Effects of gemcitabine on *cis*-platinum-DNA adduct formation and repair in a panel of gemcitabine and cisplatin-sensitive or -resistant human ovarian cancer cell lines

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Abstract. Gemcitabine (dFdC) can increase the sensitivity of both cisplatin (CDDP)-sensitive and -resistant cell lines. It has been postulated that both formation and repair of platinum-(Pt)-DNA adducts are related to these effects. Therefore, we investigated the effects of dFdC on the formation and repair of Pt-DNA adducts in the human ovarian cancer cell line, A2780, and its CDDP- or dFdC-resistant variants, ADDP and AG6000, which have a different expression of various repair enzymes. Cells were exposed for 1 h to CDDP alone or combined with dFdC in IC_{50} concentrations, followed by a 1-h exposure to thiourea and, subsequently, by a drug-free period of 1, 3 or 23 h (i.e. 2, 4 or 24 h after CDPP ± dFdC removal). Pt-DNA adducts were quantified with ^{32}P-post-labeling. The gene expression of the repair enzymes, XPA and XRCC1, was the same in all 3 cell lines but ERCC1, ERCC3 and XPC were 2-6 times higher in AG6000 compared to A2780 cells. In contrast, both ERCC1 and ERCC3 were 10- and 1.5-fold lower in ADDP cells compared to A2780. The mismatch enzyme, MLH1, was lower in ADDP cells. At equally toxic CDDP concentrations, all cell lines formed comparable peak levels of total Pt-DNA adducts (36-48 fmol/μg DNA). However, the time at which peak levels were reached showed large variation. The repair of the adducts was very efficient in the resistant cell lines whereas, in A2780 cells, plateau levels were retained until 24 h after CDDP exposure. In A2780 cells, dFdC shifted the adduct peaks from 4 h to directly after CDDP exposure and increased peak levels by >3.9-fold. dFdC also enhanced the repair of adducts by >1.7-fold and increased the Pt-GG:Pt-AG ratio compared to CDDP alone by >1.4-fold. Overall, dFdC decreased the area under the Pt-DNA adduct-time curve ($AUA_{0\text{-}25\ h}$) in A2780 cells by 2.7-fold. In ADDP cells, dFdC shifted the adduct peaks from 2 to 4 h and increased them by >2.2-fold. dFdC also increased the Pt-GG:Pt-AG ratio during the repair process by 1.4-fold. Overall, dFdC increased the $AUA_{0\text{-}25\ h}$ in ADDP cells by 1.7-fold. In AG6000 cells, dFdC increased the Pt-GG:Pt-AG ratio by 1.6-fold directly after exposure but did not clearly affect the $AUA_{0\text{-}25\ h}$. In conclusion, dFdC can affect both Pt-DNA adduct formation and repair, depending on the initial sensitivity of the cells.

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Introduction

cis-Diamminedichloroplatinum (cisplatin, CDDP) is an established anticancer drug with activity in a variety of solid tumor types, including head and neck cancer (HNC), ovarian cancer and non-small cell lung carcinoma (NSCLC). Its major disadvantage, however, is a relapse in most tumors after an initial response (1). CDDP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in mono-adducts and various types of bifunctional platinum (Pt) adducts (2). The main adduct is *cis*-$Pt(NH_3)_2$d(GpG) (Pt-GG), with Pt bound to two adjacent guanines. Another major intrastrand crosslink is *cis*-$Pt(NH_3)_2$d(ApG) (Pt-AG), in which the Pt is bound to adenine and an adjacent guanine. A tentative relationship between Pt-DNA adduct levels and anti-tumor response in cultured cells (3) and in patients has been postulated (4,5).

Resistance to CDDP has been associated with alterations in either nucleotide excision repair (NER) or mismatch repair (MMR) enzymes (6). Although an increased expression of the NER enzyme, ERCC1, has been associated with CDDP resistance (7-10), a deficiency of the MMR enzyme, MLH1, has also been described (11). ERCC1 has also been implicated in the interaction between dFdC and CDDP (12).

2',2'-Difluorodeoxycytidine (gemcitabine, dFdC) is a deoxycytidine analogue (13) with clinical activity against several solid tumors, such as ovarian cancer, NSCLC, HNC and pancreatic cancer (14). After entering the cell, dFdC is phosphorylated by deoxycytidine kinase (dCK) to its tri-phosphate (dFdCTP), which can be incorporated into DNA,

followed by one more deoxynucleotide, after which DNA-polymerization stops (15). The latter probably determines its cytotoxic effect. Besides this effect, dFdCDP is capable of inhibiting ribonucleotide reductase (RR) (16), an enzyme with a key role in DNA-repair mechanisms.

