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ORIGINAL ARTICLE

Targeting superoxide dismutase 1 to overcome cisplatin resistance in human ovarian cancer

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Abstract
Purpose Clinical drug resistance to platinum-based chemotherapy is considered a major impediment in the treatment of human ovarian cancer. Multiple pathways associated with drug resistance have been suggested by many previous studies. Over expression of several key proteins involved in DNA repair, drug transport, redox regulation, and apoptosis has been recently reported by our group using a global quantitative proteomic profiling approach. Superoxide dismutase 1 (SOD1) is one of these proteins consistently over-expressed in cisplatin-resistant ovarian cancer cells as compared to their sensitive counterparts, but its precise role in drug resistance is yet to be defined.
Method In the current study, we examined the role of SOD1 in drug resistance by inhibiting its redox activity in cisplatin-resistant ovarian cancer cells using a small-molecule inhibitor, triethylenetetramine (TETA). The effect of TETA was determined by the cell proliferation assay, clonogenic cell survival assay, and SOD1 activity assay.
Results The inhibition of the SOD1 activity enhanced the cisplatin sensitivity in the resistant cells supporting the hypothesis that SOD1 is a key determinant of cisplatin resistance and is an exploitable target to overcome cisplatin drug resistance.
Conclusion SOD1 plays an important role in cisplatin resistance and modulation of its activity may overcome this resistance and ultimately lead to improved clinical outcomes.

Keywords Ovarian cancer · Cisplatin resistance · Superoxide dismutase 1 · Reactive oxygen species · Mass spectrometry

Abbreviations

ACN	Acetonitrile
CDDP	Cisplatin or *cis*-diaminedichloroplatinum
MS	Mass spectrometry
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1
SRM	Selected-reaction-monitoring
TETA	Triethylenetetramine

Introduction

Today ovarian cancer is the fourth leading cause of death from cancer in women [1]. New and improved therapeutic treatment has been a target of much research [1, 2]. Cisplatin is one of the most effective and commonly used chemotherapeutic agents in the treatment of ovarian cancer [3]. It is believed that cisplatin-induced cell death is a result of

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the formation of cisplatin-DNA adducts that inhibit DNA replication and transcription [4]. However, a significant number of ovarian cancer patients fail chemotherapy due to acquired drug resistance that occurs in most patients over time [5]. Drug resistance has become a major impediment in cancer treatment today, and there is a dire need for the finding of novel approaches to overcome it [6].

The acquisition of cisplatin resistance is believed to be multifactorial in nature [2]. Cisplatin resistance has been attributed to decreased cellular drug accumulation, increased intracellular thiols such as glutathione and thioredoxin [4, 7, 8], altered expression of regulatory genes [7, 8], and increased DNA repair activities [2]. However, the precise mechanism of cisplatin resistance remains poorly understood. It has been suggested that cisplatin's cytotoxic effect is closely associated with increased generation of reactive oxygen species (ROS) [4, 9]. The ROS are small molecules that are formed as natural byproducts of normal metabolism of oxygen, and play an important role in cell signaling [10]. However, because of their highly reactive character, they tend to become involved in unwanted reactions that cause damage to cells that may ultimately lead to diseases. Several lines of evidence have implicated that cisplatin-induced oxidative stress is indeed associated with increased ROS levels and DNA damage, leading to cell death [3, 11]. ROS generation following exposure to drugs and radiation has long been associated with nephrotoxicity, tumorigenesis, and tissue injury in the preventative medicine field [11–13]. Together these results suggest that there is a close relationship between ROS and cisplatin-induced cytotoxicity.

