Gemcitabine Potentiates Cisplatin Cytotoxicity and Inhibits Repair of Cisplatin-DNA Damage in Ovarian Cancer Cell Lines

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ABSTRACT

Synergistic cytotoxicity between cisplatin and the nucleoside analog gemcitabine was observed in a panel of cisplatin-sensitive (2008, A2780) and -resistant (2008/C13*5.25, A2780/CP70) human ovarian cell lines. Previous studies have suggested a role for DNA repair in the mechanism of synergy between the two drugs. We therefore further investigated the hypothesis that the synergistic cytotoxicity between gemcitabine and cisplatin in these cell lines may be caused by gemcitabine-mediated inhibition of cisplatin intrastrand adduct (IA) and interstand cross-link (ICL) repair. The effect of gemcitabine on the accumulation and repair of cisplatin IA and ICL in each cell line was then measured directly using gene-specific quantitative polymerase chain reaction and denaturation/renaturation techniques, respectively. Pretreatment of 2008 cells with 1 μM gemcitabine for 2 h before exposure to cisplatin for 7 h enhanced the accumulation of cisplatin IA and ICL by 50 and 40%, respectively ($P < 0.05$), above that induced by cisplatin alone. To investigate the possibility that the increased accumulation of cisplatin lesions was caused by inhibition of removal of cisplatin damage, 2008 cells were incubated with 200 μM cisplatin for 5 h in the presence and absence of gemcitabine and then a further 8 h in the absence of cisplatin. Only 57% IA were removed in the combination treated cells compared with 74% in cisplatin control cells. Similarly, repair of cisplatin ICL was inhibited in the gemcitabine-treated cells compared with the cells treated with cisplatin only (60 versus 72%). These findings demonstrate a direct inhibitory effect of gemcitabine on the repair of cisplatin IA and ICL and suggest a mechanistic basis for the cytotoxic synergy between the two drugs.

Cisplatin (cis-diamminedichloroplatinum II) is one of the most frequently used anticancer drugs. It is used as a single agent and in combination regimens to treat a variety of solid tumor types, including head and neck, ovarian, and non–small-cell lung (NSCLC) cancers (Cooley et al., 1994; Highley and Calvert, 2000). The therapeutic efficacy of cisplatin derives from its ability to form complexes with DNA (Cohen and Lippard, 2001), where it binds the N7 reactive center on purine residues to form both monofunctional and bifunctional DNA adducts. The 1,2-intrastrand GG cross-link (65%) and the 1,2-intrastrand AG cross-link (25%) are the predominant lesions; minor lesions include the 1,3-intrastrand GNG cross-link (6%), the interstrand GG cross-link (1–3%), monoadducts, and protein-DNA cross-links (Eastman, 1986). Cisplatin-DNA adducts are suggested to exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis (Sorrenson et al., 1990; Meyn et al., 1995).

A major clinical problem associated with cisplatin therapy is that although many tumors may be initially responsive to platinum therapy, these often relapse and become refractory to further platinum agent treatment (Perez, 1998). Several mechanisms of cisplatin resistance have been identified, including reduced intracellular drug accumulation, elevated levels of cellular thiols, increased tolerance to cisplatin DNA damage, and enhanced DNA repair (Masuda et al., 1990; Perez et al., 1993; Akiyama et al., 1999). The increased capacity of cells to repair and/or tolerate cisplatin-induced DNA damage has been proposed as a major mechanism of acquired resistance to cisplatin (Reed, 1998). Several studies have shown that cisplatin resistance is associated with the increased capacity of cells to repair drug-induced DNA damage both in the overall genome (Johnson et al., 1994) and in active genes (Zhen et al., 1992). Furthermore, elevated levels of DNA repair proteins have also been shown both in cell lines and in patient tumors resistant to cisplatin (Reed, 1998). Inhibition of DNA repair pathways may therefore increase the sensitivity of a tumor to cisplatin therapy.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a nucleoside analog with clinical activity against various solid tumors including ovarian, NSCLC, head and neck cancer, and pancreatic cancers (Sandler and Ettinger, 1999; Storniolo et al.,

ABBREVIATIONS: NSCLC, non–small-cell lung cancer; CI, combination index; kb, kilobase(s); DRI, dose-reduction index; PCR, polymerase chain reaction; QPCR, quantitative polymerase chain reaction; ICL, interstrand cross-link; IA, intrastrand adduct; DHFR, dihydrofolate reductase.

1999). Upon entering the cell, gemcitabine is anabolized to its triphosphate form by deoxynucleoside salvage pathways. The triphosphate can then become incorporated into DNA, where it blocks further DNA synthesis by inhibiting DNA polymerase activity (Plunkett et al., 1995). In vitro studies have shown that the cytotoxicity of gemcitabine correlates directly with the level of incorporation of the analog into cellular DNA (Kufe et al., 1980; Huang et al., 1990, 1991). Gemcitabine also inhibits ribonucleotide reductase, hence depleting the deoxynucleotide pools required for DNA repair and replication, thereby potentiating its incorporation into newly synthesized DNA (Tseng et al., 1982; Heinemann et al., 1990).

Cisplatin and gemcitabine are ideal candidates for use in combination regimens because of their different but complementary mechanisms of action, similar antitumor activity profiles, and nonoverlapping side effect profiles (Braakhuis et al., 1995; Carmichael, 1998). Although the clinical utility of platinum agents and gemcitabine combinations has been demonstrated (Carmichael, 1998; Stewart, 1998), the molecular basis for this interaction has yet to be defined.

