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Oxaliplatin-Induced Damage of Cellular DNA

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ABSTRACT

Damage to cellular DNA is believed to determine the antiproliferative properties of platinum (Pt) drugs. This study characterized DNA damage by oxaliplatin, a diaminocyclohexane Pt drug with clinical antitumor activity. Compared with cisplatin, oxaliplatin formed significantly fewer Pt-DNA adducts (e.g., 0.86 ± 0.04 versus 1.36 ± 0.01 adducts/10^6 base pairs/10 μM drug/1 h, respectively, in CEM cells, $P < .01$). Oxaliplatin was found to induce potentially lethal bifunctional lesions, such as interstrand DNA cross-links (ISC) and DNA-protein cross-links (DPC) in CEM cells. As with total adducts, however, oxaliplatin produced fewer ($P < .05$) bifunctional lesions than did cisplatin: 0.7 ± 0.2 and 1.8 ± 0.3 ISC and 0.8 ± 0.1 and 1.5 ± 0.3 DPC/10^6 base pairs/10 μM drug, respectively, after a 4-h treatment. Extended postincubation (up to 12 h) did not compensate the lower DPC and ISC levels by oxaliplatin. ISC and DPC determinations in isolated CEM nuclei unequivocally verified that oxaliplatin is inherently less able than cisplatin to form these lesions. Reactivation of drug-treated plasmids, observed in four cell lines, suggests that oxaliplatin adducts are repaired with similar kinetics as cisplatin adducts. Oxaliplatin, however, was more efficient than cisplatin per equal number of DNA adducts in inhibiting DNA chain elongation (~7-fold in CEM cells). Despite lower DNA reactivity, oxaliplatin exhibited similar or greater cytotoxicity in several other human tumor cell lines (50% growth inhibition in CEM cells at 1.1/1.2 μM, respectively). The results demonstrate that oxaliplatin-induced DNA lesions, including ISC and DPC, are likely to contribute to the drug's biological properties. However, oxaliplatin requires fewer DNA lesions than does cisplatin to achieve cell growth inhibition.

Oxaliplatin [l-OHP, oxalato(*trans-l*-1,2-diaminocyclohexane)platinum(II)] is a third generation platinum (Pt) antitumor compound in which diaminocyclohexane (DACH) ligand replaces the amine groups present in cisplatin (Fig. 1) (Chaney, 1995; Raymond et al., 1998). Oxaliplatin has demonstrated a broad spectrum of antitumor activity (Rixe et al., 1996) with a partial or a non-cross-resistance with cisplatin in a wide range of human tumors in vitro and in vivo (Weiss and Christian, 1993; Kelland and McKeage, 1994; Chaney, 1995; Raymond et al., 1998). Ongoing clinical European phase II trials have reported encouraging activity and manageable toxicity in a variety of tumors usually resistant to cisplatin (for review, see Cvitkovic, 1998). Oxaliplatin is now approved, in combination with 5-fluouracil, for the treatment of advanced colorectal cancer in Europe.

The critical role of DNA-Pt adducts in the antiproliferative effects, well documented for cisplatin, is generally accepted for all antitumor Pt drugs (for review, see Sanderson et al., 1996). The knowledge of oxaliplatin-induced DNA lesions is largely based on extrapolation of findings for cisplatin and DACH compounds other than oxaliplatin. However, the analogy between oxaliplatin and cisplatin should not be overinterpreted. Oxaliplatin is typically at least as potent as cisplatin in inhibiting the growth of cancer cells (Rixe et al., 1996). Thus, oxaliplatin would be expected to damage DNA to a similar extent as cisplatin. However, various methodologies suggested that oxaliplatin induced fewer lesions in naked and cellular DNA than did equimolar cisplatin (Saris et al., 1996; Woynarowski et al., 1998).

These paradoxical findings suggest that oxaliplatin-induced DNA damage may differ in various aspects from that of cisplatin. Although the structures of diaminocyclohexane (DACH)-Pt DNA adducts formed by oxaliplatin and *cis*-diam-

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ABBREVIATIONS: DACH, diaminocyclohexane; oxaliplatin, l-OHP, oxalato (*trans-l*-1,2-diaminocyclohexane)platinum(II); Pt, platinum; GI_{50}, drug concentration inhibiting cell growth by 50%; ISC, interstrand DNA cross-links; DPC, DNA protein cross-links; (k)bp, (kilo)base pair.

mine-Pt DNA adducts (formed by cisplatin) are similar, the bulky DACH moiety that protrudes into the minor groove (Scheeff et al., 1999) may possibly lead to different biological properties of DACH-Pt-DNA adducts (Chaney, 1995; Scheeff et al., 1999). DACH-Pt adducts, for instance, appear to be more effective at inhibiting DNA synthesis (Gibbons et al., 1990, 1991; Schmidt and Chaney, 1993). On the other hand, the possibility that DACH moiety in oxaliplatin affects the localization of drug adducts in DNA has been ruled out because sequence and region specificities of oxaliplatin adducts are similar to those of cisplatin (Woynarowski et al., 1998).

Another unstudied possibility is that oxaliplatin might generate a greater proportion of highly lethal lesions, compensating in that way for the lower overall DNA adduction compared with cisplatin. Whereas intrastrand cross-links are probably the main type of oxaliplatin adducts, infrequent interstrand cross-links (ISC) were also detected with naked DNA (Woynarowski et al., 1998). ISC are regarded as lethal DNA lesions for cisplatin (Bedford et al., 1987; Roberts and Friedlos, 1987; Roberts et al., 1988). However, ISC induction by oxaliplatin in cellular DNA has never been reported. Also, the ability of oxaliplatin to form another likely lesion, DNA-protein cross-links (DPC), remains unknown.

Our current study explored the leads suggesting that oxaliplatin could differ from cisplatin in the ability to damage DNA. The results verify that oxaliplatin, like cisplatin, forms ISC and DPC in tumor cells. However, oxaliplatin-induced total Pt-adducts, ISC, and DPC are significantly lower than the respective lesions induced by equimolar concentrations of cisplatin, despite a similar or greater cytotoxicity of oxaliplatin.

Materials and Methods

Drugs, Cell Culture, and Cytotoxic Activity. Oxaliplatin was obtained from Sanofi-Synthelabo Research (Great Valley, PA). Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO). Drug stock solutions were made in water (oxaliplatin) or in saline (cisplatin) and stored at −20°C.

