Association between Cisplatin Resistance and Mutation of p53 Gene and Reduced Bax Expression in Ovarian Carcinoma Cell Systems

Paola Perego, Monica Giarola, Sabina C. Righetti, Rosanna Supino, Claudia Caserini, Domenico Delia, Marco A. Pierotti, Toshiyuki Miyashita, John C. Reed, and Franco Zunino

ABSTRACT

p53 status may be a determinant of chemosensitivity of tumor cells; however, its involvement in cellular resistance to cisplatin remains uncertain. To investigate the relationships between p53 and the development of resistance to cisplatin, the p53 gene status was studied in ovarian carcinoma cell systems which included two cisplatin-resistant variants (IGROV-1/Pt 0.5 and IGROV-1/Pt 1) selected in vitro after prolonged drug exposure of the cisplatin-sensitive parental IGROV-1 cell line. IGROV-1/Pt 0.5 and IGROV-1/Pt 1 cell lines exhibited a degree of resistance of approximately 6 and 14, respectively, following 96-h exposure to the drug and were cross-resistant to other DNA-damaging agents (ionizing radiation and melphalan). Resistance to cisplatin paralleled a reduced cell susceptibility to cisplatin-induced apoptosis. DNA single-strand conformation polymorphism analysis of exons 5–9 demonstrated the presence of two mutant alleles at exon 8 in the two resistant cell lines, in contrast to the parental IGROV-1 cell line which exhibited the wild-type p53 gene. Direct DNA sequencing revealed that the mutations consist of two nucleotide changes in the DNA-binding domain at codons 270 (T/A) and 282 (C/T). The constitutive levels of p53 protein were markedly enhanced only in the parental IGROV-1 cells. Following exposure to ionizing radiation or cisplatin, accumulation of the p53 protein was markedly enhanced only in the sensitive cells. Concomitantly, the expression of WAF-1 protein was strongly induced in the parental IGROV-1 cells, whereas WAF-1 protein remained undetectable in the IGROV-1/Pt 1 subline after DNA-damaging treatment. Consistent with this finding is the observation that ionizing radiation caused a different pattern of cell cycle perturbation in sensitive and resistant cells. Northern blot analysis demonstrated a marked reduction in bax mRNA levels in IGROV-1/Pt 1 cisplatin-resistant cells. Co-transfection assays with wild-type or mutant p53 expression plasmids and a reporter gene plasmid that utilized the bax gene promoter to drive transcription of chloramphenicol acetyltransferase were consistent with the role of p53 in regulation of bax expression in these cells. Taken together, these observations support a role for mutations of the p53 gene in the development of cisplatin resistance in ovarian cancer as a consequence of loss of the ability of p53 to transactivate bax, an apoptosis-inducing gene.

INTRODUCTION

Cisplatin is an effective drug in the treatment of a variety of human tumors, including ovarian carcinoma. Unfortunately, development of resistance is a major obstacle to the success of cancer therapy. The molecular basis of cisplatin resistance has not been conclusively defined (1–3). In cells selected in vitro for resistance to cisplatin, protective and detoxification mechanisms have been postulated to be operative, including decreased drug accumulation (4, 5), increased content of intracellular thiol s such as glutathione and metallothioneins (3, 6), reduction of DNA cross-linking as a consequence of decreased drug accessibility to DNA, and/or increased repair (2, 3). Although these proposed mechanisms may contribute to the development of a variable degree of cellular resistance, it is possible that the cell response following interaction between the cytotoxic agent and DNA also plays a role in determining cellular chemosensitivity. Cisplatin is known to induce programmed cell death or apoptosis (7, 8). The inability to activate the apoptotic program could result in a cell insensitivity to cytotoxic treatment. Several lines of evidence indicate that p53 participates in DNA-damaging agents and particularly in drug resistance remains uncertain. The complexity of p53 functions and the observation that p53 mutations can produce a gain of new functions (11) suggest that p53 might have a role during the development of resistance. Gene transfer studies have documented an important role for p53 in modulating the relative sensitivity of some types of p53-deficient tumor cells to drug and radiation (12). Recent data suggest that p53 exerts its role as inducer of apoptosis by transactivating expression of the bax gene (13). The major product of the bax gene is a Mr ~21,000 protein with homology to Bel-2, an important regulator of apoptosis (14). The Bax protein can form heterodimers with Bel-2 and promotes cell death (15, 16). The promoter of the human bax gene contains typical p53-binding site sequences and can mediate p53-dependent transactivation on heterologous reporter genes (13).

