

Acute application of cisplatin affects methylation status in neuroblastoma cells

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Abstract. The pharmacological mechanism of the anti-cancer effect of cisplatin is well known to be DNA intercalation, but the direct or indirect effects of cisplatin on protein expression in cancer cells remain to be explained. In this study, we used a proteomic approach to clarify the early impact of cisplatin on protein expression. In a 2-dimensional gel electrophoresis proteomic experiment, the application of cisplatin for 24 h increased the expression of four proteins and decreased the levels of one protein in neuroblastoma IMR-32 cells. Levels of S-adenosyl-L-homocysteine hydrolase, a key enzyme in methylation metabolism, were increased the most. Therefore, we examined the methylation status of histone proteins. Histone H3K9 methylation was reduced by the application of cisplatin for 24 h. These results suggest that acute cisplatin treatment alters methylation status. Thus, these data can help clarify the unknown pharmacological mechanisms of cisplatin, including the anticancer effect, adverse effects and/or the mechanism of drug resistance.

Introduction

Neuroblastoma is one of the most common solid tumors in children, and it is a major cause of death from neoplasia in infancy. Most patients over 1 year of age with neuroblastoma are diagnosed at advanced stages with distant metastasis, and their prognosis is very poor despite multidisciplinary treatment, which includes surgery, high-dose chemotherapy, radiation and bone marrow transplantation (1). Moreover, relapsed neuroblastoma after intensive chemoradiotherapy

and bone marrow transplantation often acquires resistance to cytotoxic drugs, such as etoposide, doxorubicin and cisplatin (2).

Multidrug resistance is one of the major obstacles in effective cancer chemotherapy. In neuroblastoma, expression of P-glycoprotein, an ATP-dependent drug-efflux pump, was found in metastatic tumors at clinical recurrence (3). The multidrug resistance-associated protein 1 (MRP1) gene is a MYCN target gene involved in the development of multidrug resistance (4). Furthermore, loss of p53 function confers multidrug resistance (5). However, the comprehensive mechanisms causing chemotherapy-induced multidrug resistance are still unclear. Recently, changes in methylation status were revealed to underlie drug resistance in many types of tumors. In neuroblastoma, overexpression of DNA methyltransferase is associated with cisplatin resistance (6). Additionally, epigenetic silencing of caspase-8 by hypermethylation of regulatory sequences is related to tumor aggressiveness and drug resistance (7,8). However, mechanisms of acquiring drug resistance at early phases are still unclear.

In the present study, we focused on cisplatin as a key drug used to treat neuroblastoma. To clarify the impact of acute cisplatin exposure, we comprehensively examined cisplatin-induced up- or down-regulation of proteins in neuroblastoma cells using proteomic analysis. Because we observed up-regulation of S-adenosyl-L-homocysteine (AdoHcy) hydrolase, which regulates methylation, we also determined whether cisplatin could modulate the methylation status of histones in the neuroblastoma IMR-32 cell line.

Materials and methods

Cell line. Human neuroblastoma IMR-32 cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen) at 37°C in a humidified incubator containing 95% air + 5% CO₂ atmosphere.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. IMR-32 cells (1×10⁴ cells/well) were

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spread onto a 96-well culture plate with phenol red-free RPMI-1640 medium (with 10% FBS) and maintained for 24 h. Then, cisplatin (final concentration 10^{-7} - 10^{-4} M) or vehicle were applied for 24 h. After the addition of 0.5% MTT solution as 10% of the volume of medium in the well, incubation was continued for an additional 3 h at $37^{\circ}\text{C}/5\% \text{CO}_2$. An equal volume of stop solution (0.04 N HCl in isopropanol) was added to the culture medium in each well, and the absorbance at 570 nm (peak) and 655 nm (trough) was measured after thorough pipetting to disperse the generated blue formazan.

