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Research Article

Methyl Protodioscin Downregulates SORT1 and Enhances Carboplatin Sensitivity in A2780 Ovarian Cancer Cells

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Abstract

Background: Ovarian cancer is the most common lethal cancer of the female reproductive system and is often diagnosed in advanced stages. The *Sortilin 1 (SORT1)* gene is overexpressed in ovarian cancer cells. Methyl protodioscin, an herbal bioactive compound, has significant antitumor activities.

Objectives: Considering the necessity of identifying new medicinal agents and treatments in ovarian cancer, this project investigated the effects of methyl protodioscin on the expression of the *SORT1* gene and the response to chemotherapy in the ovarian cancer cell line (A2780 S).

Methods: The viability of ovarian cancer cells (A2780) after treatment with methyl protodioscin (800, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.12 µM) was evaluated by MTT assay, trypan blue staining, and lactate dehydrogenase activity measurement. The expression level of the *SORT1* gene was investigated by real-time PCR. The effect of simultaneous treatment with methyl protodioscin and the chemotherapy drug carboplatin on cell viability was measured.

Results: After 24, 48, 72, and 96 hours of treatment, methyl protodioscin significantly decreased cell viability in a concentration- and time-dependent manner ($P < 0.05$). After 24 hours, treatment with the IC_{50} concentration of methyl protodioscin (14.5 µM) caused a significant decrease in *SORT1* expression in ovarian cancer cells by 33% ($P < 0.05$). The combination of methyl protodioscin with carboplatin decreased cell viability with a synergistic effect.

Conclusions: Methyl protodioscin had an inhibitory effect on the survival of ovarian cancer cells in a concentration- and time-dependent manner and reduced the expression of the *SORT1* gene. Additionally, this herbal compound synergistically increased the toxicity of carboplatin.

Keywords: Methyl Protodioscin, *SORT1* Gene, Ovarian Cancer

1. Background

Due to difficulties in early diagnosis, ovarian cancer is associated with a high mortality rate in women (1, 2), with a 5-year survival rate of less than 30% (3, 4). Cisplatin or carboplatin are the most widely used chemotherapeutic agents against ovarian cancer. Ovarian cancer inevitably acquires resistance to these drugs when cancer cells become insensitive to the effects of platinum-based chemotherapy, leading to treatment failure and disease progression. Cancer recurrence after initial chemotherapy is very common in this cancer. One of the challenging issues in the successful treatment of ovarian cancer is the development of drug resistance (5-9).

Sortilin 1 (SORT1) is a multifunctional protein involved in cancer development. The expression of the *SORT1* gene is increased in human tumors, including ovarian cancer, and its siRNA-mediated gene silencing in ovarian cancer cells leads to increased apoptosis and decreased proliferation. This suggests that *SORT1* is a potential therapeutic target for tumor therapy. Some studies have shown that inhibition of *SORT1* expression and function can slow the growth of tumors (10).

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Methyl protodioscin, an herbal bioactive compound, was extracted from the rhizome of *Dioscorea collettii var. hypoglauca* (Dioscoreaceae) and shows some medicinal potential. Its anticancer activities were demonstrated in in vitro and in vivo studies. However, the underlying anti-cancer mechanisms remain unknown. Additionally, a preclinical pharmacodynamic study showed that 80 mg/kg i.v. of methyl protodioscin had no serious side effects (11).

2. Objectives

Considering the necessity of identifying new treatment methods, this study was designed to determine the effects of methyl protodioscin on *SORT1* gene expression and the response to chemotherapy in ovarian cancer cells.

3. Methods

3.1. Cell Culture

The A2780s cell line was prepared from the Pasteur Institute, Iran, and cultured in RPMI-1640 medium (Gibco, Germany) containing 10% fetal bovine serum (FBS) and 1% antibiotic. The cells were maintained in a 37°C incubator with 5% CO_2. Passage was performed after the cells reached a concentration level of 70%.

3.2. Viability Measurement

The cells were cultured in 96-well plates and treated with concentrations of 0, 0.09, 0.18, 0.37, 0.75, 1.5, 3, 6, and 12 µM methyl protodioscin for 24, 48, 72, and 96 hours. For the trypan blue assay, after trypsinizing and separating the cells from the surface, the cell suspension (about 10^6 cells/mL) was mixed (ratio of 1:1) with trypan blue (Sigma, USA) 4% solution. After 2 minutes, cells were checked using a neobar lam and an inverted phase optical microscope. Living cells have clear cytoplasm, while dead cells have dark blue cytoplasm. The percentage of cell viability was calculated by dividing the number of unstained cells by the total number of cells.