In *in vitro* and *in vivo* studies, a synergistic effect of both drugs was found in both CDDP-resistant and -non-resistant tumors and tumor cell lines (17-20). The main mechanism of this synergism is an increase in Pt-DNA adduct formation, which is directly related to the incorporation of dFdC into DNA (18). Large differences were observed between the cell lines, which might be related to the retention and repair of Pt-DNA adducts. These cell lines have profound differences in the expression of enzymes of both the NER and MMR DNA repair systems.

The purpose of the present investigation was to further elucidate the effects of dFdC on the formation and repair of specific Pt-DNA adducts in wild-type and CDDP- and dFdC-resistant ovarian cancer cell lines.

Materials and methods

Drugs and chemicals. *cis*-Diamminedichloroplatinum (CDDP, Cisplatin) was obtained from Bristol-Myers Squibb (Woerden, The Netherlands) and solubilized with phosphate-buffered saline (PBS) to a concentration of 3 mM. 2',2'-Difluoro-deoxycytidine (dFdC, gemcitabine) was a kind gift from Eli Lilly Inc. (Indianapolis, IN, USA) and was solubilized with PBS to a concentration of 10 μM. Final dilutions of both drugs were made in culture medium. All antibodies were murine and were purchased from various sources: XPA, ERCC1, Ku-70 and DNA-PK were from Neomarkers (Fremont, CA, USA); MSH2 from Oncogene (Cambridge, MA, USA); and MLH1 from Zymed (San Francisco, CA, USA). The antibody against MRP2 was a kind gift from Dr R. Scheper from VUMC. All other chemicals were of analytical grade and commercially available.

Cell culture. The experiments were performed with three different human ovarian cancer cell lines. A2780 was the parental cell line (21,22), ADDP was the variant with induced resistance to CDDP (>45-fold resistant compared to A2780 (18,21), and AG6000 was the variant with induced resistance to dFdC (>50000-fold compared to A2780) (22). These cell lines also had a cross-resistance to the other drug although less pronounced; ADDP had a cross-resistance to gemcitabine of 289-fold, and AG6000 of 3.9-fold, compared to A2780 cells. The doubling times of the cell lines were 21, 32 and 37 h, respectively. A2780 and AG6000 cells were cultured in Dulbecco's medium with 5% heat inactivated fetal calf serum (FCS) and ADDP cells were cultured in RPMI medium with 5% heat inactivated FCS. All cell lines were growing exponentially as monolayers during the course of all experiments.

Cells were exposed for 1 h to the IC_{50} (drug concentrations giving 50% growth inhibition) of CDDP alone or in simultaneous combination with dFdC: A2780, 3 μM CDDP and 12.4 nM dFdC; ADDP, 200 μM CDDP and 240 nM dFdC; AG6000, 17 μM CDDP and 1 mM dFdC). These IC_{50} concentrations were based on previous growth inhibition experiments with a 4-h exposure and a total culture time of 72 h, in which the growth inhibition was determined using the sulforhodamine B assay. The 1-h drug exposure was followed by a 1-h incubation with 10 mM thiourea (TU, JT Baker Chemicals BV, Deventer, The Netherlands) in culture medium to prevent further formation of bifunctional Pt-DNA adducts from the reactive monofunctional Pt-DNA adducts (23,24). Cells were isolated by rapid trypsinization directly after the 1-h drug-exposure (before TU treatment) or at 1, 3 or 23 h of culture in drug-free medium after TU treatment; i.e. 2, 4 or 24 h after CDDP (± dFdC) removal. Cells were immediately put on ice and stored at -20˚C until Pt-DNA adduct measurement.

To study whether these treatments would affect the cell number during the course of the experiments, cells were counted after exposure to TU alone, CDDP alone, or the combination of CDDP and dFdC with or without post-exposure to TU. All exposures were performed under the same conditions and concentrations were as described above. After washing away the drugs, the cells were grown for another 23 h in drug-free medium and isolated by rapid trypsinization. The cells were counted using a Sysmex microcellcounter CC-110 (Charles Goffin, De Bilt, The Netherlands).

