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DNA Repair in Cisplatin-Sensitive and Resistant Human Cell Lines Measured in Specific Genes by Quantitative Polymerase Chain Reaction

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ABSTRACT. More than 80% of patients with testicular germ cell tumours (TGCT) are cured using cisplatin-based combination chemotherapy, and resistance to cisplatin is the final barrier to the cure of nearly all patients with this disease. In this study, we used quantitative polymerase chain reaction (Q-PCR) to investigate the role of DNA repair in cisplatin resistance in two genes, one transcribed and one not transcribed. Three pairs of cisplatin-sensitive and resistant cell lines were used, two derived from TGCT and one from a bladder cancer. In these pairs of sublines, we observed no major differences between the repair of cisplatin-induced damage in the transcribed and nontranscribed genes, nor did there appear to be any relationship between DNA repair capacity and the development of cisplatin resistance. Despite the strong indication that the sensitivity of testis tumour cells to cisplatin is related to their reduced ability to repair cisplatin-damaged DNA, these cells apparently do not become resistant to cisplatin by acquiring DNA repair capacity. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1729–734, 1996.

KEY WORDS. DNA repair; testis tumours; cisplatin; drug response; quantitative PCR

Cisplatin is a chemotherapeutic drug that is used in the treatment of many cancers, including those of the ovary, testis, head and neck, bladder and lung [1]. Its addition to combination chemotherapy for TGCTs§ has resulted in response rates approaching 100% and cure in more than 80% of patients with metastatic disease. Despite this remarkable success, for those patients who develop cisplatin-resistant disease following cisplatin-based combination chemotherapy, there is little more therapeutic benefit that can be offered. Thus, cisplatin resistance is the last major obstacle to the cure of all patients with TGCTs.

Knowledge of the mechanisms controlling cisplatin resistance has been derived predominantly from *in vitro* studies of drug-resistant cell lines. In such cells, resistance to cisplatin is due to different mechanisms, including decreased cellular accumulation of cisplatin, increased drug detoxification and enhanced DNA repair capacity [2–4].

There is considerable interest in the role of DNA repair in drug resistance. Using cell lines with acquired resistance to cisplatin, an increase in DNA repair has been observed both at the level of the whole genome [5, 6] and in individual genes [7, 8]. DNA repair is not only involved in cisplatin resistance, it can also control sensitivity to cisplatin and other DNA-damaging agents. Cell lines derived from patients with DNA repair disorders (such as xeroderma pigmentosum and Fanconi's anaemia) are hypersensitive to cisplatin [9]. Thus, drug sensitivity is associated with defective DNA repair. There is analogy between testis tumour cells and cells derived from patients with these DNA repair disorders, as both are inherently sensitive to cisplatin (and other DNA-damaging agents) and show a low capacity for DNA repair [9, 10]. Consequently, the development of resistance to cisplatin by patients with metastatic TGCT may be related to changes in their capacity to repair cisplatin-damaged DNA.

In the present study, we have looked for evidence of enhanced DNA repair capacity in cisplatin-resistant testis tumour and bladder cancer cell lines. Recent technical developments have made it possible to measure DNA repair in selected regions of specific genes by using Q-PCR. Three pairs of cisplatin-resistant and -sensitive cell lines were selected, two derived from TGCT and one from a bladder cancer. Cisplatin-induced damage and repair were measured in selected sequences of both the actively transcribed N-*ras* gene and the inactive *CD3δ* genes. In addition, cellular uptake of cisplatin and its binding to DNA were mea-

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§ *Abbreviations*: Q-PCR, quantitative polymerase chain reaction; TGCT, testicular germ cell tumour; IC_{50}, inhibitory concentration reducing colony-forming ability by 50%; RNase, ribonuclease; TCA, trichloroacetic acid; ICL, interstrand cross links.

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sured to detect any differences in the rate of intracellular accumulation of cisplatin and the number of cisplatin–DNA adducts because these two features can also be associated with cisplatin resistance [11, 12].