SOD1 is a 32-kDa cytosolic metalloenzyme that requires cofactors Cu(II)/Zn(II) to be active. It functions to prevent unwanted oxidative damage to the cell by converting highly reactive superoxide to less reactive hydrogen peroxide, which can be later reduced by catalase or glutathione peroxidase [14, 15]. SOD1 is an abundant enzyme in liver, kidney, adrenal, and red blood cells [15]. Mutations and altered expression of SOD1 have been implicated to be primarily involved in familial amyotrophic lateral sclerosis (ALS) [16]. Investigators have also shown that SOD1 deficient yeast and drosophila were found to have a reduced life span, infertility, and hypersensitivity to oxidative stress [17–19]. Blander et al. found that knockdown of SOD1 using RNAi induced senescence in normal cells and cell death in cancerous cells [10], while resistance to oxidative stress was accompanied by over-expression of SOD1 [20, 21]. More recently, built-in cellular defenses have been found to play important roles in the protection of tumor cells against cancer chemotherapy and the studies carried out along these lines suggest that SOD1 may be involved in conferring resistance to cisplatin [8, 22].

In this study, we investigated the hypothesis that SOD1 is a key determinant involved in cisplatin resistance and its up-regulation resulted in the inhibition of cisplatin-induced apoptosis based on our previous findings [23]. Our results suggest that inhibition of the SOD1 activity can lead to sensitization of cisplatin-resistant human ovarian cancer cells.

Materials and methods

Reagents

cis-Diammineplatinum (II) dichloride (CDDP or cisplatin), triethylenetetramine dihydrochloride (TETA), DMSO, iodoacetamide, iodoethanol, ACN, dithiothreitol (DTT), EGTA, mannitol, sucrose, and crystal violet were all purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Modified trypsin was purchased from Promega (Madison, WI, USA). RPMI-1640 media was purchased from Cambrex Bio Science (Walkersville, MD, USA).

Cell lines and cell culture

Two pairs of cell lines were used in this study: A2780 and its cisplatin-resistant counterpart A2780/CP, and 2008 and its cisplatin-resistant counterpart 2008/C13*5.25. Resistant sublines were developed from each sensitive cell line by repeated exposure to increasing amounts of cisplatin over time [19–21, 23–26]. The cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2 in RPMI1640 media supplemented with 15% FBS and 1% penicillin-streptomycin as previously described [23].

Relative SOD1 expression in sensitive and resistant cell lines

Relative expression of SOD1 in cisplatin-sensitive and cisplatin-resistant cell lines was specifically monitored using a mass spectrometry-based method called Selected-Reaction-Monitoring (SRM). Briefly, cell lysates were prepared and digested as previously described [27, 28]. Tryptic peptides were analyzed by SRM using a linear ion-trap mass spectrometer (LTQ, Thermo Electron, San Jose, CA, USA) interfaced with an HPLC system containing a binary pump and thermostated autosampler. The tryptic peptides (20 μg) were separated using a C18 microbore column (Zorbax 300SB-C18, 1 mm × 5 cm) at a flow rate of 50 μL/min. Peptides were eluted with a 5–45% ACN gradient developed over 142 min. A unique SOD1 (SwissProt annotation No. P00441) tryptic peptide: ^{11}GNGPVQGIINFEQK^{24} was monitored using three SRM transitions: *m/z* 751.8 → 778.4, *m/z* 751.8 → 948.4, and *m/z* 751.8 → 1,077.4, respectively. Relative SOD1 expression levels were calculated

using the area-under-the-curve (AUC) of each SRM transition after normalization from an internal standard (40S ribosomal protein S12) and a spiked external standard (chicken lysozyme).

TETA inhibition assays

Drug cytotoxicity was determined using the Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche Diagnostics, Indianapolis, IN, USA) [23]. Briefly, the cells were harvested, counted, and seeded at ~2,000–4,000 cells per well in a 96-well plate with 100 μL of cell suspension solution. The plates were placed in an incubator at 37°C, 5% CO_2, and cells were allowed to attach overnight. The cells were then treated with various concentrations of cisplatin (0–30 μM) and TETA (0–50 mM). Plates were incubated for additional 48 h after treatment. Cell proliferation was quantified based on BrdU incorporation during DNA synthesis in proliferating cells at an absorbance of 370 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instructions. IC_{50} values were determined from dose-response curves using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA) as described previously [23].