For the MTT assay, about 10 µL of MTT solution (0.5 mg/mL) (Sigma, USA) was added to each well and incubated for 3 hours in the dark at 37°C. Then, DMSO (200 µL) was added to each well, and the absorbance of the wells was read at 570 nm by an ELISA reader. A row of plate wells without drugs was considered as a control. The viability percentage was calculated from the following equation:

$$\left(Cell\ viability\ Abs\ test\frac{cells}{Abs}control\ cells\right)\ \times\ 100 = Cell\ viability\ (\%)$$

To investigate the effect of methyl protodioscin on carboplatin toxicity, cells were first treated with 800, 400, 200, 100, 50, 25, 12.5, and 6.5 µM of carboplatin for 24 hours. After measuring viability and calculating the IC_{50} using GraphPad Prism 6.0 (GraphPad Software, USA), combined treatment was performed at IC_{50} values and two concentrations higher and two lower than the IC_{50}. CompuSyn software (ComboSyn Inc, USA) was used to analyze the cytotoxicity data to determine synergy, additivity, and antagonism between methyl protodioscin and carboplatin.

3.3. Cytotoxicity Assay

The cells were cultured in 96-well plates and treated according to the procedures described in the viability assay. Cytotoxicity testing was performed using the Pierce LDH assay kit (Sigma Aldrich, USA) according to the manufacturer's instructions. The cytotoxicity of the extract was calculated using the following formula.

$$\%\ Cytotoxicity\ = \frac{(Experimental\ OD_{490} - Control\ OD_{490})}{Maximum\ lactate\ dehydrogenase\ (LDH)\ releas\ OD_{490}}\ \times\ 100$$

3.4. Gene Expression Analysis

After treating the cells and collecting them with trypsin/EDTA solution (Gibco, Germany), RNA extraction was performed according to the TRIZOL reagent kit (Life Technologies Invitrogen, USA). For the quantitative analysis of the extracted RNA, the optical absorption ratio of 260/280 and 230/260 nm was obtained using a NanoDrop. The quality of RNA was tested with agarose gel electrophoresis. The cDNA synthesis was done according to the kit protocol (Bioneer, Korea), and the quality of the synthesized cDNA was verified using a nanodrop device. For real-time PCR, SYBR Green master mix (Takara, Japan) was used. Gene expression was measured by the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the

housekeeping gene. The sequence of primers used is as follows.

(1) Glyceraldehyde 3-phosphate dehydrogenase:

- Forward: TCCTCCACCTTTGACGCTG

- Reverse: CACCACCCTGTTGCTGTAGC

(2) *Sortilin 1:*

- Forward: TCAGAGCCGAATGCCGTAG

- Reverse: CCTTCCAGCATCTTTGTCCAG

3.5. Statistical Analysis

Data were shown as mean ± SE of three independent tests (each conducted three times, with each time in technical triplicate). Statistical analysis was conducted using SPSS software version 16.0 (SPSS Inc., USA) and a one-way ANOVA test. P-values below 0.05 were considered statistically significant.

4. Results

4.1. Methyl Protodioscin Effects on Cell Viability

After treatment, the morphology of cells changed, becoming small, round, and granular. The effects of different concentrations of methyl protodioscin on cell viability after 24, 48, 72, and 96 hours were tested using trypan blue and MTT assays, as shown in Figure 1A and B. Treatment with methyl protodioscin significantly decreased viability after 24 hours at concentrations of 0.75, 1.5, 3, 6, and 12 µM ($P < 0.05$). After 48, 72, and 96 hours, the reduction in cell viability was significant at all concentrations ($P < 0.05$). This decrease occurred in a concentration- and time-dependent manner. The IC_{50} values calculated in the trypan blue test were 7.17 ± 0.12, 3.16 ± 0.17, 0.99 ± 0.08, and 0.31 ± 0.05 µM, and in the MTT test were 14.58 ± 0.55, 4.05 ± 0.01, 0.75 ± 0.08, and 0.38 ± 0.01 µM for 24, 48, 72, and 96 hours, respectively.

4.2. Cytotoxic Effects of Methyl Protodioscin

Methyl protodioscin exhibited cytotoxicity against ovarian cancer cells in a concentration- and time-dependent manner. After 24 hours, significant toxicity was observed at concentrations of 3, 6, and 12 µM. After 48 hours, significant toxicity was observed at concentrations of 0.75, 1.5, 3, 6, and 12 µM. After 72 hours, significant toxicity was observed at concentrations of 0.18, 0.37, 0.75, 1.5, 3, 6, and 12 µM. After 96 hours, significant toxicity was observed at all concentrations used ($P < 0.05$) (Figure 2).