In this report, we describe the molecular analysis of the p53 gene in ovarian cancer cell lines selected in vitro for resistance to cisplatin. We present evidence that the selection of two variants with a different degree of resistance is associated with p53 mutations and reduced susceptibility to cisplatin-induced apoptosis. In addition, we show that bax mRNA levels were markedly reduced in IGROV-1-resistant cells that harbor p53 mutations and demonstrate by use of bax promoter-CAT reporter gene assays reduced transactivation of the bax promoter in p53 mutant cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The IGROV-1 ovarian carcinoma cell line was derived from a moderately differentiated ovarian carcinoma of an untreated patient. The tumor model was originally obtained by Dr. Benard (Villejuif, France). The two cisplatin-resistant variants, designated as IGROV-1/Pt 0.5 and IGROV-1/Pt 1, were generated by continuous exposure of the IGROV-1 cell line to increasing concentrations of cisplatin and were cultured in medium always containing cisplatin 0.5 or 1 μg/ml, respectively. All of the cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS (GIBCO-BRL, Gaithersburg, MD).

Cytotoxicity Studies. Cells (1.4 × 10^6/cm2) were seeded in 6-well cluster plates. After 24 h, cells were irradiated with a 137Cs source or treated for 1 h with cisplatin (Bristol-Myers Squibb, Rome, Italy) or melphalan (Sigma, St. Louis, MO) and then incubated in drug-free medium for an additional 72 h. In long-term treatments, cells were maintained in the presence of the cisplatin through the period of the experiment. At the end of the experiments cells were trypsinized and counted with a ZBI Coulter counter (Coulter Electronics Ltd.,...
Luton, England). The IC_{50} values were calculated by plotting the surviving fraction on the drug concentration.

**Assessment of Apoptosis.** Apoptosis was assessed by fluorescence microscopy in floating cells. At different time intervals after treatment, floating and adherent cells were collected, washed in PBS, and fixed in 70% ice-cold ethanol. Samples were then stored at −20°C until analysis (2−5 days). After rehydration in PBS, cells were stained with propidium iodide solution (50 μg/ml propidium iodide and 66 units/ml RNase A in PBS) and stored in the dark for 30 min. At least 100 cells in two different smears were examined for their nuclear morphology changes (chromatin condensation and DNA fragmentation). The percentage of apoptotic cells was referred to the cell number of the whole population (floating + adherent cells).

**Alkaline Elution.** Cisplatin-induced IC_{50} values were determined using the alkaline elution method developed by Kohn et al. (17). Cellular DNA was labeled with 0.08 mCi/ml [2-^3H]thymidine (Amersham) for 24 h, and the labeled nucleoside precursor was removed 24 h before exposure to the drug. Cells were exposed to cisplatin for 1 h. To produce a known frequency of γ-ray-induced DNA-single-strand breaks before analysis, approximately 5 × 10^5 cells were irradiated with a 1^{37}Cs source (10 Gy). A comparison of the frequency of ICLs in sensitive and resistant cells was possible because the same dose of γ-irradiation caused a similar frequency of DNA single-strand breaks in the three cell lines. Cells were then deposited on a 2.0-mm pore polycarbonate filter (25 mm in diameter; Nucleopore, Pleasanton, CA) and lysed with 2% SDS, 0.02 M disodium EDTA (pH 10), and 0.5 mg/ml proteinase K. The DNA was then eluted at a flow rate of 0.035 ml/min with a 0.02 M EDTA-0.1% SDS solution adjusted to pH 12.15 with tetrapropylammonium hydroxide. During a 15-h elution, the lysate was collected in fractions and counted by liquid scintillation. ICL frequency was calculated using the following formula (17):

[(1 - r_f)/(1 - r)]^2 - 1 (1)

where r_f is the fraction of [^3H]DNA remaining on the filter in control irradiated cells, and r is the fraction of [^3H]DNA remaining on the filter in drug-exposed irradiated cells, calculated after 15 h of elution.