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Details concerning the derivations of the cisplatin-sensitive and resistant cell lines have been published previously [13, 14]. All the cell lines were grown routinely as monolayers in 25-cm^2 tissue culture flasks in RPMI medium (ICRF) with 10% heat-inactivated foetal calf serum and 2 mM l-glutamine at 36.5°C in a humidified atmosphere of 5% CO_2 in air. Each cell line was used for a maximum of 15 passages to minimise changes that might occur during long-term culture. Cisplatin (Sigma, Poole, UK) was prepared immediately before use by dissolving in water to a stock concentration of 1 mg/mL^{-1} and sterilized by using a 0.22-μ filter.

Cisplatin Sensitivity Measurements

Cytotoxicity was determined by plating 2500 cells (testis tumour cell lines) or 750 cells (bladder tumour cell lines) in 5-cm Petri dishes containing 5 mL medium and incubated overnight at 36.5°C. Four dishes were prepared for untreated controls and 3 dishes for each concentration of cisplatin. After a 1-hr exposure to a range of concentrations of cisplatin, the cells were washed twice with PBS and incubated in fresh medium for 10–14 days. Colonies were fixed with methanol, stained with 10% Giemsa and those consisting of 50 or more cells were counted. Inhibition of colony formation in treated dishes was expressed as a percentage of colony formation in untreated controls. The results are derived from a minimum of three independent experiments on each cell line. The IC_{50} values were calculated by using linear regression analysis of the dose-response data, taking the points in the exponential region of the curve. The data in Table 1 show the mean value of the three independent calculations of the IC_{50} and the standard error.

Intracellular Platinum Accumulation

The 1.5×10^6 cells were seeded in 5-cm dishes and allowed to grow overnight. The cells were treated with cisplatin (50 μM) for 1 hr, washed free of drug with PBS, scraped and harvested in 0.5 mL PBS. The samples were then sonicated (Soniprep 150, MSE). The total intracellular platinum content was determined by atomic absorption spectroscopy.

DNA Platination

Between 5×10^7 and 10^8 cells growing exponentially in 175-cm^2 culture flasks were incubated with cisplatin (50 μM) for 1 hr and harvested immediately after drug exposure. The DNA was isolated by treatment with proteinase K, 0.5% SDS and lysis buffer (150mM NaCl, 10mM Tris pH 8.0, 10mM EDTA, pH 8.0) overnight at 37°C. After phenol/chloroform extraction (2×) and chloroform extraction (1×), the DNA solution was treated with RNase (10 mg/mL) at 37°C for 1 hr followed by phenol/chloroform (1×) and chloroform extraction (1×). DNA was precipitated with a 1/10 volume of sodium acetate (3 M) and 2 volumes of cold ethanol (absolute), washed twice with ethanol (70%), dried and dissolved in 0.1 M nitric acid. The amount of platinum bound to DNA was measured by atomic absorption spectroscopy [14], and the amount of DNA was determined by measuring the absorbance at 260 nm.

Gene-specific Damage and Repair of Cisplatin-induced Lesions

Cell culture and drug treatment were performed in 6-well plates (Nunc); 10^6 cells were treated with a range of concentrations of cisplatin for 5 hr, washed with PBS and either trypsinised for DNA isolation or incubated for another 24 hr in fresh medium for repair studies. DNA isolation and quantitative PCR were performed as previously described [15]. Briefly, cells were lysed with a solution composed of 340 μL lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% w/v SDS) and 100 μL of 5 M sodium perchlorate. After vortexing, the cell lysate was shaken at 37°C for 20 min and then at 65°C for 20 min; 580 μL of chloroform was added to the solution and the mixture was rotated for 20 min at room temperature before centrifugation at 13,000 rpm in a microfuge. Of the upper layer, 330 μL was transferred to a fresh eppendorf and the DNA precipitated with 660 μL ethanol (absolute), washed twice with ethanol (70%), dried and resuspended in 400 μL H_2O. PCR was performed with 25 μL DNA suspension (equivalent to 5×10^4 cells) in a 100-μL reaction mixture containing 50 pmol of each primer (Pharmacia), 2 units *Taq* polymerase (Pharmacia), 120 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 0.75 mM $MgCl_2$, buffer IV (Advanced Biotechnologies) and 1 μCi (α-^{32}P)dCTP (Amersham). To amplify the *CD3δ* gene fragment, we used the forward primer 5′-TGA GGA CAG AGT GTT TGT GAA-3′ and the reverse primer 5′-AGA GTA ACT CCC AGC TGA GAC-3′. The amplification product was 934 bp in length and contained part of exon 2, intron 2 and exon 3. The amplification for the N-*ras* gene showed a 1053-bp fragment covering the first intron and was performed with the forward primer 5′-GCC TGG TTA CTG TGT CCT GT-3′ and the reverse primer 5′-GCG AGC CAC ATC TAC AGT AC-3′. 5% DMSO was added to the reaction mixture for the amplification of the fragment of the N-*ras* gene. The mixture was overlaid with 100 μL mineral oil and the reaction was done on a Perkin-Elmer 480 Thermal Cycler. For the N-*ras* fragment, an initial denaturation step of 2′ at 94°C was followed by 25 cycles of 94°C for 1′, 59°C for 1′ and 72°C for 1′, with a final incubation of 4′ at 72°C. The *CD3δ* gene fragment was amplified after an initial denaturation step of 5′ at