SOD1 activity assay

SOD1 activity was determined using the Superoxide Dismutase Assay Kit II (Calbiochem, San Diego, CA, USA). Briefly, cells were harvested and washed with PBS, collected by centrifugation at 1,000×*g* for 10 min at 4°C. The cell pellet was resuspended in cold HEPES buffer (20 mM, pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, and sonicated (10 s intervals). The lysed cells were centrifuged at 1,500×*g* for 5 min at 4°C. The Bradford assay was performed to determine protein concentration of the whole cell lysate (WCL). Varying amounts of purified SOD1 enzyme were placed in a 96-well plate and monitored in parallel with the WCL as standards of SOD1's ability to scavenge superoxide radicals. SOD1 activity was then determined in the presence of various concentrations of TETA (0, 0.5, 5, 20, and 50 mM, respectively) by tetrazolium salt detection of superoxide radicals generated by xanthine oxidase and hypoxanthine, according to manufacturer's instructions. Using an ELISA plate reader, the reaction was recorded at an absorbance of 450 nm, in which fluorescence increase was indicative of decreased SOD1 activity.

Clonogenic cell survival assay

To determine the capacity for cell survival and whether it is consistent with cell proliferation after drug treatment, clonogenic cell survival assays were also performed. Cisplatin-resistant ovarian cancer cells were cultured in an incubator at 37°C, 5% CO_2 with or without drug treatment for 48 h. There were four groups for each cisplatin-resistant cell line (A2780/CP and 2008C13*5.25, respectively): (1) control—no drug treatment; (2) with 15 μM cisplatin only, (3) with 10 mM TETA only, and (4) with a mixture of 15 μM cisplatin, and 10 mM TETA. After 48 h, a varying range of 200–1,000 cells from each treatment group was seeded onto T25 mm tissue culture flasks containing culture medium for colony formation. Ten days later, the cells were stained with crystal violet and colonies were counted. Colonies with less than 50 cells were discarded. The surviving fraction was determined as the ratio of the number of colonies in the drug treated samples to that of the non-treated control samples. Six flasks were set up for each condition.

Results

SOD1 is consistently over-expressed in cisplatin-resistant ovarian cancer cells

In our previous global quantitative proteomic study [23], SOD1 was found to be over-expressed in cisplatin-resistant ovarian cancer cell lines as compared to its sensitive counterparts. To validate the observed differential SOD1 expression, we chose to use a targeted MS-based analytical platform to selectively quantify SOD1 expression levels in both cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. A unique peptide of SOD1, ^{11}GNGPVQGIINFEQK^{24}, was selectively monitored using very sensitive and specific SRM scans, which consist of the *m/z* of a specific precursor ion (*m/z* 751.8, $M + 2H^+$) and three product ions (*m/z* 778.4, *m/z* 948.4, and *m/z* 1,077.4, respectively). The relative expression levels were quantified using the AUC of the SRM transitions from the extracted ion chromatogram (XIC). As shown in Table 1 and Fig. 1, ~1.6-fold increase in expression of SOD1 in the cisplatin-resistant ovarian cancer cell line was observed by SRM, consistent with what were observed by the LC/MS-based global quantitative proteomic approach [23]. In this SRM assay, we used three SRM transitions to determine the relative expression levels of SOD1, and all three SRM transitions showed a similar degree of fold change, consistent with our previous finding by a global LC/MS-based method [23].

TETA sensitizes cisplatin-resistant ovarian cancer cells

To investigate that the over-expression of SOD1 is closely associated with cisplatin resistance in ovarian cancer, cell

Table 1 SOD1 expression changes (Fold-Change ± standard deviation) in cisplatin-resistant human ovarian cancer cells (A2780-CP and 2008-C13*5.25) as compared to its sensitive counterpart (A2780 and 2008), respectively, as determined by either label-free protein quantification technology (Global) or SRM