**Cell Cycle Analysis.** Cell cycle distribution was assessed by flow cytometry. In brief, fixed cells were washed twice in PBS and resuspended in PBS containing 50 μg/ml propidium iodide and 66 units/ml RNase A. Thirty min to 2 h later, cell fluorescence was measured by FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**SSCP and Sequencing Analysis.** SSCP analysis for the detection of p53 gene mutation in the most affected exons of the gene (5−9) has been previously described (18). A modification was introduced: gels were not fixed in acetic acid and methanol before being vacuum dried. SSCP bands were cut from dried gels, placed in 100 μl distilled water, shaken, and, after a brief centrifugation, used as template for PCR amplification with the same primer pair used in SSCP. PCR products were gel purified and utilized as templates in direct sequencing reactions. Sequencing was performed by reamplification with Taq polymerase and 5'-32P-labeled primers using the AmpliTaq Cycle Sequencing kit (Perkin Elmer/Cetus, Norwalk, CT).

**Western and Northern Blot Analysis.** Cell lysates from exponentially growing cells were prepared according to Laemmli (19). In brief, samples (100 μg/lane) were fractionated by SDS-PAGE and blotted on nitrocellulose sheets. Blots were probed for 4 h at room temperature in PBS containing 5% (w/v) dried nonfat milk. Filters were incubated overnight at 4°C with DO-7 antibody to human p53 (DAKO, Glostrup, Denmark); a rabbit anti-actin antibody (Sigma) was used as a control for loading. Antibody binding to the nitrocellulose membranes was detected using peroxidase-conjugated secondary antibodies and visualized using alkaline phosphatase-conjugated secondary antibodies and ECL reagents (Amersham, Little Chalfont, United Kingdom). For the Northern blot analysis, total RNA and the murine bax probe (Millipore, Bedford, MA). Blots were treated as described above and reacted with a monoclonal antibody to human p52^{WAF1/CIP1} (catalogue no. 15091A, PharMingen, San Diego, CA) and with a rabbit antisem to recombinant human HAP1 (Ref. 20; a kind gift from Professor A. Harris, Institute of Molecular Medicine, Imperial Cancer Research Fund, Oxford, United Kingdom).

**RESULTS**

**Cross-resistance Pattern and Apoptosis Induction.** Cytotoxic effects of cisplatin on the IGROV-1 ovarian cell line and cisplatin-resistant sublines following a 1- or 96-h drug exposure are shown in Table 1 as IC_{50} values. Apparently, the degree of resistance was dependent on the exposure time. Under both conditions of exposure (1 and 96 h), IGROV-1/Pt 0.5 (selected with 0.5 μg/ml cisplatin) exhibited a resistance index lower than that of the IGROV-1/Pt 1 subline (selected with 1 μg/ml cisplatin). A comparable degree of cross-resistance was also observed for the alkylating agent melphalan. A moderate resistance to ionizing radiation (resistance index, 2) was evident for both cisplatin-resistant sublines.

The level of spontaneous apoptosis was very low and similar in the three cell lines (0.3−0.6%), and no modifications during the 4 days of the experiments were observed in untreated cells. The time course of apoptosis induction by equitoxic cisplatin concentrations in different cell lines was determined using the Alamar Blue assay (Invitrogen, Carlsbad, CA). Cells were exposed to equitoxic concentrations of cisplatin, and the fluorescence was measured at different time points after treatment. The fluorescence was normalized to the cellular DNA content of the untreated control cells.

**Table 1 Cross-resistance pattern toward DNA-damaging agents of IGROV-1 cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin (μg/ml)</th>
<th>Radiation (Gy)</th>
<th>Melphalan (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h^a</td>
<td>96 h^a</td>
<td></td>
</tr>
<tr>
<td>IGROV-1</td>
<td>3 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>2</td>
</tr>
<tr>
<td>IGROV-1/Pt 0.5</td>
<td>60 ± 5 (20)^c</td>
<td>2.5 ± 1 (6)</td>
<td>4</td>
</tr>
<tr>
<td>IGROV-1/Pt 1</td>
<td>100 ± 6 (33)</td>
<td>5.6 ± 0.8 (14)</td>
<td>4</td>
</tr>
<tr>
<td>IGROV-1/Pt 1</td>
<td>22.5 ± 6 (32)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a IC_{50} values assessed by cell count, 72 h after h-1 exposure to the drug or to γ-rays.