96°C, followed by 25 cycles of 96°C for 1', 60°C for 1' and 70°C for 1' and finished by a final incubation of 4' at 70°C. These conditions ensured that the reaction was still in exponential phase when the PCR stopped. The PCR product was quantitated by precipitating 40 μL of PCR mixture with 1 mL TCA (5% w/v trichloroacetic acid, 20 mM tetrasodium pyrophosphate). The precipitate was captured on Whatman filter discs held in a 12-position vacuum manifold (Millipore). The filters were washed free of unincorporated α-^{32}P-dCTP with 10 mL TCA (5%) and 10 mL absolute ethanol, placed in vials containing 5 mL scintillation fluid (Ecoscint, National Diagnostics) and counted by using a Beckman LS1800 scintillation counter. The lesions per region were determined according to the formula -1n (Ad/A), where A = PCR product from the undamaged template and Ad = product from the damaged template. To demonstrate that the PCR reaction was specific and produced one product only, 10 μL of PCR product were subjected to electrophoresis on a 1.5% agarose gel overnight at 42 V. The gel was then dried and exposed to autoradiographic film (Kodak).

RESULTS

Cisplatin Sensitivities

The survival curves of the parental cell lines and their cisplatin-resistant sublines after treatment with a range of concentrations of cisplatin for 1 hr are shown in Fig. 1 and the IC_{50} values are given in Table 1. The parental testis tumour cell lines SuSa and GCT27 showed similar IC_{50} values (5.4 and 4.8 μM), whereas the bladder cancer cell line RT112 was approximately 3× more resistant, with an IC_{50} value of 13.5 μM (Table 1). The sublines were more resistant than their parental cell lines by factors of 4.1 (GCT27cisR), 3.5 (SuSa-CP) and 4.5 (RT112-CP).

Cisplatin Uptake

Cisplatin uptake was measured after treatment of the cell lines with cisplatin (50 μM) for 1 hr. Platinum accumulation was similar in SuSa and its subline SuSa-CP. In contrast, the resistant sublines RT112-CP and GCT27cisR had a 3–4-fold reduction of platinum accumulation as compared with their parental cell lines (Table 1).

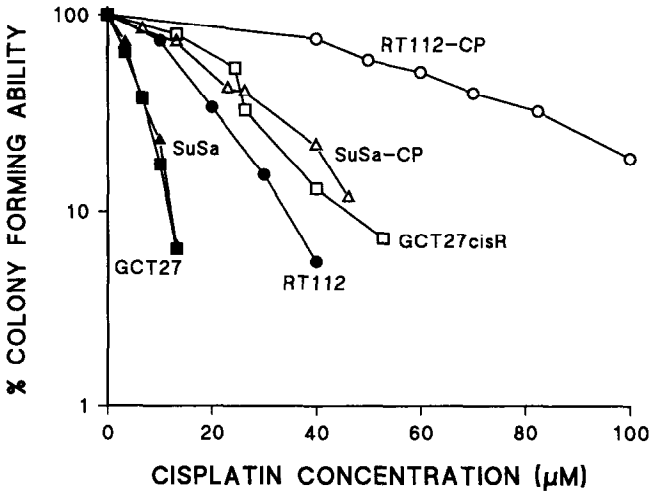

FIG. 1. Clonogenic cell survival curves of parental and cisplatin-resistant tumour cell lines after a 1 hr exposure to a range of concentrations of cisplatin.