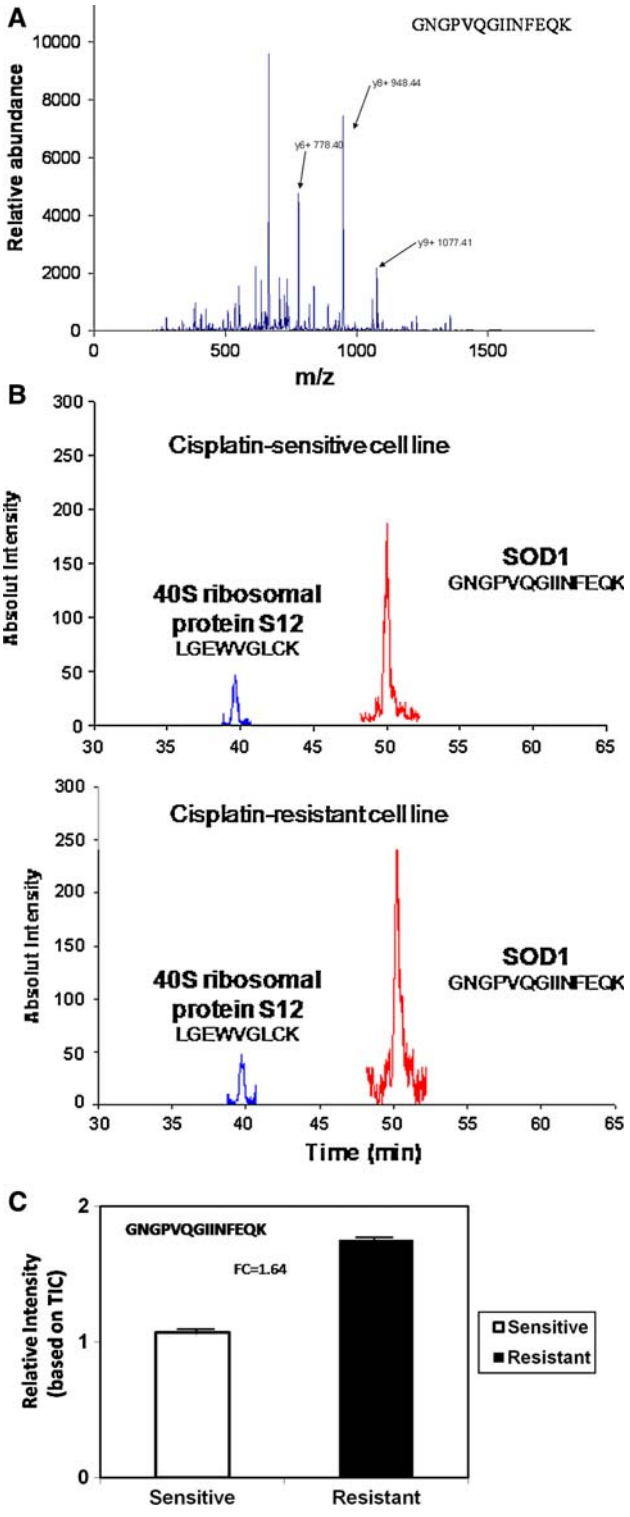
Cell line	Fold-change (Global)	Fold-change (SRM)
A2780-CP/A2780	1.38 ± 0.15	ND
2008-C13*5.25/2008	1.53 ± 0.46	1.64 ± 0.15

ND not determined

proliferation assays in the presence of cisplatin and an SOD1 inhibitor, TETA, were performed. Dose-response study by incubating the sensitive (A2780 and 2008, respectively) and resistant (A2780-CP and 2008-C13*5.25, respectively) cell lines with increasing concentrations of cisplatin and TETA showed that the IC_{50} values for the resistant cell lines A2780-CP and 2008-C13*5.25 were decreased ~50% from 15.7 and 11.2 μM to 7.3 and 6.5 μM, respectively, when cells were treated with both drugs (Table 2), suggesting a two-fold increase in cisplatin sensitivity. To confirm the effect of the combination treatment is not additive, both the sensitive and resistant cells were treated with cisplatin and TETA separately. As shown in Table 2, the sensitive cell lines were between ~12-fold and ~35-fold, respectively, more sensitive to cisplatin than their resistant counterparts, while the IC_{50} values for TETA are ~25 mM for both resistant cell lines. At ~10 mM, which is the concentration used for combination treatment, TETA had almost no cytotoxic effect to either cell lines (data not shown). These results suggest that combination treatment with both cisplatin and TETA can markedly increase the cisplatin sensitivity in these two cisplatin-resistant cell lines we tested.

