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ORIGINAL ARTICLE

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In vitro studies on the mechanisms of oxaliplatin resistance

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Abstract *Purpose*: We have previously reported that elevation of glutathione mediated by γ-glutamyl transpeptidase is one mechanism of oxaliplatin resistance. This study explored other potential oxaliplatin resistance mechanisms with two aims: (1) to identify the differences between cisplatin and oxaliplatin in terms of drug accumulation, DNA-Pt adduct formation and repair, and (2) to determine whether defects in drug accumulation and enhanced repair of the DNA-Pt adduct contribute to oxaliplatin resistance. *Methods*: The human ovarian carcinoma cell line A2780, an oxaliplatin-resistant variant A2780/C25 and a cisplatin-resistant variant A2780/CP along with an inherently cisplatin-resistant HT-29 colon carcinoma cell line were used in the study. The methods consisted of sulforhodamine-B assays, atomic absorption spectrophotometry and real-time quantitative RT-PCR. *Results*: Significantly higher drug accumulation and DNA-Pt adduct formation were observed after exposure to cisplatin compared to after oxaliplatin in the parent A2780 cells and the oxaliplatin-resistant A2780/C25 cells. The DNA-Pt adduct formed after treatment with either drug was repaired with equal efficiency by all cell lines except A2780/CP, which repaired the DNA-cisplatin adduct more efficiently than the DNA-oxaliplatin adduct. Relative to the parent line, the oxaliplatin-resistant A2780/C25 cells showed reduced Pt accumulation and DNA-Pt adduct levels following exposure to oxaliplatin, but only reduced accumulation after exposure to cisplatin. The cisplatin-resistant A2780/CP cells showed reduced accumulation and DNA-Pt adduct levels after exposure to cisplatin, but only reduced DNA-Pt adduct after exposure to oxaliplatin. In comparison to A2780 cells, the inherently cisplatin-resistant HT-29 cells showed lower accumulation and DNA-Pt adduct levels after exposure to cisplatin, but displayed no difference after exposure to oxaliplatin. An enhanced repair of the DNA-cisplatin adduct was observed only in A2780/CP cells relative to A2780 cells in an 8-h period. The steady-state levels of ERCC-1 mRNA, but not of XPA, were moderately elevated in the resistant cells. Exposure to either one of the drugs resulted in an induction of XPA in all the cell lines and of ERCC-1 in cisplatin-resistant cells. There was no relationship between the level of expression of the repair genes and the DNA-Pt adduct levels or repair. *Conclusions*: Relative to cisplatin a lower intracellular concentration and fewer DNA-Pt adducts are sufficient for oxaliplatin to exert its cytotoxicity. Resistance to oxaliplatin is mediated by similar mechanisms of reduced drug accumulation and DNA-Pt adduct formation as resistance to cisplatin. There is no clear evidence that enhanced repair is a mechanism of oxaliplatin resistance in the cell line (A2780/C25) studied here. The findings are suggestive of yet unidentified differences between the two drugs with respect to cellular uptake and/or efflux and repair of DNA-Pt adducts.

Keywords Oxaliplatin · Cisplatin · Resistance · Accumulation · Adducts

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Introduction

Oxaliplatin (*trans-l*-1,2-diaminocyclohexane oxalato platinum II) is a third generation platinum (Pt) complex that is active in metastatic colorectal cancer [37]. The drug also shows activity in the treatment of ovarian cancer [3]. Oxaliplatin is non-nephrotoxic and hematologically less toxic than carboplatin [7, 8, 20]. Preclinical studies have shown synergy between oxaliplatin and drugs that are thymidylate synthase inhibitors such as 5-fluorouracil (5-FU) and AG-337 [25]. Phase III clinical studies indicate that the oxaliplatin/5-FU/leucovorin combination has superior activity in advanced colorectal

cancers than 5-FU/leucovorin [5, 12]. Oxaliplatin is an approved drug in Europe for first-line therapy of colorectal cancer.

Oxaliplatin shows activity in a number of cell lines which exhibit intrinsic or acquired resistance to cisplatin [22, 26, 29]. Studies carried out in cell lines of the NCI anticancer drug screening panel comparing oxaliplatin and other Pt agents have shown that cisplatin and oxaliplatin have different sensitivity profiles, suggesting that the two complexes may have different mechanism(s) of action and/or resistance [29].

In vitro studies have indicated that oxaliplatin produces qualitatively similar DNA-Pt adducts as cisplatin with predominantly intrastrand crosslinks (GG > AG) and low levels of interstrand crosslinks [38]. These studies have also indicated that at equimolar concentrations, oxaliplatin produces fewer DNA lesions than cisplatin [32, 38, 39].

Resistance to cisplatin has been studied extensively and found to be multifactorial, consisting of decreased drug accumulation, increased detoxification mediated by glutathione (GSH) or metallothionein, and increased repair of DNA damage [4, 13]. Preclinical and clinical studies have shown a relationship between cisplatin resistance and elevated expression of the nucleotide excision repair genes ERCC-1 and XPA [28]. Also implicated in cisplatin resistance are enhanced replicative bypass [19, 26] and loss of mismatch repair [9, 10, 36]. These latter studies were aimed at gaining an insight into the relationship between the DNA adducts produced by Pt complexes of differing carrier ligands, the replicative bypass and/or the mismatch repair and resistance or sensitivity to the Pt complexes.