Platinum-DNA adduct measurement. Pt-DNA adduct levels were analyzed using ^{32}P-postlabeling as described previously (25-27). In short, DNA was digested to unmodified mono-nucleosides and Pt-containing (di)nucleotides. The platinated dinucleotides were than purified on a strong cation-exchange column coupled to an FPLC system (Mono-S, Pharmacia Biotech Benelux, Roosendaal, The Netherlands) after adjusting the pH to ~3. The amount of DNA per sample was calculated by comparison of the total peak areas of the unmodified nucleosides with those of known samples of digested salmon sperm DNA. The platinated dinucleotides were collected in siliconized tubes which already contained 1 pmol of thymidylyl (3'→5') thymidine (dTpT) as an internal standard and 1.2 μmol NaCN, to prevent digestion of the internal standard by nucleases that co-elute with the adducts from the column. After drying in a vacuum, the mixture was dissolved in 12 μl of 0.1 M NaCN and incubated for 2 h at 65˚C, in order to remove Pt from the adducts. In 12 μl of the deplatinated-adduct solution containing 0.2 M NaCN, the pH was adjusted with 3 M sodium acetate (NaAc; pH 5.4) to ~8.2, which is the optimal pH for the enzyme, T4-polynucleotide kinase (PNK). To each sample, 3 μl of labeling-mixture containing 0.5 μl [γ-^{32}P]ATP (3000 Ci/ mmol, 3.3 pmol/μl; Amersham Life Science, Amersham, UK), 2 μl 10 times concentrated kinase buffer (Boehringer) and 0.5 μl PNK (10 U/μl; 3'-phosphatase free; Boehringer), was added followed by a 40-min incubation at 37˚C. The separation of the radioactively labeled products was achieved by Polygram Cell 300 PEI thin layer chromatography (TLC) sheets that had been thoroughly prewashed with 50% MeOH and air-dried. After labeling, 5 μl of the mixtures were applied onto the sheets and run for ~5 h with 1.5 M NH_4-formate buffer (pH 4.0). After the sheets were dried, the amount of radioactivity of each spot was quantified using a Phosphor Imager 425 with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The amounts of Pt-GG and Pt-AG were determined by comparison to the internal standard (1 pmol dTpT in the same

Table I. Gene expression of repair enzymes in A2780 variants.

Repair enzyme	A2780	ADDP	AG6000
ERCC1	8253±511	12217	182056
ERCC3	3320±376	1845±28	10807±1214
XPA	125±24	113±17	170±16
XPC	835±125	784±27	3708±506
XRCC1	6746±1714	4862±523	5872±109
MSH	3639	2959	7510
MSH6	35431	28509	24471

Gene expression is relative to that of ß-actin. Values are means ± SE of at least 3 separate experiments, unless otherwise indicated.

TLC lane) and external standard (1 pmol of dApG and dGpG in a separate lane). The adducts, thus measured, account for >80% of all adducts formed by CDDP after the full completion of the mono- to di-adduct formation (normally 4-6 h after CDDP exposure). Therefore, we refer to the sum of the Pt-AG and Pt-GG adducts as the total Pt-DNA adduct level.

Competitive template RT-PCR. Gene expression of various NER and MMR enzymes was determined by RT-PCR using competitive templates (CT) which were added to each RT-PCR assay as an internal standard. CTs encode a similar region as that covered by the forward and reverse primer but are about 20-25% shorter (28,29). They are designed to be amplified with a similar efficiency as the native templates. Design and optimization of CT-RT-PCR for ERCC1, ERCC3, XPA, XPC and XRCC1 have been described previously (28) as well as isolation of mRNA and reverse transcription with random primers to cDNA (30).

Western blotting. Protein expression of various repair enzymes was also investigated using Western blotting. Isolation of protein and subsequent immunoblotting were performed essentially as described previously for various targets (31) at an antibody concentration of 1 μg/ml, except for Ku70 (0.25 μg/mol).

Expression of ß-actin was included as a control for protein loading.

Immunohistochemistry. In addition to Western blotting, intracellular localization of the repair enzymes was investigated using immunohistochemistry. Cytospins of the cells were prepared, acetone fixated and incubated with the primary and secondary antibodies as described above and visualized using the horseradish peroxidase labeled avidin biotin complex with 3-amino-9-ethylcarbazole as a chromogen.

HeLa cells were included as a positive control for the various repair enzymes.

Statistical evaluation. Areas under the Pt-DNA adduct-time curves ($AUA_{0\text{-}25\ h}$) were calculated using the trapezoidal rule with the Topfit program (version 2.0, Gustav Fischer Verlag, Stuttgart, Germany). Results were evaluated using the two-tailed Wilcoxon Signed Ranks test. P-values are only given when <0.15 and are considered significant when <0.05.