Human leukemia CEM cells were cultured as described previously (Woynarowski et al., 1997) in minimal essential medium Eagle, Joklik's modification (Sigma). Human ovarian carcinoma A2780 and its cisplatin-resistant clone A2780PR were cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD; Mamenta et al., 1994). Human colon adenocarcinoma HT29 cells were purchased from and were cultured as recommended by the American Type Culture Collection (Rockville, MD). Human colon carcinoma HCT 116 cells were kindly provided by Dr. R. Boland (University of California, La Jolla, CA) and were cultured in Iscove's modified Dulbecco's medium (Life Technologies). All media were supplemented with 10% fetal bovine serum. All cell lines were grown in humidified 5% CO_2 at 37°C and were regularly screened for mycoplasma.

Growth inhibitory activity against CEM cells was assayed based on cell counting with an electronic counter after a 48-h continuous treatment (approximately 3 × generation time) with drugs at 0.01 to 100 μM or a 1-h treatment followed by a 48-h postincubation. Cytotoxicity against other cell lines was determined by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Woynarowski et al., 1997) after 72 h of continuous drug treatment (approximately 3 × generation time). The results are expressed as drug concentrations that inhibit net cell growth by 50% (GI_{50}).

Cellular Pt-DNA Adducts. Platination levels were monitored by atomic absorption as previously described (Gibbons et al., 1990; Schmidt and Chaney, 1993). Pilot studies had shown that the Pt-DNA levels were proportional to the drug concentration used over the range of 50 to 250 μM. For each experiment, cells (10^6/10 ml medium in replicate 100-mm dishes) were exposed to 100 μM cisplatin or oxaliplatin as indicated in Fig. 2 and Table 2. DNA was purified from each dish separately using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) and analyzed for Pt levels by AA in triplicate on a Perkin Elmer (Norwalk, CT) Cetus model 560 atomic absorption spectrophotometer with an HGA 500 graphite furnace and an AS-1 autosampler. DNA preparations from different culture dishes and on different days were considered repeats and were as follows: $n = 4$ for 15/45 min, $n = 6$ for 1 h, and $n = 3$ for 4 h. The data for Pt adducts were normalized per 10^6 base pairs (bp)/10 μM drug, to allow the comparison with other types of DNA lesions (Table 2).

DNA Repair and Host Cell Reactivation Assay. Plasmid reactivation assays were performed using a strategy similar to that described by others (Chao et al., 1991; Ali-Osman et al., 1994). Two plasmids from Promega were used: pGL3 control vector containing the luciferase construct was used as the drug damage reporter, whereas pSV β-galactosidase control vector served as an internal control for transfection efficiency. Plasmids were prepared and purified as recommended by Promega. The pGL3 control vector (100 μg/ml) in TE buffer (10 mM Tris, 0.5 mM EDTA, pH 7.4) was treated with 0.25 to 5 μM cisplatin or 0.5 to 50 μM oxaliplatin for 22.5 h at 37°C. For oxaliplatin treatment, the reactions were supplemented with NaCl (100 mM final concentration) to accelerate formation of drug active species. After ethanol precipitation to remove unreacted drug, plasmid DNA was redissolved in TE buffer, and its concentration was quantitated by standard Hoechst 33258 fluorescence assay. Platination levels were also directly assessed in drug-treated plasmids by atomic absorption as described for cellular Pt adducts. One batch of drug-treated plasmids was used in all the transfection experiments.

Transfections of A2780, A2780PR, and HCT116 cells were carried out using the CalPhos Mammalian Transfection Protocol from Clontech (Palo Alto, CA), according to the manufacturer's recommendations. Transfection of HT29 cells used the CLONfectin Mammalian Transfection kit and protocol from Clontech at a ratio of 1:2.5 of plasmid to CLONfectin liposomes (w/w). Cells were plated at 0.2 to 0.4 × 10^5 per well of a 24-well plate 24 h before transfection. Generally, a 1:1 or 1:1.5 ratio of luciferase:galactosidase plasmids was used in the dual transfections with 2 to 2.5 total μg of plasmid DNA added per well. After all transfections, cells were incubated an additional 48 h before harvesting. Cell extracts were prepared by lysing phosphate buffered saline-washed cells in 100 mM potassium phosphate, pH 8.7, and 0.2% Triton X-100, followed by centrifuging at 14,000*g* for 2 min. Extracts were flash frozen and stored at −20°C until use.

Reporter detection was based on chemiluminescence and used a Packard (Meridian, CT) TopCount scintillation counter as a luminescence counter. Detection of luciferase used Steady-Glo Luciferase Assay System from Promega, and β-galactosidase was detected using the Galacto-Star β-Galactosidase Chemiluminescent Reporter Gene Assay System detection kit from Tropix (Bedford, MA). For each transfection, mock-transfected cells were used as background controls, and non-drug-treated plasmids were used as a positive control.

ISC by Alkaline Sucrose Gradient Sedimentation. The procedure for sedimentation analysis of cellular DNA was used essentially as described elsewhere (Woynarowski et al., 1999). Briefly, cells prelabeled with [^{14}C]thymidine were treated with drugs and, in some experiments, postincubated in drug-free medium as indicated. Aliquots of 10^5 harvested cells in 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.2% Triton X-100 were loaded onto preformed alkaline sucrose gradients composed of 0.5 ml of 60% sucrose cushion, 10 ml of 5 to 20% sucrose, and 0.4 ml of lysing layer (1% Sarkosyl, 2.5% sucrose). All the solutions were in a gradient buffer (0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA). After the sample was applied, an additional volume (200 μl) of lysing solution was laid on the top, and lysis was allowed to proceed for 20 h. Sedimentation was carried out in an

SW41 rotor (Beckman Instruments, Fullerton, CA) at 20°C for 20 h at 10,000 rpm (A2780 cells) and 9200 rpm (CEM cells). Gradients were fractionated and the fractions processed as described previously (Woynarowski et al., 1995).

Frequencies of interstrand cross-link were estimated according to the equation of Roberts and Friedlos (1982):

$$f = \frac{-\ln(1 - FD)}{MW_n/640} \quad (1)$$

where MW_n is the number average molecular weight for control DNA, 640 is the average molecular weight of 1 bp, and FD, fractional difference, is the sum of the differences between the control and treated DNA profiles (eq. 2):

$$FD = \sum(Treated_i - Control_i) \quad (2)$$

where $Control_i$ and $Treated_i$ are percentages of recovered radioactivity in gradient fractions for control and drug-treated samples, respectively. To minimize the influence of strand breaks, the original treatment (Roberts and Friedlos, 1982) was modified in that only gradient fractions corresponding to the area of cross-linked DNA (bottom portion of the gradients, where the signal for drug-treated samples was equal to or greater than for control samples) were included in FD calculations.