DNA Platination

The amount of platinum bound to DNA after treatment with cisplatin for 1 hr was 2.6-fold greater in RT112 cells than in the resistant subline (108.1 and 41.1 nmol Pt/gDNA, respectively) and 1.6-fold greater in GCT27 than in its resistant subline (68.6 and 41.4 nmol Pt/gDNA, respectively). In contrast, the amount of cisplatin bound to the DNA of the cisplatin resistant subline, SuSa-CP, was 1.4-fold greater than that in the parental line SuSa.

DNA Repair

To investigate DNA damage induction and repair within selected genes, cells were treated with cisplatin at concentrations up to 100 μM for 5 hr. Incorporation of ^{14}C-thymidine showed that under these conditions there was minimal new DNA synthesis (<1%) at 100 μM cisplatin in all the cell lines except RT112-CP, in which the incorporation was 16% of that of untreated control cells.

The bladder cancer cell line RT112 showed the expected pattern of DNA platination and its removal at 24 hr (Table 2 and Fig. 2A,B). The cisplatin-resistant subline showed less platination immediately after cisplatin exposure, but the proportion of lesions removed during the 24-hr recovery period was similar in both lines. In complete contrast, GCT27 and its cisplatin-resistant subline showed no evidence of repair. Furthermore, the number of lesions tended to have increased after the "recovery" period. Levels of platination were similar in both genes and in both cell lines. SuSa appeared to have a low repair capacity, but no evidence of repair was observed in the resistant subline.

DISCUSSION

Cisplatin is the most effective single agent for the treatment of bladder and TGCTs, and development of cisplatin

TABLE 1. Cisplatin sensitivity, cisplatin accumulation and total DNA platination in three pairs of sensitive and resistant cell lines

Cell line	IC_{50} (μM)	Pt Accumulation (ng Pt/10^6 cells)	DNA Platination (nmol Pt/gDNA)
GCT27	4.8 ± 0.8	43.0 ± 12.9	68.6 ± 14.7
GCT27cisR	20.1 ± 0.8	10.9 ± 3.5	41.4 ± 16.7
Ratio	4.1	0.25	0.60
SuSa	5.4 ± 1.0	39.3 ± 6.4	45.9 ± 7.7
SuSa-CP	18.9 ± 1.9	31.6 ± 7.1	65.1 ± 9.6
Ratio	3.5	0.80	1.42
RT112	13.5 ± 3.0	56.1 ± 6.9	108.1 ± 33.1
RT112-CP	60.7 ± 2.9	17.2 ± 1.5	41.1 ± 10.3
Ratio	4.5	0.31	0.38

TABLE 2. Number of lesions in a region of the *CD3δ* gene and *N-ras* gene in three pairs of cisplatin-sensitive and -resistant cell lines after treatment with cisplatin for 5 hr

Cell line	Cisplatin (μM)	*CD3δ* (lesions/kb)		*N-RAS* (lesions/kb)	
		0 hr	24 hr	0 hr	24 hr
RT112	25	0.69 ± 0.22	0.06 ± 0.01	0.42 ± 0.06	0.09 ± 0.01
	50	1.28 ± 0.59	0.32 ± 0.14	0.98 ± 0.37	0.42 ± 0.06
	100	1.87 ± 0.07	0.59 ± 0.16	1.56 ± 0.04	0.84 ± 0.18
RT112-CP	25	0.32 ± 0.42	0.10 ± 0.10	0.28 ± 0.09	0.09 ± 0.09
	50	0.85 ± 0.53	0.10 ± 0.10	0.65 ± 0.28	0.18 ± 0.28
	100	0.96 ± 0.42	0.32 ± 0.10	0.84 ± 0.37	0.37 ± 0.28
SuSa	25	0.18 ± 0.02	0.32 ± 0.14	0.09 ± 0.01	0.46 ± 0.38
	50	0.69 ± 0.22	0.37 ± 0.07	0.47 ± 0.13	0.56 ± 0.13
	100	1.19 ± 0.27	0.74 ± 0.53	1.03 ± 0.09	0.75 ± 0.09
SuSa-CP	25	0.42 ± 0.10	0.64 ± 0.10	0.37 ± 0.09	0.47 ± 0.01
	50	0.74 ± 0.10	0.85 ± 0.10	0.47 ± 0.01	0.75 ± 0.09
	100	1.17 ± 0.53	1.07 ± 0.32	1.03 ± 0.65	1.22 ± 0.37
GCT27	25	0.16 ± 0.20	0.67 ± 0.84	0.23 ± 0.32	0
	50	0.37 ± 0.07	1.60 ± 1.28	0.51 ± 0.46	1.17 ± 0.32
	100	0.38 ± 0.21	1.07 ± 1.07	1.09 ± 0.28	1.88 ± 0.18
GCT27-cisR	25	0.21 ± 0.21	0	0.31 ± 0.13	0.47 ± 0.39
	50	0.37 ± 0.19	0.32 ± 0.28	0.50 ± 0.47	0.63 ± 0.31
	100	0.53 ± 0.10	0.85 ± 0.21	0.56 ± 0.18	0.94 ± 0.01