Proteomics. Cells (2×10^6 cells/5 ml) were seeded onto a 60-mm dish and treated with $10 \mu\text{M}$ cisplatin, or DMSO as a control, for 24 h. For preparing protein samples, cells from 8 dishes per group were collected into one tube, washed with Tris buffered saline (TBS) and lysed in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100 and 0.5% IPG-buffer (GE Healthcare). Protein samples were diluted with rehydration buffer [7 M urea, 2 M thiourea, 2% CHAPS, 0.5% Triton X-100, 0.5% IPG-buffer and trace bromophenol blue (BPB)] at 1 mg protein/250 μl . The diluted sample solutions were loaded onto Immobiline™ DryStrips (pH 3-10, 11 cm; GE Healthcare) through rehydration at room temperature overnight. After rehydration, isoelectric focusing was started at 300 V, and the voltage was gradually increased to 3500 V for 2.5 h and further held at 3500 V for 5 h using a Multiphor II electrophoresis system (GE Healthcare). The strips were equilibrated in an initial equilibration buffer containing 1% dithiothreitol (DTT), 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS) and trace BPB for 15 min, followed by a second equilibration buffer containing 2.5% iodoacetamide, 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and traces of BPB for 15 min. The second-dimension electrophoresis was conducted on 12.5% SDS-polyacrylamide gel at 300 V at 4°C . Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R-350 (GE Healthcare) solution overnight at room temperature. After washing away the excess dye with 30% methanol/10% acetic acid solution, the gels were scanned with an Image Scanner (GE Healthcare). The spots were analyzed by Image Master 2D Platinum ver. 5.0 software (GE Healthcare), according to the manufacturer's protocol. To normalize spot volumes, the volume of each spot was divided by the total volume of all the spots in the gel. In-gel digestion, MALDI-TOF-MS analysis and protein identification were performed as described previously (9). Preliminary amino acid sequences for human ORF data were obtained from NCBI.

Western blotting. Cells (5×10^6 cells/5 ml) were seeded in a 60-mm dish and treated with $10 \mu\text{M}$ cisplatin for 0-48 h. Cells were collected, washed with TBS and lysed in an extraction buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP-40, 10% glycerol, protease inhibitor cocktail I (1:200; Sigma), phosphatase inhibitor cocktail II (1:100; Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM DTT. The cells were twice disrupted by sonication for 30 sec, and the supernatants were obtained by centrifugation at $9000 \times g$ for 10 min at 0°C . The supernatants were mixed with 3X sample buffer [0.24 M Tris-HCl (pH 6.8),

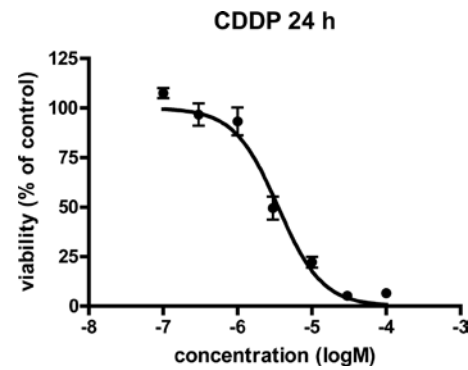


Figure 1. Cisplatin induces concentration dependent cytotoxicity. Cell viability was measured using the MTT assay. IMR-32 cells were treated with indicated concentrations of cisplatin or DMSO (vehicle control) for 24 h. Each graph shows the survival rate relative to the vehicle control (mean \pm SEM; $n=3$).

9% SDS, 30% glycerol, 15% 2-mercaptoethanol and traces of BPB] at 2:1. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (GE Healthcare). After blocking, the membranes were probed with primary antibodies, including monoclonal anti-AdoHcy hydrolase 1E11-1A7 (Abnova, 1:1000), Histone H3K4 monomethyl (Upstate, 1:1000), H3K4 dimethyl (Upstate, 1:5000), H3K4 trimethyl (Abcam, 1:1000), H3K9 monomethyl (Upstate, 1:5000), H3K9 dimethyl (Upstate, 1:1000), H3K9 trimethyl (Upstate, 1:1000), H3K27 monomethyl (Upstate, 1:5000), H3K27 dimethyl (Upstate, 1:2500), H3K27 trimethyl (Upstate, 1:2500), pan-histone (Chemicon, 1:500) and monoclonal anti- β -tubulin TUB2.1 (Sigma, 1:10000), and secondary antibodies (anti-mouse IgG peroxidase conjugate, 1:10000). The blots were detected with an ECL system (GE Healthcare).