DPC. DPC were assayed using the K^+/SDS precipitation technique. For DPC determinations in intact cells, [^{14}C]thymidine-prelabeled cells were incubated with drugs as indicated. After drug treatment, cells were lysed, and DNA coprecipitable with proteins was determined as described previously (Woynarowski et al., 1989, 1997). For DPC determination in isolated nuclei, prelabeled cells were lysed in nuclei isolation buffer (2 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, pH 6.9) supplemented with 0.3% (v/v) Triton X-100 for 20 min at 4°C, and then centrifuged for 13 min at 300*g*. The nuclear pellets were resuspended in the isolation buffer at 0.3 to 0.5 $\times$ 10^6 nuclei/ml and incubated with drugs, followed by DPC determination as for intact cells (Woynarowski et al., 1989).

The results were first expressed as a fraction of total DNA coprecipitating with proteins (FP), calculated as the ratio of radioactivity in protein pellets to the total radioactivity of an equivalent cell number, corrected for the precipitable radioactivity in untreated cells (Woynarowski et al., 1989). Next, the frequency of DPC (*f*) was estimated based on a Poisson distribution analogous to ISC frequency:

$$f = \frac{-\ln(1 - FP)}{MW_n/640} \quad (3)$$

where MW_n is number average molecular weight for DNA under the conditions of the assay and 640 is the average molecular weight of 1 bp. The MW_n values used (5×10^7 and 4×10^7 Da for intact cells and isolated nuclei, respectively) are based on separate determinations by neutral sedimentation (Woynarowski et al., 1988) of DNA in cell and nuclear lysates sheared by vortexing and processed in the same way as in the K^+/SDS assay (not shown). Molecular weights of DNA in gradient fractions and the MW_n values were calculated as described previously (Woynarowski et al., 1995) using parameters for neutral sedimentation conditions. Assuming the MW_n value of 5×10^7 Da, 1.35 and 2.86 DPC/10^6 bp correspond to a fraction of protein-bound DNA (FP) equal to 10 or 20% of total cellular DNA, respectively.

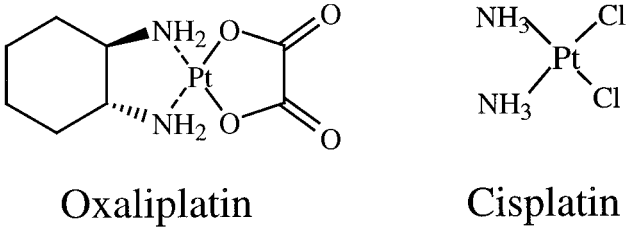

Fig. 1. The structures of oxaliplatin and cisplatin.

Chain Elongation Assay. The size distribution of radiolabeled nascent DNA was determined by velocity sedimentation analysis as described previously (Gibbons et al., 1991; Mamenta et al., 1994). Briefly, CEM or A2780 cells were plated on 60-mm dishes at an initial concentration of 5×10^5 cells/dish and uniformly labeled with [^{14}C]thymidine for 48 h. The old medium was then replaced with a medium containing various concentrations of oxaliplatin or cisplatin followed by a 15-min incubation. The drug-medium was then removed and replaced with a drug-free medium followed by a 30-min incubation. The cells were then pulsed for 15 min with 6 μCi/ml [^{3}H]thymidine (specific activity, 85 Ci/mmol). The radioactive medium was removed, and the cells were harvested into 1 ml of ice-cold harvest buffer (0.1 M NaCl-0.01 M EDTA, pH 8.0). Cells were harvested and lysed and the lysates subjected to sedimentation in alkaline sucrose gradients. The gradients were fractionated, and acid-precipitable ^{3}H counts were determined and normalized to the total amount of proteins in each sample. The percentage of chain elongation (relative to control) was calculated from the areas under the curve in the region of the profiles corresponding to the chain elongation.

Results

Cytotoxic Activities. The cytotoxic activities (GI_{50} values, drug concentration inhibiting cell growth by 50%) of oxaliplatin and cisplatin against various cell lines differing in cisplatin sensitivity, p53, and mismatch repair status are summarized in Table 1. Oxaliplatin and cisplatin showed similar levels of growth inhibition in CEM cells with mutated p53 with the GI_{50} ~ 1.1/1.2 and 11/11 μM after continuous drug treatment and a 1-h pulse treatment, respectively. A2780 cells (with wild-type p53) were more sensitive to both drugs than CEM cells, with oxaliplatin being nearly 2-fold more cytotoxic than cisplatin. Furthermore, oxaliplatin was ~2-fold more cytotoxic than cisplatin against the cisplatin-

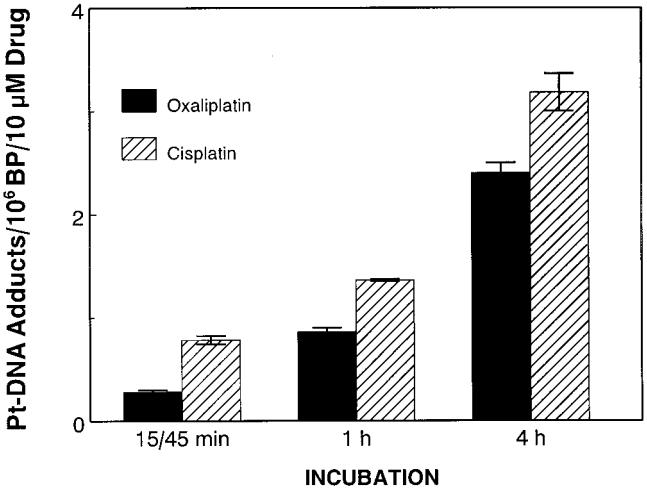

Fig. 2. Pt adducts formed by oxaliplatin (solid bars) and cisplatin (hatched bars) in CEM cells. Cells were treated with 100 μM drugs for 15 min, followed by a 45-min chase in drug-free medium (15/45 min), or for 1 or 4 h of continuous treatment as indicated, followed by DNA purification and determination of DNA platination levels by atomic absorption. The results are reported as means ± S.E.M. of data obtained from three to six replicate cell cultures/DNA preparations and are normalized per 10^6 bp/10 μM drug. The differences between cisplatin and oxaliplatin were significant ($P < .01$ for 15/45 min and 1 h and $P < .05$ for 4-h treatments).

TABLE 1
Cytotoxicity of oxaliplatin and cisplatin

Cell Line	Treatment	GI_{50}	
		Cisplatin	Oxaliplatin
		μM	
CEM	Continuous[a]	1.11 ± 0.05	1.19 ± 0.17
CEM	Pulse 1 h[b]	11.37 ± 1.48	11.38 ± 1.12
A2780	Continuous[c]	0.38 ± 0.16	0.21 ± 0.04
A2780PR	Continuous[c]	3.82 ± 0.31	2.22 ± 0.74
HCT116	Continuous[c]	11.73 ± 0.30	1.79 ± 0.19
HT29	Continuous[c]	8.27 ± 1.90	1.28 ± 0.29

[a] Determined based on cell counts after a continuous incubation for 48 h (approximately 3 × cell generation time) with drug concentrations ranging from 0.01 to 100 μM.
[b] Determined based on cell counts after a 1-h pulse treatment with drug concentrations ranging from 0.1 to 100 μM, followed by a 48-h postincubation in drug-free medium.
[c] Determined by the MTT assay after a 72-h incubation (approximately 3 × cell generation times) with drug concentrations ranging from 0.075 to 200 μM.

resistant subline A2780PR and 6- to 7-fold more cytotoxic against a mismatch repair-deficient line, HCT116, and against a generally drug-resistant line, HT-29 (Table 1).