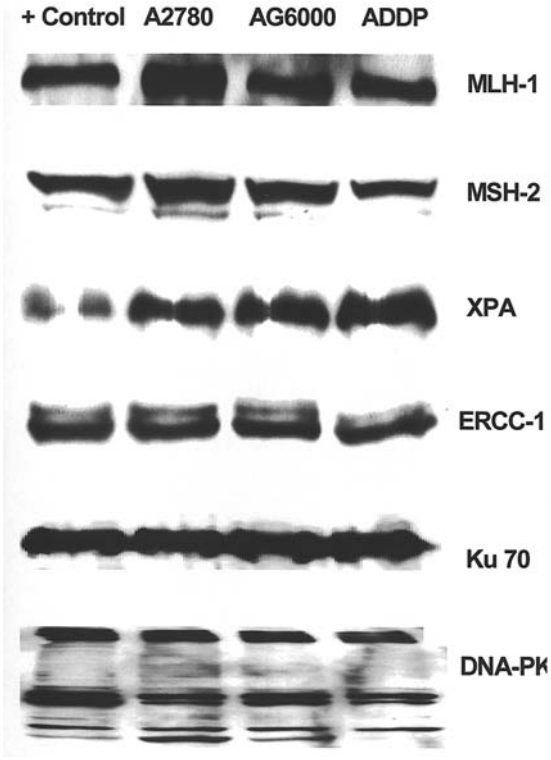

Figure 1. Representative Western blot scan of various repair enzymes in A2780, ADDP and AG6000 cells. Hela cells were used as a positive control, ß-actin was used as a loading control and was similar in the cells.

Results

In order to be able to relate differences in repair of Pt-DNA adducts in either CDDP- or dFdC-CDDP-treated cells, we measured the expression of various NER and MMR enzymes in the parent and resistant cell lines. For gene expression we used CT-RT-PCR. Although AG6000 was selected for dFdC resistance and associated with dCK deficiency, it also showed a cross resistance to other non-related drugs targeted to DNA, including CDDP (32). AG6000 showed an increase in the expression of the NER enzymes, ERCC1, ERCC3 and XPC, but ERCC3 was decreased in ADDP compared to A2780 (Table I). The expression of XPA and XRCC1 was in a comparable range. Using RT-PCR it was not possible to get a signal for MLH1 with the current primers, although the CTs showed a band in the assay. Therefore, expression of this and some other enzymes was also measured using Western blotting (Fig. 1). MLH1 showed a decreased expression in ADDP cells; the expression of several other repair enzymes did not change. In addition, the expression of MRP2/c MOAT which has been reported to play a role in CDDP efflux (33), was increased in ADDP cells but absent in A2780 and AG6000 cells.

In order to study cellular localization, we also performed immunohistochemistry. Although most repair enzymes showed a similar pattern as when using Western blotting we observed

Table II. Peak levels, retention and $AUA_{0\text{-}25\ h}$ of Pt-DNA adducts for sensitive and resistant human ovarian cancer cell lines after exposure to CDDP alone or in combination with dFdC.

Cell line	Exposure	Pt-DNA at 1 h (fmol/μg DNA)	peak Pt-DNA (fmol/μg DNA)	T_{max}[a] h	Pt-GG:Pt-AG at T_{max} Ratio	25-h levels (fmol/μg DNA)	Pt-GG:Pt-AG at 25 h Ratio	$AUA_{0\text{-}25h}$[b] (pmol Pt x h/μg DNA)
A2780	3 μM CDDP	14.5±3.7	48.0±17.3	5	2.0±0.6	42.3±10.8	2.0±0.5	0.96±0.34
	12.4 nM dFdC +3 μM CDDP	61.7±12.1[d]	61.7±12.1[c]	1	2.1±0.5	24.9±7.1[c]	3.5±1.2[c]	0.36±0.20[c]
ADDP	200 μM CDDP	17.9±4.4	37.2±12.3	3	1.3±0.2	12.5±5.6	1.7±0.3	0.46±0.26
	240 nM dFdC +200 μM CDDP	15.8±6.5	50.4±19.3[c]	5	2.1±0.6[c]	13.1±5.1	2.0±0.4	0.79±0.27[d]
AG6000	17 μM CDDP	36.7±8.2	36.7±8.2	1	1.5±0.4	6.0±3.5	1.7±0.1	0.29±0.15
	1 mM dFdC +17 μM CDDP	16.4±3.2[c]	37.5±7.9	3	3.4±1.8[c]	7.5±3.5	0.8±0.2[d]	0.40±0.13

Cells were exposed to equitoxic concentrations of CDDP alone or in combination with dFdC. [a]T_{max} is the time-point after drug addition at which Pt-DNA adduct peak levels were achieved. [b]The $AUA_{0\text{-}25\ h}$ for total Pt-DNA adduct levels was calculated using non-compartmental analysis with the Topfit program. Values are means of 3-5 experiments ± SEM. [c]Significantly different from CDDP alone at $p<0.05$ and [d]$p<0.01$ (Student's t test for paired samples).

