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Deficient Repair of Cisplatin–DNA Adducts Identified in Human Testicular Teratoma Cell Lines Established From Tumours From Untreated Patients

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Germ cell tumour lines appear generally more sensitive *in vitro* to cisplatin than other cultured cell lines, reflecting their clinical responsiveness. We proposed (*Cancer Res* 1988, 48, 3019–3024) that cisplatin hypersensitivity, expressed by a testicular teratoma line (SuSa), might be explained by an inability to repair platinated DNA. We have now quantitated cisplatin cytotoxicity by clonogenic assay, and platinum (Pt)–DNA adduct formation and removal immunochemically in four other testicular teratoma continuous cell lines (GCT46, GCT27 clone 4, H32 and H12.1), all established from tissue from non-drug-treated patients. For 1-h *in vitro* drug exposures, the cisplatin concentration required to reduce survival by 50% (IC_{50}) ranged from 0.09 to 0.42 μg/ml (0.3–1.4 μM). Immediately following a 1-h exposure to 5 μ/ml cisplatin, total cellular platination levels ranged from 4.5 to 36.8 fmol Pt per μg DNA, with lower platination occurring in the most sensitive lines. Following an 18-h post-treatment incubation period, the levels of the major *cis*-Pt-$(NH_3)_2$d(pGpG) (Pt–GG) adducts were not significantly reduced in any of the four lines, indicating a general deficiency in either the rate or extent of removal of these lesions. Deficient removal of the *cis*-Pt-$(NH_3)_2$d(pApG) adducts was also noted in two of the lines. DNA polymerase β gene expression was comparable in all the tested testicular lines established from previously untreated patients, but markedly lower than that identified in the 833K testicular line, established from a drug-treated patient and identified earlier as proficient in Pt–GG adduct removal (*Cancer Res* 1988, 48, 3019–3024). Expression of the DNA excision repair genes *ERCC-1* and *XPBC/ERCC-3* was not significantly different in any of the five lines tested, including the 833K cell line. These data provide evidence of the apparent inability of testicular cell lines, derived from untreated tumours, to repair the major platinum-DNA intrastrand crosslinks, and so provide a biological basis for their hypersensitivity to cisplatin.

Key words: germ cell tumour lines, cisplatin, platinum–DNA adducts, repair deficiency, DNA polymerase β, *ERCC-1*, *XPBC/ERCC-3*

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INTRODUCTION

CLINICAL AND experimental laboratory data indicate that human testicular non-seminomatous germ cell tumours are unusually sensitive to the antineoplastic drug cisplatin [1–6]. Studies aimed at establishing the basis for this hypersensitivity have (i) provided evidence that human embryonal carcinoma cells are inherently sensitive to DNA-bound platinum adducts [4] and (ii) proposed, on the basis of investigating one testicular teratoma cell line (SuSa) established from a previously untreated patient, that an inability to repair platinated DNA may be involved [7]. To extend this initial observation [7], we have now quantitated cisplatin cytotoxicity by clonogenic assay, and platinum (Pt)–DNA adduct formation and removal in a panel of four additional human testicular tumour cell lines established from tissue from non-drug-treated patients. In addition, we have studied the expression of several repair-associated genes, including DNA polymerase β [8] and the DNA nucleotide excision repair genes, *ERCC-1* and *XPBC/ERCC-3*, which are involved in repair of several types of DNA lesions, including cisplatin–DNA adducts [9–11]. Our results provide evidence of an apparent inability of these testicular tumour cell lines,

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established from untreated tumours, to repair the major DNA–DNA intrastrand crosslinks in the sequence pGpG.

MATERIALS NAD METHODS

Cell lines and culture techniques

The testicular tumour cell lines used and details of their fundamental growth characteristics are listed in Table 1 [12–14]. GCT27 clone 4 is a nullipotent clonal derivative of the GCT27 cells described earlier [4] with essentially identical biological characteristics. All media and sera were purchased from Gibco-Biocult (Reinfrewshire, U.K.). Population doubling times were calculated from cell counts of duplicate 3-cm dishes (Nunc, Roskilde, Denmark) containing 10^4–10^5 logarithmically-growing cells, at daily intervals, using a model ZM Coulter Counter (Coulter Electronics Ltd, Luton, U.K.). Cell volumes were also determined using this Coulter Counter.