Cisplatin sensitivity is associated with SOD1 activity

To confirm the effect of TETA is specifically due to the inhibition of the SOD1 activity, we examined the SOD1 activities under the same conditions the cell proliferation assays were performed using the Calbiochem Superoxide Dismutase Assay Kit II [29]. This SOD1 activity assay uses tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine [29]. Because SOD1 is a scavenger of superoxide radicals, thus with the higher SOD1 activity, the less superoxide radicals will be detected. Figure 2 shows that the decreased activity of SOD1 is indeed consistent with the increased TETA concentration, suggesting that the observed sensitization of the cisplatin-resistant ovarian cancer cells is primarily due to the inhibition of the SOD1 activity by TETA, not the TETA cytotoxic effect to the cells.



Clonogenic cell survival assays are consistent with cell proliferation assays

To confirm the Cell Proliferation ELISA Assay results and that the combination of cisplatin and TETA treatment leads

◀ **Fig. 1** Relative protein expression levels of SOD1 as characterized by selected-reaction-monitoring (SRM). **a** MS/MS spectrum of the unique SOD1 peptide ($M + 2H^+$) showing three selected SRM transitions: *m/z* 751.8 → 778.4, *m/z* 751.8 → 948.4, and *m/z* 751.8 → 1,077.4. **b** normalized internal standard peptide for quantification. "LGEWVGLCK" represents a unique peptide from an expression level unchanged protein, 40S ribosomal protein S12, in sensitive and resistant cells, while the unique peptide "GNGPVQGIINFEQK" represents SOD1. **c** a bar-graph showing the relative expression levels of a SOD1 peptide between cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. All the experiments were carried out in duplicate with the whole cell lysates

to cell death rather than cell cycle delay, we performed clonogenic cell survival assays with a similar condition to that of cell proliferation assays. We observed that 10 mM TETA alone has no cytotoxic effect on these resistant cells, while 15 μM cisplatin reduced cell survival to ~75 and ~50%, respectively, in these resistant cells. However, a combination of cisplatin (15 μM) and TETA (10 mM) significantly increased cell killing and reduced survival to 45 and 30%, respectively (Fig. 3), which is a significant improvement (30–40%) of the cisplatin sensitivity in these cisplatin-resistant ovarian cancer cells ($p < 0.01$). Statistical analysis was performed by Student's *t* test for paired comparison (cisplatin alone versus combination of cisplatin and TETA). Probability values of <0.05 were considered statistically significant.

Discussion

Resistance to anticancer agents has been a pressing problem in the success of ovarian cancer treatment [22]. Though platinum-based drugs remain as one of the leading chemotherapeutic drugs for cancer treatment, the acquired drug resistance has become an obstacle in cancer patient care [30, 31]. The precise mechanism of cisplatin drug resistance remains unclear. Many previous studies have suggested drug transport and DNA repair as the major potential mechanisms for acquired drug resistance [2], even though a line of evidence has suggested the involvement of the redox pathway [9, 11, 32–34]. Up-regulation of antioxidants has been associated with stress resistance, resulting in an increase in lifespan of some organisms [17].

