Comparative Evaluation of Small-molecule Chemosensitizers in Reversal of Cisplatin Resistance in Ovarian Cancer Cells

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Abstract. *Cisplatin-resistance is one of the major challenges in the treatment of epithelial ovarian cancer. Small-molecule chemosensitizers provide a therapeutically feasible approach to overcome cisplatin resistance in ovarian cancer. However, proper selection of chemosensitizer is of prime importance owing to phenotypic differences in cisplatin-resistant ovarian cancers. The resistance reversal activity of chemosensitizers buthionine sulfoximine (BSO), triethylenetetramine (TETA), genistein, rapamycin and colchicine was investigated in various cisplatin-resistant ovarian cancer cells, 2008 C13, CP70 and OVCAR 8 using MTT assays. Cellular accumulation of cisplatin in the presence of chemosensitizers was analyzed by inductively-coupled plasma-mass spectroscopy (ICP-MS). Chemosensitizers exhibited resistance reversal activity in 2008 C13 and CP70 cells in the following order; colchicine> genistein>TETA> rapamycin≥BSO ($p<0.05$), which is in correlation with cellular accumulation of cisplatin. In conclusion, our study demonstrates that resistance reversal activity of chemosensitizers varies with phenotypic behavior of cisplatin-resistant ovarian cancer cells. Data from our study can be utilized to choose a specific chemosensitizer for individualized combination therapy for cisplatin-resistant ovarian cancer.*

Cisplatin was inluded into the chemotherapeutic arsenal against ovarian cancer 30 years ago and is currently widely used as a first-line therapy in epithelial ovarian cancer. Cisplatin induces cell death by forming cisplatin-DNA adducts that subsequently inhibit DNA replication and transcription (10). Despite of the fact that approximately 80% of ovarian cancer patients are highly responsive to initial cisplatin treatment, drug resistance develops in almost 75% of these patients within 2 years of initial treatment (19). The mechanism of acquired cisplatin resistance is believed to be multifactorial in nature and has been attributed to reduced cellular drug accumulation, detoxification of cisplatin by intracellular thiols such as glutathione, aberrant expression of regulatory genes and enhanced DNA repair activities (2). Even though mechanisms of cisplatin resistance have been studied during the past several decades in great detail, there is no effective pharmacological manipulation to overcome this form of complex resistance. Use of small-molecule chemosensitizing agents provides a therapeutically achievable approach to sensitize tumor cells to cytotoxic drugs (14). These agents generally overcome cisplatin resistance by modifying resistance mechanisms involving glutathione (12), generation of reactive oxygen species (7), the ubiquitin-proteasome pathway (9), overexpression of elongation factor alpha (23) and inhibition of platinum efflux transporters (17). It is of prime importance in ovarian cancer research to identify selective, potent, safe and effective chemosensitizing agents for optimal reversal of resistance to cisplatin.

Several key factors in cisplatin cytotoxicity and resistance were identified and small-molecule chemosensitizers were designed and investigated. For example, the cytotoxic effect of cisplatin was reported to be associated with an increased generation of reactive oxygen species (ROS). However, superoxide dismutase 1 (SOD1), a 32-kDa cytosolic metalloenzyme, converts ROS to less reactive hydrogen peroxide, inhibiting the cytotoxic activity of cisplatin. Moreover, up-regulation of SOD1 is a key factor in acquired cisplatin resistance in ovarian cancer (3). Thus, inhibition of SOD1 activity by a small-molecule inhibitor such as triethylenetetramine (TETA), could potentially lead to sensitization of cisplatin-resistant ovarian cancer cells (22). Glutathione (GSH), a predominant intracellular thiol, is implicated in cisplatin resistance as it inhibits the conversion of cisplatin-DNA monoadducts into cytotoxic crosslinks and exports adducts through ATP-dependent pumps (16). Synthesis of GSH involves enzyme [γ]-glutamylcysteine synthetase ([γ]-GCS), and inhibition of [γ]-GCS results in a reduction of GSH levels. Buthionine sulphoximine (BSO), a specific inhibitor of

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Key Words: Cisplatin, chemosensitizers, rapamycin, glutathione, superoxide dismutase, ovarian cancer cells drug resistance.