Cytotoxicity assays

Cisplatin was purchased from Sigma Chemicals (Poole, U.K.) and dissolved in 0.9% sodium chloride solution immediately prior to use. Sensitivity to cisplatin was determined after a 1-h exposure to a range of drug concentrations, with the colony-forming ability of surviving cells being assessed for each line as listed in Table 1. These different conditions optimised for clonogenic survival. All assays were performed in triplicate on duplicate cultures on a minimum of two independent occasions.

Quantitation of Pt–DNA (Pt–DNA) adducts

For quantitation of Pt–DNA adducts, cells were exposed for 1 h to 5 μg/ml of cisplatin (16.6 μM) dissolved in 0.9% sodium chloride solution. After drug exposure, the cells were washed and harvested either immediately or 18 h after incubation in drug-free medium. DNA isolation was carried out, using a combined phenol and chloroform-isoamyl alcohol technique [7, 14]. Lysis of cells was carried out overnight at 50°C in the presence of 100 mM ammonium bicarbonate solution, to inactivate mono-functionally bound drug, and proteinase K, to digest the protein in DNA–protein crosslinks and destroy nuclease activity. DNA was then digested to nucleotides and Pt-containing oligonucleotides as described previously [16], except that sodium azide was omitted from the incubation medium. Separation of platinated digestion products was carried out using an anion exchange chromatography column (Mono Q; Pharmacia, Sweden). Details of the enzyme-linked immunosorbent assay used to quantitate the Pt–DNA adducts has been described in an earlier publication [15, 16]. Since the occurrence of Pt–DNA adducts is quantitated per gram of DNA isolated, correction for dilution by DNA synthesis during the 18-h post-treatment incubation period was carried out using parallel cell cultures, following the exact procedure detailed previously [7]. The specific activity of radiolabelled DNA decreased in all four cell lines during the 18-h post-treatment incubation period to approximately 80% of the values immediately following drug treatment. Calculated dilution factors were 0.75 for GCT27 clone 4, 0.77 for H12.1, and 0.79 for H32 and GCT46 cells.

RNA isolation and northern blotting

Total RNA was extracted from 5×10^7 cells using guanidinium isothiocyanate [16], and separated by isopyknic centrifugation on a caesium trifluoracetate gradient (Pharmacia GB Ltd, Milton Keynes, U.K.). Polyadenalate-containing (poly A^+) mRNA was extracted from approximately 10^8 cells using the Invitrogen fastTrack mRNA isolation kit (AMS Biotechnology, Burford, U.K.), involving purification by oligo(dT)-cellulose column chromatography. The resultant RNA was denatured in 1 M glyoxal and 50% demethyl sulphoxide, fractionated on 1% agarose gels containing 6.7% formaldehyde, and transferred to nitrocellulose membranes (Zeta-probe, Bio-Rad Laboratories, Watford, U.K.) by capillary transfer in 10 × standard saline citrate as detailed previously [17]. Quantitation of the RNA samples was performed by ethidium bromide staining of the gels, and reprobing the filters with either phosphoglycerate kinase (PGK) [18] or enolase [19]. Hybridisation conditions have been previously described [16]. Dr S. Wilson (National Cancer Institute, Bethesda, Maryland, U.S.A.) supplied the human DNA polymerase β probe in the plasmid PuC9, and the

Table 1. Testicular tumour cell line characteristics

Cell line	Origin [ref.]	Media used	PDT (h)	Cell volume (μ^3)	CFA used	CFE (%)
GCT27 (clone 4)	Primary testicular teratoma carcinoma [4]	DMEM + 10% FCS + 1 μg/ml HC	23 ± 4	2726 ± 56	Cloning on plastic	5–20
GCT46	Lung metastasis from embryonal carcinoma with yolksac elements [4]	DMEM + 10% FCS + 1 μg/ml HC	28 ± 5	2910 ± 16	Cloning on plastic + 3T3 feeders	1–2
H12.1	Primary embryonal carcinoma with immature teratoma [12]	RPMI 1640 + 10% FCS	38 + 3	2491 + 25.5	Cloning in 0.17% agarose	1–5
H32	Primary embryonal carcinoma [12]	RPMI 1640 + 10% FCS	43 ± 5	2836 ± 24	Cloning in 0.17% agarose	1–5
SuSa	Primary testicular teratoma [13]	RPMI 1640 + 10% FCS	27 ± 2		Cloning in 0.3% agar	5–15
833K	Abdominal metastasis from a testicular germ cell tumour [14]	RPMI 1640 + 10% FCS	23 ± 3		Cloning on plastic	5–10

CFA, colony forming assay; CFE, colony forming efficiency; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HC, hydrocortisone; PDT, population doubling time of cells in logarithmic growth.

