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Cisplatin treatment in EMT6 murine breast cancer cells: Impact on cell viability and molecular pathways

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ABSTRACT

Breast cancer is a prevalent malignancy that requires tailored treatments. Cisplatin, a platinum-based chemotherapy agent, is widely used for its anti-proliferative and pro-apoptotic properties. Understanding its molecular mechanisms is crucial for optimizing its efficacy. We investigated cisplatin's effect on the EMT6 breast cancer cell line across various doses and durations. Using MTT assay and qPCR, we examined cell survival and gene expressions of *PTEN, MAPK, NFEL2L2*, and *Survivin* after 24 h and 48 h of cisplatin treatments. The highest viability was at 5 µM after 24 h and at 1 and 5 µM after 48 h, with significant decreases at higher concentrations. Significant changes were observed in *MAPK, NFEL2L2* and *Survivin,* while *PTEN* remained unaffected. Notably, *Survivin* was upregulated at lower doses, while *NFEL2L2* and *MAPK* showed no significant changes. Our findings indicate that cisplatin induces apoptosis and alters gene expression in a dose-dependent manner, providing insights into its molecular mechanisms in EMT6 cells.

Keywords: breast cancer, ROS, gene expression, cisplatin, cell survival

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INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers and is the second-highest ranked cancer type causing death in women[1]. Breast cancer occurs in different forms, including estrogen receptor-positive (ER-positive) breast cancer, progesterone receptor positive (PR-positive) breast cancer, HER-positive (HER2+) breast cancer, triple-negative breast cancer (TNBC), and advanced breast cancer. Different treatments are preferred according to the specific subtype of breast cancer: hormone therapy is often used for hormone-based breast cancers, while chemotherapeutic drugs are preferred for TNBC and advanced breast cancer[2]. Combining these treatments can increase efficiency of the chemotherapeutic drugs by targeting different pathways and/or reduce serious side effects by lowering the drug doses.

Cisplatin is a platinum-based and one of the commonly used chemotherapy drugs[2,3]. It functions by forming DNA adducts that create crosslinks between DNA strands[4]. These crosslinks can lead to DNA replication errors and subsequent DNA damage, which, if not repaired, results in cell death due to apoptosis[3,5]. Therefore, cisplatin exhibits anti-proliferative properties and induces apoptosis. Cisplatin has been used to treat several cancer types including breast[2], ovarian[6], lung[7], head, and neck cancer[8]. It is a cost effective, and easily accessible chemotherapy drug, making its application preferrable for different cancer types. However, it also causes serious side effects, limiting its application[9]. It has been shown that cisplatin treatment effects the levels of reactive oxygen species (ROS), the activity of mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathways in various cancer cells types[2,9-13]. These pathways are believed to contribute to cisplatin-induced cytotoxicity[14,15].

In this article, we aimed to investigate the cytotoxic effects of cisplatin on the EMT6 murine breast cancer cell line across a range of doses, from low to high. We also sought to analyze cisplatin resistance and cisplatin-induced cytotoxicity in these cells, utilizing EMT6 as a model which is well-suited for such investigations, given that it is an ER-negative and triple negative breast cancer cell line. To gain insights into the molecular mechanisms underlying cisplatin response, starting from very low concentrations, we analyzed the expression profiles of key genes involved in cell survival, drug resistance and cytotoxicity—namely phosphatase and tensin homolog (*PTEN)*, mitogen-activated protein kinase (*MAPK)*, nuclear factor-erythroid 2-related factor 2 (*NFEL2L2*), and *Survivin*—following cisplatin treatment for 24 and 48 hours.

METHODOLOGY

Cell culture

EMT6 cells[16] (ATCC, CRL-2755) were cultured in RPMI-1640 (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37°C, with 5% CO_2 and subcultured at 80-90% confluency.