4.3. Methyl Protodioscin Effects on Sortilin 1 Gene Expression

Treatment with the IC_{50} concentration of methyl protodioscin (14.5 µM) after 24 hours caused a significant decrease in *SORT1* expression in ovarian cancer cells by 33% ($P < 0.05$) (Figure 3).

4.4. Carboplatin Effect on Cell Viability

The effects of different concentrations of carboplatin on cell viability after 24 hours were measured by the MTT test and are shown in Figure 4. At concentrations of 25, 50, 100, 200, 400, and 800 µM, a significant decrease in viability was observed ($P < 0.05$). This decrease occurred in a concentration-dependent manner. The IC_{50} value was determined to be 228.90 ± 16.10 µM.

4.5. Carboplatin and Methyl Protodioscin Combination Effect on Cell Viability

The dose-effect diagram showed that the combination of these two agents has more toxic effects on cancer cells than either one alone. The Combination Index (CI) values calculated for all five treatments were less than 1, indicating a synergistic interaction between carboplatin and methyl protodioscin in reducing cell viability. Dose Reduction Index (DRI) values for both carboplatin and methyl protodioscin were greater than 1, indicating a dose reduction to produce a specific therapeutic effect in both cases. Finally, the isobologram plot was drawn. In the isobologram diagram, the concentrations of carboplatin and methyl protodioscin alone and the simultaneous treatment that cause a decrease of 50, 75, and 90 percent of the cell population are shown. In this diagram, the placement of compound points on the chord, below, and above it respectively indicates additive, synergistic, and antagonistic interactions (Figure 5).

5. Discussion

The results of the present study showed that methyl protodioscin had a lethal effect on ovarian cancer cell lines. By increasing the concentration of methyl protodioscin, the percentage of cell viability decreased significantly. Additionally, with the increase in the duration of treatment, the percentage of live cells decreased significantly compared to the control group. Previous studies have shown that dioscin had significant inhibitory effects on HL-60 human leukemia cell growth, differentiation, and apoptosis. Moreover,

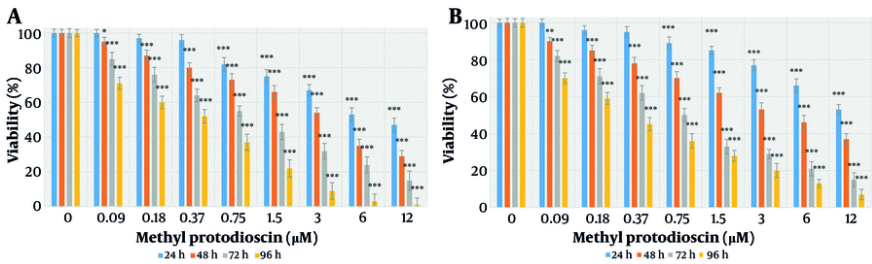

Figure 1. The effect of methyl protodioscin on the viability of ovarian cancer cells after 24, 48, 72 and 96 (h) was A, evaluated by trypan blue staining; and B, MTT test. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$ compared to the control.

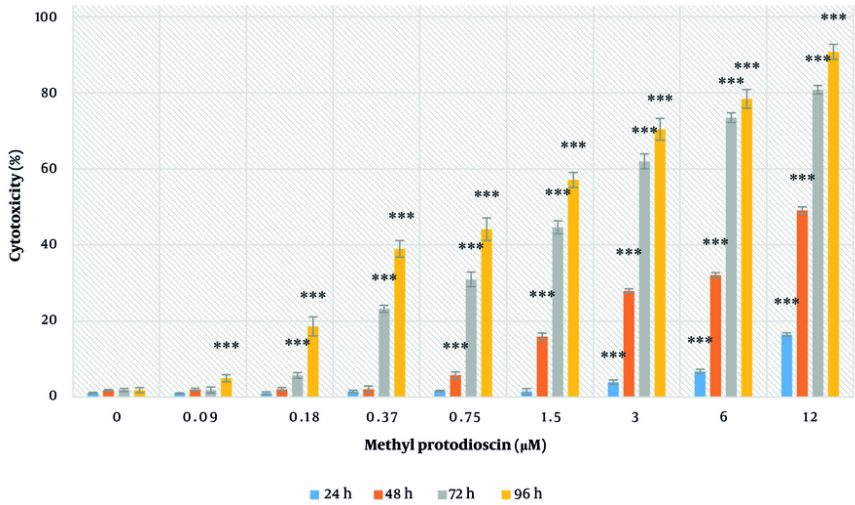

Figure 2. The cytotoxic effect of methyl protodioscin on the ovarian cancer cells after 24, 48, 72 and 96 (h) *** $P < 0.001$ compared to the control.