resistance is the most common reason for the failure of cancer chemotherapy in both these diseases. Using three pairs of sensitive parent and cisplatin-resistant cell lines derived from bladder and TGCTs, we have compared their capacity for repair of cisplatin-damaged DNA within defined regions of selected genes by using Q-PCR.

Development of resistance to cisplatin is associated with reduced intracellular accumulation of the drug in some resistant sublines [5, 16]. In agreement, we found that following exposure to cisplatin for 1 hr the intracellular accumulation of cisplatin was significantly decreased in the 4.1-fold resistant testis tumour subline GCT27cisR as compared with its parental line and in the 4.5-fold resistant bladder tumour subline RT112-CP when compared with RT112 (Table 1). Cisplatin resistance has been associated with increased efflux as a result of functional overexpression of an ATP-dependent glutathione S-conjugate pump [17, 18], and a similar mechanism may operate in GCT27cisR and RT112-CP. In contrast, there was no significant difference in intracellular accumulation of cisplatin between SuSa and its 3.4-fold-more resistant subline SuSa-CP.

The decrease in drug accumulation in RT112-CP as opposed to RT112 was reflected by a decrease in the platination of DNA. Reduction in DNA platination is associated with the development of cisplatin resistance in many cell lines [5, 12]. The same pattern was observed in GCT27 and GCT27cisR, although there was a relatively small reduction in DNA platination in the resistant line, in agreement with previous findings in this cell line [14].

Enhanced DNA repair capacity is one of the mechanisms associated with the development of resistance to cisplatin [3, 4]. Using cisplatin-sensitive and -resistant cell lines, we measured the disappearance of cisplatin from whole geno-

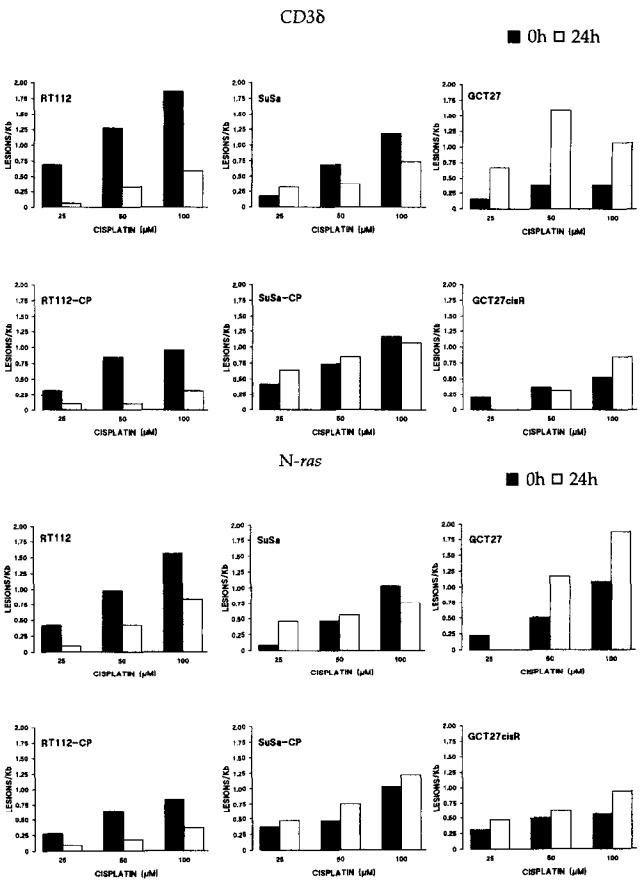

FIG. 2. Histograms showing the number of lesions per kilobase of DNA in *CD3δ* N-*ras* genes following a 5 hr exposure to a range of concentrations of cisplatin and following a 24-hr recovery period in three pairs of sensitive parental and cisplatin-resistant sublines.