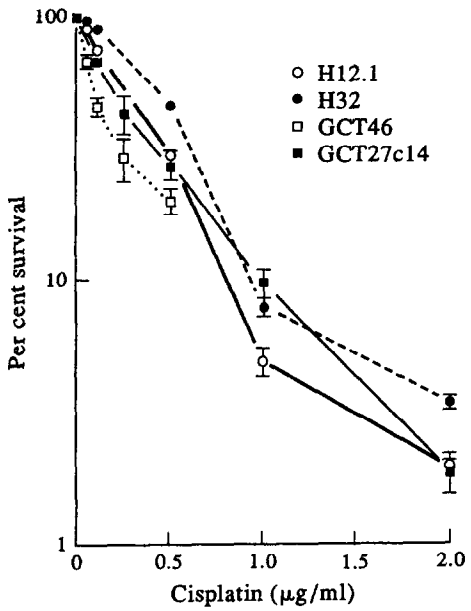

Figure 1. Survival curves for GCT46, GCT27 clone 4, H32 and H12.1 cells exposed to a range of cisplatin concentrations for 1 h, as judged by colony-forming assays. Each point represents the mean ± S.E. of two to three experiments in which duplicate cultures were tested.

cDNA (0.6 kb) was isolated by PstI digestion. Dr J.H.J. Hoeijmakers (Erasmus University, Rotterdam, the Netherlands) kindly provided the plasmids pE12-12 containing the *ERCC-1* cDNA [9] and pCD1-1 with the *EPBC/ERCC-3* cDNA [10]. The cDNA clone for human PGK/5 was obtained from A. Riggs (Beckman Research Institute, City of Hope National Medical Center, Duarte, California, U.S.A.) [20]. The cDNA coding for human α-enolase [19] was also used as a reference probe. Densitometry of the blots was carried out using an LKB laser densitometer with LKB software.

RESULTS

Figure 1 illustrates the dose–response curves following a 1-h exposure of logarithmically growing cultures of the four newly studied testicular tumour cell lines to a range of cisplatin concentrations. From repeat experiments, mean IC_{50} values (drug concentration required to reduce survival by 50%) were calculated and these are listed in Table 2. The different assay procedures were used so as to optimise for clonogenic cell survival in each cell line, and in this way it was considered appropriate to compare the IC_{50} values obtained. In all cases, IC_{50} values for cisplatin were below 0.5 μg/ml, and these values are even lower than those reported earlier for the SuSa and 833K cell lines, namely 0.9 ± 0.1 and 1.4 ± 0.1 μg/ml, respectively [7].

Levels of total platination of DNA, measured by competitive ELISA immediately and 18 h following exposure to 5 μg/ml for 1 h, are listed in Table 2, and histograms of specific adduct levels are shown in Figure 2. The two most cisplatin sensitive cell lines (GCT 27 clone 4 and GCT 46) had considerably lower levels of total platination than the two least sensitive lines. The major Pt–DNA adduct formed in all the cells lines was Pt–GG, comprising approximately 50–60% of the total platination immediately following drug exposure, with Pt-AG providing the next most abundant adduct (i.e. approximately for 20%) in two of the lines, H12.1 and H32, only.

Following an 18-h period of post-treatment incubation, none of these testicular tumour cell lines appeared proficient in adduct removal (Table 2). More specifically (see Figure 2), three of these cell lines appeared totally deficient in removing the major adduct Pt–GG, whilst the amount of loss by the GCT46 cells was very low, being less than 20% over this 18-h period. Two of these lines also appeared deficient in removing Pt–AG lesions, namely GCT46 and H32, and similarly the bifunctional lesion *cis*-$Pt(NH_3)_2d(GMP)_2$(Pt-(GMP)2) was not removed by three lines, with the fourth (H12.1) again showing only minimal removal (i.e. 18%). In the H32 cells, the total platination level following the 18-h post-treatment incubation period was considerably higher than that detected immediately following drug treatment (i.e. 1.8 times higher), which suggests that additional lesions were formed and not removed during that time period. Overall these data, using the four newly derived testicular teratoma cell lines established from untreated patients, are consistent with our earlier report of deficient removal of the major platinated intrastrand crosslinks by the SuSa testicular tumour cell line, also originating from a non-drug treated tumour [7].