Results

To clarify the mechanism of cisplatin-induced protein alteration at early time points, a time course study over 24 h was performed. First, to determine the optimal concentration of cisplatin to be applied to the cells in the proteomics study, we determined the cytotoxicity of cisplatin applied for 24 h to neuroblastoma IMR-32 cells using the MTT method (Fig. 1). The IC_{50} value was 3.52×10^{-6} M. Considering that a proportion of tumor cells remain, leading to residual disease and tumor recurrence in cisplatin-treated patient tumors, $10 \mu\text{M}$ cisplatin, a concentration causing cell death but leaving 22.2% viable cells, was selected to examine the proteomic study.

To elucidate the acute effect of cisplatin on neuroblastoma cells, especially in the mechanisms of drug resistance acquisition, protein samples were prepared from IMR-32 cells treated with $10 \mu\text{M}$ cisplatin or DMSO (vehicle control) for 24 h. Samples treated with cisplatin or control were subjected to 2-DE (pH range 3-10). This process was performed 3 times per sample. The 2-DE map for cisplatin-treated cells was then compared with the map for control cells (Fig. 2). In these gels, 287 spots were matched between the two groups, and 13 spots were changed by cisplatin in comparison to the control (>1.5 -fold and $p < 0.05$ by Student's

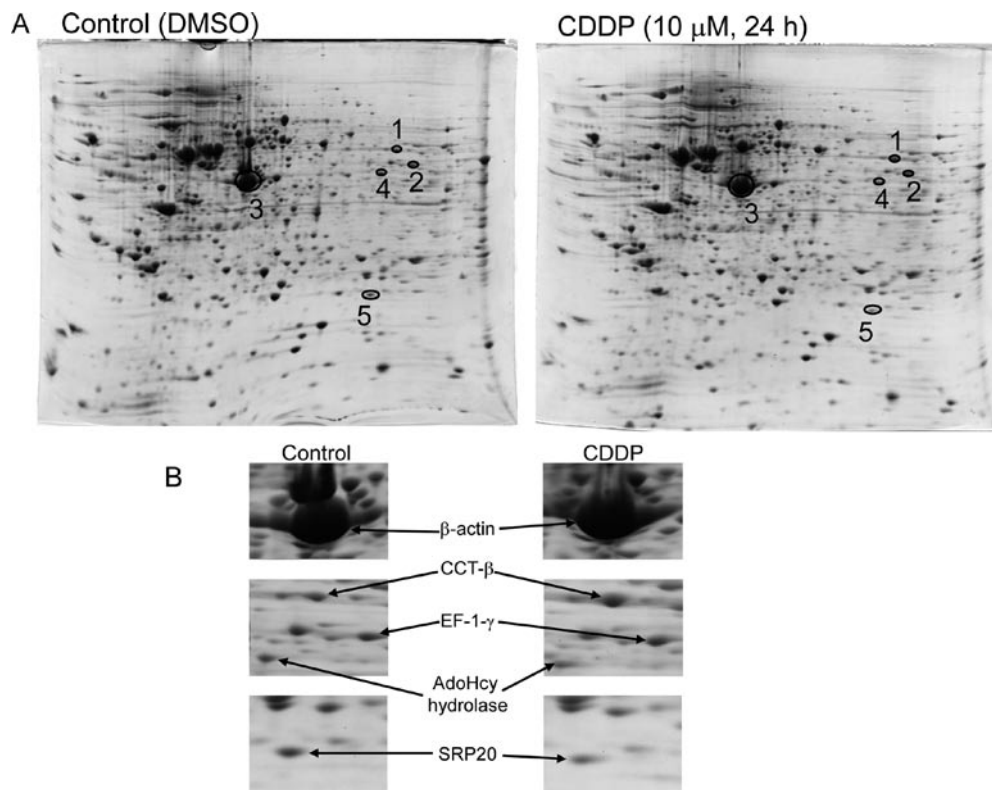


Figure 2. Proteomic analysis of cisplatin-treated neuroblastoma cells. (A) The two-DE map from cisplatin-treated IMR-32 cells. Proteins from IMR-32 cells treated with 10 μ M cisplatin or DMSO (control) for 24 h were separated by pI (horizontal) and molecular weight (MW) (vertical) and stained with CBB solution. Circles indicate spots that were up-regulated (No. 1-4) or down-regulated (No. 5) by cisplatin. (B) These proteins are shown in detail.