Previous studies have shown that the cytotoxic interaction between the nucleoside analog, fludarabine and cisplatin is accompanied by an inhibition of repair of cisplatin-induced interstrand cross-links (Yang et al., 1995). Because gemcitabine is readily incorporated into newly synthesized DNA, the treatment of cells with cisplatin in combination with gemcitabine may potentially inhibit the repair of cisplatin DNA damage, resulting in the persistence of DNA damage and increased cytotoxicity. Indeed, previous studies have shown that the nucleoside analog fludarabine synergizes with cisplatin in vitro.

The aim of this study was to investigate the cytotoxic interaction between cisplatin and gemcitabine in cisplatin-sensitive and -resistant ovarian cancer cell lines. A synergistic interaction between the drugs was demonstrated in each of the cell lines. Gemcitabine was shown to inhibit the repair of both inter- and intrastrand cross-links induced by cisplatin and to potentiate the accumulation of these lesions in ovarian cells. These results suggest that the synergistic interaction between these drugs may be because of the inhibitory effect of gemcitabine on the repair of the major cytotoxic lesions induced by cisplatin.

Materials and Methods

Drugs and Chemicals

Gemcitabine was kindly supplied by Eli Lilly Inc. (Indianapolis, IN) and was dissolved in phosphate-buffered saline to a concentration of 10 mM. Cisplatin was obtained from the Institute of Drug Technology (Melbourne, Australia) and was dissolved in phosphate-buffered saline to a concentration of 5 mM. All other chemicals were of analytical grade and commercially available.

Cell Culture

The 2008 human ovarian adenocarcinoma cell line and the cisplatin-resistant subline C13*5.25 (2008/R) (Professor S Howell, Cancer Center, University of California, San Diego, CA) were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 20 μg/ml gentamicin. The A2780 human ovarian carcinoma cell line (Professor L. R. Kelland, Institute of Cancer Research, Sutton, UK) and the resistant subline CP70 (Dr. V. A. Bohr, National Institute on Aging, National Institutes of Health, Baltimore, MD) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal

bovine serum, 20 μg/ml gentamicin and nonessential amino acids. All cells were maintained in a humidified incubator with 5% CO_2 in air at 37°C and were routinely tested for mycoplasma. All cells were grown as monolayers in tissue culture flasks and were passaged twice weekly.

Cytotoxicity Assay

The cytotoxicity of cisplatin and gemcitabine as single agents and in combination was evaluated in the four ovarian cell lines by a clonogenic assay. On day one, 2×10^5 cells were seeded in 25-cm^2 flasks containing 10 ml of growth medium. On day 3, the cells were exposed to drugs alone or in combination for 24 h and then washed twice with phosphate-buffered saline, harvested, and plated into 60-mm dishes at various cell densities such that 50 to 100 colonies/dish would be obtained after 8 days of incubation. The plates were then fixed in neutral formalin (40% formaldehyde, 30 mM NaH_2PO_4, and 45 mM Na_2HPO_4, pH 7) and stained with 0.01% (w/v) crystal violet. Colonies consisting of 50 cells or more were counted in four replicate plates. The survival fractions were calculated after setting the plating efficiency of untreated control cells at 100%.

To study the cytotoxic effects of the two drugs in combination, the cells were treated with increasing concentrations of cisplatin (0.23, 0.3, and 0.45 μM for 2008 and A2780 cells; 5.63, 7.5, and 11.25 μM for C13 and CP70 cells) or gemcitabine (11.25, 15, and 22.5 nM for 2008 and C13 cells; 2.25, 3, and 4.5 nM for A2780 and CP70 cells) as single agents or with a combination of the two agents at concentrations in a fixed molar ratio (molar ratio of cisplatin to gemcitabine of 20:1 for 2008 cells, 100:1 for A2780 cells, 500:1 for C13 cells, and 2500:1 for CP70 cells). The cytotoxic interactions between the two drugs in each cell line was determined using the median effect method of Chou and Talalay (1984). A drug concentration dependence plot was generated for each drug alone and multiple dilutions of a fixed ratio combination of the drugs. The mutually nonexclusive combination index (CI) was then determined using a computer program developed by Chou and Chou (1988) (Biosoft, Cambridge, UK). The CI is defined as the ratio of the combination drug concentration to the sum of the single-agent concentrations at an equitoxic level. A CI <1 indicates synergy, CI >1 indicates antagonism, and CI = 1 indicates additivity.

The dose-reduction index (DRI) (Chou and Chou, 1988; Yang et al., 1995), which represents the -fold cisplatin concentration reduction in the combination, compared with the drug as a single agent at given level of effect (x), was calculated using the equation DRI = $(D_x)/(D)$. In the equation, D_x is the concentration of cisplatin as a single agent required for x% effect, and D is the concentration of cisplatin that, in combination with gemcitabine, is required to cause x% effect.

Detection of Cisplatin-DNA Damage

DNA Probes. All probes were genomic inserts labeled by random priming. The 1.8-kb *Eco*RI fragment containing exons I and II of the *DHFR* gene was isolated from pBH31R1.8 plasmid probe (Dr. V. A. Bohr, National Institute on Aging, National Institutes of Health, Baltimore, MD), and labeled using [α-^{32}P]dATP. The probe was used to detect a 22-kb fragment of the *DHFR* gene, including the 5′ end of the gene. The δ-globin probe (Dr. V. A. Bohr) was used to detect the entire 18 kb gene.

