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# Research Article
# Dimethyl Sulfoxide Potentiates the Anticancer Activity of Cisplatin Against the Growth of Lung Cancer Cells

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## Abstract

**Background and Objective:** Optimal clinical usefulness of Cisplatin is limited due to its nephro and neurotoxicity. The current study investigated whether dimethyl sulfoxide (DMSO) could enhance Cisplatin cytotoxic effects against the growth of A549 human lung carcinoma cell line. **Materials and Methods:** Cytotoxicity was determined using sulforhodamine-b (SRB) method, the changes of apoptosis induction using annexin V reagent, cell cycle phase distribution using flow cytometry. Moreover, Cisplatin cellular uptake was analyzed by Inductively Spectrometry (ICP-OES) and P glycoprotein activity by means of Rhodamine-123 dye assay. These parameters were measured in A549 lung cancer cells after treatment with Cisplatin and/or DMSO. **Results:** The DMSO treatment increased the cytotoxic activity of cisplatin manifested by a significant decrease in the $IC_{50}$ (from 28.6 to 15.7 μg $mL^{-1}$). Cisplatin (5 or 20 μg $mL^{-1}$) and DMSO (10%) significantly decreased MDR activity with a significant increase in induction of apoptosis (81 and 84%) and accumulation of the cells in G0/G1 phase to 61.9 and 70.3%, respectively, compared to cisplatin treated cells. **Conclusion:** The DMSO treatment enhanced the cytotoxic activity of cisplatin against the growth of A549 cells by increasing Cisplatin cellular uptake, decreasing MDR function and disturbance of cell cycle.

Key words: Dimethyl sulfoxide, cisplatin, flow cytometry, p-glycoprotein, lung cancer cells

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and is considered as the leading cause of death (18.4% of the total cancer deaths worldwide)[1]. According to the National Saudi Cancer Registry (SCR), the number of lung cancer patients in Kingdom of Saudi Arabia amounted to 5.5 male and 1.8 female in every 100,000 patients[2].

Cisplatin is a cytotoxic drug listed under platinum-based chemotherapy with proved efficacy, either alone or in combination with other cytotoxic drugs, against different types of human malignancies, including lung cancer[3]. However, the clinical usefulness of Cisplatin is restricted by its detrimental effects including nephrotoxicity and neurotoxicity[4]. Sensitization of tumor cells to the cytotoxic effect of Cisplatin is an important strategy in order to decrease the dose of Cisplatin required to achieve the maximum cytotoxic effect while diminishing its adverse effects. One of these approaches is to use natural compound with chemopreventive properties to deliver the synthetic chemicals with low toxicity and delay carcinogenesis, such as resveratrol (a flavonoid compound extracted from grape peel) that has been used in combination with Cisplatin to decrease its adverse effects and enhance its anti-cancer efficacy[5,6]. Another approach is to use Dimethyl Sulfoxide (DMSO) (an organosulfur compound) which has a chemoprotective effect and could sensitize tumors cells to the chemotherapeutic drugs[7,8]. Dimethyl Sulfoxide is an organosulfur compound derived from wood pulp and it dissolves both polar and non-polar compounds. The DMSO enhances Cisplatin cytotoxicity by facilitating its entry into cells and increase probability of binding to DNA and reduces resistance in solid tumor[9]. From the above, DMSO may consider as a promising chemosensitizing agent. Therefore, the goal of this research was to investigate whether DMSO would enhance the cytotoxic effects of Cisplatin against the growth of A549 lung cancer cells. The current study examined the potential mechanisms of DMSO and Cisplatin interaction through various parameters, including cytotoxicity, induction of apoptosis, Cisplatin cellular uptake, multi-drug resistance (MDR) activity and disturbance of cell cycle phases.

## MATERIALS AND METHODS

This research was done at Pharmacology Department, Faculty of Medicine, KAU, Jeddah in collaboration with National Cancer Institute Cairo University from September, 2016-2018.

**Drugs and chemicals:** Cisplatin "Ebewe" and DMSO were purchased from Aldrich Chem. Corp., USA. The DMEM media, Trypsin/EDTA mixture, Penicillin G and streptomycin antibiotics, PBS, Nitric acid, cell cytotoxicity assay kit and Annexin V-FITC apoptosis detection kit were purchased from Aldrich Chem. Corp., USA. The cell cycle determination kit was purchased from Cayman Chemical Company, USA. All other chemicals were of analytical grade.