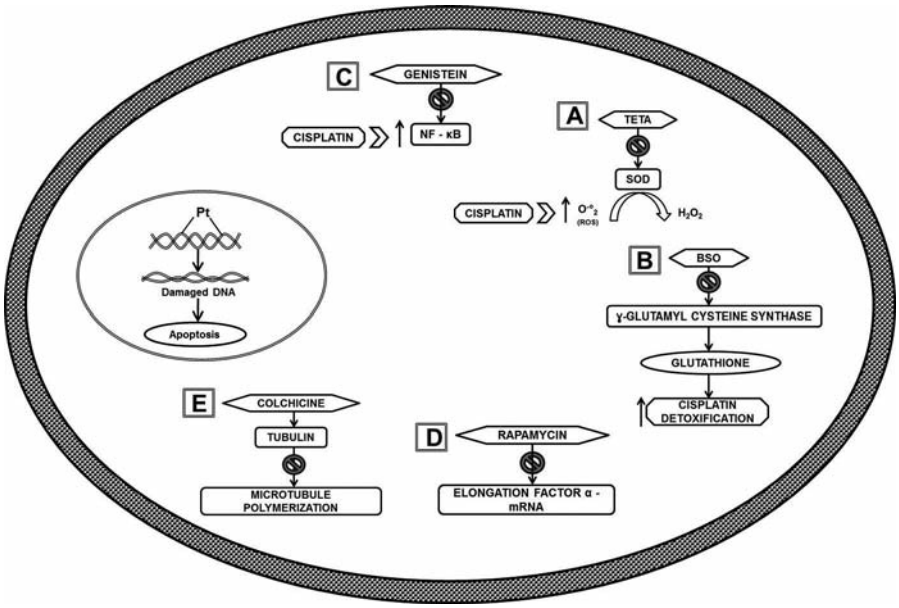

Figure 1. *Schematic depiction of the mechanism of chemosensitizers in reversal of platinum resistance. A: Triethylenetetramine (TETA). B: Buthionine sulfoximine (BSO). C: Genistein. D: Rapamycin. E: Colchicine.*

γ-GCS, was shown to reduce intracellular levels of GSH and can be used to overcome resistance in cisplatin-resistant ovarian cancer cells (8). Activation of the nuclear factor-kappa B (NF-κB) survival pathway was shown to prevent apoptosis by cisplatin in ovarian tumors leading to chemoresistance of cisplatin (6). Genistein, a natural soy isoflavone, has been shown to down-regulate NF-κB activity and sensitize chemoresistant ovarian tumor cells to cisplatin (18). Many cisplatin-resistant cell lines often overexpress the elongation factor alpha, a key gene involved in DNA repair after insult due to cisplatin. Elongation factor alpha, along with ribosomal proteins, are crucial for the translational process of repair/survival proteins and therefore inhibiting them would be a good rationale for restoring cisplatin sensitivity. Rapamycin, a well-known mTOR (mammalian target of rapamycin) inhibitor, was reported to restore cisplatin sensitivity by selectively inhibiting translation of mRNA encoding elongation factor alpha (21, 23). Furthermore, colchicine a plant-based microtubule destabilizer was shown to exhibit a concentration-dependent synergism in cytotoxic activity in cisplatin-resistant cells when administered in combination with cisplatin (24). All of the above mentioned small-molecule chemosensitizers have potential clinical benefit in the reversal of cisplatin resistance in ovarian tumors, when used in combination therapy with cisplatin. A summary of mechanisms of action of the above mentioned chemosensitizers is provided in Figure 1. Nonetheless, the phenotypes associated with cisplatin resistance in ovarian cancer cells largely influence the efficacy of chemosensitizing agents.

In ovarian cancer, techniques such as comparative genomic hybridization (CGH), suppression subtractive hybridization, microarray expression profiling, and label-free LC/MS based proteomics, were used to identify phenotypes associated with acquisition of cisplatin resistance (1, 7). Phenotypes, such as cells with loss of wild-type *p53* activity which results in inhibition of apoptosis, down-regulation of *MLH1* which causes loss of mismatch repair and eventually loss of apoptosis, and overexpression of oncogenic transcription factor c-JUN, which alters the threshold for apoptosis, were implicated in various cisplatin-resistant ovarian cancer cells (4, 5, 15). However, every cancer-resistant cell line has some uniqueness in its magnitude of expression of a particular phenotype. Besides, this variation in phenotypes also influences the response of cisplatin-resistant cell lines to chemosensitizers and their cisplatin-resistance reversal activity. Thus, the present study is an attempt to compare the chemosensitizing efficacy of five small-molecule chemosensitizing agents in cisplatin-sensitive ovarian cancer cell lines 2008, and cisplatin-resistant ovarian cancer cell line expressing p53 mutations (OVCAR-8), cell line expressing wild-type p53 and defective in hMSH2 (2008 C13), cell line expressing p53 mutations and defective in hMLH1 (CP70) (7, 13). It is anticipated that the results from this study may be utilized for choosing the appropriate chemosensitizer based on the type and magnitude of the phenotype exhibited by a cisplatin-resistant ovarian cancer cell. Furthermore, successful therapeutic application of these chemosensitizers is largely dependent on their toxicity in