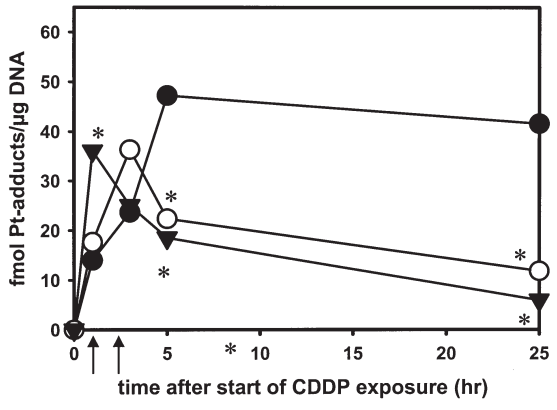

Figure 2. Total Pt-DNA adduct levels (total of Pt-GG and Pt-AG) in the A2780 (•), ADDP (○) and AG6000 (▾) cell lines after exposure of cells to IC_{50} concentrations of CDDP for 1 h followed by 1-h TU exposure [the time-points of washing away the CDDP (± dFdC) and TU are indicated with arrows] and by a 2-, 4- or 24-h CDDP (± dFdC)-free period. All values are means of 3-5 experiments. For clarity, the SEM has been omitted from the figure but varied between 25-52%. *Significantly different from A2780 cells ($p<0.05$; Student's t test).

several differences. ERCC1 expression was moderate in wild-type A2780 cells, approximately 2-fold increased in AG6000, but showed a very strong staining in the nucleus of ADDP cells. Similarly, DNA-PK staining in wild-type A2780 cells was very weak, but showed a somewhat higher cytoplasmic staining in AG6000 cells and a very strong nuclear staining in ADDP cells.

In order to study Pt-DNA adduct formation and repair, we exposed cells to IC_{50} concentrations of CDDP alone for 1 h and measured the total Pt-DNA adduct (total of Pt-GG and Pt-AG) levels of the human ovarian cancer cell lines, A2780, ADDP and AG6000, at equitoxic concentrations of CDDP. Since we used a very short incubation period, a substantial part of the DNA-bound CDDP would be present as mono-adducts (Pt-G) that carry an active Pt-binding site (34) and can still be converted to bifunctional adducts. In order to be able to separate the repair process from this new formation of bifunctional adducts, it was important to inactivate the mono-adducts to prevent their conversion into di-adducts. Therefore, we used TU post-incubation (23,24), which competitively inhibits this process. However, TU might reduce the cytotoxic effect of CDDP by prevention of the formation of di-adducts (24) and, thus, possibly interfere with our assay. Therefore, we studied whether TU post-incubation would affect CDDP, dFdC, and CDDP plus dFdC-induced growth inhibition during the course of the experiments. TU on its own tended to inhibit growth in the CDDP-sensitive cell line, A2780, by ~50% ($p=0.06$), whereas it had no effect on the resistant cell lines. Furthermore, TU slightly decreased the growth inhibitory effect of CDDP in the A2780 and ADDP cell lines by ~20% ($p=0.06$ and 0.11) but not in the AG6000 cell line. TU had no effect on the growth inhibition of CDDP and dFdC combined.

After exposure to equitoxic concentrations of CDDP, all cell lines formed comparable peak levels of total Pt-DNA adducts (Fig. 2, Table II). However, the time at which peak levels were reached showed variation (4 and 2 h after washing away CDDP and directly after exposure to CDDP for A2780, ADDP and AG6000 cells, respectively). Conversion of the mono- to di-adducts was very fast in CDDP-sensitive A2780 cells, preventing TU from completely inhibiting the process. Furthermore, clear differences were found in the ability of Pt-DNA adduct repair; A2780 cells did not repair Pt-DNA adducts and, after a 24-h CDDP-free period, >88% of the peak levels were still present. In contrast, the ADDP and AG6000 cells had a high capacity to repair Pt-DNA adduct levels, resulting in 34 and 16% of the peak Pt-DNA adducts, respectively, 24 h after washing away CDDP.

