RESEARCH ARTICLE
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Effect of diosgenin in the inhibition of gastric cancer cell proliferation and its mechanism in combination with TRAIL to induce apoptosis

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ABSTRACT: Clinical observations suggest that identifying novel curative and preventive approaches by targeting the tumor cells without affecting the normal cells is appreciable. Diosgenin (DG) is a natural sapogenin that has been reported of having pro-apoptotic and anti-cancer properties against various neoplasia. Hence, the present study investigated the effect of combined DG/TNF-related apoptosis-inducing ligand (TRAIL) on gastric cancer cell lines (BGC-823) *in vitro*. Cell viability and IC_{50} for DG and TRAIL alone or in combination were determined using MTT. The apoptosis rate was assessed by ELISA cell death assay and Caspase 8 activity assays. The gene expression evaluation of candidate genes, including survivin, Bcl-2, XIAP, c-IAP1, c-IAP2, and c-FLIP, were accomplished before and after the treatment by quantitative real-time PCR. Our results demonstrated that DG synergistically enhanced the cytotoxic effects of TRAIL ($p < 0.01$). DG could exaggerate cell apoptosis through TRAIL-induced apoptosis and amplify the Caspase 8 activity. These results were confirmed by decreasing anti-apoptotic genes' expression levels. Overall, our findings shed light on a novel strategy of TRAIL-induced apoptosis in combination with DG for the treatment of gastric cancer.

KEYWORDS: apoptosis, diosgenin, gastric cancer, TRAIL

INTRODUCTION

Gastric cancer (GC), the fourth rank in cancer-related mortality worldwide [1], is more prevalent among individuals with lower socioeconomic status [2]. It is a multifactorial disorder resulting from complicated etiology including genetic, environmental, and personal factors [3]. In the case of localized disease, radical surgery is still the only curative method. The most crucial obstacle in GC treatment is the advanced stage of the tumor [4]. Newly introduced methods in screening these patients, including cell-free plasma DNA, can have promising results in the prognosis of the disease [5]. However, despite many attempts in diagnostic and therapeutic studies and improvements in clinical outcomes of patients with GC, the 5-year survival rate of these patients has not shown significant improvement due to the higher rate of metastasis and tumor recurrence in advanced-stage patients [6–8].

In the fight against cancer, innumerable molecules with natural or chemical origins, were investigated. Among the wide variety of anti-cancer molecules, Diosgenin (DG) has attracted the attention of researchers. DG, as a plant-based steroidal saponin extracted from *Trigonella foenum-graecum* is known as an effective molecule against inflammation [9], oxidants [10], and cancer [11]. Recent studies proved that DG is also active in the induction of DNA damage and apoptosis as well as the activation of mitochondrial signaling pathways [12, 13].

In the path of combating solid tumors, the induction of DNA damage and apoptosis in cancer cells is a considerable approach. To this purpose, numerous agents were tested, including ginkgolic acid [14], phytochemicals [15], alantolactone, and thioredoxin reductase 1 [16].

While the two mitochondrial death pathways of the cells regulate cell proliferation and viability, the death receptors (DRs) pay attention to therapeutic targets [17]. The DR pathway is involved in cell death through apoptosis using ligand-receptor interactions [18]. There are three types of DRs, TNF-related apoptosis-inducing ligand receptors (TRAIL-Rs), DR4, and DR5, and theTRAIL-Rs have a unique ability to target and eradicate malignant cells without damaging the normal cells [19]. The target death protein for the TRAIL pathway is Caspase 8, which may activate either caspase-3 or mitochondrial pathway through the activation of Bid [20, 21]. Despite the expression of functional TRAIL-Rs in most cancer cells, the resistance to the apoptosis induced through these receptors is prevalent [22]. In the path of this

resistance, P53 and MAPK pathways, NF-κB signaling, c-Myc, and PI3K/AKT pathway modulate the sensitivity of TRAIL [23, 24]. Moreover, several therapeutic agents, including chemotherapeutic compounds or natural molecules, can improve the apoptotic role of TRAIL through the upregulation of its receptors or blocking the survival pathways [25–29].