**Cells and cell culture:** Human Lung Cancer cell line (A549) was used in this study. Cells were grown as monolayer cultures and maintained in DMEM tissue culture medium at 37°C in a humidified 5% $CO_2$ air and collected by trypsinization.

### Methods

**Assessment of cytotoxicity:** Cytotoxicity was determined by using sulforhodamine-b (SRB) methods previously described by Skehan *et al.*[10]. Cells seeded in 96 well microtiter plates at concentration of $40\times10^3$ cells/well in DMEM medium. About 24 h later, the cells incubated with different concentrations of Cisplatin and 10% of DMSO (3 wells for each concentration). After 48 h, cells fixed, washed, air dried, stained with 0.4% of SRB for 30 min and unbound dye removed by washing three times with 1× dye wash solution. Bound stain solubilized with 200 µL/well SRB solubilization buffer for 10 min. The optical density was read in ELx808 absorbance microplate reader (Bio Tek, U.S.A.) at wavelength of 490 nm. Surviving fraction was calculated as following:

- **Surviving fraction:** Optical density of treated cells/ optical density of control cells. The $IC_{50}$ (the concentration of Cisplatin necessary to produce 50% inhibition of cells growth) was calculated from liner equation of the survival fraction curve:

$$Y = mX + b$$

Where:

Y = 0.5 (The surviving fraction when there is a 50% inhibition of cell growth)
m = Slop
X = Dose of CIS induce 50% inhibition
b = The y-intercept

**Apoptosis assay:** Cells were seeded in T25 flasks at cell density of $5\text{-}8\times10^5$ cells/flask in DMEM media and then the flasks were incubated for 24 h at humidified air containing 5% $CO_2$. After that the cells were incubated with Cisplatin concentration (5 and 20 µg $mL^{-1}$) alone and combined with

10% DMSO for 48 h (3 flasks for each concentration). Medium was removed and the flasks were washed with PBS. Afterwards, the cells were harvested with Trypsin/EDTA. After trypsinization, cells were washed with cold PBS and suspended in 100 µL annexin V incubation reagent prepared by mixing (binding buffer 10x, PI, annexin V-FITC and deionizes water ) for each sample . After that, the solution was incubated at room temperature in the dark for 15 min. Then 400 µL of binding buffer was added to each sample and process by flow cytometry (NAVIOS Beckman Coulter, U.S.A).

**Cell cycle analysis:** Cells were seeded in T25 flasks and proceeded as above. Following trypsinization, cells were washed twice with cold assay buffer. The cell pellet was then re-suspended to a density of $10^6$ cells $mL^{-1}$ in assay buffer. About 1 mL of fixative agent was added to each sample to fix and permeabilize the cells for at least 2 h. The fixed cells pellet were suspended in staining solution and incubated for 30 min at room temperature in the dark. Cell cycle analysis was performed by using flow cytometry[11] (Becton DICNSON (BD) FACS Calibur, U.S.A).

**Assessment of cisplatin cellular uptake:** Cells were seeded in T25 flasks at cell density of 5-8×$10^6$ cells/flask in DMEM medium and proceeded as mentioned above. Afterwards, cells were incubated with Cisplatin concentration (5 and 20 µg $mL^{-1}$) alone and combined with 10% DMSO simultaneously for 48 h. After 48 h treatment the cells medium was removed and the flasks were washed with PBS, the cells were harvested and washed twice with cold PBS. For drug uptake analysis, cells (1×$10^6$) were suspended in 2 mL of 1% $HNO_3$ for 24 h at 70°C to be digested. About 3 mL of PBS was then added to each sample to complete the volume. Lysed cells were analyzed by means of ICP-OES (OPTIMA 7000 DV ICP-OES PerkinElmer, Inc., Waltham, USA), according to Scott *et al.*[12].

**Determination of the activity of multidrug resistance (MDR) via rhodamine-123 dye:** Accumulation of rhodamine-123 in the cells is inversely related to MDR activity[13]. In brief, cells were seeded in T25 flasks at cell density of 5-8×$10^6$ cells/flask in DMEM medium and proceeded as above. About 2.62 µM (100 µL form working solution) of Rhodamine-123 was added and was kept it in $CO_2$ incubator at 37°C for 1 h. Cells were incubated with Cisplatin concentrations alone and combined with 10% DMSO for 10 min. Cells were then harvested and washed once with iced PBS. For p-glycoprotein analysis, cells (1×$10^6$) were suspended in 1 mL of PBS to each sample and shaking. Lysed cells were analyzed by spectrofluorometric at wavelength (485-590 nm).