^b IC_{50} values assessed by cell count 96 h after exposure to the drug.

^c Numbers in parentheses, resistance index.
cell lines (10, 200, and 300 μg/ml in IGROV-1, IGROV-1/Pt 0.5, and IGROV-1/Pt 1, respectively, corresponding to IC₅₀ following 1-h exposure) is consistent with the possibility that the acquisition of resistance to cisplatin was the result of a reduced cell susceptibility to drug-induced apoptosis (Fig. 1).

Cisplatin Induction of ICLs. The frequency of ICLs was studied in IGROV-1 and IGROV-1/Pt 0.5 cells exposed for 1 h to the same cisplatin concentration (300 μg/ml). The extent of this type of DNA lesions was not related to cellular response to cisplatin, since the frequency is apparently higher in the resistant subline (0.342 ± 0.032) than in the sensitive line (0.167 ± 0.012).

Cell Cycle Perturbations after DNA Damage. Perturbations in cell cycle progression were assessed by flow cytometric analysis of DNA content 24 and 72 h following irradiation (Table 2). A marginal increase in the G₁:G₂ ratio was observed only for the parental IGROV-1 cell line 24 h following exposure to ionizing radiations. This radiation-specific cell response is consistent with only a transient reduction in the rate of progression of cells from G₁ to S-phase. At 72 h after treatment, flow cytometric profiles showed no differences between control and irradiated cells. Resistant cell lines showed an increase of cell population in the G₂ + M phase compared to control untreated cells both at early and late posttreatment times (Table 2).

During 4 days after cisplatin treatment with equitoxic concentrations (causing a 80% growth inhibition; Fig. 2) in the sensitive cell line, the percentage of cells in G₁ was similar to that of the control untreated cells. On the contrary, a marked decrease of the G₁ population in advantage of G₂ population, starting at 2 days after treatment, was observed in the two resistant cell lines. The G₂ + M accumulation induced by cytotoxic treatment was related to the degree of drug resistance. The marked perturbation of the cell cycle following induction of DNA damage in resistant cells is consistent with a loss of G₁ delay. However, a lack of marked differences in the cell cycle distribution of irradiated sensitive cells as compared with control cells suggests the presence of a G₁ delay related to the functional wild-type p53. In contrast, the marked increase of cells in G₂ in the resistant cell lines is consistent with a loss of the G₁ checkpoint (see below).

p53 Gene Analysis. p53 gene analysis was undertaken by SSCP analysis. Exons 5–9 were analyzed because they are localized in the region of the p53 gene most often mutated. As shown in Fig. 3, the occurrence of p53 gene alterations involving exon 8 in the two cisplatin-resistant variants was revealed, as documented by the altered mobility of the PCR products derived from amplification of DNA in comparison to control DNA. IGROV-1 cells were homozygous for the wild-type p53 allele.

To characterize the p53 gene mutations detected using SSCP analysis, the nucleotide sequence of p53 exon 8 from cisplatin-sensitive and -resistant cells was determined. Direct sequencing of the PCR-amplified exon revealed the simultaneous presence of two mutations...
CISPLATIN RESISTANCE AND p53 MUTATION

Fig. 4. Nucleotide sequence analysis of a portion of p53 exon 8. The codons at which the mutations occur are indicated. Arrows, bands corresponding to the nucleotides involved in the mutations. A, reactions of different samples are grouped so that mutations can be easily recognized. In sample 1 (IGROV-1/Pt 0.5, SSCP gel-excised band a) at codons 270 and 282, both mutated and wild-type alleles are present. Sample 2 (IGROV-1/Pt 0.5 gel-excised band b) is mutated only at codon 282. Sample 3 (IGROV-1 genomic DNA) is wild type at both codons as is sample 4 (control DNA). B, the sequence of conformer C is shown, which resulted in wild-type at codon 282 and mutated 270 (the mutated nucleotide is indicated in boldface).

