the upregulation of CHOP expression (41-45), these correlated to the upregulation of CHOP expression in goniothalamin treatment. Another mechanism which is involved in sensitization to TRAIL-induced apoptosis is downregulation of cFLIP and Mcl1. cFLIP is the major protein that prevents caspase-8 from activation by death receptors through binding to FADD and/or caspase-8 and TRAIL receptor DR5 in a ligand-dependent and -independent manner and forms an apoptosis inhibitory complex (AIC), then prevents death-inducing signaling complex (DISC) formation and subsequently suppress the activation of caspase cascade (46-53). Mcl1 is an antiapoptotic protein involved in death receptor mediated pathway cross-link to mitochondrial mediated pathway by interacting with truncated Bid (tBid) and then strongly inhibits tBid-induced cytochrome c release in mitochondrial mediated apoptosis pathway (54-57). Downregulation of cFLIP and Mcl1 expression sensitizes TRAIL-induced apoptosis in various TRAIL refractory cancers. Thus, we speculated that goniothalamin plus TRAIL might play a critical role in goniothalamin-stimulated TRAIL-mediated apoptosis in LoVo cells through both upregulation of DR5 and CHOP and downregulation of cFLIP and Mcl1 expression enhancing TRAIL ability to be selectively cytotoxic to TRAIL-refractory LoVo cells. Moreover, DR5 translocation to cell surface was increased by goniothalamin treatment that may increase potent death receptor-mediated apoptosis induction by TRAIL through binding to DR5 (58-60).

Caspase-dependent pathways of these TRAIL-mediated apoptosis are involved in this combination treatment, resulting in a strong enhancement of PARP, caspase-3, -8 and -9 activation. TRAIL triggered death receptor mediated apoptosis pathway via binding to death receptor DR5 resulting in induction cleavage of Bid to tBid, then crosslinking to
mitochondria-mediated apoptosis activation (4-7,61), supported by downregulation of Mcl1 in goniothalamin treatment. Our results showed that apoptotic related molecules triggered activation in both death receptor- and mitochondrial-mediated apoptosis pathways under combination treatment of goniothalamin and TRAIL. Moreover, the increased accumulation

Figure 4. Effects of goniothalamin on induction of death receptor mediated apoptosis pathway associated with TRAIL-induced apoptosis. (A) LoVo cells were treated with various concentrations of goniothalamin for 24 h or (B) LoVo cells were treated with 15 µM goniothalamin for various time of treatment and the whole protein lysate was separated on SDS-PAGE followed by immunoblot analysis, actin was used as loading control. (C) Expression of apoptosis mRNA genes were determined by reverse transcription quantitative PCR. Significant values were defined as *p<0.05, and **p<0.001, compared with 0.5% DMSO treatment as a control in each gene. (D) Goniothalamin increased cell surface DR5 presentation in LoVo cells upon treatment with various goniothalamin concentrations for 24 h. The cells were stained with PE-conjugated antibodies against DR5 and detected with a flow cytometer.
of subG1 phase population in cell cycle, increased cell surface phosphatidyl-serine presentation and increased phosphorylation of H2AX were observed under the combined treatment of TRAIL and goniothalin, indicating apoptosis induction in LoVo cells as compared to a single treatment with TRAIL or goniothalin alone (62-65). Similar reports of other compounds sensitize TRAIL-induced apoptosis include inostamycin (19), delphinidin (66), and parthenolide (67). Therefore, the combined treatment of TRAIL and goniothalin enhanced cytotoxicity in TRAIL refractory LoVo cells through caspase-dependent apoptosis pathway in both death receptor- and mitochondrial-mediated pathways.

In conclusion, this is the first report that the combination of TRAIL and goniothalin was able to effectively enhance TRAIL mediated apoptosis induction in TRAIL refractory colorectal cancer, LoVo cells. In addition, we found that goniothalin enhanced TRAIL sensitization in LoVo cells associated with caspase cascade activation via induction of the DR5 pathway and decreased expression level of antiapoptotic proteins which related to DR5 pathway and subsequently increased apoptosis. From these results, we speculate that combined treatment of TRAIL and goniothalin provides a possible therapeutic application for treatment of colorectal cancer that are resistant to TRAIL.

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