Total DNA Platination. Platination levels were used as an overall measure of adduct formation after various incubation conditions in CEM cells (Fig. 2). Oxaliplatin consistently formed significantly fewer adducts than cisplatin at equimolar external concentrations (Fig. 2 and Table 2). For instance, after a 1-h treatment, oxaliplatin and cisplatin formed 0.86 and 1.36 Pt adducts/10^6bp/10 μM, respectively (Fig. 2 and Table 2). The difference between both drugs remained significant after a 4-h incubation, with the respective platination values of 2.4 versus 3.18 Pt adducts/10^6 bp/10 μM. Similar differences in oxaliplatin and cisplatin adduct levels were observed in A2780 cells (data not shown).

Repair of Oxaliplatin Lesions-Host Cell Reactivation Assay. To address the possibility that oxaliplatin adducts are less prone to removal by repair processes, we analyzed the reactivation of drug-treated plasmids in several cell lines. The initial levels of plasmid platination showed a good dependence on drug concentration during plasmid treatment ranging from 1.3 ± 0.1 up to 180 ± 2.3 Pt atoms/kbp for 0.5 to 50 μM oxaliplatin and from 1.6 ± 0.1 up to 36.9 ± 0.7 Pt atoms/kbp for 0.25 to 5 μM cisplatin. The detected cisplatin platination levels are in reasonable agreement with literature data for similar treatment conditions (Chao et al., 1991).

The results of plasmid reactivation during 48-h post-transfection of A2780 and HT-29 cells are plotted against the initial number of adducts per plasmid molecule (Fig. 3). For both drugs, there was a progressive abrogation of the plasmid signal dependent on the initial level of Pt adducts. For example, reporter activity remained profoundly inhibited in both cell lines transfected with plasmids containing, respectively, ~130 and 190 oxaliplatin and cisplatin adducts/plasmid. The differences in the reactivation of oxaliplatin- and cisplatin-treated plasmids were small and were within the range of experimental variation in A2780 and HT-29 cells (Fig. 3) and also in A2780PR and HCT116 cells (data not shown). Thus, the magnitude of cellular repair of oxaliplatin DNA lesions seems similar to the repair of cisplatin DNA lesions.

ISC in Intact Cells. Induction of ISC by oxaliplatin and cisplatin was examined to assess whether the lower total Pt adducts formed by oxaliplatin might be compensated by a greater proportion of highly lethal lesions such as ISC. Sedimentation analysis after a 4-h incubation of CEM cells with 25 μM cisplatin produced the characteristic pattern for interstrand cross-links indicated as shifts in DNA sedimentation profiles toward the bottom of the gradients (fractions 19–24, brackets in Fig. 4). DNA from oxaliplatin-treated cells also revealed fast-sedimenting material, although less prominent than for cisplatin. A similar pattern was observed in A2780 cells (data not shown). These results confirm that oxaliplatin forms interstrand cross-links but at markedly lower levels than equimolar cisplatin.

The estimates of ISC frequency based on the shifts in the sedimentation profiles suggest that oxaliplatin formed 0.7 ± 0.2 ISC/10^6 bp/10 μM compared with 1.8 ± 0.3 ISC/10^6 bp/10 μM for cisplatin (Table 2). The respective numbers for oxaliplatin and cisplatin in A2780 cells were 1.4 (±0.4, range) versus 3.4 (±0.4, range) ISC/10^6 bp/10 μM. It should be noted, however, that these values might be underestimated because of the strand breaks that were apparent in some experiments. This strand breakage was more clearly noticeable at 50 μM drug levels as a material sedimenting near the top of the gradients (data not shown).

The strand breaks, which probably reflect early apoptotic DNA fragmentation (Faivre and Woynarowski, 1998), affect the size distribution of DNA fragments. Thus, the quantitation of ISC may not be accurate, particularly after longer incubation times. Still, additional experiments with a 1-h

TABLE 2
Summary of normalized lesion frequencies induced by oxaliplatin and cisplatin in CEM cells

Treatment (h)	Normalized Lesions/10 μM/10^6 bp					
	Continuous 4 h			Pulse 1 h		
Postincubation (h)	None			0	6[d]	12
Drug	Total Pt-DNA Adducts[a]	ISC[b]	DPC[c]	Total Pt-DNA Adducts[a]	ISC[b]	DPC[c]
Oxaliplatin	2.4 ± 0.10	0.7 ± 0.2	0.8 ± 0.1	0.86 ± 0.04	0.14 ± 0.03	0.19 ± 0.02
Cisplatin	3.18 ± 0.18	1.8 ± 0.3	1.5 ± 0.3	1.36 ± 0.01	0.35 ± 0.02	0.37 ± 0.04
Oxaliplatin to cisplatin ratio (P value)[e]	0.76:1 (<.05)	0.39:1 (<.05)	0.53:1 (<.05)	0.63:1 (<.01)	0.40:1 (.06)	0.51:1 (<.01)

[a] By atomic absorption, based on data in Fig. 2.
[b] By sedimentation analysis in alkaline sucrose gradients, such as data in Fig. 4, for 25 to 50 μM oxaliplatin and cisplatin for 4-h treatment and 78 to 156 μM and 34 to 68 μM oxaliplatin and cisplatin, respectively, for 1-h pulse treatment.
[c] By K^+/SDS precipitation, based on data in Fig. 5 for 10 to 100 μM and 5 to 50 μM oxaliplatin and cisplatin, respectively, for 4-h treatment and 78 to 156 μM and 34 to 68 μM oxaliplatin and cisplatin, respectively, for 1-h pulse treatment.
[d] These ISC estimates can be distorted due to interference by apoptotic strand breaks noticeable under these conditions.
[e] Significance of the difference between cisplatin and oxaliplatin based on t statistics.

pulse drug treatment followed by a 6-h postincubation in drug-free medium suggested that oxaliplatin forms similar or lower levels of ISC than does cisplatin (0.14 and 0.35 ISC/10^6 bp/10 μM, respectively, Table 2).