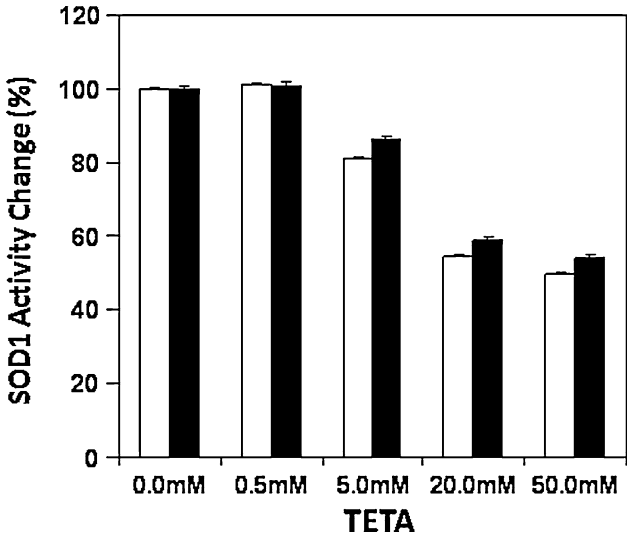


Fig. 2 SOD1 activity assay in cisplatin-resistant ovarian cancer cell lines. These assays were performed under the same experimental conditions as that for the cell proliferation assays, suggesting a correlation between SOD1 activity and drug resistance. *Open bars* represent A2780-CP cisplatin-resistant cell line, *solid bars* represent 2008-C13*5.25 cisplatin-resistant cell line

Therefore, to overcome drug resistance in cancer cells, it is critically important to identify the factor(s) and the mechanism(s) involved. Previous studies conducted by our lab showed increased levels of protein expression in many proteins including two well-known antioxidant proteins SOD1 and thioredoxin in cisplatin-resistant cell lines [23]. These proteins were proposed to be major contributors in cisplatin resistance due to their abilities to neutralize or decrease the cellular ROS levels resulting from cisplatin treatment [3, 7–11]. The role of thioredoxin in cisplatin resistance in ovarian cancer has been previously suggested [35]. Inhibition of thioredoxin with various drugs decreased oxidative stress and increased cisplatin-induced apoptosis [3, 33]. However, the contribution of SOD1 in cisplatin resistance in ovarian cancer cells has not been demonstrated previously. We first reported the SOD1 up-regulation in cisplatin-resistant cells using a global quantitative proteomic approach [23]. To verify our results from the global proteomic study, that SOD1 is over-expressed in the cisplatin-resistant ovarian

Table 2 Dose-dependent BrdU Cell Proliferation Assay results displaying IC_{50} values of drug cytotoxicity in ovarian cancer cell lines used in this study

Drug	IC_{50} (A2780)	IC_{50} (A2780-CP)	IC_{50} (2008)	IC_{50} (2008-C13*5.25)
CDDP	1.3 μM	15.70 ± 0.15 μM[a]	0.32 μM	11.20 ± 0.10 μM[a]
Combination[b]	–[c]	7.30 ± 0.15 μM[a]	–[c]	6.50 ± 0.04 μM[a]

Fold-change (FC ± standard deviation) in cisplatin sensitivity represents the degree of resistance in different cell lines

[a] IC_{50} values of cisplatin cytotoxicity

[b] 15 μM cisplatin and 10 mM TETA

[c] Drug effects were too toxic for IC_{50} calculation for these sensitive cell lines