Fig. 5. Western blot analysis of p53 protein level in IGROV-1 (Lane 1), IGROV-1/Pt 0.5 (Lane 2), and IGROV-1/Pt 1 (Lane 3) cells. An anti-actin antibody was used as a control loading.

at codon 270 and 282 in genomic DNA of cisplatin-resistant cells. The localization of these two mutations on different alleles was demonstrated by sequencing all of the SSCP bands cut from the dried gel (Fig. 3, a-c). Sequence analysis revealed the presence in conformers b and c of a unique mutation at codon 282 and 270, respectively (Fig. 4). Both mutations were present in conformer a (Fig. 4).

Analysis of p53 and WAF-1. The results of Western blot analysis of p53 protein are shown in Fig. 5. A reduced antibody reactivity was observed in the IGROV-1 cell line compared with the two cisplatin-resistant variants. Since the antibody used reacts with a conserved domain present in both wild-type and mutant p53, this observation indicates that mutations are associated with p53 protein accumulation. The levels of p53 were increased after challenge by DNA-damaging agents (irradiation or cisplatin) in sensitive cells (Fig. 6). To verify the functional role of p53 in the IGROV-1 cells, we measured the levels of p21WAF-1 after DNA damage. The parental IGROV-1 cells exhibited an appreciable level of WAF-1 protein, with a marked increase of expression already 4 h after irradiation as expected in cells expressing wild-type p53. In cells treated with cisplatin, the increase was evident at 24 h after treatment. In contrast, resistant cells showed no detectable levels of WAF-1 before and after irradiation. In addition, p53 expression was examined by flow cytometry in sensitive and resistant cells after DNA-damaging treatment (Table 3). In the sensitive cell line starting at 24 h after treatment, the protein expression was increased both after cisplatin and, at a lower extent, after radiation. No appreciable changes in p53 expression were observed in the resistant cell lines.

Bax Expression. Northern blotting indicated lower levels of bax mRNA in the drug-resistant than in drug-sensitive cells (Fig. 7). This was confirmed by Western blot analysis of cell lysates, which revealed a marked reduction of bax expression in IGROV-1/Pt 0.5 and IGROV-1/Pt 1 cells in comparison to IGROV-1 cells (data not shown). To explore whether the reduced expression was related to p53 status, a CAT reporter gene construct containing the bax promoter was used for transient transfection experiments. The results shown in Fig. 8 indicated that the basal CAT activity was higher in the IGROV-1 cells than in the cisplatin-resistant variant IGROV-1/Pt 1. In parental cells, cotransfection with reporter gene plasmid and plasmid DNA encoding wild-type p53 protein showed an almost 4-fold increase of CAT activity compared with plasmid encoding a mutant p53 (179His→Glu) or no p53 protein (Fig. 7, columns 1–3). A strong transactivation (around 50-fold) by wild-type but not mutant p53 was observed in IGROV-1/Pt 1 (Fig. 7, columns 4–6).

DISCUSSION

Mutation of p53 is one of the most frequently occurring genetic lesions found in a variety of human cancers, including ovarian carcinoma (26). The p53 gene has been reported to be mutated, with consequent overexpression of p53 protein, in 30–50% of ovarian carcinomas. This alteration has been more frequently associated with tumor progression (27). The p53 gene encodes a phosphoprotein, which functions as a transcriptional enhancer of several DNA dam-

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age- and growth arrest-induced genes via sequence-specific DNA binding (28). p53 is recognized as an important component of the pathway leading from DNA damage to apoptosis (9, 10). Several studies have suggested that p53 is involved in activation of the apoptotic program induced by DNA-damaging chemotherapeutic agents (29). A relationship has been proposed between wild-type p53 expression and the ability of cells to undergo apoptosis (30). However, the effect of p53 in mediating cellular responses to DNA damage may be cell type specific and/or lesion specific. Conflicting results have been reported on the role of mutant p53 in determining the sensitivity/resistance status (31—34). Brown et al. (31) reported that introduction of a mutant p53 into a cisplatin-resistant ovarian carcinoma (A2780) cell line results in a sensitization of these cells to cisplatin. Modulation of drug resistance by the mutant p53 may be dependent on the cell type, since the resistant cells overexpressed wild-type p53. It remains to be determined whether the different mutations observed in various cell systems are all associated with a loss of normal functions of p53.