Detection of Gene-Specific Cisplatin Intrastrand Adducts. Cells were seeded in 15-cm dishes at a density of 5×10^6 cells/dish for 16 h before the experiment to ensure exponential growth of the cells at the time of drug treatment. 2008 and C13 cells were incubated in the absence or presence of 1 μM gemcitabine for 2 h before exposure to increasing concentrations of cisplatin for 7 h. Cells were subsequently washed twice with phosphate-buffered saline, trypsinized, and pelleted.

Total genomic DNA was isolated (QIAamp blood kit; QIAGEN, Valencia, CA) and the DNA concentration of the samples was determined by fluorometric quantitation using Hoechst 33258. The DNA

was then restriction-digested with *Hin*dIII at 37°C for 2 h, and extracted using phenol/chloroform followed by ethanol precipitation. The pellet was resuspended in Tris/EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and the final DNA concentration recovered was determined as described above. Quantitative PCR of an 1858-bp fragment in the *DHFR* gene was performed as described previously (Shahin et al., 2001). Briefly, each PCR amplification mixture (25 μl) consisted of 100 ng of total genomic DNA and contained 0.625 U of *Taq* polymerase (Promega, Madison, WI), primers (0.5 μM for each primer; sense primer, 5′-AAACGTAGCTCGTCCTCAAA; antisense primer, 5′-TTCCAGTCTACGGGAAGCC), dNTPs (0.2 mM of each dNTP), 1× QIAGEN PCR buffer, 1× Q solution (QIAGEN), 2.6 mM MgCl$_2$, and made up to 25 μl with Milli-Q water. The PCR conditions employed involved an initial denaturation at 96°C for 5 min, 29 cycles of 96°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, and a final equilibration for 5 min at 72°C. The amplification products were then resolved on a 1% agarose gel in 1× Tris/acetate/EDTA buffer for 1 h at 80 V. The gel was then stained with ethidium bromide (0.5 μg/ml) and then destained in water.

Band quantitation was performed using a Molecular Imager FX and QuantityOne software (version 4.2.1; Bio-Rad, Hercules, CA). The Poisson distribution was used to calculate the number of adducts in the amplified fragment of the gene and this value was normalized to lesions/2 kb.

Detection of Gene-Specific Cisplatin Interstrand Cross-Links. To study the effect of gemcitabine on the accumulation of cisplatin-induced ICL in specific DNA genes, DNA (6 μg) from treated cells was restricted with *Hin*dIII (to release the 22-kb DHFR fragment and the 18 kb δ-globin gene) for 2 h at 37°C. The DNA was extracted once with phenol and once with chloroform and precipitated with ethanol. The pellet was resuspended in 15 μl of Tris/EDTA buffer and denatured by the addition of an equal volume of 100 mM NaOH and incubated for 20 min at 37°C. Loading buffer (4 μl of 10× buffer: 10 mM EDTA, 26% Ficoll, 0.25% bromocresol green) was added to each sample and the DNA was then electrophoresed through a 0.5% agarose gel in Tris-acetate/EDTA buffer at 27 V overnight. The DNA was transferred to nylon (Hybond N$^+$; Amersham Biosciences, Piscataway, NJ), fixed to the membrane by baking for 2 h at 80°C, and probed for the gene fragment of interest. Band visualization and quantitation were performed by PhosphorImager analysis. The Poisson distribution was used to calculate the number of cross-links in the gene fragment and this value was normalized to cross-links/10 kb.

Detection of Gene-Specific Repair of Cisplatin Interstrand and Intrastrand Cross-Links. Cells were treated with cisplatin (200 μM in 2008, A2780; 400 μM in CP70) for 5 h in the absence or presence of gemcitabine, followed by treatment with 0.1 M thiourea for 1 h to block the conversion of monoadducts to ICL during the post-treatment incubation. The cells were then incubated for times up to 8 h in drug-free medium or in 1 μM gemcitabine-treated medium. Cells were harvested, and the DNA was extracted and analyzed for total adducts and ICLs as described above. The repair efficiency was expressed as the percentage of initial cross-links that remained at that time point.

Statistical Analysis. Statistical significance was determined using Student's *t* test calculated with SigmaStat software (SPSS Science, Chicago, IL).

Results

Cytotoxic Synergy between Cisplatin and Gemcitabine. Clonogenic survival assays were used to investigate the sensitivity of two matched pairs of cisplatin-sensitive and -resistant cell lines to gemcitabine and cisplatin. The IC$_{50}$ for each drug after a 24-h exposure was determined in each line. The C13*5.25 (IC$_{50}$ = 7.5 μM) and CP70 (IC$_{50}$ = 5.5 μM) cell lines demonstrated 19- and 13-fold resistance to cisplatin

compared with their 2008 (IC$_{50}$ = 0.4 μM) and A2780 (IC$_{50}$ = 0.4 μM) parental lines, respectively. The 2008/C13 (IC$_{50}$ = 15 nM) and A2780/CP70 (IC$_{50}$ = 3 nM) pairs had the same sensitivity to gemcitabine.