DPC in Intact Cells. DPC are another type of previously unstudied DNA lesion, in which oxaliplatin might possibly compensate for its lower overall reactivity with DNA, compared with cisplatin. The induction of DPC was analyzed based on direct physical separation of protein-bound and protein-free DNA by the K^+/SDS precipitation technique (Woynarowski et al., 1989, 1997). After a continuous 4-h treatment, oxaliplatin formed significantly fewer DPC than did cisplatin (Fig. 5A). Normalized per 10 μM drug, oxaliplatin and cisplatin formed an estimated 0.8 ± 0.1 and 1.5 ± 0.3 DPC/10^6 bp, respectively (Table 2). The difference between two drugs was diminished but still noticeable in A2780 cells (data not shown).

DPC determinations are less affected by strand breakage, because of the intentional DNA shearing in the DPC assay. Therefore, DPC can be reliably monitored in prolonged incubations as illustrated by the time course of DPC formation after a 1-h pulse drug treatment of CEM cells (Fig. 5B). The number of DPC increased with the postincubation time for both drugs, although the effects appeared to be reaching a plateau by 12 h postincubation (Fig. 5B). Thus, corroborating the findings with the 4-h continuous drug incubation, cisplatin induces approximately twice as many DPC as oxaliplatin after normalization to equimolar drug levels (Fig. 5C and Table 2).

ISC and DPC in Isolated Nuclei. To unequivocally establish whether oxaliplatin is inherently less efficient than cisplatin in the formation of both ISC and DPC, these lesions were measured in isolated nuclei from CEM cells. In this system, the drugs react with intact nuclear chromatin but the induction of secondary effects, including strand breaks, which affect the determinations in intact cells, was unlikely. Also, potential differences in drug uptake can be ruled out in the nuclear system. The results provide a clear-cut answer that there are markedly fewer ISC and DPC induced by oxaliplatin than cisplatin (Fig. 6). Sedimentation profiles observed at 25 μM drugs (Fig. 6A) correspond to 5.5 and 15.6 ISC/10^6 bp, for oxaliplatin and cisplatin, respectively. Similarly, oxaliplatin remained less efficient in DPC induction

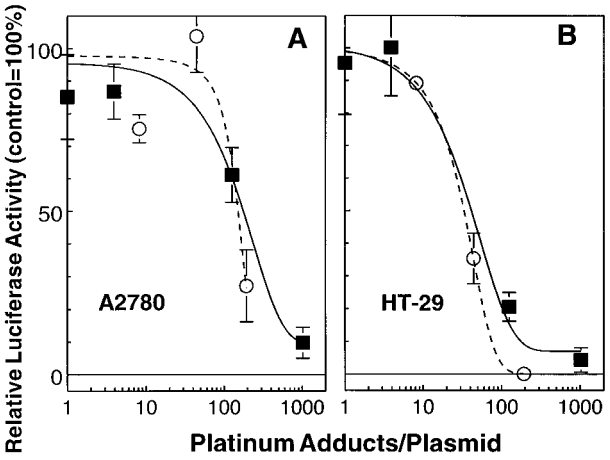

Fig. 3. Repair of DNA lesions in oxaliplatin-treated (■) or cisplatin-treated (○) plasmids in A2780 (A) and HT29 (B) cells. The luciferase reporter plasmid was treated with various concentrations of drugs as described under *Materials and Methods*, followed by the determinations of platination levels (abscissa). Cells were cotransfected with the drug-damaged luciferase plasmids and undamaged β-galactosidase plasmid. After 48 h, luciferase levels were measured and normalized to their respective β-galactosidase levels. Relative luciferase activity is expressed as a percentage of activity of the undamaged control plasmid. Each point represents the mean ± S.E.M. of two to four different transfections of the damaged plasmids.

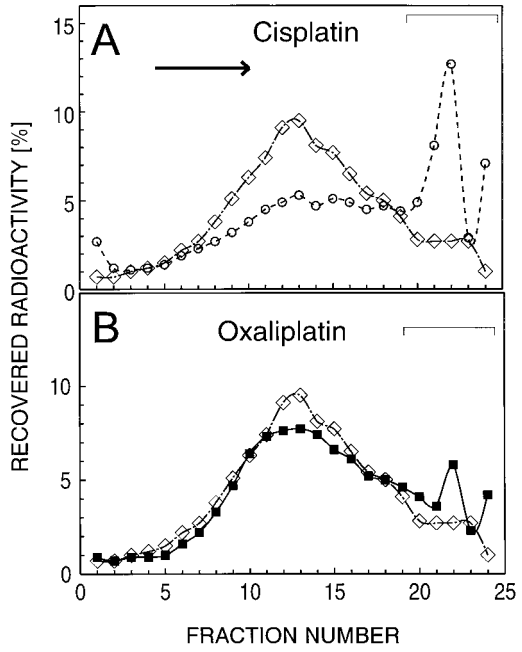

Fig. 4. ISC induced by cisplatin (A) and oxaliplatin (B) in CEM cells treated continuously with drugs for 4 h. The figure depicts examples of sedimentation on alkaline sucrose gradients of DNA from control cells (◇) and cells treated with oxaliplatin at 25 μM (■) and cisplatin at 25 μM (○). The arrows show the direction of sedimentation, and the brackets show the area of the sedimentation profiles that is indicative of inter-strand cross-links. The sedimentation profiles shown were averaged from three to five independent determinations. Average S.E.M. values, omitted for clarity, ranged from 1 to 1.5% of recovered radioactivity.

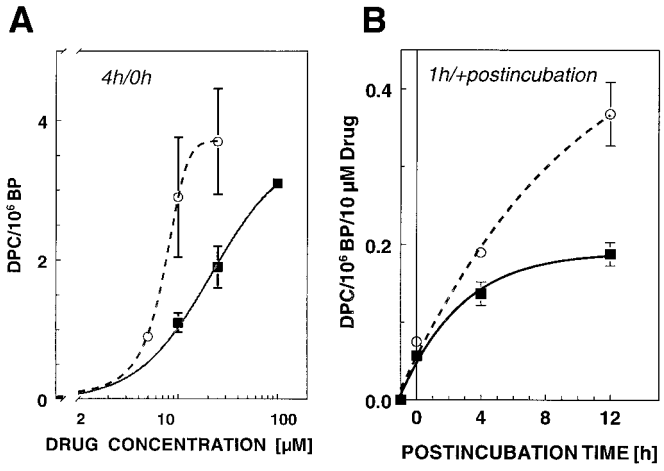

Fig. 5. DPC induced by oxaliplatin and cisplatin in intact CEM cells. A, cells were treated for 4 h with oxaliplatin (■) or cisplatin (○) at the indicated concentrations. B, CEM cells were treated with drugs for 1 h, followed by a post-treatment incubation as indicated. The data shown in B are normalized per 10 μM drug based on determinations for 79 and 158 μM oxaliplatin (■) and 34 and 68 μM cisplatin (○). DPC were determined by the K^+/SDS precipitation method. DPC frequency was estimated as described under *Materials and Methods*. Data represent means ± S.E.M. from two to three determinations.

than did cisplatin (Fig. 6B). For instance, 10 μM oxaliplatin and cisplatin produced ~8 and ~20 DPC/10^6 bp, respectively, in the nuclear system. Moreover, the DPC induction reached a plateau for both drugs after ~4 h (data not shown), but the levels of oxaliplatin-induced DPC were significantly lower than cisplatin-induced DPC in their steady state.