Evaluation of cell viability by MTT Test

The MTT colorimetric assay was used to assess cell viability[17]. EMT6 cells were seeded into 96-well plates, at a density of 1.0 x 10^4 cells/ml in 0.1 ml complete medium. 24 hours (h) after seeding, the cells were treated with different concentrations of cisplatin. After incubation for 24h or 48h, 100 µl of MTT solution [0.5 mg/ml in DMEM w/o phenol red] was added to each well and cells were incubated for 4h at 37°C. After removal of MTT solution, the purple-blue MTT formazan precipitates were dissolved in 100 µl DMSO. The absorbance was measured at 540 nm using absorbance microplate reader. The relative cell viability was expressed as the ratio (%) of the absorbance in the cisplatin treated wells to that of non-treated control wells. The IC_{50} values for 24h and 48h cisplatin treatments were determined from the dose-response curves.

Cisplatin treatment

1.0 x 10^5 cells/ml were seeded to six-well plates. 24h after seeding, cells were treated with cisplatin with various concentrations in addition to non-treated control cells: 0.1 µM, 1 µM, 5 µM, 10 µM, and 50 µM. Cells were incubated for 24h and 48h before being collected for gene expression analysis.

qRT-PCR analysis

EMT6 cells were collected, total RNA was extracted and synthesized into cDNA. qPCR reaction was performed according to the following protocol: (a) for preincubation: 95°C for 10 minutes (min), (b) for amplification: 95°C for 10 seconds (sec), 57°C for 20 sec and 72°C for 30 sec, for 45 cycles. Samples were assayed in BioRad CFX Connect Real-Time System. $^{\Delta\Delta C}$t value was then calculated by subtracting the average Ct from the corresponding average Ct. Relative expression levels were analyzed by calculating $2^{-\Delta\Delta Ct}$. GAPDH was used as an internal control.

Statistical analysis

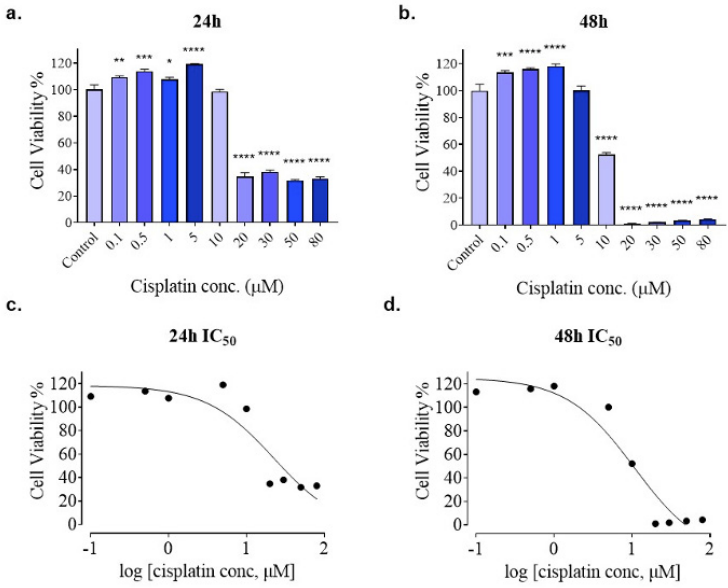
Data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, California). Statistical analysis of the data was performed with two-tailed unpaired student's t-test. For multiple comparisons, one-way anal-

ysis of variance (ANOVA) followed by Dunnett's multiple comparison. Data were normalized to GAPDH expression levels. P-values less than 0.05 were considered statistically significant.

RESULTS and DISCUSSION

Elevated cell viability at lower doses of cisplatin treatment

To assess the cytotoxic effects of cisplatin on EMT6 cancer cells at different concentrations and treatment durations, we treated the cells with varying concentrations of cisplatin and measured cell viability using MTT assay after 24 h and 48 h (Figure 1). In both 24 h and 48 h treatment groups, we observed highly significant change in cell viability in almost all concentrations of cisplatin, except 10 μM in 24 h, and 5 μM in 48 h treatment groups. The highest cell viabilities were recorded at 5 μM after 24 h treatment (Figure 1[a]), and at 1 μM and 5 μM after 48 h treatment (Figure 1[b]). In 24 h and 48 h treatment groups, cell viability dramatically decreases after 10 μM and 5 μM; respectively (Figure 1[a] and [b]). Thus, similar levels of cell survival are observed at higher doses (Figure 1[a] and [b]).