**Statistical analysis:** Statistical analysis of data was calculated by using statgraphic computer package (Excel, 2010) and computer program package (SPSS, version 18). All date was expressed as mean with their standard error of mean (SEM) of three separate experiments, each one in triplicate. One way analysis of variance (ANOVA) was used to test for difference between experimental groups. It was followed by the least significance difference (LSD) test. However, two-sample t-test and its p-value were used to analyze the significance of the difference in the sample means. Differences were considered significant at $p<0.05$.

## RESULTS

**Effect of Cisplatin and/or DMSO on proliferation of A549 lung cancer cells:** Figure 1 show the effects of Cisplatin, DMSO and their combination on the survival of A549 lung cancer cells. When cells exposed to different concentrations of Cisplatin (0, 10, 20 and 40 µg $mL^{-1}$ ) for 24 h, a concentration-dependent cytotoxicity was observed and according to linear regression line $IC_{50}$ was 28.6 µg $mL^{-1}$. Although, 10% of DMSO did not show any cytotoxicity against A549 cells, it increased the cytotoxicity of Cisplatin manifested as a significant decrease in the $IC_{50}$ to 15.7 µg $mL^{-1}$.

**Effect of Cisplatin and/or DMSO on induction of apoptosis in A549 cells:** The effect of Cisplatin treatment with or without DMSO (10%) on the percentage of cells in early apoptosis (annexin V-positive cells) can be observed in Table 1. Treatment with Cisplatin 5 µg $mL^{-1}$ alone showed 74.5% of early apoptotic cells while combined treatment with Cisplatin 5 µg $mL^{-1}$ and DMSO (10%) significantly increased

Table 1: Effect of Cisplatin, DMSO (10%) and their combination on the induction of early apoptosis in A549 cells

| Treatments | Early apoptosis (%) |
|---|---|
| Control | 52.2±0.10 |
| DMSO (10%) | 73.3±0.15[a] |
| Cisplatin (5 µg $mL^{-1}$ ) | 74.5±2.05[a] |
| Cisplatin (5 µg $mL^{-1}$) +DMSO (10%) | 81.0±0.60[a,b] |
| Cisplatin (20 µg $mL^{-1}$) | 77.8±1.25[a] |
| Cisplatin (20 µg $mL^{-1}$)+DMSO (10%) | 84.0±1.20[a,b] |

Apoptosis was analyzed when the cells exposed to drug for 48 h then staining with annexin-FITC and PI. Data are expressed as mean±SEM of three separate experiments, each one in triplicate. [a]Significantly different from the control at p-value 0.05. [b]Significantly different from corresponding Cisplatin at p-value 0.05

Table 2: Effect of DMSO on the cellular uptake of Cisplatin in A549 cells

| | Cisplatin concentrations ( ng/$10^6$ cells) | |
|---|---|---|
| | Treatments | |
| Cisplatin cellular levels | 24 h after treatment | 48 h after treatment |
| Cisplatin (5 μg $mL^{-1}$) | 0.07±0.02 | 0.80±0.05 |
| Cisplatin (5 μg $mL^{-1}$)+DMSO (10%) | 0.15±0.03[a] | 2.84±0.05[a] |
| Cisplatin (20 μg $mL^{-1}$) | 2.13±0.40 | 6.40±0.80 |
| Cisplatin (20 μg $mL^{-1}$)+DMSO (10%) | 6.07±0.34[a] | 9.04±0.10[b] |

A549 growing cells were treated with Cisplatin and/or DMSO (10%) for both 24 and 48 h, then the cells were washed once with PBS, harvested, counted and digested by (1%) $HNO_3$. Cisplatin level was measured by using ICP-OES. Data are expressed as mean±SEM of three separate experiments, each one in triplicate. [a]Significantly different from corresponding Cisplatin at p-value 0.05. [b] Significantly different from corresponding Cisplatin after 24 or 48 h of treatment at p-value 0.05

Table 3: Effect of Cisplatin, DMSO and their combination on the accumulation of Rhodamine-123 in A549 cells

| Treatments | Rho-123 concentrations (μg/$10^6$ cells) |
|---|---|
| Rhodamine-123 (100 μg $mL^{-1}$) | 0.010±0.01 |
| DMSO (10%) | 0.103±0.01[a] |
| Cisplatin (5 μg $mL^{-1}$) | 0.122±0.01[a] |
| Cisplatin (5 μg $mL^{-1}$+DMSO (10%) | 0.194±0.01[a,b] |
| Cisplatin (20 μg $mL^{-1}$) | 0.169±0.01[a] |
| Cisplatin (20 μg $mL^{-1}$+DMSO (10%) | 0.222±0.01[a,b] |