Enhanced replicative bypass, which is an enhanced ability of the replication complex to synthesize DNA beyond the lesion, has been demonstrated for cisplatin-DNA adducts in cisplatin-resistant cells compared to parent cells, but not for oxaliplatin-DNA adducts [19, 26]. Other studies from this group have indicated that DNA polymerases, the mismatch repair system and damage recognition proteins could all influence replicative bypass and therefore may impact on resistance to Pt agents with different carrier ligands [2]. Loss of mismatch repair as a mechanism of cisplatin, but not oxaliplatin, resistance has been found in both in vitro and in vivo studies [10]. It is postulated that a functional mismatch repair complex recognizes cisplatin-DNA adducts, but not oxaliplatin-DNA adducts and that mismatch repair proteins are involved in mediating apoptotic responses to DNA damage [10, 11]. Molecular modeling studies have revealed distinct differences between the structures of cisplatin and oxaliplatin DNA adducts, supporting this hypothesis [33]. In mismatch repair-proficient and -deficient cells, a differential activation of damage response pathways has been shown for cisplatin but not oxaliplatin, again supporting the postulate [21]. Other studies have indicated that mismatch repair defects may contribute to increased replicative bypass of the cisplatin adduct [36].

The majority of studies carried out to explore mechanisms of resistance to Pt complexes have used cisplatin-resistant cell lines. We have developed oxaliplatin-resistant sublines of the A2780 ovarian carcinoma cell line in our laboratory by incremental exposure to oxaliplatin [6]. Two of these cell lines, C10 and C25, were 8- and 12-fold resistant to oxaliplatin and have increased cellular GSH and increased mRNA expression and activity of the GSH salvage pathway enzyme γ-glutamyl transpeptidase (γGT), but not the biosynthetic pathway enzyme γ-glutamylcysteine synthetase (γGCS) [6]. These cell lines have not been characterized with respect to potential defects in drug accumulation and DNA-Pt adduct formation after exposure to oxaliplatin.

The present study was undertaken to gain further understanding of the oxaliplatin resistance phenotype with respect to Pt accumulation and DNA-Pt adduct levels, and at the same time an insight into the differences between oxaliplatin and cisplatin. Repair of the DNA-Pt adduct and the mRNA expression of ERCC-1 and XPA were also studied to evaluate their potential relationship to oxaliplatin resistance. The human tumor cell lines used in this study included the ovarian carcinoma cell line A2780, the oxaliplatin-resistant subline A2780/C25 and a cisplatin-resistant variant A2780/CP. The A2780/CP subline was used in our previous studies evaluating isomers of oxaliplatin [23]. Also included in this comparative study was HT-29, a colon carcinoma cell line that is inherently cisplatin-resistant and oxaliplatin-sensitive [22]. In all the experiments oxaliplatin and cisplatin were compared simultaneously. Thus, the results presented here, with respect to drug accumulation, DNA-Pt adduct levels, repair and the mRNA expression of ERCC-1 and XPA delineate (1) the differences between oxaliplatin and cisplatin in these different cell lines and (2) the differences between sensitive and resistant cell lines with respect to each of the drugs.

Materials and methods

Drugs

Oxaliplatin was kindly provided by Dr. Paul Juniewicz of Sanofi-Synthelabo (Malvern, Pa.) and previously by Dr. Yoshinori Kidani (Fujisawa, Japan). Cisplatin was purchased from Sigma Chemical Company (St. Louis, Mo.).

Cell culture

All cell lines used are of human origin. The HT-29 colon carcinoma cell line is from the American Type Tissue Collection (Rockville, Md.). The A2780 and A2780/CP cell lines were a gift from Dr. R. Ozols (Fox Chase Cancer Center, Philadelphia, Pa.). The oxaliplatin-resistant A2780/C25 cell line was developed at RPCI by repeated exposure of A2780 cells to increasing concentrations of oxaliplatin over a 10-month period [6]. All cell lines were maintained in drug-free RPMI-1640 medium (Life Technologies, Grand Island, N.Y.) supplemented with 10% fetal bovine serum and 1% L-glutamine and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity

The cytotoxicity experiments were carried out using the sulforhodamine B (SRB) microculture colorimetric assay as previously described [23, 30]. The cells were exposed to oxaliplatin or cisplatin for 72 h after which they were fixed and subjected to the SRB assay.

Pt Accumulation and DNA binding

Cells in log phase (6×10^6 cells per 75-cm^2 culture flask) were exposed to 3, 10, or 30 μM oxaliplatin or cisplatin. Cells in triplicate flasks were used each for the accumulation and DNA-Pt adduct measurements at every concentration. After 20 h, the drug-containing medium was removed and cells were washed thoroughly with phosphate-buffered saline (PBS). The drug concentrations and exposure times chosen were of pharmacological relevance to oxaliplatin and reflected peak concentrations of Pt in plasma and ultrafiltrate in clinical use and average terminal phase half-life for Pt in ultrafiltrate, respectively [18, 34].

For accumulation experiments, cells were trypsinized and centrifuged into a pellet and lysed in 0.5 ml 0.1% Triton X-100 and 0.2% nitric acid overnight and sonicated for 1 min [14, 24]. Pt in the cell lysate was measured by graphite furnace atomic absorption spectrophotometry (GFAA) with Zeeman background correction (PE 4100ZL, Norwalk, Ct.). Protein was measured using the Bradford assay [1].