Northern blots of poly A^+ mRNA (Figure 3), probed for the expression of DNA polymerase β, provide evidence of comparable levels in all three of the newly established lines tested. A similar level of expression was also identified in the SuSa cells, used as a control. Interestingly, however, these values were all considerably lower (by a factor of 4 determined by

Table 2. Cycotoxicity data and levels of total DNA platination measured by competitive ELISA immediately/zero time and 18 h following exposure to 5 μg/ml cisplatin for 1 h

Cell line	Cisplatin IC_{50} values (μg/ml)	Total platination* (fmol adducts μg of DNA) Zero time	18 h	% Removal
GCT27 clone 4	0.06 ± 0.05	7.5 ± 0.2	9.4 ± 0.4	0
GCT46	0.09 ± 0.05	4.5 ± 0.1	4.1 ± 0.1	8
H12.1	0.28 ± 0.03	36.8 ± 1.5	39.2 ± 1.4	0
H32	0.42 ± 0.03	32.9 ± 0.8	59.7 ± 0.9	0

*Total platination was calculated by adding together the amount of each of the four individual adducts. Values are the mean ± range of two different ELISAs utilising two samples independently derived, each performed on four dilutions in duplicate wells.

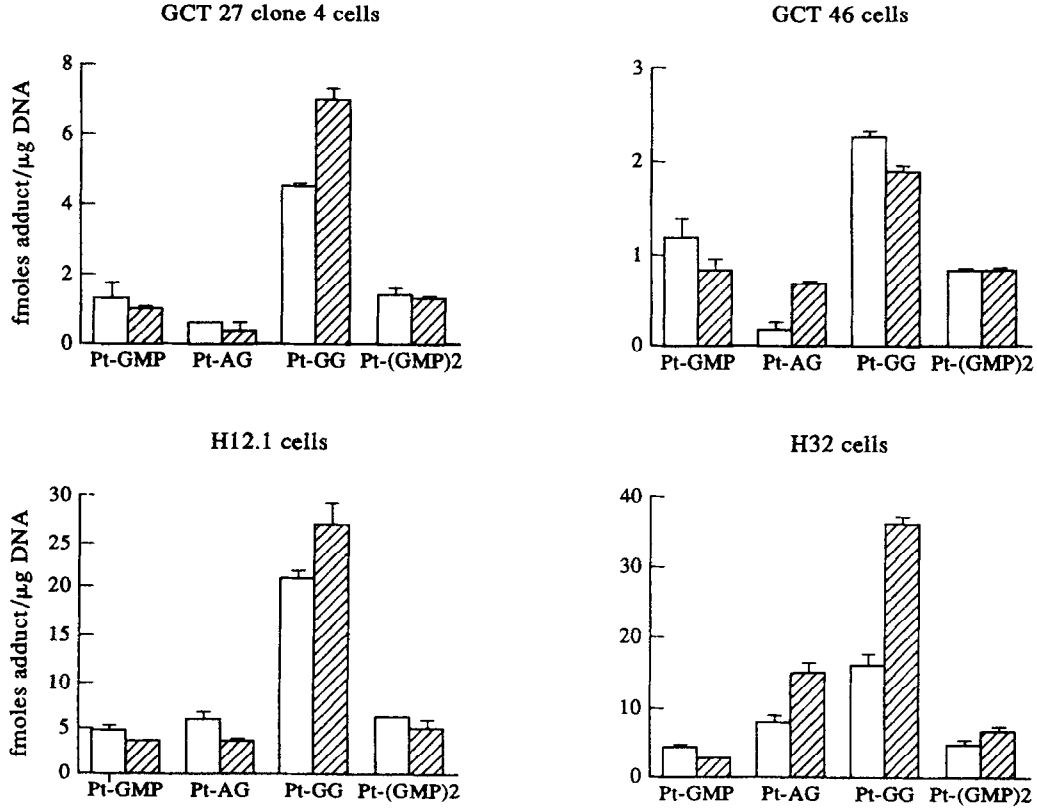

Figure 2. Induction and removal of Pt–GMP, Pt–AG, Pt–GG and Pt–(GMP)2 in the human testicular teratoma cell lines GCT27 clone 4, GCT46, H12.1 and H32. The number of adducts was determined immediately after a 1-h exposure to 5 μg/ml cisplatin (open boxes) and following an 18-h post-treatment incubation period (hatched boxes). Columns (± range) represent the mean (normalised for DNA content) of two different competitive ELISAs, utilising two samples independently derived and each performed in four dilutions on duplicate cells.

densitometry) than the level of expression of DNA polymerase β in the repair-proficient 833K cell line established from a tumour from a drug-treated patient [14].

Results of quantitation of northern blot analyses of total RNA for expression of *ERCC-1* and *XPBC/ERCC-3* by densitometry are listed in Table 3. These results show that the level of expression of both these genes in all the testicular tumour cell lines tested, including the 833K cells, were not significantly different, with all values falling within a 2-fold range.

DISCUSSION

In this study, we have used four human testicular tumour cell lines, established from samples taken from previously untreated patients, and shown that they all retained a high degree of sensitivity to cisplatin in culture. We, and others [5, 7, 21], have documented the fact that cell lines established from testicular tumours are generally more sensitive to cisplatin than lines derived from other tumour types. For