mic DNA and from selected regions of two genes, the actively transcribed N-*ras* and the inactive *CD3* gene. None of the sublines showed an increase in repair within either of the two selected genes compared with their respective parental line. RT112-CP and RT112 were both repair proficient, and the proportion of the lesions removed during the 24 hr recovery period was similar in both the lines.

SuSa showed some inconsistency in that there was a small reduction at some concentrations in the numbers of lesions following the 24-hr recovery period. The differences were not significant and probably reflect the inherent variability between experiments of the technique. There was no removal of lesions from SuSa-CP DNA in either gene. There was also no increase in the ability to remove cisplatin lesions in GCT27cisR compared with GCT27. In an earlier study using these two cell lines, there was some evidence for increased DNA repair in GCT27cisR [14], but this was at later time points (48 and 72 hr) following a shorter exposure (2 hr) and at the level of the whole genome. In GCT27 and GCT27cisR, there was much higher variability between experiments and, unexpectedly, cisplatin lesions appeared to accumulate during the recovery period. At the supralethal doses of cisplatin used, there may have been some degradation of the damaged DNA during the recovery period, thus giving the appearance of more lesions. However, this explanation seems unlikely given the results of an earlier study, which also observed an increase in the number of lesions during the repair period in some testis tumour cell lines following exposure to cisplatin, but at the much lower dose of 5 μg/mL (16.7 μM) for 1 hr [19]. This result indicates that further lesions may have been formed [19], which could be the result of intracellular redistribution of cisplatin during the recovery period. Another possibility is that cisplatin monoadducts were converted to cross links during the recovery period because cross links may block *Taq* polymerase more readily.

In contrast with our findings, enhanced repair of cisplatin-damaged DNA has been observed in other cisplatin-resistant cell lines. At the level of the whole genome, an increase in DNA repair capacity was seen in cisplatin-resistant colon and ovarian cancer cell lines [5, 6]. Gene-specific repair can also contribute to the development of cisplatin resistance [7]. In another study, although no increase in whole genome or gene-specific repair of cisplatin-induced intrastrand cross links was observed in cisplatin-resistant ovarian cancer cells, ICL were removed more efficiently [8]. ICL account for only about 1% of all cisplatin-induced lesions [20], and Q-PCR does not distinguish between intra- and interstrand cross links. A decrease in the frequency of ICL after a 24-hr repair period would therefore not be detectable by Q-PCR.

Increased tolerance to DNA damage can be another explanation for increased resistance to cisplatin. Cells can achieve damage tolerance by bypassing DNA lesions during replication or transcription. This ability to bypass cisplatin-induced adducts has been described in bacteria and eukaryotic cells [21] and has recently been attributed to DNA polymerase-β [22]. Replicative bypass of platinum adducts is associated with resistance to cisplatin in murine leukemia cells [23].

The response of testis tumour cells to stress may be a key component of their sensitivity to chemotherapeutic drugs. Compared with bladder cancer cells, testis tumour cells contain low levels of heat shock protein 27, and overexpression of the human gene for hsp27 following its transfection into testis tumour cells resulted in increased resistance to heat shock, cisplatin and doxorubicin [24]. Testis tumour cells also contain high levels of wild-type p53 protein and low levels of bcl-2 protein, and these characteristics may make them more susceptible to undergo apoptosis [25].

Testis tumour cells, as confirmed in this study, have a limited capacity to repair cisplatin-damaged DNA. This limited capacity is associated with their hypersensitivity to cisplatin, a feature they share with cells derived from patients with inherited DNA repair disorders. However, we have failed to demonstrate any connection between the development of cisplatin resistance and enhanced DNA repair capacity in three pairs of cisplatin-sensitive and -resistant cell lines.

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