Inhibition of DNA Chain Elongation. To assess the potential consequences of DNA damage, additional experiments explored the relationship between drug adducts and DNA chain elongation. The short time of drug treatment needed for these determinations necessitates the use of relatively high drug concentrations (Gibbons et al., 1991; Mamenta et al., 1994). However, the parallel determinations of the platination levels (cf. Fig. 2) allowed us to express drug effects on chain elongation as a function of adduct frequency (Fig. 7). Oxaliplatin adducts seemed to be approximately 7-fold more effective in inhibiting DNA chain elongation than cisplatin adducts in CEM cells, considering the number of Pt-DNA adducts per 10^6 bp (Fig. 7). Somewhat less profound (approximately 2-fold) difference in the adduct inhibitory efficiency was found in A2780 cells (data not shown).

Discussion

In contrast to extensive studies of DNA damage by cisplatin, little is known about damage to cellular DNA by oxaliplatin. It is commonly accepted that DNA damage by antitumor Pt drugs is responsible for their cytotoxic properties. According to this central paradigm, oxaliplatin should be more proficient in damaging DNA than cisplatin, given that oxaliplatin is at least equally cytotoxic or frequently more cytotoxic than cisplatin (Table 1) (Rixe et al., 1996). Systematic characterization of oxaliplatin-induced DNA lesions in this study suggests that oxaliplatin declines from the predictions of the central paradigm. In addition to total platination, we quantified oxaliplatin-induced ISC and DPC in comparison to analogous effects of cisplatin. The results demonstrate that oxaliplatin is undoubtedly a DNA-reactive drug in cellular systems and resembles closely cisplatin with regard to the types and proportions of specific DNA lesions. Yet, oxaliplatin consistently forms markedly fewer total adducts and specific type of cross-links than cisplatin, despite at least equal or greater cytotoxicity.

The determinations of the total levels of DNA platination demonstrate that oxaliplatin formed significantly fewer adducts with cellular DNA than did cisplatin (Fig. 2 and Table 2). This observation that platination levels produced by oxaliplatin are consistently lower than those induced by cisplatin is in agreement with the determinations by other researchers (Saris et al., 1996), who found a 10-fold lower level of oxaliplatin adducts, compared with cisplatin adducts, in A2780 cells by immunochemical detection. Also, our recent studies showed that the level of Pt adducts induced by oxaliplatin in specific regions of DNA from drug-treated A2780 cells was 2 to 6 times lower than that of cisplatin (Woynarowski et al., 1998). Clearly, in various systems, oxaliplatin needs to form fewer adducts than cisplatin for comparable cytotoxicity.

The lower overall DNA adduction by oxaliplatin might still be reconciled with the equal or higher cytotoxic activities, if oxaliplatin-DNA adducts were considerably more difficult to repair compared with cisplatin adducts. Adduct repair is an important factor in Pt drug action (Petersen et al., 1996; Damia et al., 1998; Hibino et al., 1999; Koeberle et al., 1999). However, oxaliplatin-induced DNA damage appears to be no

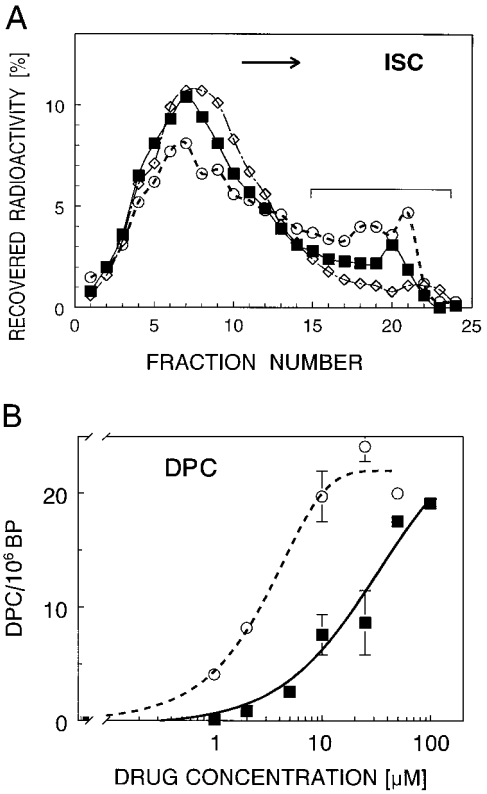

Fig. 6. ISC and DPC in isolated nuclei from CEM cells. A, sedimentation profiles for DNA from nuclei incubated for 4 h with no drugs (◇), oxaliplatin (25 μM, ■), and cisplatin (25 μM, ○). The arrow shows the direction of sedimentation, and the bracket shows the areas of sedimentation profiles, which are indicative of interstrand cross-links. The profiles shown correspond to 5.5 and 15.6 ISC/10^6 bp/25 μM, for oxaliplatin and cisplatin, respectively. B, DPC in nuclei treated for 4 h with oxaliplatin (■) or cisplatin (○) and analyzed by the K^+/SDS precipitation method. The ordinate gives the frequency of DPC estimated as described under *Materials and Methods*. Data represent means ± S.E.M. from two determinations carried out in duplicate.

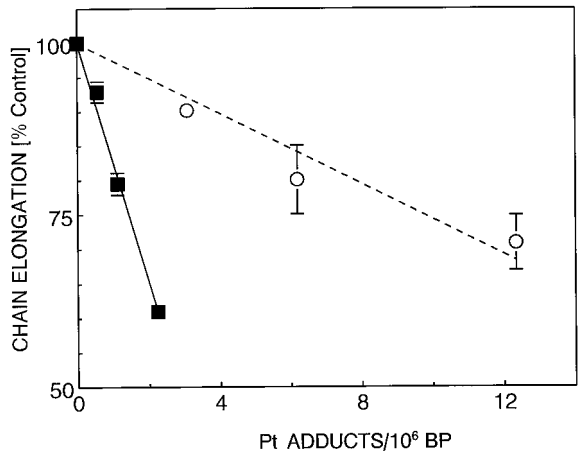

Fig. 7. Inhibition of chain elongation by oxaliplatin-treated (■) or cisplatin-treated (○) CEM cells as a function of Pt adducts. Cells were treated with drugs for 15 min, followed by a 30-min chase in drug-free medium and a 15-min pulse of [^{3}H]thymidine. The percentages of chain elongation (relative to control) were obtained from sedimentation profiles of pulse-labeled nascent DNA as described under *Materials and Methods* and plotted as a function of DNA-Pt adducts using data from Fig. 2. Each point represents the mean ± S.E.M. of three different experiments.

more difficult to repair than cisplatin-induced damage as judged by plasmid reactivation in several cell lines (Fig. 3), which reflects mainly excision repair processes. This result is consistent with the observation that both cisplatin and oxaliplatin adducts are similarly removed in a cell-free excision repair system (Reardon et al., 1999). Thus, the lower total platination in the case of oxaliplatin is unlikely to be compensated, compared with cisplatin, by impeded adduct removal.