In the present study, we investigated two cisplatin-resistant ovarian cancer cell lines selected by continuous exposure to the cytotoxic drug. The resistant sublines were cross-resistant to other DNA-damaging cytotoxic agents, including an alkylating agent and ionizing radiation. A preliminary pharmacological characterization of the selected cell lines indicated that cisplatin resistance is not related to alterations in drug accumulation (data not shown) or accessibility to the primary target (DNA). Since, in the examined cell systems, the reduced sensitivity to cisplatin treatment paralleled a resistance to apoptosis induction (i.e., equitoxic doses are required to induce comparable level of apoptosis), we have tested the hypothesis that p53 alterations could lead to the relative resistance to cisplatin. To our knowledge, this is the first report describing the occurrence of cisplatin resistance in association with mutation of the p53 gene. Whatever the process of cisplatin resistance development (selection or induction) may be, this finding is indirect evidence that ovarian cancer cells with a mutant p53 gene have a reduced sensitivity to cisplatin, and, thus, wild-type p53 may have a role as a determinant of tumor cell responsiveness. A peculiar feature of these variants is the simultaneous presence of two mutations on different alleles found at codons 270 and 282 in the cisplatin-resistant cells. These mutants belong to class I (35), since the involved residues are in the DNA-binding domain. Whether the mutations are associated with a conformational change remains to be determined. Class I mutants are known not to bind antibody P Ab 240 (35); thus, it is also likely that the mutations at codons 270 and 282 found in cisplatin-resistant ovarian cancer cells have no marked influence on protein conformation. Indeed, mutations in the same region (i.e., Arg273 and Arg280) are P Ab 2401P Ab 1620 as expected for the native status of p53 (36, 37). This possibility is consistent with a modest accumulation in p53 protein levels in resistant cells. However, these mutations are expected to eliminate critical DNA contacts resulting in p53 inactivation. Loss of normal functions

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Table 3 Changes of p53 expression after challenge by DNA-damaging agents

<table>
<thead>
<tr>
<th></th>
<th>IGROV-1</th>
<th>IGROV-1/Pt 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h   48 h  72 h  96 h</td>
<td>24 h   48 h  72 h  96 h</td>
</tr>
<tr>
<td>Cisplatin a</td>
<td></td>
<td>148  162  134  161</td>
</tr>
<tr>
<td>Radiation b</td>
<td>131</td>
<td>137  114  86   60</td>
</tr>
</tbody>
</table>

a Values are expressed as percentage of the control untreated cells.
b Cells were exposed to equitoxic concentrations for 1 h (i.e., 10 and 300 µg/ml in IGROV-1 and IGROV-1/Pt 1 cells, respectively).
c Cells were exposed to equitoxic doses (i.e., 2 and 4 Gy for IGROV-1 and IGROV-1/Pt 1, respectively).

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D. Delia, unpublished observation.
Evidence is provided that p53 mutations are associated with the in vitro developed drug-resistant phenotype in an ovarian carcinoma cellular model. The most relevant repercussion of the mutations in p53 appears to be a marked reduction in transcription of the bax gene, a promoter of apoptosis. The parental IGROV-1 cells, carrying a wild-type p53, showed an increase of p53 and WAF-1 expression and a control of the G1 population after DNA damage and a high level of apoptosis induction at pharmacologically relevant concentrations of cisplatin. On the contrary, in the resistant cells, carrying a mutated p53 and characterized by lack of expression of WAF-1 and reduced expression of bax, DNA-damaging drugs induced no modifications of p53 protein levels, cell accumulation in G2, nor a reduced apoptotic response. Additional studies are in progress to clarify whether the above-described mutations arise as a consequence of selection of a preexisting cell subpopulation carrying mutated p53 in IGROV-1 cell lines or whether they are de novo induced during continuous exposure to cisplatin.

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