Table I. Proteins up- or down-regulated by cisplatin in proteomic analysis.

Spot no. ^a	Name ^b	Volume control (average)	Volume cisplatin (average)	Increase (+)/decrease (-)	Ratio cisplatin to control (average)	MW	pI
1	T-complex protein 1, β subunit (CCT- β)	0.140	0.279	+	1.989	57545	6.02
2	Elongation factor 1- γ (EF-1- γ)	0.082	0.139	+	1.697	50036	6.27
3	Actin, cytoplasmic 1 (β -actin)	6.744	11.760	+	1.744	41982	5.29
4	Adenosylhomocysteinase (AdoHcy hydrolase)	0.049	0.101	+	2.061	47794	5.92
5	Splicing factor, arginine/serine-rich 3 (SRP20)	0.108	0.068	-	0.631	19334	11.64

^aThe protein spots indicated in Fig. 2A were analyzed using MALDI-TOF-MS. ^bProteins were identified using MASCOT with a custom human ORF database obtained from NCBI.

t-test). Then, 5 of these spots were successfully identified by MALDI-TOF-MS. As shown in Fig. 2 and Table I, 4 proteins [T-complex protein 1 β subunit (CCT- β), elongation factor 1- γ (EF-1- γ), β -actin and AdoHcy hydrolase] were up-regulated, and 1 protein [splicing factor arginine/serine-rich 3 (SRP20)] was down-regulated. Among these proteins, AdoHcy hydrolase was the most changed relative to the control (Fig. 2 and Table I). To confirm the results, Western blot analysis for AdoHcy hydrolase was performed 0-48 h after the application of cisplatin (Fig. 3). Consistent with the proteomic results, AdoHcy hydrolase was up-regulated

significantly at 24 h. These results imply that the methylation status was affected by cisplatin because AdoHcy hydrolase degrades AdoHcy, a strong competitive inhibitor of the methyl donor, AdoMet.

Subsequently, 0-48 h after the application of 10 μ M cisplatin by Western blotting, we examined the methylation status of histones H3K4, 9 and 27 (Fig. 4). Levels of mono-, di- and tri-methylated histone H3K9 and monomethylated histone H3K27 were diminished time-dependently. On the other hand, the methylation status of histone H3K4 was not changed.

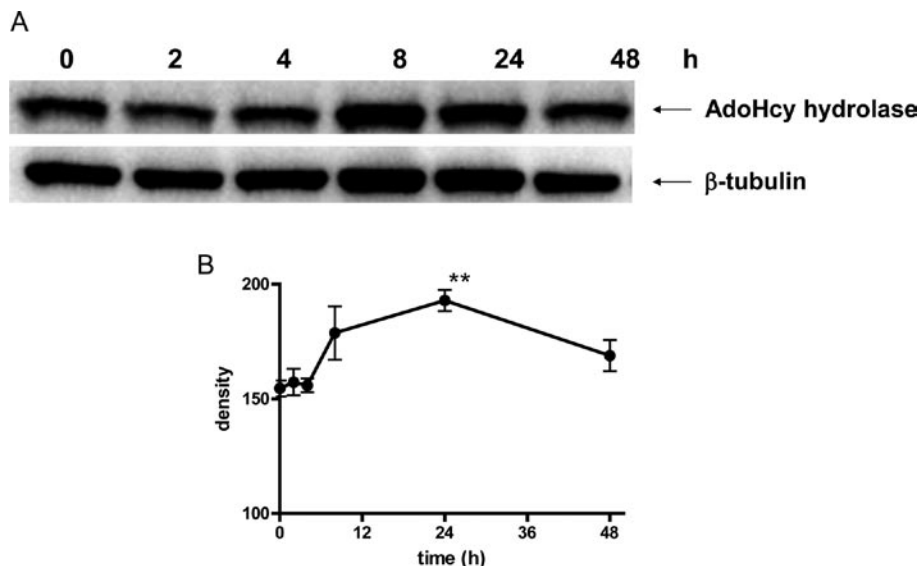


Figure 3. Cisplatin up-regulates AdoHcy hydrolase. (A) AdoHcy hydrolase bands detected by Western blotting are shown. IMR-32 cells were treated with 10 μ M cisplatin for 0-48 h. The band density of AdoHcy hydrolase is shown in B.