Considering the anti-cancer effects of TRAIL and DG, we hypothesized that a combination of these two molecules could have more potent anti-cancer effects.

MATERIALS AND METHODS

Cell culture and reagent preparation

The human gastric cancer cell line (BGC-823) and Hs 738.St/Int normal cells were provided from the American Type Culture Collection (ATCC; Manassas, VA, USA). The BGC-823 cell line was cultured in RPMI-1640 medium Gibco (Carlsbad, CA, USA) containing 10% of fetal bovine serum (FBS) (Gibco), followed by incubation at 37 °C in a humidified atmosphere with 5% carbon dioxide. The Hs 738 normal cells were cultured in DMEM plus 10% FBS at 37 °C. To treat the cells with specific concentrations of test compounds, stock solutions of DG (Sigma-Aldrich; St. Louis, MO, USA) and TRAIL (Sigma-Aldrich) were prepared in 50 μM and 800 ng/ml, respectively. Then, each of the stocks was serially diluted.

Cell cytotoxicity assay

To perform the toxicity assay, the cells were seeded into a 96-well cell culture plate with a density of 2×10^4 cells/well. After 24-h incubation, the cells were categorized into 4 groups; control, TRAIL treated, DG treated, and combined TRAIL/DG treated groups. The cells were exposed to TRAIL with final concentration of 25, 50, 100, 200, 400, and 800 ng/ml; and DG with final concentration of 0.8, 1.6, 3.1, 6.2, 12.5, 25, and 50 μM.

After incubation with defined concentrations of TRAIL and DG for 24 h, the culture medium in each well was replaced with 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) following washing twice with phosphate-buffered saline (PBS). After 4-h incubation at 37 °C, the liquid phase was replaced with 100 μl of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm.

Apoptosis assay

The BGC-823 cells were seeded in a density of 4×10^4 cells/well in a 24-well cell culture plate. 24 h after exposure to the defined TRAIL, DG, and TRAIL/DG concentrations, the cells were harvested, and apoptosis was assessed using an ELISA cell death detection kit (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's protocol. Briefly, the BGC-823 cells were lysed and centrifuged at $200\times g$, and 20 μl of the cell supernatant and 80 μl of the mixed solution of DNA peroxidase and anti-histone biotin were added to each biotin coated well. Then, the plate was incubated for 2-h; and, after a washing step with incubation buffer, 100 μl of 2,2-azinobis (3-ethylbenzthazoline-6-sulfonic acid) solution was added to each of the wells. Finally, the absorbance was measured at 405 nm (with a reference wavelength of 450 nm).

Caspase 8 activity assay

Caspase 8 activity was assessed using a Caspase 8 cholera metric assay kit (BioVision K113; Milpitas, CA, USA). BCG-238 cells were cultured in a 6-well plate at a density of 3×10^5 cells/well for 24-h and exposed to the TRAIL, DG, and combined TRAIL/DG concentrations. Subsequently, the cells were trypsinized and centrifuged at 1500 rpm for 10 min, followed by a washing step using PBS containing 0.01 M EDTA. The cellular pellet was re-suspended in 0.055 ml of lysis buffer, stirred, and kept on ice for 10 to 20 min. The cell lysate solution was centrifuged at 1200 rpm and 4 °C for 10 min. For each test, a mixture of 50 μl cell lysate and 50 μl reaction buffer containing dithiothreitol and IEDT-Pna (a Caspase 8 substrate) was used. The prepared samples were kept at 37 °C in the dark. Eventually, the samples were diluted to reach the volume of 900 μl, and the absorbance was measured at 405 nm.