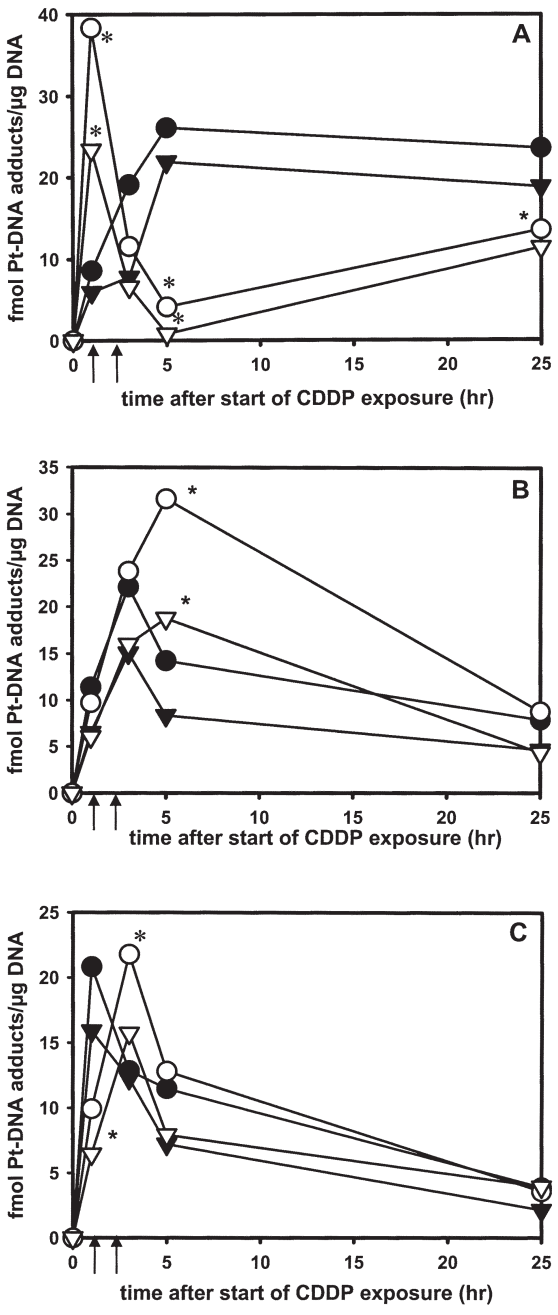

Figure 3. Effect of dFdC on Pt-GG (•, ○) and Pt-AG (▾,▽) adduct levels in DNA after exposure of cells to IC_{50} concentrations of CDDP alone (filled symbol) or combined with IC_{50} concentrations of dFdC (open symbol) for 1 h, followed by 1-h TU exposure [the time-points of washing away the CDDP (± dFdC) and TU are indicated with arrows] and by a 2-, 4- or 24-h CDDP (± dFdC)-free period in A2780 (A), ADDP (B) and AG6000 (C) cell lines. All values are means of 3-5 experiments. For clarity, the SEM has been omitted from the figure but varied between 33-67%. *Significantly different at $p<0.05$ and ** $p<0.01$ from CDDP alone, paired Student's t test.

To study the effect of dFdC on the formation and repair of Pt-DNA adducts, cells were exposed to the same equitoxic concentrations of CDDP in simultaneous combination with IC_{50} concentrations of dFdC and were post-incubated with TU. The Pt-GG and Pt-AG levels after exposure of A2780 cells to CDDP with or without dFdC are shown in Fig. 3A. dFdC clearly enhanced the Pt-DNA adduct peak formation: it shifted the peaks of both Pt-GG and Pt-AG in A2780 from 4 h after the washing away of CDDP to directly after CDDP exposure and increased the peak levels (total Pt-DNA adduct levels, 1.4-fold; Table I). The increase compared to levels directly after CDDP exposure was 4.5- ($p=0.07$) and 3.9-fold ($p=0.07$), respectively. However, dFdC also enhanced the repair of these Pt-DNA adducts by 6.4- and 28.1-fold (4 h after CDDP removal: $p=0.11$ and 0.11, respectively) and 1.7- and 1.7-fold (24 h after CDDP removal: $p=0.04$ and 0.14, respectively), resulting in significantly lower total Pt-DNA adduct levels in CDDP-dFdC cells after 24 h compared to cells exposed to CDDP alone. dFdC increased the ratio between Pt-GG and Pt-AG adducts by 1.4-fold during the formation of the Pt-DNA adducts (levels directly after CDDP exposure) compared to the ratio after treatment with CDDP alone (Pt-GG:Pt-AG ratio for 1-h exposure to CDDP alone: 1.4). During the repair process, this ratio increased even further; 4- and 24-h ratios were increased by 3.2- ($p=0.05$) and 1.7-fold compared to the ratios after treatment with CDDP alone (Pt-GG:Pt-AG ratio for 4 and 24 hr after the 1-h exposure to CDDP alone: 2.0 and 2.0), respectively. However, the overall net result was that dFdC decreased the area under the Pt-DNA adduct-time curve ($AUA_{0\text{-}25\ h}$) in A2780 cells by 2.7-fold ($p=0.049$) (Table II).