For DNA-Pt adduct measurement, DNA was extracted from drug-exposed cells using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.) according to the manufacturer's recommendations. DNA was hydrolyzed in 5% HCl (0.5 ml) for 30 min at 95ºC and quantitated spectrophotometrically by A_{260} relative to identically hydrolyzed calf thymus DNA standards [23]. Pt in the hydrolyzed DNA was measured by GFAA.

DNA repair

Cells (6×10^6 cells per 75-cm^2 cell culture flask) were incubated with drug for 20 h, the drug-containing medium was removed, cells were washed three times with PBS, and incubated for an additional 4 or 8 h in drug-free medium. DNA isolation, quantitation, and Pt measurements were done as for the DNA binding experiments. Repair experiments were carried out with similar initial levels of DNA-bound Pt in all cell lines after a 20-h drug exposure (35–50 pg Pt per microgram DNA). The drug concentrations used to achieve this were as follows: A2780, 15 μM cisplatin, 30 μM oxaliplatin; A2780/C25, 15 μM cisplatin, 40 μM oxaliplatin; A2780/CP, 20 μM cisplatin, 40 μM oxaliplatin; HT29, 30 μM cisplatin, 30 μM oxaliplatin.

mRNA expression

The mRNA levels of ERCC-1 and XPA were measured using real-time quantitative RT-PCR (Taqman assay) using a PE-ABI 7700 sequence detection system. β-Actin was used as the endogenous standard. The mRNA levels of the genes of interest (ERCC-1 and XPA) are expressed relative to that of the endogenous standard (β-actin) measured concurrently from the same cDNA preparations. Steady-state levels of both genes were measured in log-phase cells (24 and 48 h after plating). For induction experiments, cells were exposed to a 30 μM concentration of each of the drugs for 20 h.

Total RNA was extracted using RNeasy spin columns (Qiagen, Valencia, Calif.). cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Grand Island, N.Y.). The quantitation of the PCR product was in real time with fluorescence detection and no post-PCR processing of samples. The PE-ABI 7700 is a closed tube system, with a capacity of 96 tubes. The instrument has a built-in thermal cycler, a laser to induce fluorescence, a charge coupled device (CCD) detector and real-time sequence detection software. During the PCR, a fluorogenic probe consisting of an oligonucleotide with a reporter and a quencher dye attached to the 5′ and 3′ ends, respectively, anneals specifically between the forward and reverse primers. During the extension phase of the PCR, the reporter dye is cleaved from the probe due to the 5′ nuclease activity of the DNA polymerase (Amplitaq Gold) and separated from the quencher generating a fluorescence signal. With each cycle, additional reporter dye molecules are cleaved and the fluorescence intensity is monitored in real time throughout the PCR. The higher the initial copy number of the target gene, the sooner a significant increase in fluorescence is observed.

A parameter called C_T (threshold cycle) is used for quantitation. C_T is the fractional cycle number at which the fluorescence generated after the cleavage of the reporter dye from the probe passes a fixed threshold above the baseline. The higher the initial copy number, the lower the value of C_T. A plot of log initial copy number (e.g. serial dilution of the cDNA preparation) versus C_T is a straight line. Quantitation is carried out by the comparative C_T method [15]. For this, the efficiencies of the reactions for the target gene and the endogenous standard should be similar. With this method the differences in C_T (ΔC_T) between the gene of interest and the endogenous standard (β-actin) is determined. The ΔC_T is translated into copy number difference with the formula $2^{\Delta CT}$ which is derived from the equation that describes the exponential amplification of PCR: $X_n = X_0(1 + E_x)^n$, where X_0 is the initial number of target molecules, E_x the efficiency of amplification, and n the number of cycles. At 100% efficiency, the equation becomes $X_0(2)^n$. The efficiency of reaction for all the genes studied here was determined to be near 100%. A normalized value of expression for the target gene with reference to β-actin is then obtained by the calculation $1/2^{\Delta CT}$.

Primers and probes were designed based on the published cDNA sequences from Genbank downloaded through the National Center for Biotechnologies (NCBI) using the Primer Express program supplied with the PE-ABI 7770. ERCC-1 primers and probe were synthesized at the RPCI Biopolymer Core Facility and the XPA by PE-ABI.

Specific method development for each of the genes consisted of (1) optimizing primer/probe concentrations, (2) evaluating amplification efficiencies for different primer/probe sets, (3) evaluating relative efficiency between the target gene and the endogenous standard (β-actin) at several cDNA concentrations, (4) subjecting the PCR product to gel electrophoresis to ensure the presence of a single band of the expected size, and (5) sequencing the PCR product. During PCR for all the experiments, template-free controls and RT controls were used.

The primers and probes used in the PCR reaction were as follows. 6-Carboxy-fluorescein (FAM) was the reporter dye and 6-carboxy-tetramethylrhodamine (TAMRA) was the quencher dye on the probe:

- ERCC-1 (accession no. M28650):
- Forward primer: TACCCCTCGACGAGGATGAG
- Reverse primer: ATGGCATATTCGGCGTAGGT
- Taqman probe: 5′ FAM-TGGAGTGGCCAAGCCCTTATT-CCG-TAMRA 3′
- XPA (accession no. D14533):
- Forward primer: 5′- GCTGCCCGGCCCTACT-3′
- Reverse primer: 5′- GCCTCCTCCTGTGTCAATTATCTT-3′
- Taqman probe: 5′- FAM- ATTAGCCATGCCTCCAGTA-GCCGCA-TAMRA-3′
- β-Actin (accession no. M10278):
- Forward primer: GCTCCTCCTGAGCGCAAGT
- Reverse primer: GATGTGGATCAGCAAGCAGGA
- Taqman probe: FAM-5′-CCATCCTGGCCTCGCTGTCCA-3′-TAMRA

Statistical analysis

Student's t-test was used with the computer program 'Epistat' for the evaluation of statistical significance of differences between the drugs and cell lines.