To investigate the interactions between the drugs, each cell line was exposed to a sequential drug schedule consisting of a 4-h pretreatment with various concentrations of gemcitabine followed by a 20-h incubation with a combination of gemcitabine and cisplatin at a fixed concentrations ratio of each drug. The cells were then plated to enable assay for clonogenic survival. The nature of the interactions between cisplatin and gemcitabine was evaluated using the median-effect analysis method of Chou and Talalay (1984). The median effect plot generated from the survival data of 2008 cells treated with each drug alone and in combination is shown in Fig. 1a. The slope of the drug-alone plots are not parallel, which suggests that the two agents have different modes of action, and the equation for the conservative isobologram was therefore used to calculate the combination index (CI). All CI values at all fraction-affected levels were less than 1.0, indicating that the drug combination produced a synergistic response (Fig. 1b). The computer-simulated median effect was calculated from the survival responses at different levels
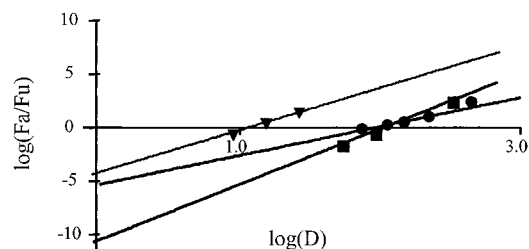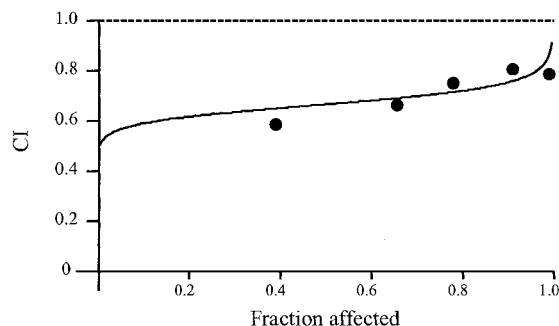
Fig. 1. Cytotoxic interactions between cisplatin and gemcitabine. A, the survival of 2008 cells was assessed by clonogenic assay after cells were treated with cisplatin (■) (0.23, 0.3, and 0.45 μM) or gemcitabine (▼) (11.25, 15, and 22.5 nM) as single agents, or with both agents in combination (●) at concentrations in a fixed molar ratio of 20:1 for 24 h (cisplatin, 75, 112.5, 150, 225, and 450 nM; gemcitabine, 3.75, 5.63, 7.5, 11.25, and 22.5 nM). The median effect plot was constructed using the data from the survival curves, where Fa is the affected fraction, Fu is the unaffected fraction, and D is the drug concentration. B, the fraction-affected CI plot was constructed by computer analysis of the data in A using the method of Chou and Talalay (1984).

TABLE 1
Evaluation of the interaction between cisplatin and gemcitabine in ovarian cancer cell lines by median drug effect analysis
Values represent CIs (non-mutually exclusive case) calculated at the fraction affected (Fa) of 0.25, 0.5, 0.75, and 0.9 of the combination of cisplatin and gemcitabine and are means ± S.E. of at least three separate experiments.

Cell Line	Average CI ± S.E.			
	Fa_{25}	Fa_{50}	Fa_{75}	Fa_{90}
2008	0.55 ± 0.03	0.61 ± 0.04	0.68 ± 0.04	0.77 ± 0.03
C13*5.25	1.13 ± 0.06	0.88 ± 0.05	0.67 ± 0.05	0.55 ± 0.04
A2780	0.45 ± 0.05	0.64 ± 0.08	0.68 ± 0.07	0.71 ± 0.07
CP70	0.89 ± 0.05	0.84 ± 0.06	0.79 ± 0.04	0.72 ± 0.04

of effect (fraction affected). The average CI values for the drug combination in each of the cell lines are summarized in Table 1. Apart from the result in the C13 cell line at 25% growth inhibition, the gemcitabine/cisplatin combination was synergistic at all levels of effect in each of the cell lines tested.

The DRI was calculated in each cell line to evaluate the extent by which the drug combination enhanced cisplatin cytotoxicity. In the 2008 and C13 cell line pairs, the DRI values were 2.57 and 2.67, respectively, suggesting that cisplatin cytotoxicity was enhanced to a similar extent in both cell lines. In contrast, DRI values of 2.47 and 3.04 were observed in the A2780/CP70 cell pair, suggesting greater enhancement of cisplatin cytotoxicity in the cisplatin-resistant CP70 cells.
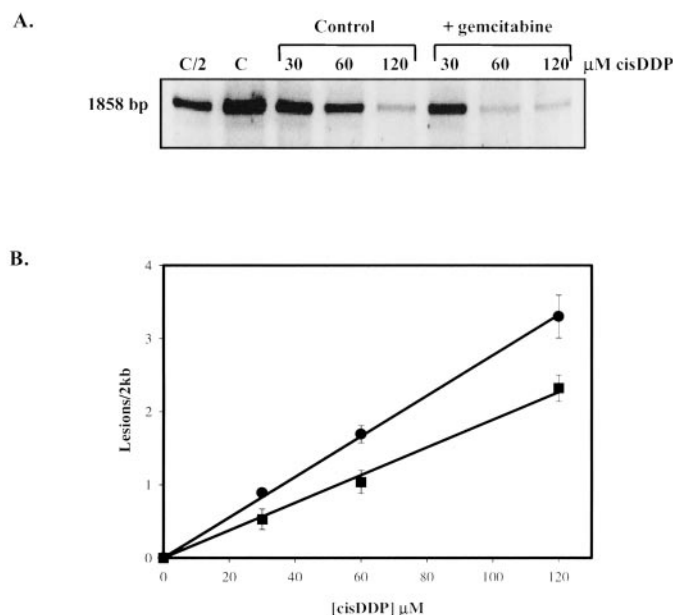
Effects of Gemcitabine on the Accumulation of Cisplatin-Induced DNA Intrastrand Adducts in the *DHFR* Gene. Subsequent studies sought to investigate the mechanistic basis of the synergy between cisplatin and gemcitabine in combination, specifically the effect of gemcitabine on the accumulation and removal of cisplatin DNA damage. A quantitative PCR (QPCR) method was first employed to investigate the effect of gemcitabine on the formation of cisplatin-induced IA in the ovarian cell lines. This method exploits the ability of drug-DNA lesions to block the progression of *Taq* polymerase, thereby inhibiting the amplification of the damaged DNA template (Ponti et al., 1991).