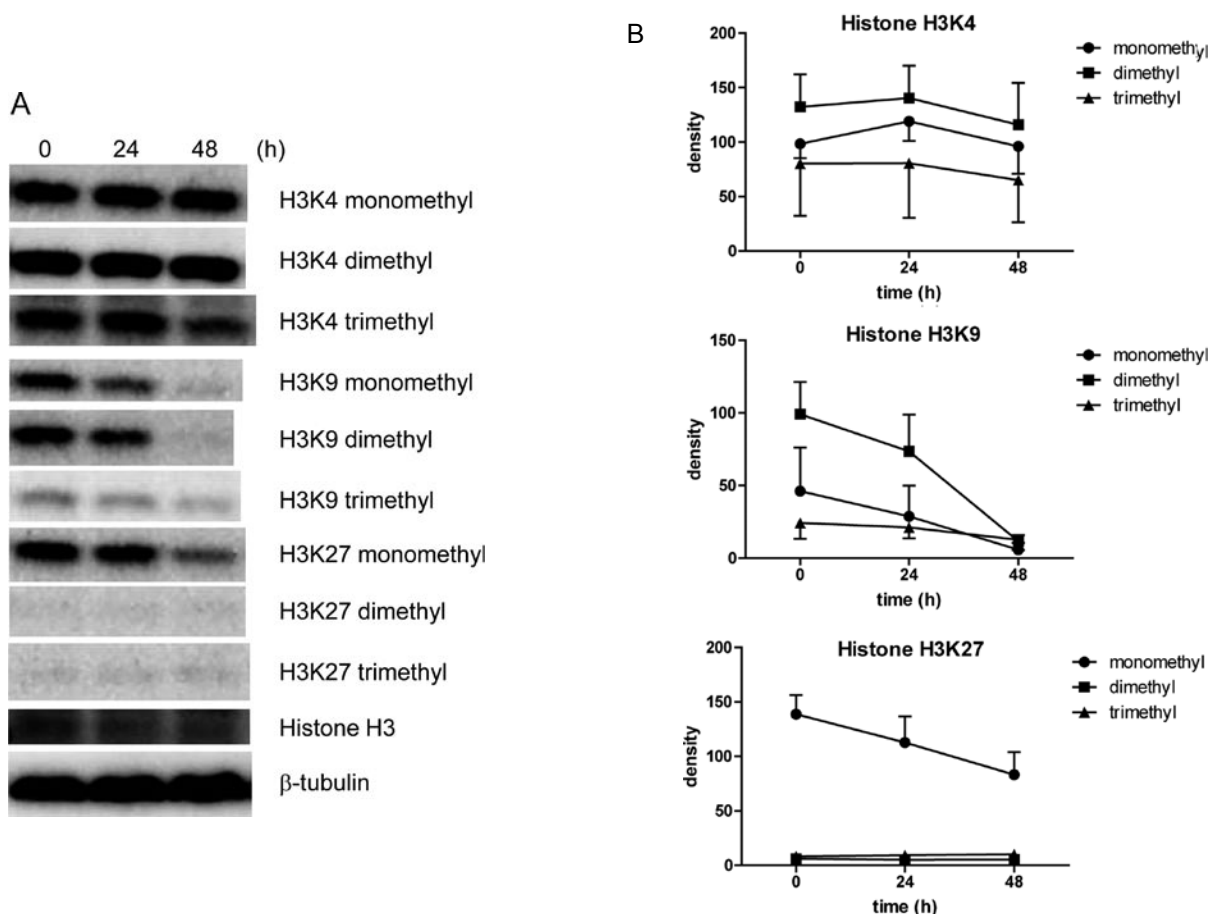


Figure 4. Effects of cisplatin on the methylation status of Histone H3. (A) Histone H3K4, 9 and 27 (mono-, di- and tri-) methylation detected by Western blotting are shown. IMR-32 cells were treated with 10 μ M cisplatin for 0-48 h. The band densities of the methylated histone H3K4, 9 and 27 are shown in B.

