Tubuloside A Induces DNA Damage and Apoptosis in Human Ovarian Cancer A2780 Cells

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**ABSTRACT**

**Objective:** Ovarian carcinoma is one of the most lethal gynecological cancers, as it responds later to diagnostic methods and therapeutic responses in advanced stages. Many phytochemical compounds have been shown to be protective against cancer. Tubuloside A (TbA) is the main compound extracted from the plant *Cistanche tubulosa*, and its pharmacological effects have been studied broadly. Until now, the role of TbA in human ovarian carcinoma is unknown. The goal of this study was to evaluate the effects of TbA on DNA damage and apoptosis in A2780 cell lines.

**Methods:** Different concentrations of TbA (1, 5, 25, 50, and 100 µM) and 5-Fluorouracil (1, 5, 25, 50, and 100 µM) treated to the human ovarian cancer cell (A2780) line for 24 h. After incubation, cell viability (MTT), genotoxicity (Comet analyses), and mRNA expression analyses of apoptotic markers (Caspase-3, Bax, Bcl-2, and p53) were determined.

**Results:** Applied doses of 50 and 100 µM of TbA and 5-Fluorouracil significantly reduced cell viability. Also, TbA increased DNA damage in A2780 cells. Additionally, TbA up-regulated the mRNA expressions of caspase-3, Bax, and p53, which are apoptosis-inducing factors, and down-regulated the expression of Bcl-2.

**Conclusion:** These results show that the p53 and caspase-3 signaling pathways may exhibit a key role in TbA-associated effects on A2780 cells and TbA may be a potential drug aspirant for ovarian cancer therapy.

**Keywords:** Tubuloside A, cell viability, genotoxicity, apoptosis, ovarian cancer

**Main Points:**
- Ovarian cancers are one of the most common cancer types in the reproductive system.
- Tubuloside A (TbA), a phenoylethanoid glycoside, significantly reduced ovarian cancer cell viability
- TbA increased DNA damage and apoptosis in ovarian cancer cell line
- TbA may be a potential drug aspirant for ovarian cancer therapy
INTRODUCTION
Ovarian cancer is one of the lethal among gynecological tumors and comes after uterine cancer in terms of incidence [1]. Ovarian cancer cases are hidden and it is very difficult to determine whether it is benign or malignant. However, diagnosing ovarian cancer at an advanced stage limits its treatment options [2]. Developing diagnostic methods and effective treatments for this disease group are promising. Statistical results report that the survival rate in ovarian cancer is 70-90% and it is increasing rapidly day by day [3].

Resistance to chemotherapeutic drugs is a frequently encountered problem in cancer treatment. One of the main reasons for this problem is the drug resistance that develops in cancer cells. This process has a very complex infrastructure and is an important cause of tumor heterogeneity. One of the important consequences of drug resistance is that cancer cells become insensitive to therapeutics and escape from apoptosis [4]. Changes in apoptotic genes (such as p53, Bcl-2, and Bax) and signal transduction pathways have been reported in drug-resistant cells [5]. Studies report that Bcl-2, together with Bcl-XL and Mcl-1, modulates resistance to chemotherapy and reduces survival in ovarian cancer patients [6,7]. There are different cancer subgroups in which at least one family member of these anti-apoptotic proteins is overexpressed, and these are more common in inherently resistant cancers [8]. Currently, available anticancer therapies contain treatments based on targeting cancer cell DNA integrity and/or replication, which indirectly triggers apoptosis in tumor cells [9,10]. The discovery of new compounds and the determination of their potential cytotoxic and apoptotic effects are important for increasing the success rate of treatment.

Tubuloside A (TbA) is a phenylethanoid glycoside obtained from Cistanche tubulosa plant and is widely used in the purpose of memory-augmentative, impotency and constipation [11]. In in vivo studies demonstrated that C. tubulosa extract exhibited hepatoprotective effects against liver damage-induced d-galactosamine (d-GalN)/lipopolysaccharide in mice [12] and hypoglycemic and hypolipidemic effects in diabetic rodents [13,14]. However, in vitro studies, it was reported that Tubuloside B (TbB) obtained from C. salsa plant antagonized TNF-α-induced apoptosis in SH-SY5Y cells and exhibited neuroprotective effect [15]. Furthermore, it was reported that acteoside (81%), echinacoside (75%), cistantubuloside A (83%), 2'-acetylacecteoside (93%), and cistanoside A (33%), which were isolated from C. tubulosa plant, showed antitumor activity on mouse skin melanoma cells [16].