Results

Cytotoxicity studies

The degree of resistance and the cross-resistance patterns for oxaliplatin and cisplatin were evaluated for the four cell lines using the SRB assay. The results are presented in Table 1. The oxaliplatin-resistant A2780/C25 cells were 8.4-fold resistant to oxaliplatin and 5.1-fold cross-resistant to cisplatin. A2780/CP cells were 10.9-fold resistant to cisplatin and 2-fold cross-resistant to oxaliplatin. Relative to A2780 cells, HT-29 cells were 5-fold resistant to cisplatin, but exhibited greater sensitivity to oxaliplatin. The differences in IC_{50} values between the resistant cell lines and A2780 cells for both drugs were statistically significant (P-values ranged from 0.05 to 7.6×10^{-5}).

Pt accumulation

The accumulation of Pt in cells after a 20-h exposure to oxaliplatin or cisplatin is shown in Fig. 1A, B, respectively. At equimolar concentrations, there was a significantly higher accumulation of Pt after cisplatin exposure than after oxaliplatin exposure in A2780 and A2780/C25 cells ($P<0.01$ for both). No significant differences in accumulation were observed for the two drugs in A2780/CP and HT-29 cells.

There were significant differences in accumulation of Pt between resistant and sensitive cell lines. Compared to A2780 cells, oxaliplatin-resistant A2780/C25 cells showed a significantly lower accumulation of both oxaliplatin and cisplatin ($P<0.01$ for both). Cisplatin-resistant A2780/CP cells, on the other hand, showed a significant decline in cisplatin accumulation ($P=0.003$), but not in oxaliplatin accumulation. In comparison to A2780 cells, inherently cisplatin-resistant HT-29 cells showed significantly less accumulation of cisplatin ($P=0.004$), but not of oxaliplatin. The significant differences between the resistant cell lines and the A2780 cell line with respect to drug accumulation after oxaliplatin or cisplatin exposure are summarized in Table 2.

Table 1 Cytotoxicity and cross-resistance patterns of oxaliplatin and cisplatin in the four cell lines. Values are means ± SD ($n=3$)

Cell line	Oxaliplatin		Cisplatin	
	IC_{50} (μM)	Fold resistance	IC_{50} (μM)	Fold resistance
A2780	0.66 ± 0.05	1	0.68 ± 0.03	1
A2780/C25	5.5 ± 0.5	8.4 ± 0.15	3.4 ± 0.66	5.1 ± 0.98
A2780/CP	1.3 ± 0.26	2.0 ± 0.27	7.3 ± 3.4	10.9 ± 4.9
HT29	0.33 ± 0.14	0.5 ± 0.24	3.4 ± 0.7	5.0 ± 1.1

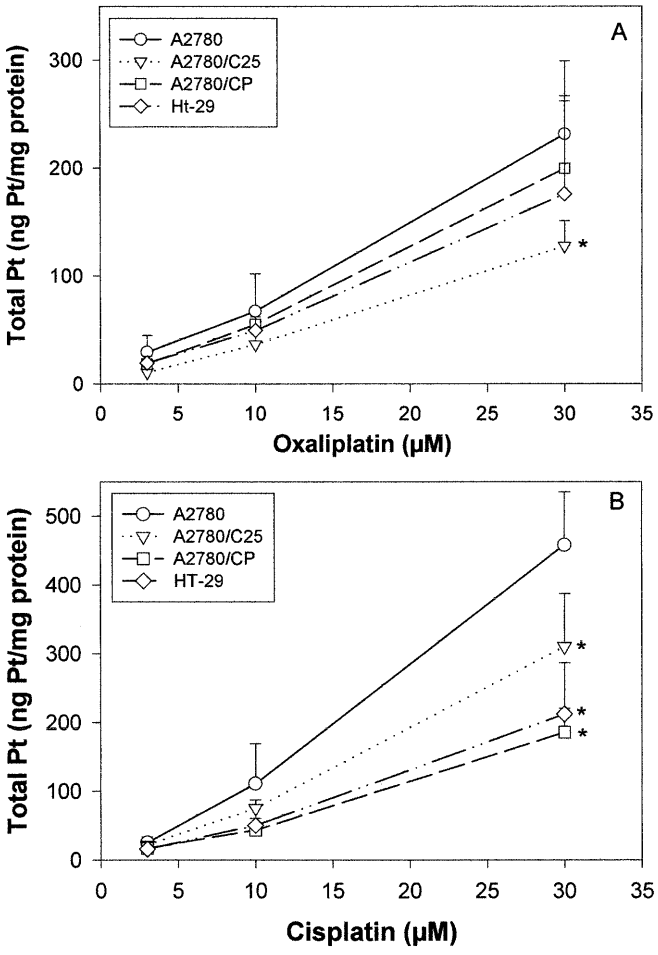

Fig. 1A, B Accumulation of Pt in cells after a 20-h continuous exposure to 3, 10 and 30 μM oxaliplatin (**A**) or cisplatin (**B**) ($n=3$). *Significant difference between this cell line and A2780

DNA-Pt adduct levels

The total DNA-Pt adduct levels after a 20-h exposure to oxaliplatin or cisplatin are shown in Fig. 2A, B, respectively, in the different cell lines. A comparison of the DNA-Pt levels between the two drugs indicate that at equimolar concentrations, there was less Pt bound to the DNA after oxaliplatin exposure than after cisplatin exposure in A2780 and A2780/C25 cells ($P<0.01$ for both). No statistically significant differences in DNA-Pt levels were seen between the two drugs in A2780/CP and HT-29 cells.