(*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001)

Figure 1. Cisplatin's effect on EMT6 cell survival. Cells were incubated with different doses of cisplatin for 24h (a, c) and 48h (b, d). Cisplatin significantly improved cell viability in all concentrations below 10 μM after 24h (a) and below 5 μM after 48h treatments (b). Cisplatin significantly decreased cell viability in all concentrations above 10 μM after 24h (a) and above 5 μM after 48h treatments (b). The data were normalized to the control and presented as mean ± SEM. (n=3). Asterisks indicate statistical significance compared with the corresponding control.

Next, we calculated the IC_{50} values for cisplatin in EMT6 cells after 24 h and 48 h treatments (Figure 1[c] and- [d]). IC_{50} value for cisplatin in EMT6 cells after 24 h treatment was determined to be 22.5 μM (Figure 1[c]), and after 48 h treatment was determined to be as 10.7 μM (Figure 1[d]) (n=3).

Based on these cell viability results and calculated IC_{50} values for both treatment durations, we selected five different cisplatin concentrations to perform gene expression analysis to understand the proliferative activity at lower concentrations and cell death at higher concentrations. Next, we analyzed the gene expression levels of *PTEN*, *MAPK*, *NFEL2L2*, and *Survivin* in both treatment groups.

Cisplatin treatment shows no effect on *PTEN* expression in EMT6 cells

PTEN gene encodes for a lipid and protein phosphatase, that acts on phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway[12,13]. PTEN acts as a tumor suppressor and has an important role in controlling cell survival, proliferation and migration[12,18,19]. In healthy cells, PTEN activity inhibits the PI3K signalling pathway, thereby supressing cell survival, proliferation and migration. Mutations in the *PTEN* gene or reduced PTEN activity have been observed in various types of cancer[12,18,20].

Here, we analyzed *PTEN* expression levels in EMT6 cells with increasing concentrations of cisplatin at 24 h and 48 h treatment durations. At both time points, we did not observe any significant decrease in *PTEN* expression compared to the control (Figure 2). This observation supports the idea that cisplatin may promote cell survival and proliferation in EMT6 cells, as shown in Figure 1 (a) and (b). This could be due to an indirect effect of cisplatin on cell proliferation pathways, or suggest that EMT6 cells begin to exhibit resistance to cisplatin within 24 hours. Considering that many studies prefer a 24 h drug treatment to study drug resistance in cancer cells[21,22], it is possible that we are observing the development of cisplatin resistance in EMT6 cells.