example, in this study IC_{50} values from colony-forming assays involving 1-hr drug exposures were all below 0.5 μg/ml, whilst figures of 1–27 μg/ml and 1.3–6.2 μg/ml have been reported for three ovarian [22] and three lung carcinoma cell lines [23], respectively, using the same clonogenic assay methodologies. Although the total platination levels, measured immunochemically immediately after a 1-h drug exposure, were lower in the two more sensitive cell lines, there was no overall direct relationship between cisplatin sensitivity and the extent of total platination. This lack of correlation was also reported earlier in two ovarian tumour cell lines, with markedly different cisplatin sensitivities (i.e. >23-fold) [24], in two testicular tumour lines [7] and in three lung carcinoma cell lines [23]. It is, therefore, of interest that a recent publication evaluating multiple biological markers in patients with germ cell tumours treated with platinum-based chemotherapy stressed that there was marked variation in the level of Pt–DNA damage, measured by atomic absorption spectroscopy in leucocyte DNA, between individuals receiving the same dose and sampled at the same time point [25]. However, in this present study, there does appear to be a correlation between the IC_{50} cytotoxicity values and the levels of total platination remaining, following an 18-h post-treatment incubation period. This result perhaps indicates that it is the level of residual damage, as opposed to the level of initial damage, which is more related to cisplatin cytotoxicity.

The most striking feature of this study is that all four human testicular tumour cell lines proved deficient in removing the major Pt–DNA intrastrand crosslinks Pt–GG, and a similar deficiency was noted for the next most abundant intrastrand adduct, Pt–AG, in two of the lines, GCT46 and H32. An abnormally slow rate of removal of platinum from DNA, as determined by atomic absorption spectroscopy, was reported earlier in the original GCT27 cell line [21]. These results, therefore, confirm and extend our original observation [7] that the hypersensitivity to cisplatin expressed by another testicular teratoma cell line (SuSa), also derived from an untreated patient, was associated with an apparent deficiency in the repair of these major Pt–DNA adducts. Certainly these data, using lines established from tumours from patients not previously treated with drugs, contrast with the proficient repair of both Pt-GG and Pt-AG adducts reported earlier [7], by the 833K teratoma cell lines, established from a patient's tumour after treatment