QPCR was used to detect cisplatin-induced adducts in a 1.8-kb fragment of the constitutively expressed *DHFR* gene. 2008 cells were incubated in the absence or presence of gemcitabine for 2 h before exposure to increasing concentrations (30 to 120 μM) of cisplatin for 7 h. Total genomic DNA was isolated and amplified using QPCR, and the amplification products were resolved on an agarose gel. The results are shown in Fig. 2a. In the control lanes, the amount of product amplified decreased with increasing drug concentration, consistent with presence of increasing drug-DNA adducts. This effect was enhanced in the amplification products from cells treated with cisplatin in the presence of gemcitabine.

The bands were quantitated and the results are summarized in Fig. 2b. Adduct levels increased linearly as a function of drug concentration, reaching maximum levels of 2.2 lesions/2 kb at 120 μM. Treatment of 2008 cells with cisplatin in the presence of gemcitabine resulted in the enhanced formation of cisplatin total adducts 1.5-fold at 120 μM ($P <$ 0.05). In the cisplatin-resistant C13 subline, maximum cisplatin adduct levels formed in the *DHFR* gene of control cells were 4-fold lower than in parental 2008 cells, consistent with reduced drug uptake as one of the major mechanisms of resistance in this cell line (Andrews et al., 1988). No poten-

tiation of intrastrand adduct accumulation by gemcitabine was observed in C13 cells (data not shown).

Effects of Gemcitabine on the Accumulation of Cisplatin-Induced DNA Interstrand Cross-Links in the *DHFR* Gene. We next sought to investigate the effects of gemcitabine on the accumulation of the other important cisplatin lesion, the interstrand cross-link. DNA from cells treated as described for QPCR was analyzed for the presence of the ICLs in a 22-kb fragment of the *DHFR* gene. As shown in Fig. 3a, DNA from control cells incubated in the absence of any drugs was completely denatured under the conditions employed and migrated as single-strand DNA. DNA from cells treated with cisplatin showed a concentration-dependent increase in double-strand cross-linked DNA. The bands were quantitated and the results summarized in Fig. 3b. ICL
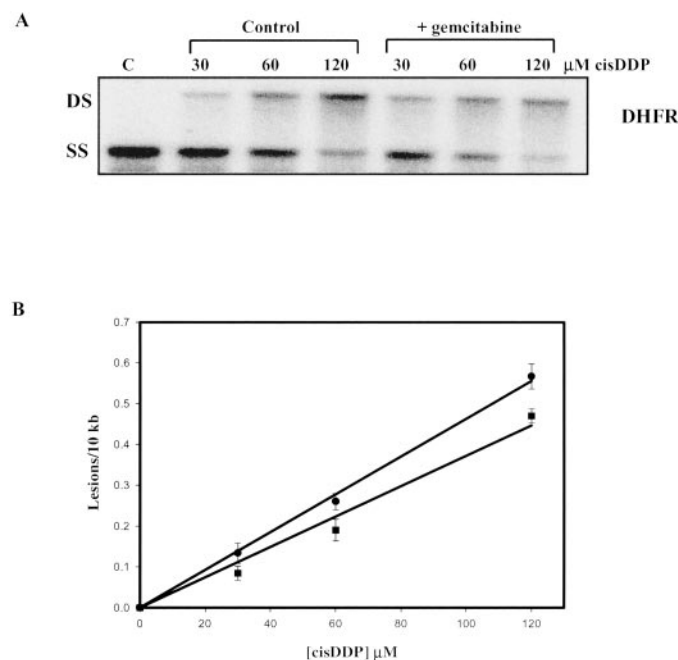
Fig. 2. Effects of gemcitabine on the cisplatin-induced DNA intrastrand adducts in the *DHFR* gene. 2008 cells were incubated in absence or presence of 1 μM gemcitabine for 2 h before exposure to increasing concentrations of cisplatin (as shown) for 7 h. Total genomic DNA was isolated and restricted with *Hind* III and an 1858-bp fragment in the *DHFR* gene was subjected to QPCR amplification. A, representative image of QPCR amplification products resolved on a 1% agarose gel. Lane C represents control DNA from untreated cells, and lane C/2 contains half the DNA concentration in lane C. 'Control' lanes represent cells treated with increasing concentrations of cisplatin in the absence of gemcitabine; and '+ gemcitabine' lanes represent cells treated in the presence of 1 μM gemcitabine. Band quantitation was performed using a Molecular Imager FX. B, The Poisson distribution was used to calculate the number of DNA adducts in the DNA fragment from cells incubated in the absence (■) or presence (●) of gemcitabine. These values were normalized to lesions/2 kb and expressed as the mean ± S.E. and plotted as a function of cisplatin concentration.

levels increased linearly as a function of drug concentration. Incubation of cells with cisplatin in the presence of gemcitabine enhanced ICL levels by 1.4-fold ($P < 0.05$) at 120 μM. Similar results were obtained after analysis of ICL levels in the transcriptionally inactive δ-globin gene (data not shown).