In the CDDP-resistant cell line, ADDP, dFdC increased and shifted the peak of Pt-DNA adduct formation from 2 to 4 h after the washing away of CDDP to a comparable peak level and formation pattern as in wild-type A2780 cells exposed to CDDP alone. dFdC enhanced the conversion of mono- to di-adducts to such an extent that it could not be inhibited by TU anymore. The peak of the Pt-GG and Pt-AG adducts increased by 2.2- and 2.3-fold, respectively (increase compared to 4 h after CDDP removal, $p=0.04$ for both) (Fig. 3B). This effect is most likely the result of both increased formation and decreased initial repair of Pt-DNA adducts. However, considering the slope of the curve, dFdC appears to enhance the long-term repair of Pt-DNA adducts, as was found in the A2780 cells. Furthermore, in this CDDP-resistant cell line, dFdC increased the ratio between the Pt-GG and Pt-AG adducts by 1.4-fold during the repair process compared to the ratio after treatment with CDDP alone (Pt-GG:Pt-AG ratio 4 h after the 1-h exposure to CDDP alone: 1.5). Overall, dFdC increased the $AUA_{0\text{-}25\ h}$ in ADDP cells by 1.7-fold (Table II).

In the dFdC-resistant AG6000 cells, Pt-DNA adduct formation and, thus, the mono- to di-adduct conversion was found to be maximal directly after CDDP exposure, allowing no apparent additional effect of dFdC. dFdC increased the Pt-GG:Pt-AG ratio (1.6-fold) directly after exposure compared to the ratio after treatment with CDDP alone (Pt-GG:Pt-AG ratio directly after exposure to CDDP alone: 1.5), but this was due to decreased Pt-AG adduct formation (2.5-fold, $p=0.04$) and not an increase in Pt-GG adduct formation (Fig. 3C). Subsequently, dFdC slightly inhibited the repair of both Pt-DNA adducts by 1.1-fold. Overall, however, dFdC did not clearly affect the $AUA_{0\text{-}25\ h}$ in AG6000 cells (Table II).

Discussion

In this study, we demonstrated that dFdC can affect both Pt-DNA adduct formation and repair. These effects were different

in each cell line and apparently related to the type of resistance to each compound. Furthermore, dFdC clearly affected the ratio between the two main Pt-DNA adducts formed, Pt-GG and Pt-AG.

At equitoxic concentrations of CDDP, the three human ovarian cancer cell lines did not show differences in peak levels. However, the times at which these peak levels were reached (T_{max}) were markedly different. These differences might be due to altered Pt accumulation, due to drug-uptake and efflux, as was described for several CDDP-resistant cell line A2780 variants (18,35), including ADDP cells. ADDP cells indeed have an increased expression of MRP2/c MOAT which can efflux CDDP from the cells (33). However, AG6000 did not show an increased expression of MRP2/c MOAT. ADDP and AG6000 cells have a decreased Pt accumulation compared to the wild-type cell line at equimolar concentrations (18). Both the resistant ovarian cancer cell lines, ADDP and AG6000, seem to be more efficient in repairing the Pt-DNA adducts, which is possibly due to the above-mentioned differences in repair enzyme status. Altered repair has been previously described for several CDDP-resistant ovarian cancer cell lines (36-38). Surprisingly, Pt-DNA peak levels in the A2780 cells were found after TU exposure. Apparently the mono- to di-adduct conversion in this CDDP-sensitive cell line was so efficient that it could not be completely blocked by TU.