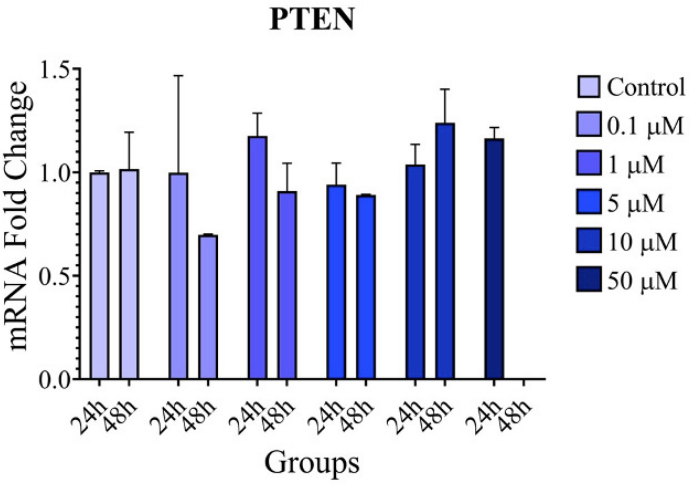
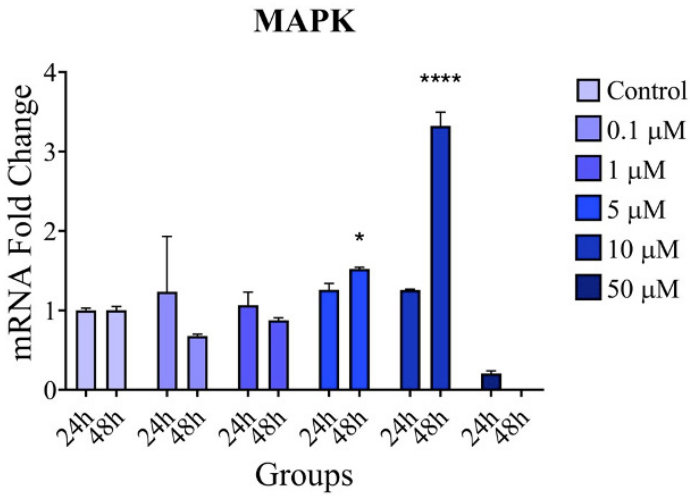


Figure 2. Relative mRNA expression levels of PTEN after 24h and 48h treatment with various cisplatin concentrations. No significant changes were observed in PTEN expression across all the tested concentrations in both treatment groups. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test relative to the control. $p<0.05$ were considered statistically significant.

MAPK expression in EMT6 cells is elevated at higher doses of cisplatin

The MAPK signaling pathway is another important intracellular pathway which plays a crucial role in regulating survival, differentiation, cell growth and apoptosis. The activation of the MAPK pathway in response to cisplatin treatment has been extensively studied in various cancer cells, and its activation has been documented in several studies[10,11,23]. Cisplatin is known to activate the MAPK pathway, increase MAPK protein levels, and induce apoptosis[23]. Conversely, some studies have suggested that cisplatin treatment leads to MAPK activation and associated autophagy, which may counteract the apoptotic effects of cisplatin[11,24].

In our study we observed a significant increase in *MAPK* levels in the 48h treatment group at concentrations of 5 μM and a notably strong expression at 10 μM (Figure 3). When we correlate this observation with the cell viability results shown in Figure 1(b), we notice an association between increased *MAPK* expression and elevated cell death at these concentrations, likely due to apoptosis induction. Although we observed minor changes in *MAPK* levels at other concentrations in both the 48h and 24h treatment groups, these changes were not statistically significant.



(*: p<0.05; ****: p<0.0001)

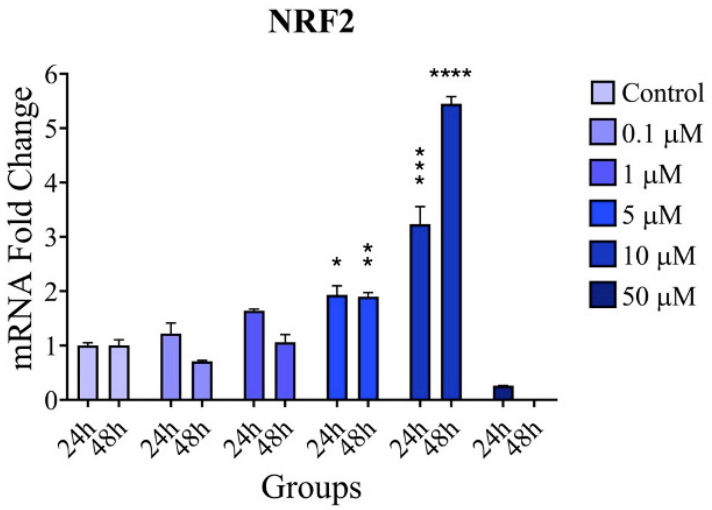
Figure 3. Relative mRNA expression levels of MAPK after 24h and 48h treatments with various cisplatin concentrations. A significant elevation in MAPK expression was observed at 5 μM, with a notably strong expression at 10 μM after 48h treatment. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test relative to the control.