normal ovary cells. Thus, the toxicity of these agents in CHO (Chinese Hamster Ovary) cells was also determined. Finally, in order to provide insight into the cisplatin transport in the presence of the chemosensitizer, cellular uptake of cisplatin after chemosensitization with the above mentioned agents was performed using inductively-coupled plasma–mass spectroscopy (ICP-MS).

Materials and Methods

Reagents. Cis-Diamineplatinum (II) dichloride (cisplatin), L-buthionine-sulfoximine (BSO), insulin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and colchicine were purchased from Sigma-Aldrich (St Louis, MO, USA). Triethylenetetramine tetrahydrochloride (TETA) was purchased from MP Biomedicals (Solon, OH, USA). Genistein was purchased from LC laboratories (Woburn, MA, USA). Rapamycin was obtained from Calbiochem® EMD biosciences (Bilerica, MA, USA). Omni Trace® nitric acid was purchased from EMD biosciences (Gibbstown, New Jersey, USA). The Plasma Cal tune A, tune B, platinum standard (1000 μg/mL) and iridium standard (1000 μg/mL) were purchased from SCP science (Baie D'Urfè, Quebec, Canada). RPMI-1640, F-12 K media, fetal bovine serum, trypsin-EDTA, penicillin-streptomycin solution were purchased from Cellgro®, Mediatech Inc, Manassas, VA, USA).

Cell lines and cell culture. Human ovarian carcinoma cell line 2008 (passage # 15-30) and its resistant variant 2008 C13 (passage # 16-30) were kindly provided by Dr. Qingxiu Zhang, Department of Molecular Therapeutics, University of Texas M.D Anderson Cancer Center, Houston, Texas. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL). Cisplatin-resistant CP70 (passage # 40-60) ovarian cancer cells were kindly provided by Dr. Steve W. Johnson, Fox Chase Cancer Center, Philadelphia, PA, USA. CP70 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/mL)-streptomycin (100 μg/mL) solution and 0.25 units/ml of insulin. Cisplatin-resistant OVCAR-8 cells (passage # 25-42) were obtained from National Cancer Institute, Bethesda, MD and were grown in RPMI 1640, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Chinese hamster ovary (CHO) cell line (passage # 14-32) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in F-12 K medium supplemented with 10 % fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL) solution. All cells were maintained at 37˚C in a humidified incubator containing 5% CO_2.

Cytotoxicity assay. *In vitro* cytotoxicity of cisplatin in the presence of chemosensitizers was measured using the MTT assay. Briefly, 2,500 tumor cells were seeded in 96-well plates in 200 μL of medium per well. After 24 h, the medium was removed and chemosensitizers at various concentrations, TETA (0.1-5 mM), BSO (5-50 μM), genistein (5-50 μM), rapamycin (0.5-5 μM) and colchicine (0.1-10 μM) dissolved in respective media, were added to the cells and the volume of solution was kept 200 μL. After treating the cells with chemosensitizers for 24 h, the medium was removed and the cells were gently washed with PBS 7.4 and fresh media containing various concentrations of cisplatin (0-50 μM) were added to the cells. Then the cells were exposed to cisplatin continuously for five days at 37˚C, after which 50 μL of MTT (0.5 mg/mL diluted in respective media) was added to each well and incubated for 4 h. The formazan crystals formed were dissolved in 150 μL dimethyl sulfoxide after aspiration of the medium and absorbance was measured at 590 nm with 650 nm absorbance as the background, using a NOVOstar® microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). The IC_{50} values for each cisplatin concentration were calculated using nonlinear regression curve in GraphPad Prism 5.03 software. The reversal activity of chemosensitizers on cisplatin resistance is expressed as the fold reversal (FR) calculated according to the following equation:

$$\text{Fold Reversal (FR)} = \frac{IC_{50} \text{ of cisplatin alone}}{IC_{50} \text{ of cisplatin+chemosensitizer}}$$