A549 cells were exposed to 100 μL $mL^{-1}$ Rho-123 for 30 min at 37°C. After washing, Rho-123 accumulation was quantified by spectrofluorometry. Data are expressed as ratio of fluorescent dye accumulation in A549 cells with mean±SEM of three separate experiments, each one in triplicate. [a]Significantly different from Rho-123 at p-value 0.05. [b]Significantly different from corresponding Cisplatin at p-value 0.05

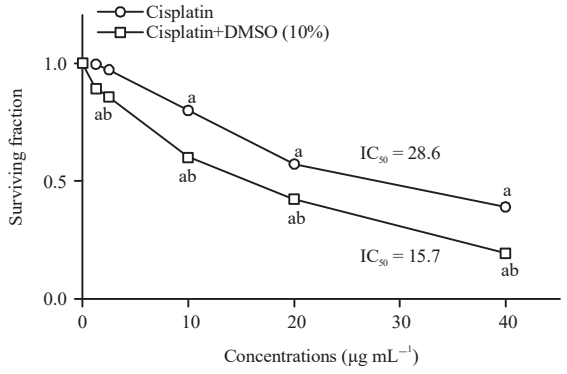


Fig. 1: Effect of Cisplatin, DMSO and their combination on survival of A549 cells

Data are expressed as mean±SEM of three separate experiments, each one in triplicate. [a]Significantly different from control at p-value 0.05. [b]Significantly different from corresponding Cisplatin at p-value 0.05

percentage of early apoptotic cells to 81%. Similar finding were observed where Cisplatin 20 μg $mL^{-1}$ showed 77.8% of early apoptotic cells while combined treatment of Cisplatin 20 μg $mL^{-1}$ and DMSO (10%) showed 84% of early apoptotic cells.

**Effect of Cisplatin and/or DMSO on cell cycle phase distribution of A549 cells:** The findings in Fig. 2 show the effect of treatment with various concentrations of Cisplatin alone and combined with DMSO on cell cycle distribution, using the flow cytometry after staining with PI. Treatment with DMSO alone showed a preferential block in $G_0/G_1$ at the expense of $G_2$/M phase cells, where there was 52% accumulation. Combined treatment with 5 μg $mL^{-1}$ Cisplatin and DMSO showed a significant 61.9% increase in percentage of cells in $G_0/G_1$ as compared to 5 μg $mL^{-1}$ Cisplatin (5%) alone which showed 47.5%. In addition, increasing the concentration of Cisplatin to 20 μg $mL^{-1}$ in presence of DMSO showed a significant 70.3% increase of cells in $G_0/G_1$ compared to 64.5% of Cisplatin alone.

**Effect of DMSO on cellular uptake of Cisplatin in A549 cells:** The Cisplatin cellular uptake concentration in A549 cells 24 and 48 h after treatment can be observed in Table 2. After 24 h exposure, Cisplatin was hardly detected after treatment with 5 μg $mL^{-1}$, while increased significantly to 0.15 ng/$10^6$ cells in the presence of DMSO. Moreover, when the concentration of Cisplatin increased to 20 μg $mL^{-1}$, Cisplatin cellular concentration significantly increased to 2.13 ng/$10^6$ cells and in presence of DMSO increased to 6.07 ng/$10^6$ cells. After 48 h, the uptake of Cisplatin was 0.80 ng/$10^6$ cells after exposure to 5 μg $mL^{-1}$. Moreover, when the concentration of Cisplatin increased to 20 μg $mL^{-1}$, Cisplatin cellular uptake concentrations were 6.40 ng/$10^6$ cells.

**Effect of Cisplatin and/or DMSO on the activity of multidrug resistance (MDR):** As shown in Table 3, non-treated control A549 cells poorly accumulated Rho 123 (0.01 μg/$10^6$ cells) which reflecting MDR-mediated efflux of the fluorescent dye. Cisplatin (5 and 20 μg $mL^{-1}$) increased dye accumulation in A549 cells to 0.122 and 0,169 μg/$10^6$ cells, respectively.