Cisplatin treatment causes dose-dependent increase in *NRF2* expression

The NRF2 transcription factor, encoded by the *NFEL2L2* gene, acts on Antioxidant Response Elements (ARE) located in the promoters of several antioxidant genes and regulates transcription of many antioxidant and detoxifying genes in different cell types[22,25]. It is an important transcription factor overseeing the maintenance of the correct oxidative balance and regulating the expression of antioxidant genes in varying oxidative stress conditions[25]. As mitochondria being the main site of energy production of the cells, free radicals are constantly generated in mitochondria[26]. If these NRF2 mediated antioxidative gene machinery will not work properly, oxidation inside the cell due to ROS production leads to several diseases, like cancer[27]. In the basal condition of normal cells, *NRF2* is expressed at lower levels. Elevated *NRF2* expression in the cancer cells decreases the efficiency and toxicity of the chemotherapeutic drug, provides cyto-protection and potentiates cancer metastasis[28,29].

In our study, we observed a dose-dependent increase in *NRF2* expression levels following cisplatin treatment. After 24h of treatment, a nearly three-fold

increase in *NRF2* expression was observed at a concentration of 10 μM of compared to the control. Moreover, overexpression of *NRF2* is at the highest level after 48h treatment with 10 μM cisplatin (Figure 4).



(*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001)

Figure 4. Relative mRNA expression levels of NRF2 after 24h and 48h treatment with various cisplatin concentrations. At the higher doses of cisplatin, that are 5 μM and 10 μM, strong increases in gene expressions were observed in both treatment groups. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test relative to the control.

It has been shown that cisplatin induces mitochondrial-ROS production in cancer cells at 24h treatment[5]. Thus, this has been proposed as a mechanism behind cisplatin-induced cytotoxicity observed in non-cancerous cells during chemotherapy with cisplatin[5]. Additionally, elevated ROS levels trigger the upregulation of *NRF2* gene, through NF-κB pathway[30]. Here, in our results, higher levels of *NRF2* expression are correlated with higher doses of cisplatin and supporting cisplatin-induced oxidative stress and cisplatin-induced cytotoxicity. In the 24h treatment group, there were tendencies for increased *NRF2* expression at lower cisplatin concentrations, but these changes were not statistically significant. At the lower doses of cisplatin in 48h treatment group, lower expression of *NRF2* is observed, although slightly higher expressions are observed at the same doses of 24h treatment group. This suggests that at these lower concentrations, cisplatin may not induce oxidative stress to the same extent and may instead promote cell survival and proliferation.

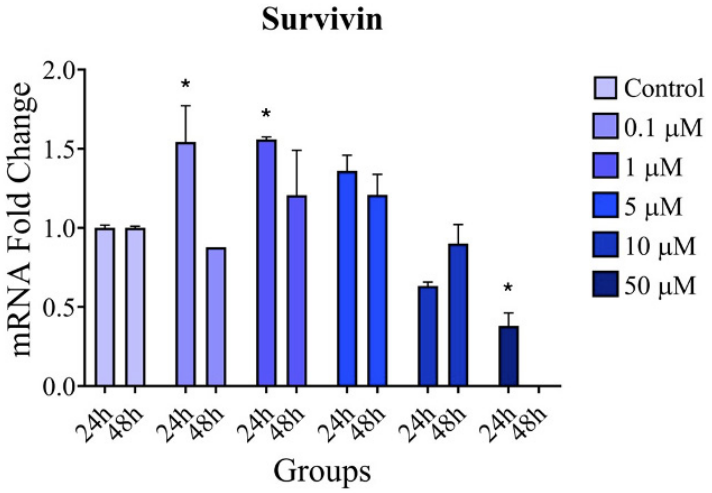
Moreover, observation of low ROS levels and oxidative stress adaptation of cancer cells in case of prolonged exposure to chemotherapeutic drugs, and subsequent occurrence of drug resistance have been suggested in the literature[31-34]. At 50 μM dose in 24h treatment of cisplatin, we surprisingly observed extreme and significant decrease in the ROS levels compared to 10 μM dose. Considering that a 24h exposure can be sufficient to induce drug resistance and that a 50 μM dose is relatively high, the result suggests that the observed low ROS levels at this concentration may indeed be indicative of cancer cells adapting to oxidative stress.