In the current studies, there has been no study on the role of TbA on ovarian cancer. This study reports for the first time whether TbA has possible cytotoxic potential in ovarian cancer cells. In this regard, the effect of TbA on A2780 ovarian cancer cells; cell viability test was determined by changes in mRNA expression of DNA damage and apoptotic markers.

MATERIAL and METHODS
Cell culture
The A2780 ovarian cancer cells were provided by Professor Süleyman Sandal from the İnönü University Faculty of Medicine. The cells were first removed from the nitrogen tank and inoculated in flasks (25 cm²). A2780 cells were nourished with 1640-RPMI medium (prepared by adding 100 U/mL penicillin, 10% FBS, and 0.1 mg/mL...
streptomycin). The medium was changed (in a twice a week) and the cells were cultured in 5% CO₂ in an incubator medium at 37°C (Thermo Forma, USA). Cells that were approximately 80% confluent in the flask, were removed by trypsin-EDTA solution, stained with trypan blue (0.4%), and counted under an inverted microscope. To determine the effect of the compounds on cell viability, seeding was done in 96-well plates with approximately 15x10³ cells in each well.

**Cell viability analyzes**

1, 5, 25, 50 and 100 µM doses of TbA (ChemFaces, Wuhan, Hubei, China) and 5-Fluorouracil (5-FU, Sigma-Aldrich, MO, USA) were prepared in dimethylsulfoxide (DMSO; the final concentration of DMSO was maintained at 0.1%) and applied for 24 hours. After incubation, the medium in the well was removed and 50 µL of the prepared MTT solution (0.5 mg/ml) was transferred to each well. After 3 hours of incubation, the MTT solution in the wells was removed and DMSO (100 µL) was transferred to well. The optical densities were measured on an ELISA (Thermo, USA) at a wavelength of 570 nm [17]. Control wells (wells containing only medium) were read and values were accepted as 100% viable cells and percent viability values were calculated.

**Genotoxicity analyses**

For genotoxicity analyses, cells were seeded in 6-well plates and 50 and 100 µM doses of TbA were applied for 24 hours. The level of damage to cell DNA was determined using the alkaline Comet assay technique according to Singh et al. with minor modifications [18]. First, the slides were coated with 1% normal melting agarose (NMA) prepared in phosphate buffer. 24 hours after application of the compounds, cells were removed from the plates and counted under a microscope. 10 µL of the suspension (approximately 10000 cells) was transferred to an tube and 80 µL of low melting agarose was added. This cell mixture was placed on slides coated with NMA and a coverslip was covered. The preparations were left at +4 °C and in the dark for 15 minutes. The coverslips were then carefully stripped and the slides were kept in lysis solution at +4 °C for 1 hour. Cold electrophoresis buffer was placed on the slides placed in the same plane on the horizontal electrophoresis tank and electrophoresis was performed at 25 volts (300 mA) for 25 minutes. After electrophoresis, the slides were washed with neutralization buffer three times for 5 minutes at +4 °C. Finally, the slides were stained with ethidium bromide and images were taken under a fluorescence microscope (Zeiss Axio1, Germany). Images were processed using Tritek Comet Score software. One hundred cells were randomly counted from slides and tail DNA (%) parameters were determined and data were given as median (10-90 percentiles). This value was considered an indicator of DNA damage [19].

**mRNA expression analyses**

For mRNA analyses, cells were propagated in 100 mm culture disks and applications were carried out. The cell medium was then aspirated and the cells were washed with cold PBS. Cells were collected into tubes with the help of a scraper. RNA was isolated from cells using the RNA Purification Kit (GeneJet, Thermo Fisher Scientific). The quantity and/or quality of the obtained RNAs were measured at A260/280 UV wavelengths using the Nanodrop (Maestrogen/MN-913) device. The amounts of RNA isolated from each group of cells were calculated to yield 1µg RNA in total for cDNA extraction. DNase I (Thermo Fisher Scientific, USA) was then
applied to remove DNA from RNA and cDNA was obtained from the RNA using cDNA Synthesis Kit (RevertAid, Thermo Fisher Scientific, USA).