RNA extraction and q-PCR

After 24-h incubation of the cells separately with 289 ng/ml of TRAIL and 9.1 μM of DG, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). The quality and quantity of the extracted RNA were assessed by agarose gel electrophoresis and Nanodrop (Thermo Fisher scientific; Washington, DC, USA). Then the complementary DNA (cDNA) was synthesized using Prime Script reverse transcriptase (Takara, Tokyo, Japan). Survivin, Bcl-2, XIAP, c-IAP1, c-IAP2, and c-FLIP primer design was performed using oligo7 (Molecular Biology Insights Inc., Cascade, CO, USA) and BLASTed in NCBI primer BLAST software. The expression of the genes was determined using Power SYBR Green master mix (ABI; Warrington, UK) and Bio-Rad IQ5 system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD), and $p < 0.05$ was accepted statistically significant. All quantitative results were processed by GraphPad Prism v7.04 software (GraphPad Software, San Diego, CA, USA). The Two-tailed Student *t*-test examined the statistical significance for two groups' comparisons.

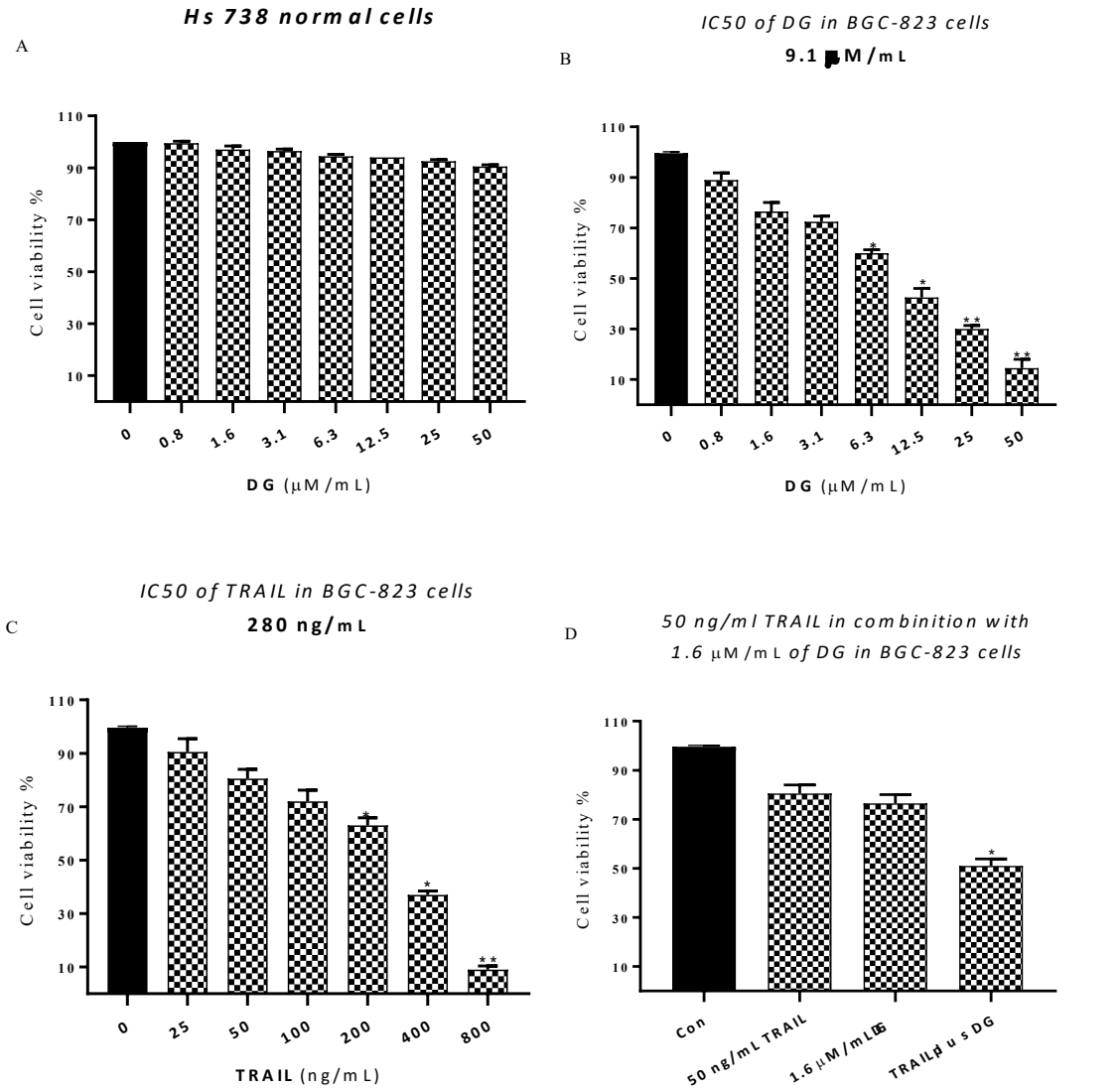

Fig. 1 Cell viability in different concentrations of DG and TRAIL alone or in combination in BGC-823 cells. A, Cell viability in various concentrations of DG in Hs 738 normal cells before and after 24-h of treatment; B, Cell viability in various concentrations of DG in BGC-823 cells before and after 24-h of treatment; C, Cell viability of various concentrations of