There was a significant difference in the amount of DNA-bound Pt between resistant and sensitive cell lines. Compared to A2780 cells, a significantly lower DNA-Pt adduct formation was seen in A2780/C25 cells after oxaliplatin exposure ($P=0.026$), but not after cisplatin exposure. A2780/CP cells, on the other hand, showed a significantly lowered DNA-Pt adduct for both drugs ($P<0.01$ for both). In comparison to A2780 cells, inherently cisplatin-resistant HT-29 cells had lowered DNA-Pt adduct after cisplatin exposure ($P=1.1\times10^{-4}$), but not after oxaliplatin exposure. The significant

Table 2 Summary of significance of differences in Pt accumulation, DNA-Pt adduct levels and repair in resistant cells relative to A2780 cells (↓ statistically significant decrease, ↑ statistically significant increase, *NS* no statistically significant change)

Cell line	Difference relative to A2780 (%)							
	Resistance		Accumulation after		DNA-Pt after		DNA repair	
	Oxaliplatin	Cisplatin	Oxaliplatin	Cisplatin	Oxaliplatin	Cisplatin	Oxaliplatin	Cisplatin
A2780/C25	8.4×	5.1×	44.9 ± 10.1↓	32.3 ± 16.9↓	32.9 ± 20.7↓	NS	NS	NS
A2780/CP	2.0×	10.9×	NS	59.5 ± 7.7↓	39.1 ± 18.5↓	69.1 ± 6.8↓	NS	81.8 ± 16.0↑
HT-29	0.5×	5.0×	NS	57.3 ± 22.2↓	NS	59.8 ± 10.0↓	NS	NS

differences observed between resistant cell lines and A2780 cells in DNA-Pt adduct levels after oxaliplatin or cisplatin exposure are summarized in Table 2.

DNA repair

Repair was measured as the time-dependent loss of Pt from cellular DNA in an 8-h period when drug-exposed cells were reincubated in drug-free medium. The 8-h period was chosen based on the fact that for all the cell lines the doubling times were more than 17 h and therefore any new DNA synthesis could be expected to be insignificant. As evident from Fig. 3A, B, with the exception of A2780/CP cells, all cell lines repaired both the DNA-cisplatin adduct and DNA-oxaliplatin adduct with equal efficiency. A2780/CP cells repaired the DNA-cisplatin adduct more efficiently than the DNA-oxaliplatin adduct. This cell line repaired about 82% of the DNA-cisplatin adduct in 8 h, compared to about 30%

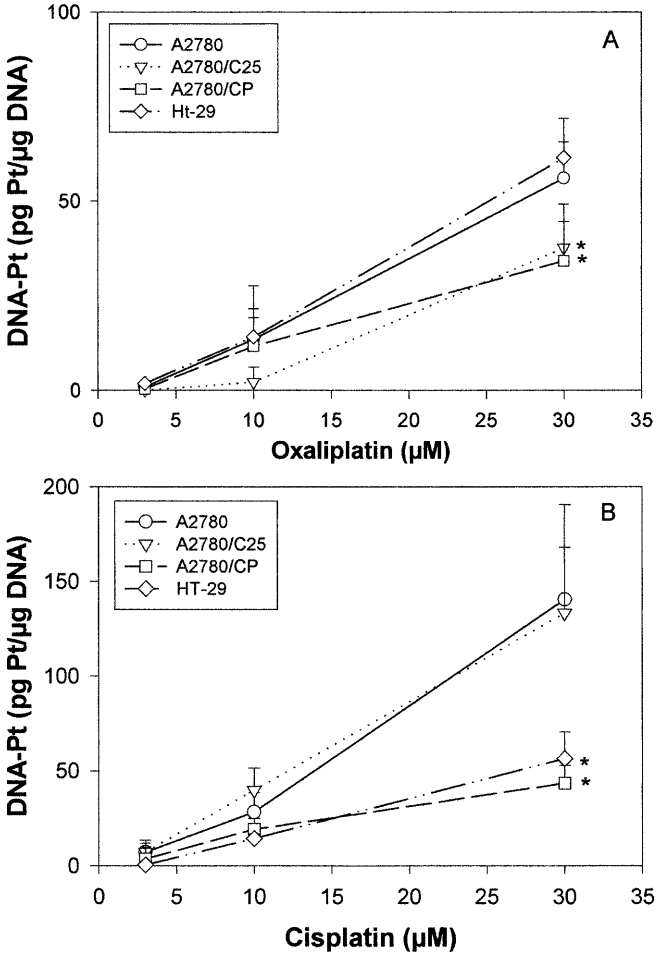

Fig. 2A, B Total DNA-bound Pt when cells were exposed to 3, 10 or 30 µ*M* oxaliplatin (**A**) or cisplatin (**B**) for 20 h ($n=3$). *Significant difference between this cell line and A2780