Discussion

Using proteomic analysis of neuroblastoma IMR-32 cells, we examined comprehensive protein alterations induced by cisplatin. We showed that levels of AdoHcy hydrolase were

increased by cisplatin. AdoHcy is a metabolite of AdoMet, and it inhibits AdoMet methylation in a negative feedback manner (10). Because the increase in levels of AdoHcy hydrolase implies that AdoHcy would be degraded more efficiently, we considered the possibility that the transmethylation

ability of AdoMet could be facilitated by cisplatin. However, histone H3K9 methylation was diminished.

Ras-association domain family 1 (RASSF1A), a tumor suppressor gene, is frequently inactivated by hypermethylation in 40-55% of neuroblastoma tumors and in 86% of cell lines (11). Promoter hypermethylation of *RASSF1A* is associated with drug resistance in germ cell tumors (12). Furthermore, it was reported that cisplatin treatment induced *de novo* hypermethylation of promoter regions, including the *RASSF1A* promoter *in vivo* but not *in vitro* (12). Our results showed that cisplatin increased levels of AdoHcy hydrolase in neuroblastoma IMR-32 cells *in vitro*, which could shift cellular conditions in favor of methylation. Therefore, increased levels of AdoHcy hydrolase may contribute to *RASSF1A* hypermethylation, and it seems that the end effect of *RASSF1A* promoter by cisplatin on hypermethylation needs additional stimulation.

Histone modification and DNA methylation are epigenetic phenomena that play critical roles in many neoplastic processes, including the silencing of tumor suppressor genes. In recent years, the association between histone methylation and cisplatin-resistance has been investigated. Cisplatin-resistant MCF-7 human breast cancer cells show a loss of global DNA methylation, a loss of histone H4K20 trimethylation and a diminished expression of Suv4-20h2 histone methyltransferase (13). The removal of H3K27 methylation resensitized drug-resistant ovarian cancer cells to cisplatin (14). However, there are no reports on the regulation of H3K9 methylation status by cisplatin treatment. Here, we show that dramatic hypomethylation of histone H3K9 occurred in response to cisplatin treatment in neuroblastoma cell lines at early time points (24-48 h). Histone methylation of H3K9 is known as a hallmark of transcriptional silencing. Histone H3K9 hypermethylation is linked to the inhibition of genes related to cell cycle, telomerase and DNA repair (15-17). Stimulation by hypoxia or nickel ion increases H3K9 methylation and represses the transcription of tumor-related genes (18,19). Taken together, cisplatin-induced H3K9 hypomethylation may contribute to the unknown additional therapeutic mechanism of cisplatin. Additionally, cisplatin may facilitate the acquisition of multidrug resistance in neuroblastoma following drug treatment.

Considering the methylation status, we hypothesized that AdoHcy hydrolase up-regulation induces methylation by reducing levels of AdoHcy and subsequently decreases the competitive inhibition of AdoMet. In our results, however, AdoHcy hydrolase overexpression coincided with hypomethylation of histone H3K9 after acute cisplatin application. Qiu *et al* reported that continuous exposure to MDL28842, an AdoHcy hydrolase inhibitor, induces drug resistance by causing overexpression of DNA-methyltransferases in neuroblastoma cells (20). The discrepancy between potential for methylation, such as the AdoHcy level, and the actual DNA methylation status are discussed (10). Thus, the systems regulating methylation are considered to be complex. Further analyses are needed to explain the overall mechanisms of methylation.

In our proteomic analysis, CCT- β and β -actin were also overexpressed in response to the application of cisplatin. CCT, a hexadecameric chaperonin composed of eight subunits,

including CCT- β , is involved in the successful folding of actins and tubulins. CCT activity is required for cell cycle progression and cytoskeletal organization (21). Reduction in CCT- β levels by siRNA results in growth arrest (21). The levels of CCT subunit synthesis have been shown to be similar to tubulin synthesis during cell cycle progression (22). These results indicate that the cell cycle was affected by cisplatin via CCT- β and β -tubulin up-regulation as well as by the direct or indirect alteration of methylation status.

In conclusion, acute cisplatin increased the levels of proteins involved in methylation status and cell cycle control. Moreover, cisplatin induced hypomethylation of histone H3K9, which is linked to gene silencing. These results imply that regulating the acute impact of cisplatin may serve as effective therapeutics for neuroblastoma patients.

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