TRAIL in BGC-823 cells before and after 24-h of treatment; D, Cell viability of BGC-823 cells exposed with 50 ng/ml of TRAIL plus 1.6 μM/ml of DG after 24-h of treatment. Con, control: BGC-823 cells without treatment 0 μM/ml of DG and TRAIL.

RESULTS

Cell cytotoxicity assay

Cell viability was measured using MTT assay for TRAIL, DG, and TRAIL/DG. Results revealed that the viability of the cells was reduced in the presence of both TRAIL and DG, but the reduction was significant only when the cells were co-treated with both TRAIL and DG. The IC_{50} was measured at 9.1 μM and 289 ng/ml of DG and TRAIL, respectively. Furthermore, the cell viability for 50 ng/ml TRAIL in combination with 1.6 μM/ml DG was 50% (Fig. 1). Indeed, this combination index revealed 50% cytotoxicity in BGC-823 cells.

Cell apoptosis assay

DG and TRAIL co-treatment increased the apoptosis in BGC-823 cells. The results of the MTT assay demonstrated that the cell viability was decreased in the lower concentrations of DG when the cells were co-treated with TRAIL; and as shown in Fig. 2, the apoptosis was significantly (4-folds) elevated when the cells were treated with combination of TRAIL and DG.

Caspase 8 activity assay

Fig. 3 shows that 24-h incubation of the BGC-823 cells with combined TRAIL/DG led to a significant increase

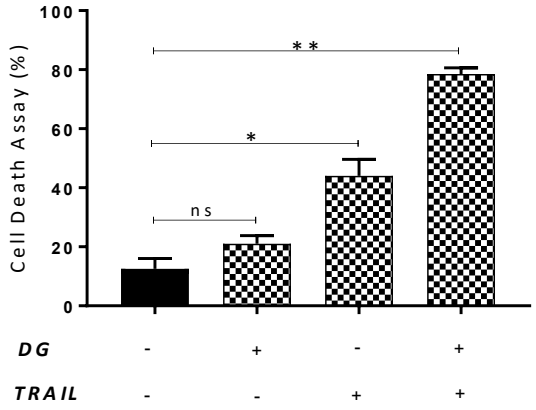

Fig. 2 Effects of DG and TRAIL alone or in combination for 24 h using an ELISA cell death assay in BGC-823 cells.