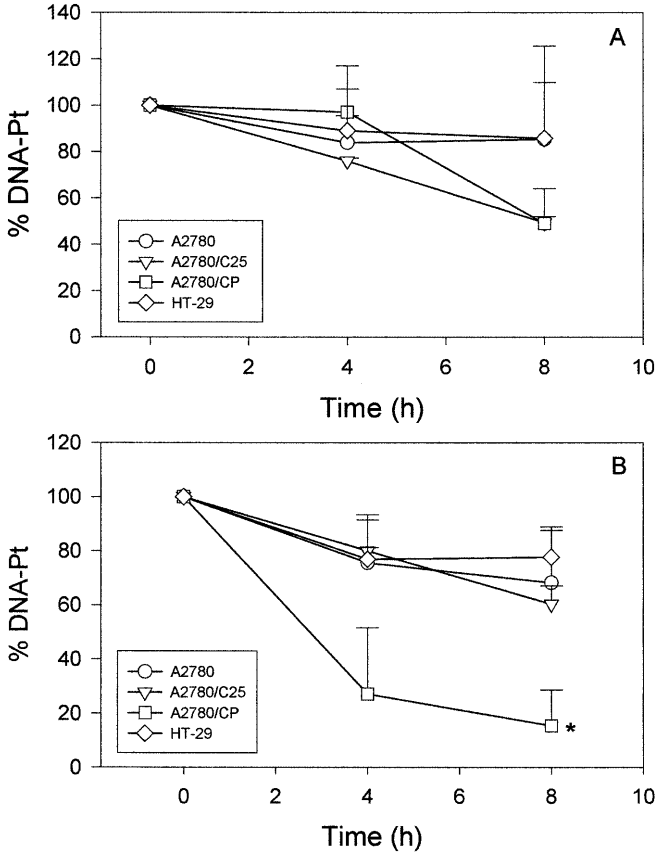

Fig. 3A, B Repair of DNA-Pt adduct measured as the loss of Pt from cellular DNA with time when cells previously exposed to oxaliplatin (**A**) or cisplatin (**B**) for 20 h were incubated in drug-free medium. The initial DNA-Pt (pg/µg DNA) at the time cells were incubated in drug-free medium were as follows: after oxaliplatin: A2780 41.4 ± 12.7, A2780/C25 35.4 ± 7.0, A2780/CP 41.3 ± 11.1, HT-29 48.9 ± 13.2; after cisplatin: A2780 54.2 ± 16.9, A2780/C25 41.8 ± 14.9, A2780/CP 21.4 ± 3.9, HT-29 48.1 ± 8.6 ($n=2$). *Significant difference between this cell line and A2780

by the parent A2780 cells ($P = 0.03$). The differences in repair for the other cell lines were not significant.

The significant differences observed between the resistant cell lines and A2780 cells are summarized in Table 2, with respect to drug accumulation, DNA-Pt adduct levels and repair.

Steady-state mRNA levels and induction of ERCC-1 and XPA

The steady-state mRNA levels of ERCC-1 and XPA are shown for the four cell lines in Fig. 4A, B, respectively. The mRNA levels of ERCC-1 showed 1.3-, 1.7- and 1.9-fold elevation in HT-29, A2780/CP and A2780/C25 cells, respectively, in relation to A2780 cells. Only the difference between A2780/C25 cells and A2780 cells was statistically significant ($P = 0.048$). The steady-state mRNA levels of XPA were not significantly different between A2780 cells and its resistant sublines.

Because the DNA binding and repair experiments were carried out following 20-h exposures to the Pt drugs, experiments to evaluate the induction of the ERCC-1 and XPA genes were carried out at the same time point (Fig. 5A, B). At a concentration of 30 μM in a 20-h drug exposure, XPA was induced in all the cell lines by both cisplatin and oxaliplatin (Fig. 5B). However, ERCC-1 was induced only in the two cisplatin-resistant cell lines HT-29 and A2780/CP, but by both drugs (Fig. 5A). The induction in HT-29 cells was to a significantly higher level than in A2780/CP cells.

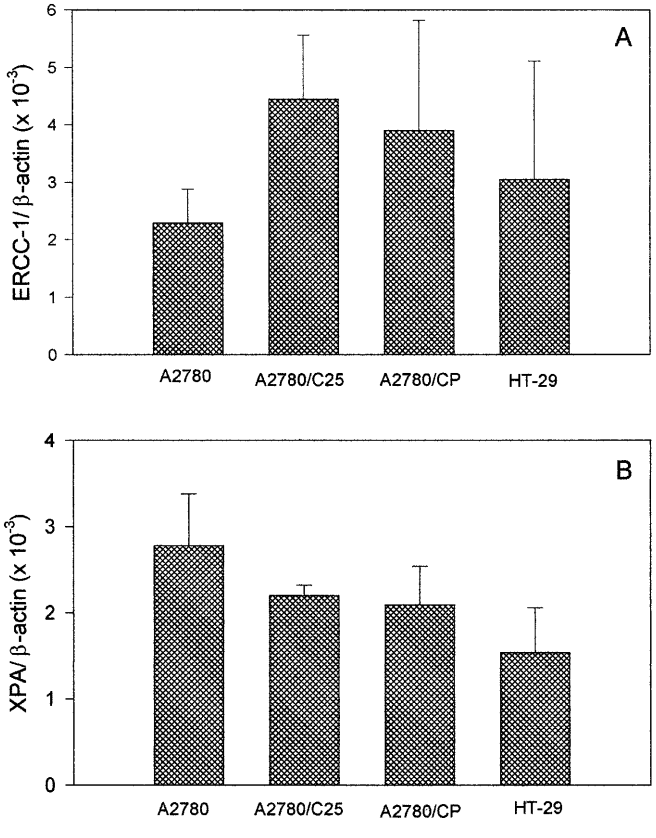

Fig. 4A, B Steady-state mRNA levels of ERCC-1 (**A**) and XPA (**B**) in A2780, A2780/C25, A2780/CP and HT-29 cells ($n = 3$)