Oxaliplatin cytotoxicity might possibly result from proportionately more highly lethal lesions, such as interstrand cross-links, or other previously uncharacterized lesions, such as DPC. Oxaliplatin forms both types of lesions in cellular DNA (Figs. 4 and 5 and Table 2). These results document for the first time that oxaliplatin is able to induce ISC and DPC in cellular DNA. Although ISC and DPC constitute a minor fraction of total adducts for either oxaliplatin or cisplatin, these lesions may contribute to oxaliplatin effects. However, the absolute levels of ISC and DPC induced by oxaliplatin were markedly lower than those induced by cisplatin, after the reduced total platination levels (Table 2). Thus, it seems unlikely that these lesion types may compensate for the overall reduction of oxaliplatin adducts.

Inherently lower reactivity of oxaliplatin compared with cisplatin is corroborated in a clear-cut way in isolated nuclei (Fig. 6). Thus, potential differences in cellular uptake of the two drugs can be ruled out as a simple explanation for the lower levels of oxaliplatin adducts in intact cells. Markedly lower reactivity of oxaliplatin was shown also with naked DNA (Woynarowski et al., 1998). Although the details of intracellular oxaliplatin activation remain unknown, the slow dissociation of the oxalate ligand may be the bottleneck in oxaliplatin-DNA reactivity (Luo et al., 1998, 1999a,b).

Our data suggest that oxaliplatin-DNA adducts may be more lethal than cisplatin adducts. In CEM cells, GI_{50} values for oxaliplatin and cisplatin are virtually identical, even though oxaliplatin treatment results in significantly fewer lesions than cisplatin treatment at equimolar concentrations. The greater lethality of oxaliplatin adducts is consistent with their greater inhibition of DNA chain elongation (Fig. 7). Similar differences in replicative bypass have been noted between DACH adducts and cisplatin adducts in other cell lines (Gibbons et al., 1991; Mamenta et al., 1994). Processing of oxaliplatin and cisplatin adducts may also elicit different downstream responses. For instance, cisplatin depends on intact mismatch repair for its maximal cytotoxicity (Aebi et al., 1996; Fink et al., 1996; Vaisman et al., 1998; Ferry et al., 1999). In contrast, oxaliplatin adducts are poorly recognized by mismatch repair proteins (Fink et al., 1996) and do not activate JNK and c-Abl (Nehme et al., 1999), and oxaliplatin retains a high cytotoxicity in mismatch repair-deficient cells (Fink et al., 1997; Vaisman et al., 1998).

Finally, it is important to recognize that DNA damage, although probably crucial, represents only one aspect of the pleiotropic effects of Pt drugs. Only 5 to 10% of covalently bound cell-associated cisplatin is found in the DNA fraction, whereas cisplatin binding to proteins is 1 order of magnitude greater (~75–85%; Akaboshi et al., 1992, 1994). An intriguing possibility is that functional protein damage (interference with enzymatic, receptor, and/or structural functions) may play a greater role in the effects of oxaliplatin than other Pt drugs. The hydrophobic DACH moiety in oxaliplatin may shift drug reactivity toward a subset of cellular proteins with hydrophobic binding pockets (Chaney, 1995). These proteins might well be different from those that react with cisplatin. Thus, enhanced and/or different protein binding might also be a factor in the disproportionately potent apoptosis induction by oxaliplatin (Faivre and Woynarowski, 1998), given the drug's modest DNA reactivity. Studies are under way to elucidate whether protein damage, in addition to DNA damage, contributes to the cytotoxic and proapoptotic properties of oxaliplatin.

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References

Aebi S, Kurdi-Haidar B, Gordon R, Cenni B, Zheng H, Fink D, Christen RD, Boland CR, Koi M, Fishel R and Howell SB (1996) Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Res* **56:**3087–3090.

Akaboshi M, Kawai K, Maki H, Akuta K, Ujeno Y and Miyahara T (1992) The number of platinum atoms binding to DNA, RNA and protein molecules of HeLa cells treated with cisplatin at its mean lethal concentration. *Jpn J Cancer Res* **83:**522–526.

Akaboshi M, Kawai K, Ujeno Y, Takada S and Miyahara T (1994) Binding characteristics of (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-2-pla in um(II) to DNA, RNA and protein molecules in HeLa cells and its lethal effect: Comparison with cis- and trans-diamminedichloroplatinums(II). *Jpn J Cancer Res* **85:**106–111.

Ali-Osman F, Berger MS, Rairkar A and Stein DE (1994) Enhanced repair of a cisplatin-damaged reporter chloramphenicol-O-acetyltransferase gene and altered activities of DNA polymerases alpha and beta, and DNA ligase in cells of a human malignant glioma following in vivo cisplatin therapy. *J Cell Biochem* **54:**11–19.

Bedford P, Walker MC, Sharma HL, Perera A, McAuliffe CA, Masters JR and Hill BT (1987) Factors influencing the sensitivity of two human bladder carcinoma cell lines to cis-diamminedichloroplatinum(II). *Chem Biol Interact* **61:**1–15.

Chaney SG (1995) The chemistry and biology of platinum complexes with the 1,2-diaminocyclohexane carrier ligand (review). *Int J Oncol* **6:**1291–1305.

Chao CC, Lee YL, Cheng PW and Lin-Chao S (1991) Enhanced host cell reactivation of damaged plasmid DNA in HeLa cells resistant to cis-diamminedichloroplatinum(II). *Cancer Res* **51:**601–605.

Cvitkovic E (1998) Ongoing and unsaid on oxaliplatin: The hope. *Br J Cancer* **77:**8–11.

Damia G, Guidi G and D'Incalci M (1998) Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. *Eur J Cancer (A)* **34:**1783–1788.

Faivre S and Woynarowski JM (1998) Oxaliplatin effects on DNA integrity and apoptosis induction in human tumor cells, in *Proceedings of the Annual Meeting of the American Association of Cancer Research*, vol 39, p 158; 1998 Mar 25–Apr 1; New Orleans, LA.