Quantification of cisplatin by ICP-MS. ICP-MS system used was an X series ICP-MS (Thermo Electron Corporation, Madison, WI, USA) equipped with a Cetac 500 auto sampler (Cetac Technologies, Omaha, NB, USA). Samples were analyzed in X-series default mode using 2.Xi + screen. The results were analyzed using the Plasma Lab software (Thermo Electron Corporation). Major isotopes of platinum and iridium were monitored at *m/z* 195 and 193, respectively. Sample nebulization was performed using a concentric nebulizer and detection modes for both isotopes were 'scanning'. Quantification was based on the mean (n=3) intensity ratios for platinum and iridium against a calibration curve using linear regression analysis. All standards and samples were prepared in 1% OmniTrace® nitric acid. A standard platinum calibration curve was prepared with concentrations from 0.1 to 1000 ng/mL. 10 ng/mL of iridium were added to all standard solutions as the internal standard.

Cellular accumulation of platinum. The quantitative estimation of platinum content in ovarian cancer cells was performed using ICP-MS. 75×10^4 cells per well were seeded in 24-well plates. After 24 h, the cells were treated for one day with chemosensitizers, TETA (5 mM), BSO (50 μM), rapamycin (5 μM), genistein (50 μM) and colchicine (10 μM), dissolved in their respective media. Cisplatin at concentrations 0, 1 and 50 μM was added to the same media containing chemosensitizers after 24 h to allow co-incubation of cisplatin with the chemosensitizers. After 4 h of co-incubation of cisplatin with chemosensitizers, the media were removed and the cells were washed with ice cold PBS (pH 7.4) three times. Cells were then detached by trypsinization and the pellet was resuspended in 200 μL of lysis buffer. The obtained pellet was digested using 70% HNO_3 and resuspended in 5 mL of 1% HNO_3 containing iridium as the internal standard. The amount of platinum was then estimated using ICP-MS, as described in the previous section and the data obtained were the average of triplicate samples for each concentration of cisplatin.

Statistical analysis. The significance of differences in the mean values between the two groups was analyzed using an unpaired two-tailed Student's *t*-test by Graph Pad Prism 5.03; *p*-values <0.05 were considered statistically significant.

Results

Effect of chemosensitizers on IC_{50} of cisplatin in various cisplatin-resistant ovarian cancer cells. The influence of chemosensitizers TETA, BSO, genistein, rapamycin and