The effect of gemcitabine on the accumulation of cisplatin-induced ICL in the *DHFR* gene was investigated in each of the four cell lines, and the results are summarized in Table 2. As observed in the 2008 cells, gemcitabine enhanced ICL formation in the A2780 and CP70 cells. No effect of gemcitabine was observed on the very low levels of ICL induced in the C13*5.25 line.

Effects of Gemcitabine on the Removal of Cisplatin-Induced Intrastrand Adducts in the *DHFR* Gene. To investigate the possibility that gemcitabine potentiates the accumulation of cisplatin damage by suppressing the repair of cisplatin-induced DNA lesions, the effect of gemcitabine on the repair of cisplatin ICL and IAs was directly examined. The QPCR assay was used to investigate the effect of gemcitabine on the removal of cisplatin-induced IA in the *DHFR* gene. Cells were incubated with cisplatin in the absence or presence of gemcitabine to induce damage, followed by treatment with thiourea (to prevent the slow conversion of mono-adducts to cross-links) before the cells were allowed to repair the damage. The DNA was then isolated and analyzed for the presence of IAs in the *DHFR* gene and the results are summarized in Table 3.

Fig. 3. Effects of gemcitabine on the cisplatin-induced DNA interstrand cross-links in the *DHFR* gene. DNA from cells treated as described in Fig. 2 was isolated and analyzed for ICL formation using a denaturation/renaturation assay as described. A, representation of *Hin*dIII-digested DNA denatured and electrophoresed through a 0.5% agarose gel, before being transferred to nylon and probed for a 22-kb *DHFR* gene fragment. DS is double-strand DNA; SS is single-strand DNA. The data shown were based on at least three independent experiments and DNA samples from each biological experiment were subjected to gel electrophoresis and Southern analysis at least twice. B, band quantitation was performed using a PhosphorImager. The Poisson distribution was used to calculate the number of cross-links in the *DHFR* gene and this value was normalized to cross-links/10 kb in the absence (■) or presence (●) of 1 μM gemcitabine, expressed as the mean ± S.E. and plotted as a function of cisplatin concentration.

In 2008 cells, as seen in Fig. 4, cisplatin IAs were repaired rapidly, with 82% of lesions removed within 8 h. In contrast, IA repair was inhibited by 30% ($P < 0.05$) in cells undergoing repair in the presence of gemcitabine. Interestingly the repair of IA was inhibited in CP70 to a greater extent than that in the 2008 and A2780 parental lines.

Effects of Gemcitabine on the Removal of Cisplatin-Induced DNA Interstrand Cross-Links in the *DHFR* Gene. The effect of gemcitabine on the repair of cisplatin ICL was also examined. DNA from cells treated as described above was analyzed for ICL in the *DHFR* gene and the results are shown in Fig. 5. In both the control (cisplatin alone) and gemcitabine-treated cells, ICLs were removed rapidly, with few cross-links remaining after 8 h. Quantitation revealed that 2008 cells efficiently repaired cisplatin ICL with 72% of damage removed within 8 h. In the presence of gemcitabine, however, the extent of repair was reduced to 60%. Similar results were observed in the A2780 and CP70 cell lines (Table 4). Analysis of ICL repair in the DHFR gene in A2780 and CP70 again revealed a greater extent of repair in the CP70 cell line. These combined data suggest that gemcitabine suppresses the repair of cisplatin-induced ICL in gene-specific DNA sequences.

Discussion

The aim of this study was to explore the mechanistic basis of the synergistic cytotoxic interaction between gemcitabine and cisplatin in cisplatin sensitive and resistant ovarian cancer cell lines. Synergistic interactions between gemcitabine and cisplatin have been described previously in ovarian cell lines using growth inhibition assays (Bergman et al., 1996; van Moorsel et al., 1999). Because such assays reflect the short-term growth inhibitory effects of a drug or drug combination and not necessarily cytotoxicity, we used clonogenic survival assays to ensure a rigorous analysis of the nature of the interaction between gemcitabine and cisplatin in the cell lines studied. A synergistic cytotoxic interaction was revealed between cisplatin and gemcitabine in both the cisplatin-sensitive and -resistant ovarian cell lines.

The greatest cytotoxic synergy, as determined by the DRI, and the maximum inhibition of cisplatin IA and ICL repair by gemcitabine were observed in the cisplatin-resistant CP70 cells. One of the major mechanisms of cisplatin resistance identified in this cell line is enhanced DNA repair (Li et al., 1998; Ferry et al., 2000). In a recent study by Yang et al. (2000), cellular inactivation of the *ERCC1* repair gene was shown to abrogate the synergistic cytotoxic interaction between gemcitabine and cisplatin. Furthermore, in NSCLC patients treated with gemcitabine and cisplatin, survival was shown to correlate with the level of expression of the ERCC1 repair gene (Lord et al., 2002). Our finding is therefore consistent with the proposal that inhibition of repair of cisplatin DNA damage by gemcitabine is critical to the cytotoxic synergy observed between the drugs.