Discussion

Clinically oxaliplatin exhibits differences from cisplatin in toxicity and activity profiles [8, 37]. While cisplatin and carboplatin are inactive in the treatment of colorectal cancers oxaliplatin is active [37]. Such observations along with the reported differences in the sensitivity profiles of oxaliplatin and cisplatin [29], raise the question as to whether resistance mechanisms between these two drugs are different. Much of our knowledge of Pt drug resistance comes from cisplatin-resistant cell lines and not from oxaliplatin-resistant lines. The goal of this study therefore was a parallel comparison of oxaliplatin- and cisplatin-resistant cells in terms of drug accumulation, DNA-Pt adduct levels and DNA repair for each of the drugs. Altered GSH

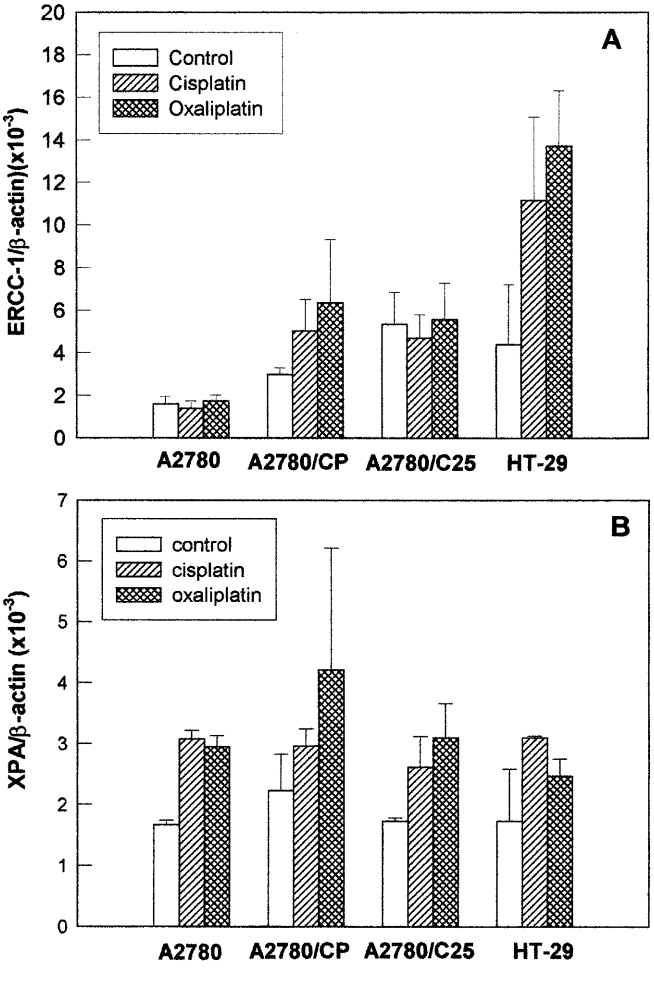

Fig. 5A, B Induction of the expression of ERCC-1 (**A**) and XPA (**B**) genes in different cell lines after a 20-h exposure to a 30 μM concentration of cisplatin or oxaliplatin ($n = 2$)

metabolism in oxaliplatin-resistant cells has been described by us previously [6].

The data obtained in this study indicate that at equimolar concentrations of treatment there was significantly more drug accumulation and DNA-Pt adduct formation after cisplatin exposure than after oxaliplatin exposure in some cell lines (e.g. A2780 and A2780/C25). For A2780 and A2780/C25 cell lines, which have IC_{50} values in the same order of magnitude for oxaliplatin and cisplatin, the Pt accumulation after oxaliplatin exposure was only 50% and 40% of that after cisplatin exposure, respectively. The DNA-Pt adducts in A2780 and A2780/C25 cells after oxaliplatin exposure were 40% and 28% of that after cisplatin exposure, respectively. These observations suggest that, relative to cisplatin, oxaliplatin can exert its cytotoxicity through a lower intracellular drug concentration and fewer DNA-Pt adducts. The bulky oxaliplatin DNA adducts presumably result in more interference with the replication and/or transcription processes affecting cell survival. The observed greater DNA-cisplatin adduct than DNA-oxaliplatin adduct in these cells is in agreement with previous studies [32, 38, 39]. It is to be noted, however, that the impaired drug accumulation and DNA adduct formation after oxaliplatin exposure in A2780/C25 cells may have contributed to the observed differences between cisplatin and oxaliplatin. Similarly, the lack of difference between the two drugs in the cisplatin-resistant cell lines A2780/CP and HT-29 appears to be due to the much reduced cisplatin accumulation and DNA-Pt adduct formation after cisplatin exposure.

In three of the four cell lines, there were no significant differences in the repair of DNA-cisplatin or DNA-oxaliplatin adducts. This finding is in agreement with an earlier report in which, based on the in vitro nucleotide excision repair assays of cell-free extracts, the authors conclude that this process does not discriminate between cisplatin and oxaliplatin [27]. However, the cisplatin-resistant A2780/CP cell line repaired the DNA-Pt adduct after cisplatin exposure much more efficiently than after oxaliplatin exposure, suggesting that other intracellular factors or mechanisms may influence the different rates of repair of these adducts.