The primers used in the experiment were designed by the study team using the FastPCR 6.0 [20] computer package program and the mRNA sequences of Homo sapiens-specific β-actin (F-AGCAAGAGAGGACATCCTCACC, R-ACAGGGATAGCAGGCTCTGGA; NM_001101.5), Bax (F-GACATTGGACTTCTCCGGGA, R- ACAAGATGGTCACCGTCTGC; NM_001291428.2) Bcl-2 (F-TGGACAAACATCGCCTGTGGA, R- TCACCTGTGCCCAGATAGGCA; NM_000633.3), caspase-3 (F-GCTCCTAGCGATGGGTGCTA, R- GATTCAAGGCGACGCCAAC; NM_004346.4), and p53 (F-AAACCTACCAGGGCAGCTACG, R-CTCACAACCTCCGTTCATGTGC; AB082923.1) genes were obtained from the NCBI website.

Differences between the expression levels of selected genes were examined using Bio-RAD CFX Manager 3.1 software on the Bio RAD real-time PCR device. The relative changes in the mRNA expression levels of target genes were calculated by the 2^ΔΔct method based on the cycle threshold (Ct) values of the amplification curves obtained after the amplification process consisting of three steps: denaturation, primer adhesion and chain extension [21]. The calculated value was substituted into the 2^ΔΔct formula for each gene and the mRNA expression level was determined as a fold decrease or increase. The β-actin gene was used as an endogenous control and the expression levels of other genes were corrected (normalization) according to the β-actin gene level of each sample.

Statistical analyses
The obtained data were analyzed in GraphPad 8 program. The Kruskal-Wallis test was used for differences between groups in MTT and Comet analysis results, and Dunn’s Multiple Comparison Test was used for multiple comparisons. In mRNA analyses, the difference between group means was evaluated with the one-way ANOVA test, and multiple comparisons were evaluated with Tukey’s test. Results were given as mean ± standard deviation and p<0.05 was considered significant in all comparisons.

RESULTS
TbA and 5-FU applications reduced cell viability
Applied doses of 50 and 100 µM of TbA significantly reduced cell viability compared to the control group (Figure 1) (p<0.05). This reduction was determined to be 25% and 35% at 50 µM and 100 µM doses, respectively. Moreover, 100 µM TbA application showed a significant cytotoxic effect compared to the solvent group (p<0.05). To determine the cytotoxic activity of TbA in A2780 cells, the activity of the standard chemotherapy drug 5-FU in cell cytotoxicity was also determined (Figure 2). Applied doses of 1-25 µM of 5-FU did not significantly affect viability in A2780 cells. On the other hand, 50 and 100 µM doses of 5-FU significantly decreased cell viability (p<0.05).
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Figure 1. A2780 cell viability level after TbA application. Data are given as mean ± SD. *p<0.05 compared to control group, #p<0.05 compared to DMSO group

Figure 2. A2780 cell viability level after 5-FU application. Data are given as mean ± SD. *p<0.05 compared to control group, #p<0.05 compared to DMSO group

TbA application induced DNA damage in cancer cells

The level of DNA damage in A2780 cells after TbA and 5-FU applications was shown in Figure 3. After 100 µM 5-FU application, the tail DNA % parameter did not alter significantly compared to the control. On the other hand, 100 µM TbA caused a significant enhancement in the DNA damage level, and this change was significant compared to the control group (Figure 3; p<0.05). These results show that TbA exerts a genotoxic effect on human ovarian cancer cell lines by inducing DNA damage and that this effect may mediate cell death.
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Figure 3. DNA damage level in A2780 cells after TbA (100 µM) and 5-FU (100 µM) application. Data are given as mean ± SD. Different letters (a,b) are statistically significant (p<0.05).

TbA increased mRNA expression levels of pro-apoptotic genes

5-FU (100 µM) and TbA (50 and 100 µM doses) treatments up-regulated Bax (Figure 4A), p53 (Figure 4B), and caspase-3 (Figure 4C) mRNA expressions compared to controls, whereas down-regulated Bcl-2 mRNA expression (Figure 4D) (p<0.05). This showed that TbA exerts a cytotoxic effect on human ovarian cancer cells by inducing apoptosis, especially at high doses.