Using an in vitro repair synthesis assay, Yang et al. (2000) also demonstrated that gemcitabine triphosphate, the active metabolite of gemcitabine, inhibits the repair of plasmid DNA containing cisplatin damage. To define the role of gemcitabine in the repair of specific cisplatin lesions in cells, gene-specific assays were used in the current study. Because DNA repair is heterogeneous throughout the genome and

TABLE 2

Effect of gemcitabine on formation of cisplatin ICL in the DHFR gene of ovarian cancer cell lines

Data are from at least two blots, each of three independent biological experiments. Standard errors were in the range of 0 to 0.07 ICL per fragment.

Cell line	[CDDP]	ICL/10 kb	
		−dFdC	+dFdC
	μM		
2008	30	0.08	0.12
	60	0.17	0.25
	120	0.39	0.52*
A2780	30	0.05	0.08
	60	0.13	0.18
	120	0.28	0.38*
CP70	100	0.07	0.09
	200	0.14	0.19
	400	0.26	0.37*

dFdC, gemcitabine; CDDP, cisplatin.
* $P < 0.05$. Significance was evaluated at the highest dose levels.

TABLE 3

Effect of gemcitabine on formation and repair of cisplatin IA in the *DHFR* gene of ovarian cancer cell lines

Values represent IA/2 kb. Data are from at least two blots, each of three independent biological experiments. Standard errors were in the range of 0 to 0.08 IA per fragment.

Cell Line	Repair Time	IA/2 kb		Repair	
		−dFdC	+dFdC	−dFdC	+dFdC
	h			%	
2008	0	1.60	1.56	0	0
	4	0.61	0.86	64	45
	8	0.44	0.66	74	57*
A2780	0	1.24	1.19	0	0
	4	0.54	0.63	56	47
	8	0.36	0.49	71	59*
CP70	0	1.16	1.12	0	0
	4	0.42	0.63	63	44
	8	0.21	0.41	82	63*

dFdC, gemcitabine.
* $P < 0.05$. Significance was evaluated at 8 h.
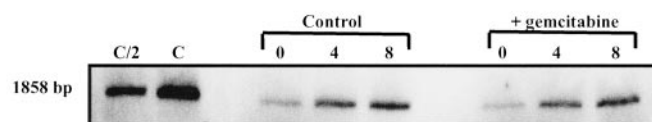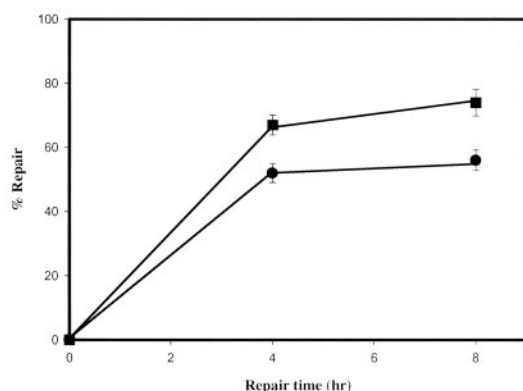
repair in transcriptionally active genes is a critical determinant in cell survival after DNA damage (Jones et al., 1991), cisplatin damage in the transcriptionally active *DHFR* gene was analyzed.

Because more than 90% of DNA lesions induced by cisplatin are IAs, the results from the QPCR analysis primarily reflect the presence of these lesions (Talarico et al., 2001). As observed previously by Zhen et al. (1992) using the *Escherichia coli* ATP-binding cassette excinuclease, cisplatin IAs are rapidly removed from the *DHFR* gene. However, in the
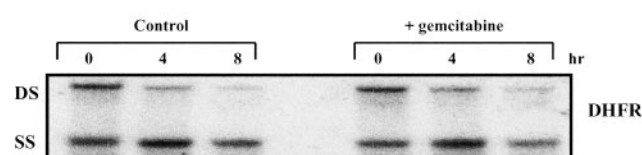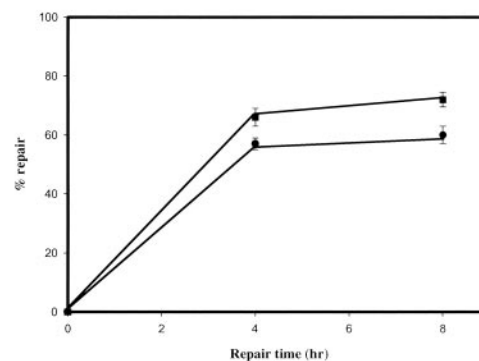
presence of gemcitabine, the repair of these lesions was reduced, consistent with an inhibitory effect of the nucleoside analog on cellular repair of these lesions.