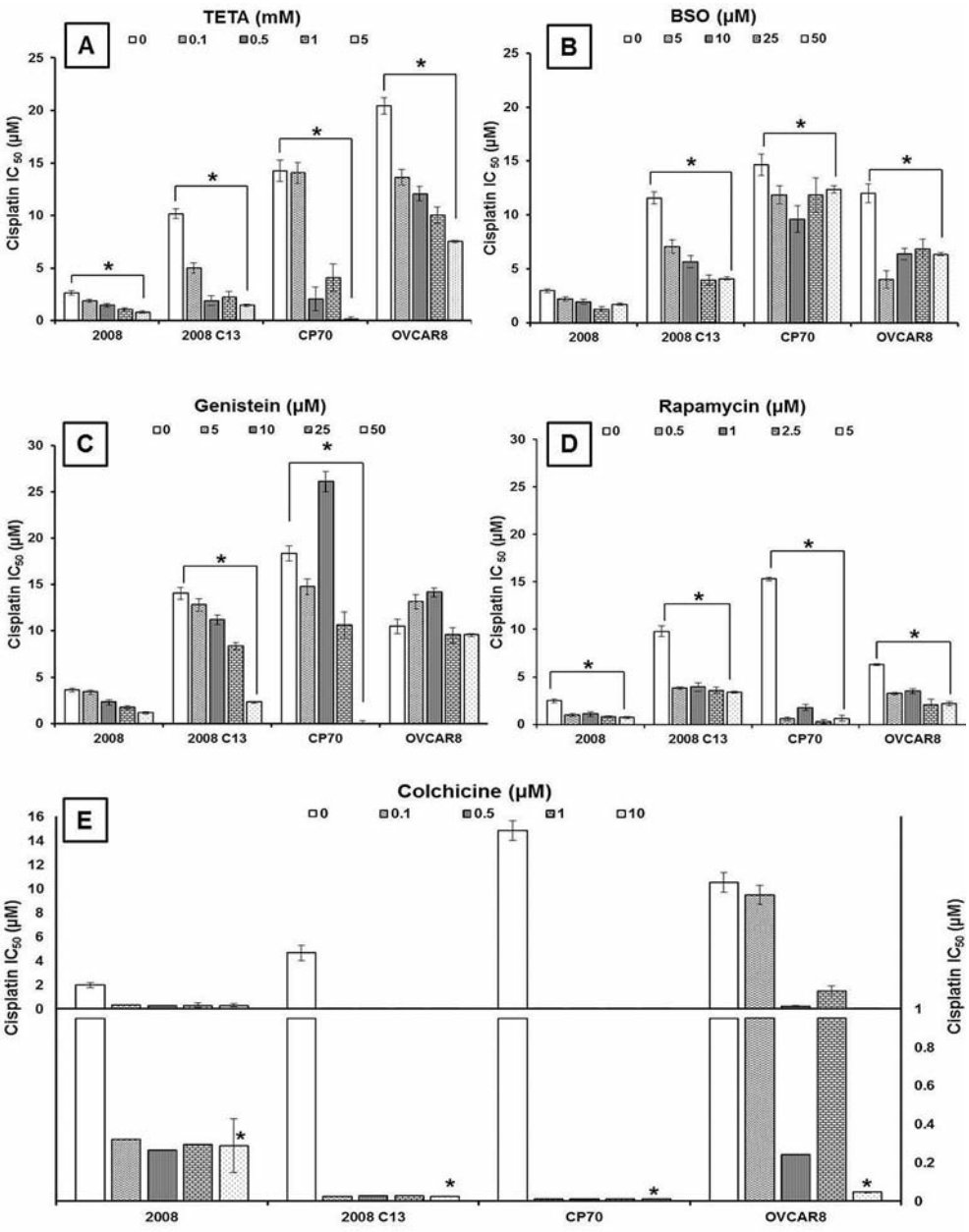

Figure 2. *Effect of chemosensitizers on the antitumor activity of cisplatin in various ovarian cancer cells. Cells were exposed to chemosensitizers at different concentrations for 24 h. Cells were then washed and treated with cisplatin (0-50 μM) for 5 days and the MTT reagent was added to evaluate the % viability. The IC_{50} values of cisplatin were calculated by non-linear regression using the Graph Pad Prism, 5.03 software. A. IC_{50} values of cisplatin after treatment with TETA (0-5 mM), B. IC_{50} values of cisplatin after treatment with BSO (0-50 μM), C. IC_{50} values of cisplatin after treatment with Genistein (0-50 μM), D. IC_{50} values of cisplatin after treatment with Rapamycin (0-5 μM), E. IC_{50} values of cisplatin after treatment with colchicine (0-10 μM). The data are presented as means±SD of the IC_{50} values of cisplatin with each concentration tested in five replicates. *p<0.05 compared with cisplatin 0 μM.*

colchicine on IC_{50} of cisplatin in various ovarian cancer 2008, 2008 C13, CP70 and OVCAR 8 cells was determined by an MTT assay. Cisplatin-resistant ovarian cancer cells exhibited approximately a 5.1- to 10.2-fold increase in IC_{50} when compared with cisplatin-sensitive ovarian cancer cells, thereby validating their use in the present study. IC_{50} values of cisplatin in all the cell lines after pre-treatment with chemosensitizers are shown in Figure 2 and resistance reversal activity represented by fold reversal (FR) is provided in Table I. Pre-treatment of chemosensitizers with cisplatin-resistant ovarian cancer cells resulted in a significant decrease in IC_{50} of cisplatin in 2008 C13, CP70 and OVCAR

8 cells. TETA at 50 mM concentration resulted in a 6.9 fold reversal in cisplatin-resistant 2008 C13 cells. Interestingly, in CP70 there was an 88.40-fold reversal, and in OVCAR 8 cells there was only a 2.71-fold reversal. BSO at 50 μM concentration also exhibited a significant reduction in IC_{50} in cisplatin-resistant cell lines 2008 C13 and OVCAR 8, with a FR of 2.8 and 1.8, respectively. Similar to TETA, BSO also did not show any significant reversal activity in CP70 cells and FR was only 1.1. Genistein at 50 μM concentration showed FR of 509.44 in CP70 cells which is much higher than TETA and BSO. However, genistein did not show a higher FR (1.099) in OVCAR 8 cells, similar to BSO and TETA. Rapamycin, at 5 μM concentration, showed very high FR of 24.71 in CP70, but did not show any significant FR in 2008 C13 cells (FR 2.89). Finally, colchicine exhibited a very high FR in all cisplatin-resistant cell lines 2008 C13, CP 70 and OVCAR 8 with FRs 185.44, 1347.27 and 221.51, respectively. However, this high FR of colchicine may be attributed to the toxicity of colchicine to the cell lines under study. The percentage of viability after colchicine treatment was <17.02% for all the cell lines under study (2008, 2008 C13, CP70 and OVCAR 8), whereas for all other sensitizers it was between 80-100 % (data not shown).