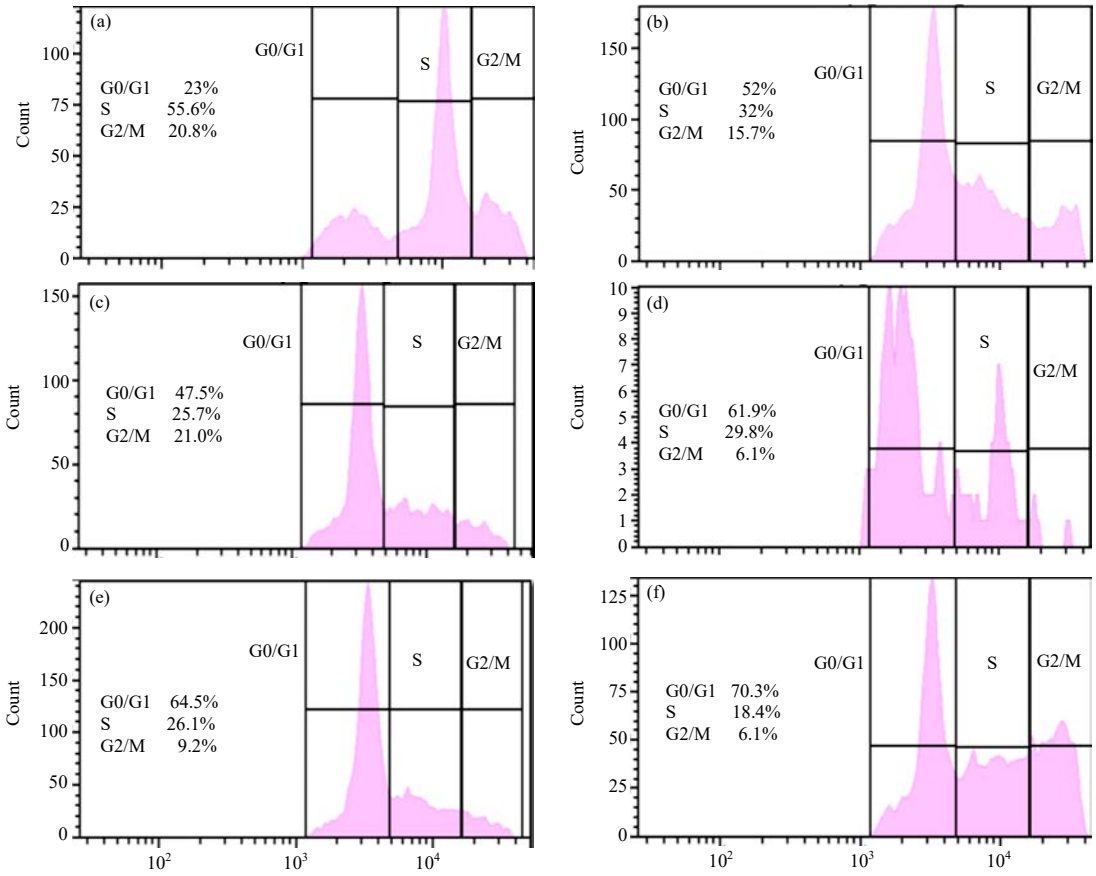


Fig. 2(a-f): Effect of Cisplatin and/or DMSO (10%) on cell cycle phase distribution of A549 cells. Cell cycle distribution was analyzed after 48 h of exposure to drug by staining with propidium iodide (PI), (a) Control, (b) Cells treated with DMSO (10%), (c) Cell treated with Cisplatin 5 μg $mL^{-1}$, (d) Cells treated with Cisplatin 5 μg $mL^{-1}$ and DMSO (10%), (e) Cells treated with Cisplatin 20 μg $mL^{-1}$ and (f) Cells treated with Cisplatin 20 μg $mL^{-1}$ and DMSO (10%). The experiment was repeated three times, each one in triplicate

Addition of DMSO (10%) to Cisplatin either 5 or 20 μg $mL^{-1}$ significantly increased accumulation of Rho-123 dye to 0.194 and 0.222 μg/$10^6$ cells, respectively.