Repair of cisplatin IAs occurs through the concerted activity of over 30 proteins comprising the nucleotide excision repair pathway. After lesion recognition, the DNA is incised on either side of the lesion, a ~29-base oligomer containing the DNA lesion is excised, and the DNA strand is resynthesized and ligated (de Laat et al., 1999). Gemcitabine is suggested to mediate its cytotoxic effects by inhibiting the repair synthesis step of this pathway. Through its actions as a ribonucleotide reductase inhibitor, gemcitabine depletes the intracellular deoxynucleotide pools, thereby enhancing the

Fig. 4. Effects of gemcitabine on the repair of cisplatin-induced DNA intrastrand cross-links in the *DHFR* gene. 2008 cells were incubated with 100 μM cisplatin in absence or presence of 1 μM gemcitabine for 5 h. The cells were then incubated with thiourea before incubation up to 8 h (as shown) in drug-free medium or in 1 μM gemcitabine-treated medium. A, representative image of amplification products resolved on a 1% agarose gel. B, quantitation of cisplatin-induced IA removal from the DHFR gene fragment. The Poisson distribution was used to calculate the number of adducts in the 1858-bp fragment of the *DHFR* gene in the absence (■) or presence (●) of gemcitabine, and this value was normalized to lesions/2 kb, expressed as percentage repair, and plotted as a function of repair time.

Fig. 5. Effects of gemcitabine on the repair of cisplatin-induced DNA interstrand cross-links in the *DHFR* gene. 2008 cells were incubated with 100 μM cisplatin in the absence or presence of 1 μM gemcitabine for 5 h. DNA from cells allowed to repair for up to 8 h was analyzed for ICL remaining. A, representative ICL blot probed for the 22-kb *DHFR* gene fragment. B, quantitation of cisplatin ICLs remaining in the *DHFR* gene as a function of repair time. The Poisson distribution was used to calculate the number of cross-links remaining after repair in the absence (■) or presence (●) of gemcitabine, and was expressed as a percentage of initial ICL levels.

TABLE 4
Effect of gemcitabine on formation and repair of cisplatin ICL in the DHFR gene of ovarian cancer cell lines

Values represent ICL/10 kb. Data are from at least two blots, each of three independent biological experiments. Standard errors were in the range of 0 to 0.06 ICL per fragment.

Cell Line	Repair Time	ICL/10 kb		Repair	
		−dFdC	+dFdC	−dFdC	+dFdC
	h			*%*	
2008	0	0.32	0.35	0	0
	4	0.11	0.15	66	57
	8	0.09	0.12	72	60*
A2780	0	0.25	0.21	0	0
	4	0.08	0.10	68	55
	8	0.06	0.07	75	64*
CP70	0	0.18	0.19	0	0
	4	0.04	0.07	78	62
	8	0.03	0.06	86	69*

dFdC, gemcitabine.
* $P < 0.05$. Significance was evaluated at 8 h.

potential for its own incorporation into newly synthesized DNA. Once incorporated into DNA, the analog causes termination of DNA synthesis and is resistant to removal by exonucleases, resulting in DNA strand breaks (Plunkett et al., 1995). Incorporation of gemcitabine into a cisplatin IA repair patch may therefore lead to a persistence of DNA damage, which leads to cell death.

The results of the current study demonstrated that gemcitabine also inhibited the cellular repair of interstrand cross-links, the other major cytotoxic lesion induced by cisplatin. Repair of interstrand cross-links in mammalian cells is not well understood but is suggested to involve recombination and aspects of the nucleotide excision repair pathway. In a recently proposed model of cross-link repair in replicating DNA, the replication fork stalls upon encountering a cross-link, resulting in a double-strand DNA break. The DNA is then incised, the cross-link unhooked, and the resulting gap in the DNA strand is repaired by recombination. The second strand is then incised on either side of the damage, the DNA fragment containing the cross-link is released, and the resultant gap is resynthesised and ligated (McHugh et al., 2001).

The denaturation/renaturation assay used to detect cisplatin ICL in the current study has been used previously by Yang et al. (1995) to investigate the effects of the nucleoside analog, fludarabine on the repair of cisplatin ICL. As observed with gemcitabine in the current study, fludarabine inhibited the repair of cisplatin damage in specific gene fragments. Because this cross-linking assay detects an early event in the repair of cross-links (the unhooking of an ICL), the results therefore indicate that the nucleoside analogs are able to block this step of ICL repair.

Because the nucleoside analogs are expected to exert their inhibitory effects on cross-link repair at the level of DNA resynthesis, these results suggest that the steps in the ICL pathway are tightly coupled. This hypothesis is supported by results that involve the nucleotide excision repair pathway. Using an in vitro reconstituted human DNA repair excision nuclease, Svoboda et al. (1993) demonstrated that incision of damaged templates is greatly inhibited in the absence of dNTPs required for repair synthesis. In that study, it was suggested that the complex of proteins comprising the human excision nuclease remains stalled on the DNA after

incision and requires displacement by the proteins involved in repair replication. Incorporation of gemcitabine into a repair patch may therefore result in the sequestering of the repair replication proteins at that site because of incomplete repair. The reduction of free repair replication proteins may result in the reduced displacement of other repair complexes from damaged DNA and inhibition at all steps of the ICL repair pathway.

The results of the current study directly demonstrate that gemcitabine inhibits the repair of both IA and ICL in ovarian cancer cell lines. These findings strongly support a role for inhibition of nucleotide excision repair and ICL repair pathways in the mechanism of interaction between gemcitabine and cisplatin in these cell lines. These results, together with other findings that gemcitabine produces synergistic cytotoxicities with cisplatin, suggest that gemcitabine may serve as a repair modulator to improve antitumor efficacy when combined with DNA-damaging agents that induce cellular repair.

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