Overall, chemosensitizers TETA, BSO, genistein and rapamycin, have shown significantly higher dose-dependent reversals of resistance in various cisplatin-resistant ovarian cancer cell lines. However, their reversal activity was different in different cell lines indicating their relative specificity for certain phenotypes. For example, one such phenotype, elongation factor 1-α2 has been reported by Fitzpatrick *et al*. (7) using a label-free LC/MS-based protein quantification method. They reported that in CP70 cells, the elongation factor 1-α2 expression is approximately 1.2-fold times higher when compared with 2008 C13 cells (7), which correlates with our data where FR in CP70 cells, when treated with rapamycin (inhibitor of elongation factor 1α-2), was 24.76 and FR in 2008 C13 under same conditions was only 2.89.

Effects of chemosensitizers on the cellular accumulation of cisplatin in ovarian cancer cells. The influence of pre-treatment with the chemosensitizers on the cellular uptake of cisplatin was evaluated in paired isogenic cisplatin-sensitive 2008 and cisplatin-resistant 2008 C13 ovarian cancer cell lines using ICP-MS. The results revealed that all the chemosensitizers increased the cellular uptake of cisplatin at 50 μM concentration in cisplatin-resistant ovarian cancer cell lines by 3.5- to 6.0-fold ($p<0.05$). Furthermore, a correlation between cellular uptake and percentage viability was observed for cisplatin at concentrations 1 and 50 μM after co-incubating with chemosensitizers. Figure 3 represents the correlation between cellular uptake and percentage viability of cisplatin in the presence of various chemosensitizers.

Table I. *Resistance reversal activity of chemosensitizers on cisplatin resistance in various cisplatin-resistant ovarian cancer cells. The values in the Table are the fold reversal (FR) calculated by the equation:*

$$\text{Fold Reversal (FR)} = \frac{IC_{50} \text{ of cisplatin alone}}{IC_{50} \text{ of cisplatin+chemosensitizer}}$$

Chemosensitizers	Cell lines		
	2008 C13	CP70	OVCAR 8
TETA (mM)			
0.1	2.038	1.014	1.500
0.5	5.341	6.922	1.696
1	4.556	3.506	2.039
5	6.900	88.407	2.712
BSO (μM)			
5	1.634	1.236	2.974
10	2.049	1.529	1.879
25	2.914	1.235	1.748
50	2.814	1.185	1.885
Genistein (μM)			
5	1.100	1.243	0.798
10	1.259	0.702	0.739
25	1.675	1.730	1.099
50	6.077	509.444	1.099
Rapamycin (μM)			
0.5	2.550	26.491	1.952
1	2.477	8.702	1.812
2.5	2.722	54.649	3.083
5	2.893	24.761	2.907
Colchicine (μM)			
0.1	178.308	1482	1.109
0.5	159.862	1482	43.568
1	171.704	1347.27	7.023
10	185.440	1347.27	221.519

Toxicity to CHO cells. The cytotoxicity of all chemosensitizers was also evaluated by an MTT assay in CHO cells. The results have shown that all the chemosensitizers were relatively non-toxic at the concentrations used in the study in CHO cells including colchicine (Figure 4). The percentage viability for all chemosensitizers in CHO cells varied from 78.68 to 101.67%.