Comparison of resistant and sensitive cell lines in terms of drug accumulation, DNA-Pt adduct levels and repair suggest that both oxaliplatin- and cisplatin-resistant cell lines showed impaired drug accumulation. The cisplatin-resistant cells (A2780/CP) showed impairment only to cisplatin, whereas the oxaliplatin-resistant cells (A2780/C25) showed impairment to both drugs, suggesting that membrane alterations produced by these drugs during the isolation of resistant variants is quite different. The oxaliplatin-resistant cell line was cross-resistant to cisplatin and this may have been due to reduced cisplatin accumulation. The findings indicate that lower DNA-Pt adducts observed after oxaliplatin and cisplatin exposure in A2780/C25 and HT-29 cells, respectively, are a reflection of the deficiencies in the accumulation of the corresponding Pt agents and not DNA repair. Whereas, an enhanced repair of DNA-cisplatin adduct was observed in A2780/CP cells, suggesting that in this cell line enhanced DNA repair along with lowered drug accumulation contribute to the resistance phenotype.

Relative to A2780 cells, A2780/CP cells showed significantly lower levels of DNA-Pt adduct after oxaliplatin exposure, and this may account for the observed low degree of cross-resistance to oxaliplatin. Since this cell line was not deficient in oxaliplatin accumulation, it is possible that the decrease in oxaliplatin-derived Pt-DNA adducts may have been due to a DNA repair process that may go on at a much slower rate than that for DNA-cisplatin adduct. The fact that cisplatin-resistant cell lines (A2780/CP and HT-29) defective in cisplatin accumulation did not show significant reduction in oxaliplatin accumulation suggests that the uptake and/or efflux mechanisms for these two drugs may not entirely be common. In A2780/C25 cells, the reduced cisplatin accumulation did not translate into decreased DNA-Pt adduct. This may have to do with the fact that at equal concentrations cisplatin makes more DNA-Pt adduct than oxaliplatin [32, 38, 39].

Decreased DNA-Pt adducts resulting from increased DNA repair has been described in different model systems [4, 16]. Elevated expression of the repair genes ERCC-1 and XPA has been reported in cisplatin resistance both clinically and preclinically [28]. The ERCC-1 gene encodes a 5′ endonuclease and XPA encodes a damage recognition protein [31]. It has been suggested that ERCC-1 may also have a damage recognition function [28]. ERCC-1 is considered to be critical for the nucleotide excision repair pathway [28]. Similarly, testicular tumors hypersensitive to cisplatin therapy have been described as having normal amounts of most nucleotide excision repair pathway proteins but lower amounts of XPA, leading to poor repair of DNA-Pt damage [17].

Because of the implicated critical nature of ERCC-1 and XPA in the repair of Pt drug-induced DNA damage, we compared the expression of these genes between the resistant and sensitive cell lines. There was a modest increase in ERCC-1 expression in the resistant cells, but the expression of XPA was about the same or slightly lower. The higher ERCC-1 levels were seen in both A2780/C25 and A2780/CP cells. Yet, relative to A2780 cells, there were differences between these cell lines in the levels of DNA-Pt adducts formed and removed from each of the drugs. This suggests that the inducibility of ERCC-1 and XPA expression following the drug exposure may be much more important than the steady-state levels present during normal growth conditions [28]. In our study, the induction of the expression of these genes following exposure to the two Pt drugs showed interesting differences. While both drugs induced XPA essentially to the same extent in all the cell lines, only the cisplatin-resistant cell lines, especially HT-29, showed significant induction of ERCC-1. However, in terms of DNA repair, we could not demonstrate a difference between A2780 and HT-29 cells, while we did see a

difference between A2780 and A2780/CP cells. Thus, no clear-cut relationship was observed either between the basal levels or induction of expression and the adduct levels in any of the cell lines.

It has been reported that cells express a full-length and an alternatively spliced form of mRNA for ERCC-1, the latter with exon 8 missing [40]. It is believed that exon 8 encodes the active site of the protein [28]. An inverse relationship between the ratio of the alternatively spliced ERCC-1 mRNA to full length and the adduct repair after cisplatin treatment has been reported [28]. The primers and probe used in our study measured the total ERCC-1 mRNA present in the cells and did not distinguish between the two forms. Future studies are necessary to gain further insight into the adduct levels and relationship to repair and the induction of total and alternatively spliced ERCC-1 mRNA. In addition, the 5′ endonuclease activity of ERCC-1 is produced by an ERCC-1/ERCC-4 (XPF) heterodimer [31, 35]. Therefore, measuring ERCC-1 and ERCC-4 together in cells may give valuable insight into the mechanisms of resistance to Pt agents.

In conclusion, these studies demonstrate that (1) relative to cisplatin a lower cellular amount of oxaliplatin and fewer DNA-Pt adducts are adequate for oxaliplatin to exert its cytotoxicity and (2) resistance to oxaliplatin is due to some of the same mechanisms of resistance as that to cisplatin, such as reduced drug accumulation resulting in reduced DNA-Pt adduct formation and the previously demonstrated elevations in GSH [6]. There are indications from this study that there are yet unidentified cellular mechanisms that distinguish these two drugs such as uptake and/or efflux and also perhaps a differential repair of the DNA-Pt adduct.

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