dioscin was found to affect many cancer cells (12). Methyl protodioscin also showed cytotoxic effects on solid tumor cell lines. An analysis utilizing the computer program COMPARE indicated that no compounds in the NCI Anticancer Drug Screen database have similar cytotoxicity patterns to methyl protodioscin, suggesting a potential new mechanism of anticancer action (13). Methyl protodioscin exhibited anti-mitotic activity and induced G2/M arrest and apoptosis in HepG2 cells via cyclin B1 and Bcl-2 expression reduction and Bax expression induction (14). Treatment of oral cancer cells with methyl protodioscin induced apoptosis and could also induce autophagy (15). Protodioscin inhibits cell viability and induces mitochondrial loss of function in apoptosis (16). Methyl protodioscin inhibits proliferation and increases apoptosis in pancreatic cancer (11). In cervical cancer cells, methyl protodioscin induces cell cycle arrest and apoptosis (17).

Despite recent advancements in cancer therapy, the life span and quality of life of patients with ovarian cancer remain unsatisfactory due to the development of drug resistance. Thus, developing new strategies to overcome this problem is needed. Recently, the use of drug combinations has been employed for lethal diseases such as cancer. The major purpose of this strategy is to achieve a synergistic therapeutic effect, reduce the dosage and toxicity, and minimize or delay

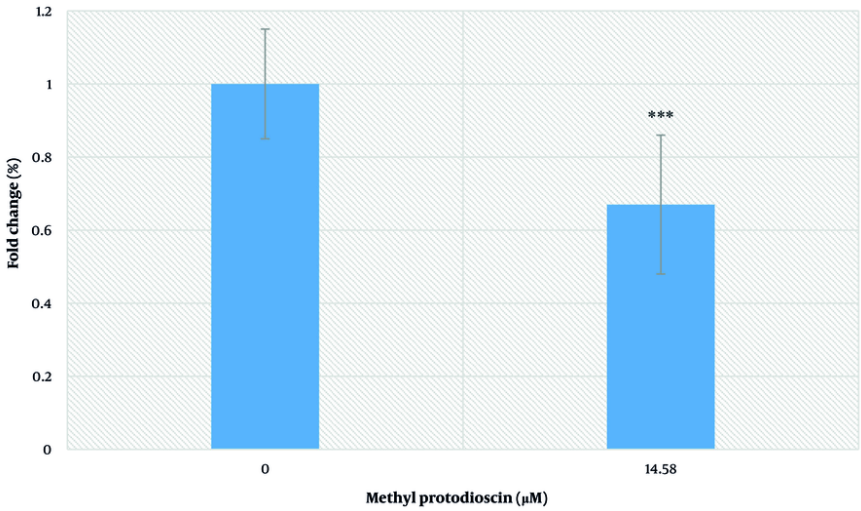

Figure 3. The effect of methyl protodioscin on *SORT1* gene expression in ovarian cancer cells after 24 (h). *** P < 0.001 compared to the control.

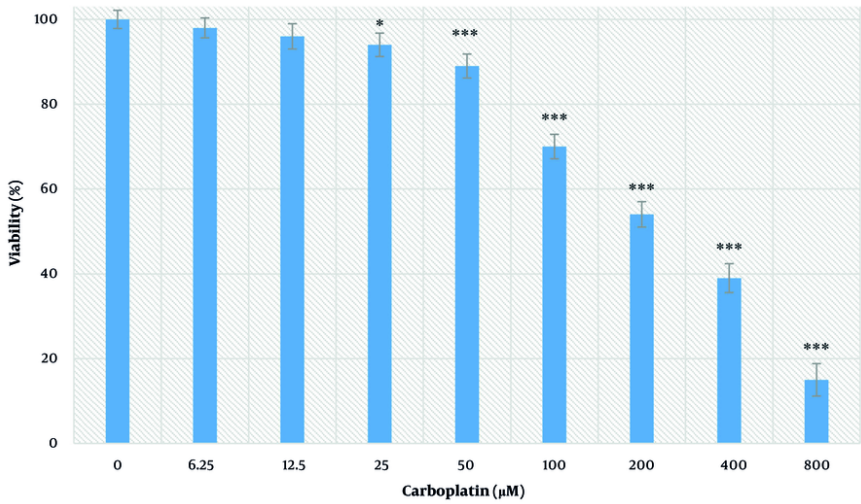

Figure 4. The effect of carboplatin on the viability of ovarian cancer cells after 24 (h)* P < 0.05 and *** P < 0.001 compared to the control.

the development of drug resistance. Today, the use of several anticancer drugs from different groups is widely practiced in cancer treatment (18).