Discussion

Platinum resistance in ovarian cancer is considered an intrinsic behavior and is clinically defined as progression of the tumor during initial treatment with a platinum-based combination chemotherapy regimen or recurrence within 6 months of completing front line therapy (18). Cisplatin resistance has become a major obstacle in the treatment of ovarian cancer and there is definitely an urgent need for finding novel strategies to overcome platinum resistance (11). One such approach is the use of small-molecule chemosensitizers for

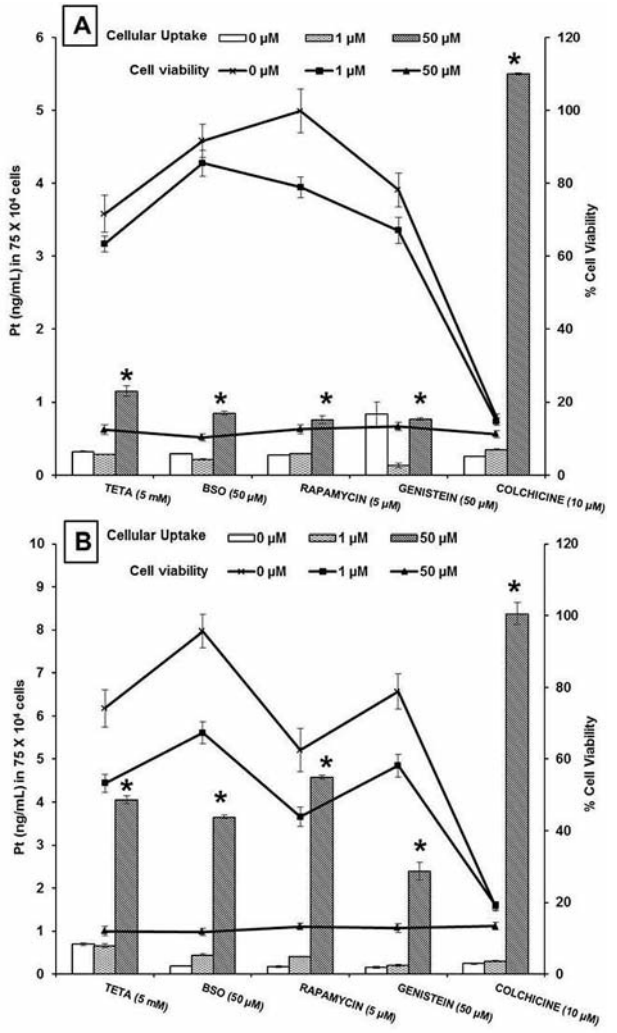

Figure 3. *Cellular accumulation of cisplatin in 2008 and 2008 C13 cells after treatment with chemosensitizers. 2008 and 2008 C13 cells were treated with chemosensitizers: TETA (5 mM), BSO (50 μM), rapamycin (5 μM), genistein (50 μM) and colchicine (10 μM) for 24 h. Cisplatin was added at 0, 1 and 50 μM concentration, to cells and after 4 h, intracellular platinum levels were determined using ICP-MS. In the graph, the left y-axis represents platinum concentration as ng/mL for 75×10⁴ cells. The right y-axis represents the % cell viability of 2008 and 2008 C13 cells after treatment with chemosensitizers and cisplatin. The data are presented as means±SD of triplicates for cellular uptake and five replicates for cellular viability experiments. *p<0.05 compared with cisplatin 0 μM.*

sensitizing platinum-resistant cancer cells. Even though the use of RNAi may be a more target-specific approach, small-molecule chemosensitizers present an approach which is clinically relevant and therapeutically feasible. Nonetheless, in recent years many small-molecule chemosensitizers with various mechanisms were investigated for their ability to reverse resistance in a diverse array of cancers (20). In this study a comparative evaluation of five chemosensitizers was performed in platinum-resistant ovarian cancer cells with varying phenotypes. Chemosensitizers TETA, genistein, and rapamycin showed significant reversal of resistance in platinum-resistant CP70 ovarian cancer cells as FR was 88.40, 509.44 and 24.76, respectively. The order of resistance reversal in CP70 was: colchicine > genistein > TETA > rapamycin > BSO. Whereas in 2008 C13 cells TETA and genistein showed a reversal of resistance with FR values 6.9 and 6.077, respectively. The resistance reversal order for chemosensitizers in 2008 C13 cells was: colchicine > genistein > TETA > rapamycin=BSO. However, in OVCAR 8 cells, except colchicine, none of the chemosensitizers showed significant reversal, as the FR was only 1.01 to 2.907. Interestingly, colchicine, a microtubule inhibitor, has shown very high reversal activity in 2008 C13, CP70 and OVCAR8 cells (FR 185.44, 1347.27, and 221.51) but its activity might be due to severe toxicity to cancer cells in the study (% viability <17.02%).