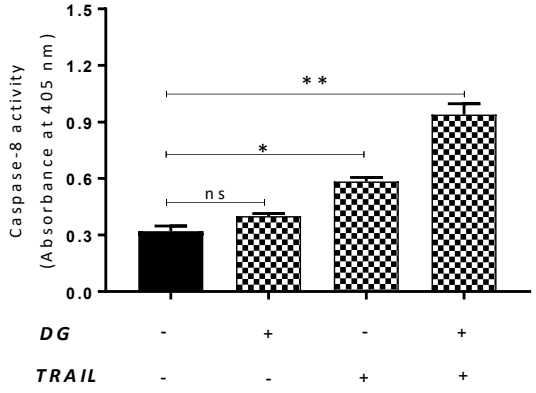

Fig. 3 Effects of DG on TRAIL-induced apoptosis through Caspase 8 dependent on BGC-823 cells of the gastric cancer line.

in Caspase 8 activity compared with the other 3 groups (3.7 folds, $p < 0.01$), resulting in increased apoptosis rate in the cancer cells.

Regulation of apoptotic genes in combined TRAIL/DG treated BGC-823 cells

Quantitative PCR analysis demonstrated a decreased expression level of anti-apoptotic genes; Survivin, Bcl-2, XIAP, c-IAP1, c-IAP2, and c-FLIP. Co-treatment of DG and TRAIL showed a significant down-regulating effect on these genes in treated cells ($p < 0.05$) (Fig. 4).

DISCUSSION

Apoptosis, as a mechanism regulating cell proliferation, controls the age-related DNA mutations in numerous species, and it is controlled by a series of orderly orchestrated steps. TRAIL has a unique characteristic of inducing apoptosis in cancer cells without affecting normal cells; however, the resistance of cancer cells to TRAIL remains an obstacle in using this compound as a therapeutic agent [30]. More specifically, the resistance to DR4/DR5-induced apoptosis has been reported in gastric cancer, which has posed challenges in targeting and intensifying TRAIL [31]. Many studies have introduced drugs and agents to induce apoptosis in cancer cells [32]. Hence, the combination of TRAIL with natural components to augment the apoptotic feature of the TRAIL has been seriously considered. According to previous studies, the combination of TRAIL with DG showed promising apoptotic results in cancer cell lines, including colorectal cancer [22, 25, 26, 33, 34]. Moreover, Lepage et al [22] reported a sensitizing effect of DG on TRAIL in colorectal cancer cells and showed improved TRAIL-induced apoptosis in HT-29 cells via the induction of DR5 and the inhibition of PI3K/AKT pathway. Following these findings, our results showed an increased apoptosis rate in the BGC-823 cells treated with the combination of TRAIL and DG.

In the present study, we demonstrated that DG induced apoptosis in BGC-823 cell line at the concentration of 9.1 μM; while in a study by Liu et al [35] the IC_{50} was observed at 20.02 μM and 17.40 μM in AGS and SGC-7901 cell lines, respectively. In addition, the IC_{50} for TRAIL was reported at 9.2 μM in a study on gastric cancer cell lines [36]. Furthermore, we showed that DG could induce apoptosis in BGC-823 cells through the downregulation of anti-apoptotic genes, including Survivin, Bcl-2, XIAP, c-IAP1, c-IAP2, and c-FLIP. Moreover, the cell viability assay results showed increased cell death in TRAIL-treated and combined TRAIL/DG-treated groups. Caspase 8 is the effector molecule of the apoptotic cascade and is activated in TRAIL-induced apoptotic cells. Caspase 8 is involved in both the intrinsic and the extrinsic pathways of apoptosis. The extrinsic pathway initiates by activating the Fas receptor followed by the activation of cas3, which is inhibited by the c-FLIP [37].

Besides, Caspase 8 activation activates Bid, the effector molecule in the mitochondrial pathway of cell death. Activation of the mitochondrial pathway can be induced by Bax activation, leading to the cytochrome-c activation resulting in Caspase 9 and, finally, Caspase 3 activation. This pathway may be inhibited by Bcl-2 and IAPs, which blocks Bax and Caspase 9/ Caspase 3, respectively [38]. On the other hand, survivin inhibits pro Caspase 9 to Caspase 9 and cytochrome-c and inhibits apoptosis [39]. Our data showed a significantly enhanced Caspase 8 activity in the cells treated with TRAIL alone ($p < 0.05$) and combined TRAIL/DG ($p < 0.01$). Polyubiquitination of Caspase 8 is involved in the extrinsic signaling of apoptosis [40]. It has been demonstrated that TRAIL could induce the ubiquitination of Caspase 8 in prostate cancer cells [41]. Similarly, our results showed an increased Caspase 8 activation via the downregulation of anti-apoptotic genes in BGC-823 cells, which was induced by both the TRAIL and the DG.