Methyl protodioscin significantly increases the cytotoxicity of carboplatin, with a CI between 0.69 and 0.92, indicating a synergistic effect across all combined concentrations used in this project. The average CI for all tests performed was 0.87, highlighting the overall synergistic effect of the combination of methyl protodioscin and carboplatin on the ovarian cancer cell line. This combination led to a dose reduction for carboplatin, which is clinically valuable as it reduces the general side effects of chemotherapy. Real-time PCR results showed that treatment of cancer cells with the

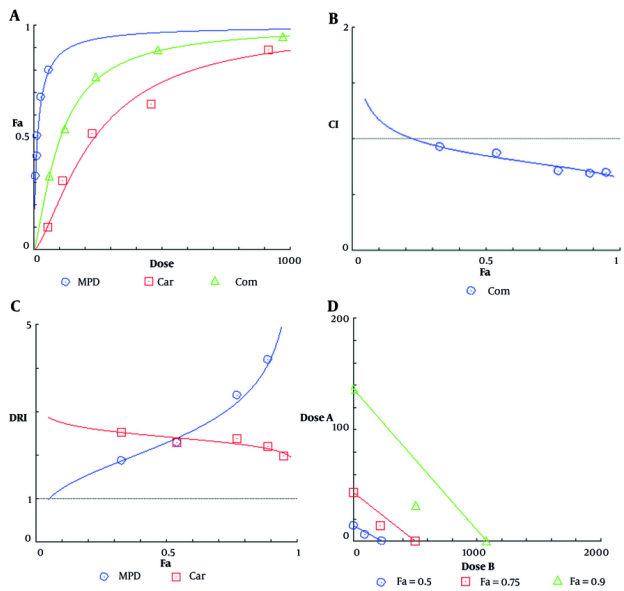

Figure 5. Diagram of A, effect-dose; B, Combination Index (CI); C, Dose Reduction Index (DRI); and D, isobologram for ovarian cancer cells after 24 hours of treatment with carboplatin, methyl protodioscin and a combination of two agents. Dose A: Carboplatin concentration, and dose B: Methyl protodioscin concentration.

IC_{50} concentration of methyl protodioscin for 24 hours significantly reduced *SORT1* gene expression compared to the control group.

The first molecular characterization of *SORT1* showed that it is expressed in the heart, brain, placenta, skeletal muscle, testis, thyroid, and spinal cord (19). The *SORT1* gene expression was increased in ovarian cancer tissues compared to non-malignant ovarian tissues (20, 21). These results may suggest the possible role of *SORT1* in ovarian tumorigenesis. The potential role of overexpressed *SORT1* in ovarian cancer cells is an interesting topic for research. It has previously been proven that decreased *SORT1* expression in ovarian tumors leads to apoptosis induction and reduced proliferation of cancer cells, so its decreased expression induced by methyl protodioscin in this study may be responsible for its anti-ovarian cancer effect.

One of the limitations of this study is the lack of examination of *SORT1* siRNA/overexpression experiments to validate its functional role in methyl protodioscin-induced chemosensitization. Currently, it is unknown whether methyl protodioscin synergizes with carboplatin in *SORT1*-silenced cells.

5.1. Conclusions

Methyl protodioscin had an inhibitory effect on the viability of ovarian cancer cells in a concentration- and time-dependent manner and reduced the expression of the *SORT1* gene. The present study also showed that this herbal compound synergistically enhances the chemotherapy effects of carboplatin. The results suggest that methyl protodioscin has a therapeutic effect on ovarian cancer cells and holds promise for use in the development of anti-ovarian cancer drugs.

Footnotes

Authors' Contribution: Study concept and design: I. R.; Acquisition of data: S. A. M.; Analysis and interpretation of data: S. B. M.; Drafting of the manuscript: M. Z.; Critical revision of the manuscript for important intellectual content: C. J.; Statistical analysis: M. Z.; Administrative, technical, and material support: I. R.; Study supervision: I. R.

Conflict of Interests Statement: The authors declare no conflict of interest.

Data Availability: No new data were created or analyzed in this study. Data sharing does not apply to this article.

Ethical Approval: This study is approved under the ethical approval code of IR.KUMS.MED.REC.1401.280 .

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