Reduction in cellular uptake of cisplatin is one of the major phenotypes with cisplatin-resistant ovarian cancer cells. Thus, cellular uptake of cisplatin was quantified using highly sensitive ICP-MS after pre-treatment and co-incubation of chemosensitizers in cisplatin-resistant ovarian cancer cells. Even though there is no evidence of involvement of these chemosensitizers with the uptake mechanisms of cisplatin, all chemosensitizers showed increased cellular uptake of cisplatin in 2008 C13 cells, in comparison with 2008 cells, providing an additional advantage. However, their detailed mechanism in increasing intracellular accumulation of cisplatin needs to be further investigated. Moreover, cellular uptake data corroborated with the cytotoxic activity of the cisplatin as pre- and co-treatment with chemosensitizers, improved the reduction of IC_{50} of cisplatin along with improved cellular uptake. The major impediment in clinical development of small-molecule chemosensitizers is their non-specific activity towards normal ovary cells. All chemosensitizers were evaluated for their non-specific activity towards CHO cells and they did not show any significant toxicity, as more than 78 % of cells were viable when treated with different concentrations of the chemosensitizers used in study. These results indicate the potential of these chemosensitizers in the clinical development of therapeutics against cisplatin-resistant ovarian cancer.

Conclusion

The present study demonstrates that all the chemosensitizers except colchicine have shown reversal of cisplatin-resistance in ovarian cancer cells, and their activity widely varies with different cell phenotypes. Thus, information obtained from the phenotypic profiling of each cisplatin-resistant ovarian cancer cell line will be applied to stratify patients according

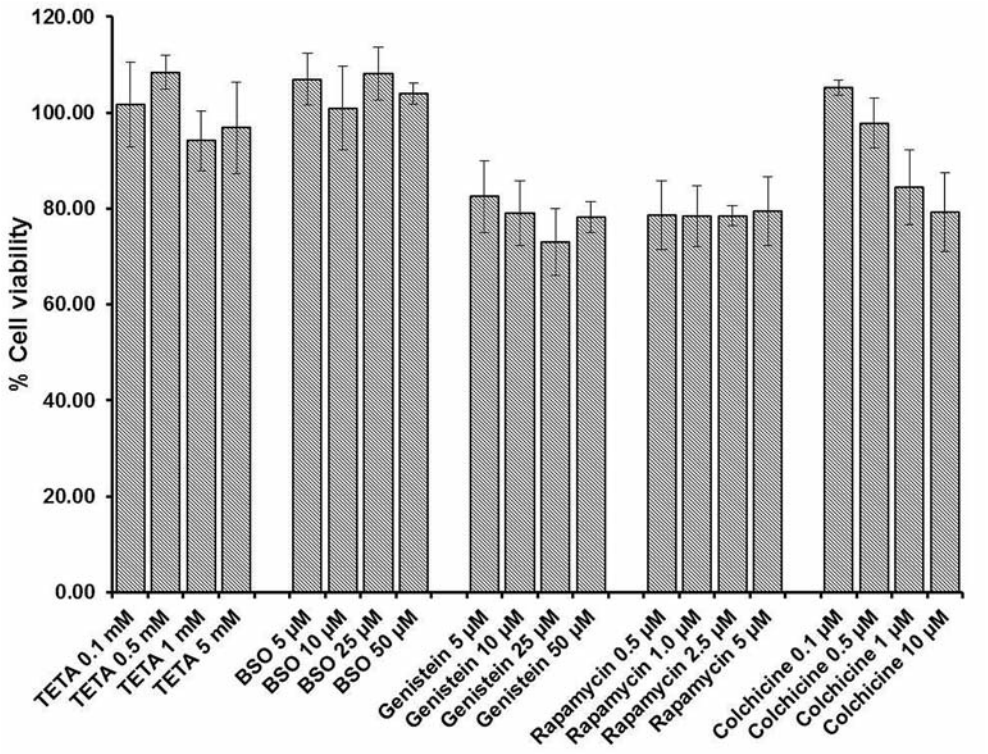

Figure 4. *Toxicity of chemosensitizers on CHO cells. CHO cells were treated with chemosensitizers at all concentrations used in the study for 24 h. The cell viability was evaluated by an MTT assay. The data are presented as means±SD of five replicates.*

to their likelihood of response to the appropriate chemosensitizer. This provides the ability to develop individualized-therapy of cisplatin-resistant ovarian cancer with suitable chemosensitizers in combination with platinum-based drugs.

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