Previously, we demonstrated that the combination of dFdC and CDDP caused different levels of synergism in these ovarian cancer cell lines (17,18). In the sensitive A2780 cell line, the combination index (CI) was previously shown to be 0.66 (average CI for 50, 75, 90 and 95% growth inhibition of the combination after 4-h exposure; CI<1 indicating synergism) (17). In this cell line, dFdC was now found to rapidly enhance and to increase Pt-DNA adduct formation, which was in agreement with our previous study in which A2780 was exposed to higher CDDP concentrations (18). In that study, we postulated that the increase in total Pt-DNA adduct formation might be due to increased dFdC incorporation into DNA. A similar mechanism was shown for the interaction between 2'-deoxy-5-azacytidine (DAC) and CDDP, DAC substituted plasmid DNA showed increased Pt binding to DNA (39), which was not hypomethylation dependent. We have now demonstrated that dFdC increased the Pt-GG:Pt-AG ratio during the formation of the Pt-DNA adducts, indicating a favored formation of Pt-GG adducts possibly due to conformational changes when dFdC is incorporated into the DNA. It is noteworthy that, in a previous study, dFdC decreased peak levels of Pt-DNA adducts in white blood cells (WBC) when administered 4 or 24 h before CDDP (27). This difference in Pt-DNA adduct levels between both studies might be explained by the fact that WBC are non-dividing, in contrast to A2780 cells.

Indeed, both ADDP and AG6000 showed pronounced differences in the expression of several repair enzymes known to be involved in resistance to CDDP. Consistent with previous findings in other A2780 variants (11), ADDP had a decreased expression of the MMR enzyme, MLH1. Gene expression of the repair enzyme, ERCC1, was not altered much in the cell lines. For ERCC1, a low expression has been associated with a good response to cisplatin-containing therapy (7-9) and, in various cell lines, a high expression has been associated with cisplatin resistance (10,12). Cellular localization seems to play an important role for this enzyme since, with immunohistochemistry, we found a much higher expression in the nucleus of ADDP cells compared to A2780 cells. In addition, it has been demonstrated that ERCC1 expression may abrogate gemcitabine-cisplatin synergism when MLH1 is deficient (12). The differences in the gene expression of repair enzymes may explain the difference in Pt-adduct accumulation, such as the decreased retention in ADDP and AG6000 cells.

In A2780 cells, dFdC enhanced Pt-DNA repair, which might be due to the additional extent of initial damage induced to the DNA, triggering the repair process. The decrease in total Pt-DNA adducts was accompanied by an increased Pt-GG:Pt-AG ratio during the repair process. These data indicate the easier removal of Pt-AG adducts from the DNA during the repair process, which might be related to the incorporation of dFdC into DNA during repair. Interestingly, an increased Pt-GG:Pt-AG ratio was previously found to be related to the response of the patients (27). The increased Pt-GG:Pt-AG ratios might thus be important for the synergistic interaction between dFdC and CDDP.

In the CDDP-resistant cell line, ADDP, the combination of dFdC and CDDP was previously shown to be more synergistic (4-h exposure, CI=0.16) than in wild-type A2780 cells (18). In the ADDP cells, dFdC appeared to increase the Pt-DNA adduct levels, 4 h after CDDP exposure, to levels comparable to the wild-type cell line. This process appeared to be so rapid that it could not be inhibited by TU exposure. These dFdC effects might be due to a combination of increased Pt-DNA adduct formation and inhibition of initial repair, which is probably very rapid directly after CDDP exposure (40,41). The enhancing effect of dFdC on Pt-DNA adduct formation exceeded the TU blockade of mono to di-adduct conversion. Apparently, dFdC changed the catalytic properties for this competitive reaction (38) by increasing either the maximal velocity of this process or the affinity for this process. These changes may be induced by conformational changes in the DNA that make DNA highly attractive for Pt adduct formation. The inhibition of the initial repair of Pt-DNA adducts might be related to the depletion of deoxyribonucleotides in the cell, caused by the inhibition of ribonucleotide reductase by dFdCDP (16,41). This process will disable the cell to repair DNA damage. Similar to the A2780 cell line, the second phase of Pt-DNA adduct repair appeared to be enhanced, which was accompanied by an increased Pt-GG:Pt-AG ratio, indicating an additional, possibly conformational, effect of dFdC at the DNA level.

In the dFdC-resistant AG6000 cells, the combination of dFdC and CDDP was previously described to be less synergistic (4-h exposure, CI=0.98) than in wild-type cells (17). In this cell line, which does not incorporate dFdC into DNA at detectable levels (42), dFdC only inhibited Pt-DNA adduct repair 24 h after removal of the drugs, without affecting the Pt-GG:Pt-AG ratio. This is in line with a potential lack of conformational changes compared to wild-type and CDDP-resistant cell lines. This may be related to a specific inhibition of Pt-AG repair in AG6000 cells. Therefore, further studies on the conformational changes in DNA caused by dFdC are warranted.

In conclusion, dFdC clearly increased Pt-DNA adduct formation. Strikingly, dFdC was found to both inhibit and enhance Pt-DNA repair, depending on the initial level of sensitivity to dFdC and CDDP and the level of synergism between these compounds.

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