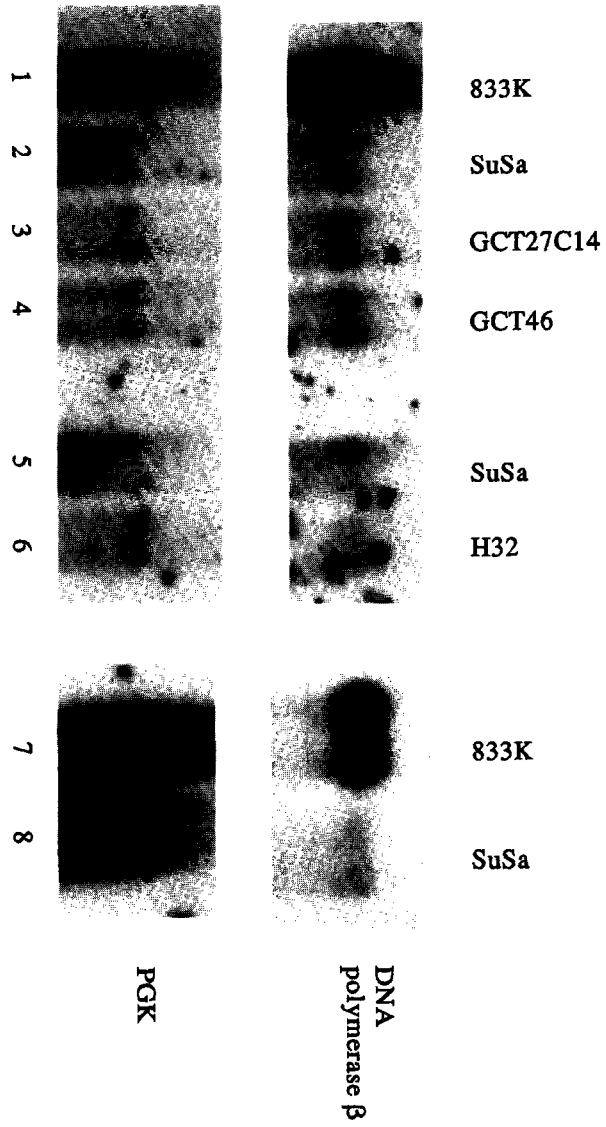

Figure 3. Results of northern blotting for DNA polymerase β and PGK gene expression using poly A-enriched RNA (2 μg per lane). Results from two separate experiments are depicted: experiment 1, lanes 1–6, and experiment 2, lanes 7–8.

Table 3. Relative expression of ERCC-1 *and* XPBC/ERCC-3 *in the various testicular tumour cell lines derived from densitometry readings of northern blots*

Cell line	*Relative expression *ERCC-1*	*XPBC/ERCC-3*
SuSa	1.0	1.0
GCT27 clone 4	0.7	0.8
GCT46	0.5	1.0
H32	0.7	1.2
833K	0.9	0.8

*Expression relative to enolase and using SuSa cells as the control.

with combination chemotherapy including cyclophosphamide, but not cisplatin [14], and by the more cisplatin resistant RT112 bladder tumour cell line. In addition, a defect in the ability to remove platinum from their DNA was reported in a cisplatin-resistant, stable variant of the teratoma line GCT27 [21].

Our analyses of certain enzymes implicated in the repair of drug-induced DNA damage, namely DNA polymerase β, *ERCC-1* and *XPBC/ERCC-3* (see reviews [8] and [11]), has shown that the inherent levels of expression of these genes are comparable in these three testicular tumour cell lines and in the SuSa line, all derived from untreated patients. However, by including in our investigation the 833K cell line, established from a patient after treatment with chemotherapy [14], and which had proved proficient in repairing all the major Pt–DNA adducts [7], we have been able to show that the level of expression of DNA polymerase β is considerably reduced in these repair deficient testicular tumour cell lines. However, no corresponding differences in *ERCC-1* and *XPBC/ERCC-3* expression were noted. The role, if any, of these various enzymes, and of those involved in glutathione synthesis [26] as well as the glutathione S-transferases [27, 28] and the metallothioneins [29, 30] in mediating cisplatin sensitivity remains to be defined. However, these testicular tumour cell lines may provide a valuable tool for examining the involvement of DNA polymerase β. For example, it is known that signal transduction from protein kinase C may genetically regulate the expression of this enzyme, and that its expression is regulated by promoter sequences from a transcription factor that may be a Fos-related protein [31].

Overall, these observations clearly support the idea that the clinical effectiveness of cisplatin in treating testicular tumours may be explained by the inefficient repair of the major Pt–DNA adducts formed by this drug. We are now extending these studies to examine DNA nucleotide excision repair, using cellular extracts [32, 33], and aim to establish whether deficient gene specific repair of cisplatin adducts, using procedures described earlier [34], can also be identified as characteristic of testicular tumour cell lines, established from untreated and generally cisplatin-responsive patients.

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