Cisplatin treatment at low doses increases *Survivin* expression in EMT6 cells

Survivin is a key member of the inhibitor of apoptosis protein (IAP) family, along with X-linked IAP (XIAP)[35,36]. These proteins play crucial roles in tumorigenesis, influencing various biological functions in cancer cells, and their expressions are found to be higher in some cancer types[35-37].

One of the significant functions of these proteins is their contribution to chemotherapeutic resistance by promoting cell proliferation, migration, and metastasis[35,36,38-41]. In the cancerous state, the interaction of XIAP and Survivin prevents XIAP from polyubiquitination and proteasomal degradation, and therefore inhibition of caspases and activation of NF-κB pathway occur[42]. These result in the occurrence of cancer cell metastasis and evasion from apoptosis[35].

In our study, we observed an increase in *Survivin* expression in almost all low doses of cisplatin in both 24h and 48h treatment groups (Figure 5). Statistically significant increases in *Survivin* expression were observed at concentrations of 0.1 μM and 1 μM in the 24h treatment group (Figure 5). Higher expression of *Survivin* correlates with increased cell viability observed in these concentrations shown in Figure 1(a), supporting the literature.



(*: p<0.05)

Figure 5. Relative mRNA expression levels of Survivin after 24h and 48h treatment with various cisplatin concentrations. A decreasing trend in gene expression levels was observed in a dose-dependent manner in both treatment groups. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test relative to the control.

Moreover, higher doses of cisplatin led to decreased *Survivin* expression after 24 h treatment, potentially inhibiting cell proliferation (Figure 5). Similar trends, although not statistically significant, were observed in the 48 h treatment group (Figure 5). We observed statistically significant decrease in *Survivin* expression at 50 μM in the 24 h treatment group. Silencing *XIAP* and *Survivin* expression using shRNA has been shown to significantly reduce cell proliferation, increase *caspase-3/7* levels, and enhance the response to chemotherapeutics, consistent with existing literature[36,43,44]. Moreover, it has been shown that partial reversion of epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial transition (MET) occurs when *XIAP* and *Survivin* expressions were inhibited, further confirming their active role in metastasis[36].

Based on these findings, we hypothesize that cisplatin may contribute to cell proliferation by upregulating *Survivin* expression at lower concentrations. These results suggest that *Survivin* plays a role in the cellular response to cisplatin, potentially influencing cell viability and proliferation in a dose-dependent manner.

In conclusion, varying doses and durations of cisplatin treatment result in differential expression of key genes regulating important molecular mechanisms in cancer cells, such as cell survival and apoptosis. Our findings indicate that

cisplatin induces apoptosis and alters gene expression levels in a dose-dependent manner. However, lower doses of cisplatin may not be sufficient to change the cancerous state of EMT6 cells, potentially supporting their survival. Looking forward, additional gene expression analyses could elucidate the molecular alterations induced by lower concentrations of cisplatin. To explore this further, low concentrations of cisplatin can be combined with nanoparticles for enhanced delivery to the cell and effects can be investigated in terms of cell survival and gene expression. Such insights could be pivotal in devising more effective treatment strategies with reduced side effects for breast cancer.

STATEMENTS OF ETHICS

No ethical approvals are required for this study.

CONFLICT OF INTEREST STATEMENT

The authors claim no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.O. and A.K. designed and performed the experiments, analyzed and interpreted the data. T.O. wrote the draft; T.O., A.K., and N.A. revised the manuscript.

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