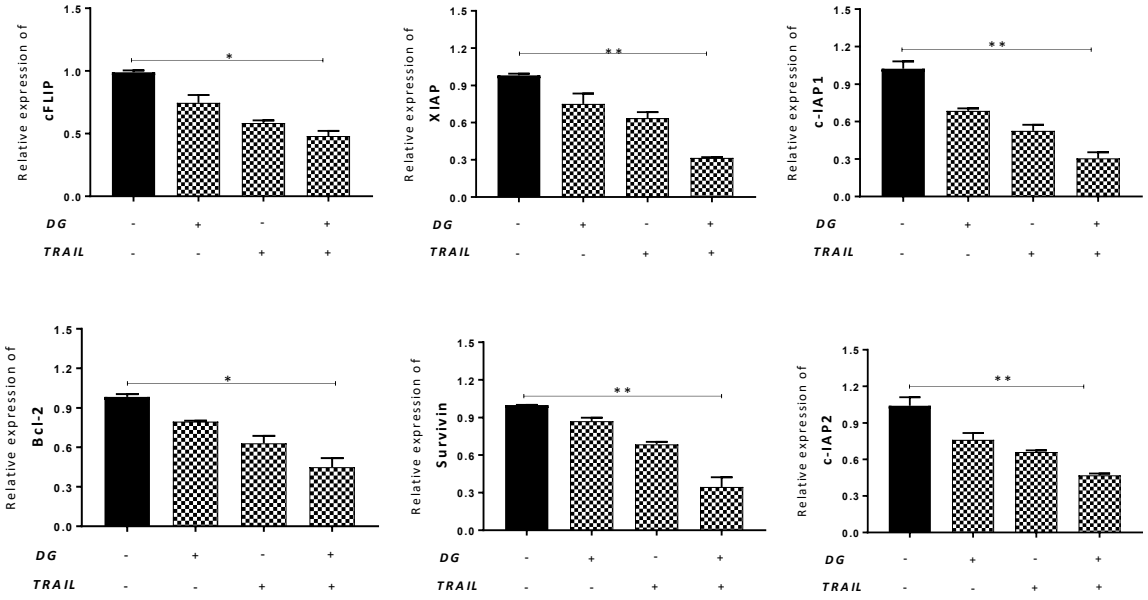

Fig. 4 Effects of co-treatment of DG plus TRAIL on the mRNA expression levels of c-FLIP, XIAP, c-IAP1, Bcl-2, survivin, and c-IAP2 in BGC-823 cells before (black bar) and after 24-h of treatment (striped bar).

CONCLUSION

The use of TRAIL in combination with the DG showed an improved the outcome of apoptosis induction in gastric cancer cells via the downregulation of anti-apoptotic genes and induction of Caspase 8 activity more than the use of each of them alone. These results have not only brought us one step closer to our goals in cancer treatment, but also raised hope on finding more effective anti-cancer treatments for GC.

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Appendix A. Supplementary data

Table S1 Abbreviation list.

Abbreviation	Expression
Bcl-2	B-cell lymphoma 2
Cas-8	Caspase 8
c-FLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
c-IAP	cellular inhibitor of apoptosis protein
DG	Diosgenin
DMSO	dimethyl sulfoxide
DR.	Death receptor
GC	Gastric cancer
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	phosphatidylinositol 3 kinase
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
XIAP	X-linked inhibitor of apoptosis protein