Ferry KV, Fink D, Johnson SW, Nebel S, Hamilton TC and Howell SB (1999) Decreased cisplatin damage-dependent DNA synthesis in cellular extracts of mismatch repair deficient cells. *Biochem Pharmacol* **57:**861–867.

Fink D, Nebel S, Aebi S, Zheng H, Cenni B, Nehme A, Christen RD and Howell SB (1996) The role of DNA mismatch repair in platinum drug resistance. *Cancer Res* **56:**4881–4886.

Fink D, Zheng H, Nebel S, Norris PS, Aebi S, Lin T-P, Nehme A, Christen RD, Haas M, MacLeod C and Howell SB (1997) In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. *Cancer Res* **57:**1841–1845.

Gibbons GR, Kaufmann WK and Chaney SG (1991) Role of DNA replication in carrier-ligand-specific resistance to platinum compounds in L1210 cells. *Carcinogenesis* **12:**2253–2257.

Gibbons GR, Page JD, Mauldin SK, Husain I and Chaney SG (1990) Role of carrier ligand in platinum resistance in L1210 cells. *Cancer Res* **50:**6497–6501.

Hibino Y, Hiraoka Y, Kamiuchi S, Kusashio E and Sugano N (1999) Enhancement of excision repair of cisplatin-DNA adducts by cell-free extract from a cisplatin-resistant rat cell line. *Biochem Pharmacol* **57:**1415–1422.

Kelland LR and McKeage MJ (1994) New platinum agents: A comparison in ovarian cancer. *Drugs Aging* **5:**85–95.

Koeberle B, Masters JRW, Hartley JA and Wood RD (1999) Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. *Curr Biol* **9:**273–276.

Luo FR, Wyrick SD and Chaney SG (1998) Cytotoxicity, cellular uptake, and cellular biotransformations of oxaliplatin in human colon carcinoma cells. *Oncol Res* **10:**595–603.

Luo FR, Wyrick SD and Chaney SG (1999a) Biotransformations of oxaliplatin in rat blood in vitro. *J Biochem Mol Toxicol* **13:**159–169.

Luo FR, Yen TY, Wyrick SD and Chaney SG (1999b) High-performance liquid chromatographic separation of the biotransformation products of oxaliplatin. *J Chromatogr Biomed Sci Appl* **724:**345–356.

Mamenta EL, Poma EE, Kaufman WK, Delmastro D, Grady HL and Chaney SG

(1994) Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines. *Cancer Res* **54:**3500–3505.
Nehme A, Baskaran R, Nebel S, Fink D, Howell SB, Wang JYJ and Christen RD (1999) Induction of JNK and c-Abl signalling by cisplatin and oxaliplatin in mismatch repair-proficient and -deficient cells. *Br J Cancer* **79:**1104–1110.
Petersen LN, Mamenta EL, Stevnsner T, Chaney SG and Bohr VA (1996) Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity. *Carcinogenesis* **17:**2597–2602.
Raymond E, Faivre S, Woynarowski J and Chaney S (1998) Oxaliplatin: Mechanism of action and antineoplastic activity (review). *Semin Oncol* **25:**4–12.
Reardon JT, Vaisman A, Chaney SG and Sancar A (1999) Efficient nucleotide excision repair of cisplatin, oxaliplatin, and bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. *Cancer Res* **59:**3968–3971.
Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K and Fojo T (1996) Oxaliplatin, tetraplatin, cisplatin, and carboplatin: Spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. *Biochem Pharmacol* **52:**1855–1865.
Roberts JJ and Friedlos F (1982) The frequency of interstrand cross-links in DNA following reaction of cis-diamminechloroplatinum(II) with cells in culture or DNA in vitro: Stability of DNA crosslinks and their repair. *Chem Biol Interact* **39:**181–189.
Roberts JJ and Friedlos F (1987) Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: Relationship to toxicity. *Pharmacol Ther* **34:**215–246.
Roberts JJ, Knox RJ, Pera MF, Friedlos F and Lydall DA (1988) The role of platinum-DNA interactions in the cellular toxicity and anti-tumour effects of platinum co-ordination compounds. *Dev Oncol* **54:**16–31.
Sanderson BJS, Ferguson LR and Denny WA (1996) Mutagenic and carcinogenic properties of platinum-based anticancer drugs. *Mutat Res* **355:**59–70.
Saris CP, Van de Vaart PJM, Rietbroek RC and Blommaert FA (1996) In vitro formation of DNA adducts by cisplatin, lobaplatin and oxaliplatin in calf thymus DNA in solution and in cultured human cells. *Carcinogenesis* **17:**2763–2769.
Scheeff ED, Briggs JM and Howell SB (1999) Molecular modeling of the intrastrand guanine-guanine DNA adducts produced by cisplatin and oxaliplatin. *Mol Pharmacol* **56:**633–643.
Schmidt W and Chaney SG (1993) Role of carrier ligand in platinum resistance of human carcinoma cell lines. *Cancer Res* **53:**799–805.
Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC and Chaney SG (1998) The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: Correlation with replicative bypass of platinum-DNA adducts. *Cancer Res* **58:**3579–3585.
Weiss RB and Christian MC (1993) New cisplatin analogues in development: A review. *Drugs* **46:**360–377.
Woynarowski JM, Chapman WG, Napier C and Herzig MCS (1999) Induction of AT-specific DNA-interstrand crosslinks by bizelesin in genomic and simian virus 40 DNA. *Biochim Biophys Acta* **1444:**201–217.
Woynarowski JM, Chapman WG, Napier C, Herzig MCS and Juniewicz P (1998) Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. *Mol Pharmacol* **54:**770–777.
Woynarowski JM, McHugh M, Gawron LS and Beerman TA (1995) Effects of bizelesin (U-77779), bifunctional akylating minor groove agent, on genomic and simian virus 40 DNA. *Biochemistry* **34:**13042–13050.
Woynarowski JM, McNamee H, Szmigiero L, Beerman TA and Konopa J (1989) Induction of DNA-protein crosslinks by antitumor 1-nitro-9-aminoacridines in L1210 leukemia cells. *Biochem Pharmacol* **38:**4095–4101.
Woynarowski JM, Napier C, Koester S, Chen S-F, Troyer D, Chapman W and MacDonald JR (1997) Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene, 6-hydroxymethylacylfulvene (HMAF, MGI 114). *Biochem Pharmacol* **54:**1181–1193.
Woynarowski JM, Sigmund RD and Beerman TA (1988) Topoisomerase II-mediated lesions in nascent DNA: Comparison of the effects of epipodophyllotoxin derivatives VM-26, and VP-16, and 9-anilinoacridine derivative, m-AMSA and o-AMSA. *Biochim Biophys Acta* **950:**21–29.

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