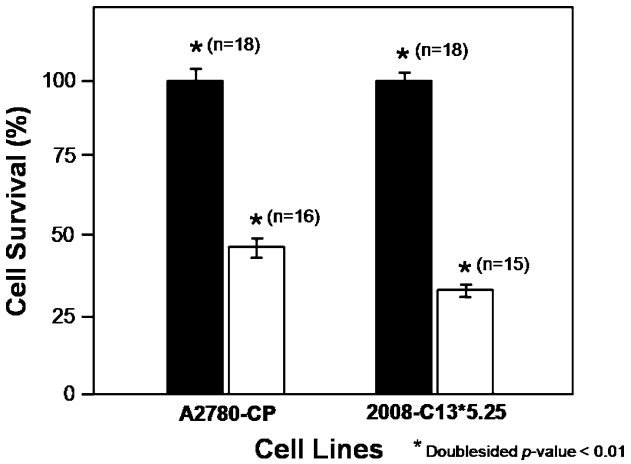


Fig. 3 Clonogenic cell survival assay. Both cisplatin-resistant cell lines A2780-CP and 2008-C13*5.25 were used for the study. The solid bars represent the normalized (to 100%) cell survival rate when they were treated with 15 μM cisplatin alone while the *open bars* indicate the cell survival rate when they were treated with a combination of 15 μM cisplatin and 10 mM TETA. Each bar represents the mean ± sd. The number of replicates is indicated by "n" and the $p < 0.01$ indicates the changes were statistically significant when their cell survival rates (cisplatin alone versus the combination of cisplatin and TETA) were compared

cancer cells, a targeted proteomic platform known as SRM was used to selectively monitor the expression levels of SOD1 in the same cell lines. The SRM results confirmed the SOD1 up-regulation in the cisplatin-resistant ovarian cancer cells when compared to their sensitive counterparts (Fig. 1). Hirose et al. [36] showed that increased SOD1 levels promoted tumor cell survival against cancer fighting methods such as drug therapy and radiation. Our findings are consistent with previously documented studies, showing the elevated level of SOD1 in cisplatin resistant cells.

Our hypothesis in the current study was that over-expression of antioxidant protein superoxide dismutase SOD1 is a key factor in acquired cisplatin resistance in ovarian cancer and modulation of its activity can lead to sensitization of the cisplatin-resistant cells. While RNAi may be a more target-specific approach, we propose that the use of a small-molecule inhibitor may be a more therapeutically achievable approach. It is known that SOD1 requires copper or zinc for its activity and loss of these ions results in its complete inactivation [37]. The use of copper binding compound tetrathiomolybdate as an inhibitor of SOD1 in other studies was found to attenuate angiogenesis and tumor cell proliferation in vitro [34, 38]. More recently, triethylenetetramine (TETA) has been used as a copper chelator to inhibit SOD1 activity [39]. Although these metal chelators may not be SOD1 specific, they can provide information about whether these inhibitors have any effect on cisplatin sensitivity when they are presented in the SOD1 over-expression cell lines. In this study, we chose TETA as a small-molecule inhibitor of SOD1 activity. We first titrated TETA to determine its cytotoxic concentration and then chose a non-toxic concentration (10 mM) to demonstrate its ability to reduce SOD1 activity through the measurement of ROS levels (Fig. 2). We then went on to show in two cisplatin-resistant ovarian cancer cell lines that inhibition of SOD activity by TETA significantly increased cisplatin sensitivity by both a decrease in IC_{50} and an increase in cell killing by clonogenic assays in Fig. 3 and Table 2. Taken together, our data suggest that SOD1 exhibits a protective function against cisplatin-induced cell death, and through modulation of its activity, we can at least, partially sensitize resistant ovarian cancer cells to cisplatin-induced cell killing.

The mechanism of cisplatin resistance is believed to be multifactorial in nature [2]. Statistics shows that although ~80% ovarian cancer patients are initially very responsive to platinum-based drug treatment, ~75% of these patients eventually develop drug resistance within 2 years of initial treatment [40]. Research has shown that this acquired cisplatin resistance stems from protein changes that may occur during various cancerous states [30, 40–42]. Combination therapies have been suggested to be a more effective way to treat cancer, where multiple targets may be manipulated in order to maintain a toxic effect. Our study demonstrates that the combination treatment with cisplatin and copper chelating agent TETA resulted in an increase of the efficacy of cisplatin in drug-resistant ovarian cancer cells and partially restored cisplatin cytotoxicity.

Conclusion

Currently, there is no clear cut consensus on how to treat ovarian cancer patients who acquired platinum-drug resistance, due to the lack of knowledge by which drug resistance occurs. As a continuation of the proteomic study aimed to understand the mechanism of cisplatin resistance, the current study not only significantly extended our understanding of the mechanism of drug resistance, but also suggested a new approach to potentially sensitize drug resistant cancer cells, which may ultimately improve treatment outcomes for ovarian cancer patients.

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