## DISCUSSION

The current study showed that DMSO increased Cisplatin cytotoxicity against the growth of A549 lung cells which manifested as a significant 45% decrease in the $IC_{50}$ compared to Cisplatin alone. This increase in the cytotoxic effect of Cisplatin by DMSO has been confirmed by the observed significant increase in the percentage of early apoptosis and arrest of the cells at $G_0/G_1$ phase compared to the control. It is well known that Cisplatin mediates its anti-tumour activity via DNA-inter and intra strand cross linking leading to severe local distortion in the DNA double helical structure and cell death[14]. In the current study, the observed increase in cytotoxicity of Cisplatin by DMSO was parallel to the increase in its cellular uptake which may point to the consideration of increased absorption and accumulation of Cisplatin by DMSO as an important mechanism whereby DMSO enhances Cisplatin cytotoxicity. Earlier studies demonstrated that the increase in Cisplatin accumulation in presence of DMSO proved the ability of DMSO to change the membrane of cancer cells and mitochondrial permeability, this might lead to increase the uptake of Cisplatin resulting in cell death[15-17]. The current results are consistent with the data presented by Uribe *et al.*[7], who reported that DMSO potentiated the effect of Cisplatin and killed more sensory hair than treatment with Cisplatin alone by facilitating Cisplatin entry into cell, rising its intracellular concentration and probability of binding to DNA. It is well documented that rhodamine-123 dye is a substrate for MDR genes and the proteins codified by these genes including p-glycoprotein[13]. In the present study, accumulation

of rhodamine-123 in lung cancer cells was increased by combined treatment of DMSO and Cisplatin compared with Cisplatin alone. These results suggested that DMSO my down regulate MDR proteins or inhibits their catalytic activity with the consequent increase in the accumulation of Cisplatin in presence of DMSO. Also, it is possible that DMSO binds to Cisplatin, where Cisplatin-DMSO adducts have greater affinity for DNA and enhances Cisplatin cytotoxicity. This hypothesis is consistent with data reported by Sundquist *et al.*[18], who documented that the use of DMSO in combination with Cisplatin increased the rate of platinum binding to calf thymus DNA. Earlier study reported that DMSO sensitized cancer cells to apoptosis, growth arrest and synergistically increased the cytotoxicity of anti-neoplastic agents[19]. On the same line, with Osman *et al.*[8] demonstrated that DMSO pretreatment enhanced the cytotoxic activity of Cisplatin against the growth of Ehrlich ascites carcinoma cells and attenuated Cisplatin-induced nephrotoxicity.

The present study showed a significant increase in percentages of early apoptosis after Cisplatin treatment which increased in presence of DMSO. This confirmed the cytotoxic study where Cisplatin was more cytotoxic in presence of DMSO. It has been reported that DMSO induced apoptosis in SV40-transformed human keratinocytes but not in normal keratinocytes[20]. Also, Aita *et al.*[21] reported that DMSO can significantly increase apoptosis in murine lymphoid organs and other cancer cells. The current study showed a significant increase in the arrested cells in $G_0/G_1$ of A549 cells treated with Cisplatin plus DMSO compared with cells treated with Cisplatin alone. This could be due to the ability of DMSO to modify several cells signaling molecules, including cell proliferative proteins, drug transporters and cell survival protein and the capability of DMSO to interfere with the expression of anti-apoptotic signals[14].

In contrary to previous results of present authors, Hall *et al.*[22] reported that there was inhibition of cytotoxicity and the ability of Cisplatin to initiate cell death when Cisplatin dissolved in DMSO. Moreover, Fischer *et al.*[23] showed that the conjugation between DMSO and Cisplatin forms a new adduct with lower nephrotoxicity and cytotoxicity. This discrepancy could be refuted due to formation of new chemical between Cisplatin and DMSO which lead to inhibition of cytotoxicity effect of Cisplatin, whereas present study depended on treatment of cells with DMSO and Cisplatin. In general the action of DMSO might be disruption of the structure and/or arrangement of cell surface receptors that regulate growth and differentiation[8]. These effects will synergistically raise the Cisplatin cytotoxicity against cancer cells. As a final point, it may be recommended that the forthcoming studies using DMSO could focus on defining and elucidating the pharmacokinetics, pharmacodynamics and pharmacogenetics interaction between DMSO and other cytotoxic drugs to open practical value in cancer therapy.

## CONCLUSION

The DMSO treatment enhanced the cytotoxic activity of Cisplatin against the growth of A549 through increasing Cisplatin cellular uptake, decreasing MDR function and disturbance of cell cycle.

## SIGNIFICANCE STATEMENT

This research investigated the role of DMSO treatment in potentiating Cisplatin cytotoxicity through decreasing the function of MDR protein with consequent increase in the accumulation of Cisplatin inside the cells. This introduces a key for the researchers and clinicians to study other mechanisms which may be involved in the cytotoxic potentiation of other chemotherapeutic drugs

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