Figure 4. Effect of TbA (50 and 100 µM) and 5-FU (100 µM) treatment on Bax (A), p53 (B), caspase-3 (C) and Bcl-2 (D) expression levels in A2780 cells. Data are given as mean ± SD. Different letters (a,b,c) are statistically significant (p<0.05).
DISCUSSION

The main active components of *C. tubulosa* are phenylethanoid glycosides [22]. In recent years, the results of various studies have emphasized that TbA shows various pharmacological and biological activities [12,23]. However, the molecular and/or cellular mechanisms underlying these effects are not fully solved. This study demonstrated for the first time that TbA has genotoxic and cytotoxic effects on a human ovarian cancer cell line by inducing DNA damage and apoptosis.

In an in vitro study investigating the protective effect of TbB on SH-SY5Y neuronal cells, it was reported that approximately 45.6% of the cells underwent apoptosis death after incubation with TNF-α, whereas TbB (1, 10 and 100 mg/L) treatment decreased the cell death rate in a dose-dependent manner (30%, 19.5% and 6.2%, respectively) [15]. Similarly, it was reported that different concentrations of TbB (5, 10, 50 and 100 µM) increased the cell survival rate decreased by 1-methyl-4-phenylpyridinium ion in PC12 neuronal cells [24]. In addition, it has been reported that acteoside (26.7µM), a phenyletenoid glycoside, can induce DNA degradation in promyelocytic leukemia HL-60 cell lines [25]. Another phenyletenoid compound, verbascoside, has been shown to inhibit the growth of human colorectal cancer cell lines (HT-29, HCT-116, LoVo, and SW62) in a time- and dose-dependent manner at a concentration of 29-67 µM after 24, 48 and 72 hours of incubation at 25-100 µM [26]. In this study, like other phenyletenoid compounds, 24 h TbA treatment inhibited cell growth and showed antiproliferative effect in A2780 ovarian cancer cells at 50 and 100 µM amounts.

Whether Echinacoside from Cistanche and Echinacea plants causes DNA damage was investigated in cancer cell lines (SW480, MCF-7, SK-HEP-1, and 48 MG-63) and non-cancer cell lines (human normal liver-LO2, human embryonic kidney-HEK 293 and mouse fibroblast-NIH/3T3). These cell lines were exposed to Echinacoside 0, 15, 30, 60, and 80 µM for 5, 12 or 24 hours and examined by fluorescent immunostaining, it was reported that doses above 60 µM increased DNA damage in cancer cell lines and did not cause any change in non-cancer cell lines [27]. Furthermore, Dong et al [28] revealed that Echinacoside occurred a significant increase in 8-oxoG, intracellular oxidized guanine, and a dramatic increase in double-stranded DNA break binding protein (53BP1) in SW480 cancer cells. Similarly, as a result of Comet analysis in this study, it was seen that TbA had a genotoxic effect by causing DNA damage in A2780 ovarian cancer cell lines.

It has been shown in some studies that *C. tubulosa* phenylethanoid glycosides exhibit antitumor effects on various tumor cells [29,30]. Yuan et al [31] reported that phenylethanoid glycosides significantly inhibited the growth of HepG2 and BEL-7404 from cancer cells through mitogen-activated protein kinase and apoptosis. Also, in the study investigating the antitumor effect of *C. tubulosa* phenylethanoid glycosides on esophageal cancer (Eca-109), it was reported that the apoptotic process was associated with Bcl-2 and showed effect by increasing caspase-3, -7 and -9 levels [32]. Some studies have shown that Bcl-2, Bax, and caspase-3 play a main role in apoptosis in A2780 cells [5,33]. In this study, it was shown that TbA could induce Bax expression and up-regulate caspase-3 while inhibiting Bcl-2 expression in A2780 cells. Besides, the majority of tumors are associated with mutation of p53 gene, and this may affect apoptosis by changing the regulation of Bcl-2 [5]. In this study, it was found that TbA increased p53 expression in A2780 cells. These results suggest that TbA may
induce apoptosis through caspase-3-dependent apoptotic signaling as well as increasing p53 expression in ovarian cancer.

CONCLUSIONS

In conclusion, it has been shown that TbA reduces cell viability in ovarian cancer and promotes apoptosis by activating p53 and caspase-3 pathways. This indicates that TbA may be a promising new choice for the treatment of ovarian cancer. Additionally, the underlying mechanism needs to be investigated in more detail in future studies.

REFERENCES


