

Inhibition of Carboplatin-Induced DNA Interstrand Cross-link Repair by Gemcitabine in Patients Receiving these Drugs for Platinum-Resistant Ovarian Cancer

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Abstract

Background: The potential of gemcitabine to interact with carboplatin was explored in a phase II trial in platinum-resistant ovarian cancer. Peripheral blood lymphocytes were sampled after drug administration to measure DNA interstrand cross-link formation and repair.

Patients and Methods: Forty patients received carboplatin target area under concentration-time curve (AUC 4) followed by gemcitabine 1,000 mg/m² with a second dose of gemcitabine on day 8. Peripheral blood lymphocytes were obtained in 12 patients before and at intervals during the first cycle of chemotherapy. DNA cross-link formation and repair (unhooking) were measured by the single-cell gel electrophoresis (comet) assay following *ex vivo* incubation.

Results: The global response rate was 47% (Response Evaluation Criteria in Solid Tumors rate, 29%; CA125 rate, 63%). Delays in treatment were seen in 24% of cycles largely due to myelosuppression; 15% of day 8 administration was omitted. Peak carboplatin-induced DNA cross-linking was seen by 24 hours. Significant reduction was seen in the repair of *in vivo* carboplatin-induced DNA cross-links following administration of gemcitabine.

Conclusion: An enhanced activity of carboplatin in platinum-resistant ovarian cancer may be due to synergy with gemcitabine through inhibition of repair of DNA cross-links. Future studies should explore coadministration of these drugs, as this may be a more effective schedule. *Clin Cancer Res*; 16(19); 4899-905.

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Platinum-containing drugs are the most active compounds for treating ovarian cancer. They are often reused for recurrent disease, but their activity diminishes as tumors become increasingly resistant. The working definition of "platinum resistance," based on observations made many years ago (1, 2), refers to tumors relapsing or progressing within 6 months of previous therapy. In this situation, the probability of a further response to platinum compounds is low and most patients are offered nonplatinum drugs. However, the activity of these agents in this situation is modest and usually short lived.

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Gemcitabine (2',2'-difluorodeoxycytidine) is one of many nonplatinum drugs that has some activity in recurrent, platinum-resistant ovarian cancer (3). Interestingly, preclinical studies suggest that gemcitabine may have an additive or synergistic effect when combined with cisplatin (4), and clinical studies in a group of women who had previously received multiple lines of therapy support this notion (5, 6). The cytotoxicity of platinum drugs results from the formation of DNA adducts. Intrastrand cross-links constitute the majority (>80%) of lesions formed on cellular DNA, and these distorting lesions are repaired by nucleotide excision repair (7). Interstrand cross-links, which link the two complementary strands of DNA together, comprise less than 5% of the total DNA lesions but are highly cytotoxic and difficult to repair (8). We have recently shown enhanced repair of DNA interstrand cross-links in ovarian cancer cells from patients following treatment with platinum-based chemotherapy (9). *In vitro* studies with cell lines have shown a direct inhibitory effect of gemcitabine on the repair of cisplatin-induced cross-links and suggested a mechanistic basis for the cytotoxic synergy between the two drugs (10), although this has not been studied in the clinic.

In this phase II study in platinum-resistant ovarian cancer, we explored the possibility that gemcitabine might have a

Translational Relevance

We have shown that the combination of gemcitabine with carboplatin is synergistic by demonstrating in patients' lymphocytes that gemcitabine, administered following carboplatin, leads to inhibition in the repair of carboplatin-induced DNA interstrand cross-links. These findings support the view that the combination of carboplatin and gemcitabine is rational and that clinically useful synergy may occur. The combination should in the future be given as a doublet to make best use of the interaction, and further studies should be done with this schedule in tumors for which these drugs are currently used.

synergistic effect on carboplatin therapy, resulting in a higher tumor response than might be expected from either drug on its own. Additionally, a potential mechanism of interaction was studied by examining the effect of gemcitabine on the formation and repair of carboplatin-induced DNA interstrand cross-links in peripheral blood lymphocytes (PBL).

Materials and Methods

Between June 2004 and June 2006, 40 women gave consent to enter this phase II open-labeled trial from four U.K. hospitals following ethical and regulatory approval.

Patient eligibility

Patients ages 18 years or older with known epithelial ovarian carcinoma, primary peritoneal carcinoma of the serous type, or fallopian tube carcinoma were included. All women relapsed or progressed within 6 months of previous platinum-based chemotherapy and started carboplatin and gemcitabine when there was a clinical indication for chemotherapy. Patients had an Eastern Cooperative Oncology Group performance score of 0 to 2; a life expectancy >12 weeks; and an assessable disease, either radiologically measurable or evaluable by serum CA125.

Treatment plan

All patients received carboplatin at area under the concentration-time curve [(AUC 4) mg/ml × min] (or AUC 5 if the glomerular filtration rate was calculated) i.v. on day 1 as a 30-minute infusion followed by gemcitabine 1,000 mg/m² i.v. over 30 minutes on days 1 and 8 for a maximum of six cycles, every 3 weeks. Treatment was delayed for up to 2 weeks if the neutrophil count was <1.5 × 10⁹/L or if platelets were <100 × 10⁹/L. Granulocyte colony-stimulating factors were not used in this study. Gemcitabine was reduced to 800 mg/m² for delays of more than 1 week, presence of neutropenic fever, or grade 4 thrombocytopenia. The dose of gemcitabine was reduced by 50% on day 8 if neutrophils were between 1.0 × 10⁹/L and 1.4 × 10⁹/L, and/or if the platelets were between 75 × 10⁹/L and 99 × 10⁹/L. For values lower than these, gemcitabine on day 8 was omitted.

Assessments

Patients were assessed at baseline, before each chemotherapy cycle, and then six weekly for the first 6 months and then three monthly until progression or death. Response was assessed either by computed tomography (CT) or CA125. CT scans were done at baseline and after the third, sixth, or final chemotherapy cycle. Serum CA125 was measured at baseline and before each chemotherapy cycle. A CA125 response was defined according to the Gynaecological Cancer Intergroup as at least a 50% decrease, confirmed with a further sample at least 28 days later (11). Patients stopped treatment after three cycles for disease progression, defined either by a >30% increase in measurable lesions, development of new lesions, or if CA125 increased beyond twice the lowest previous level. Toxicity was assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events, Version 3.0.

Statistical analysis

The primary outcome measure was a Response Evaluation Criteria in Solid Tumors (RECIST) response rate to gemcitabine and carboplatin; secondary measures included toxicity, CA125 response, time to disease progression, and (in selected centers only) measurement of DNA damage in PBLs before, during, and after therapy. We also assessed the global response rate based on having a response under either the RECIST or CA125 criteria. A two-stage Simon design (12) was used, assuming the response rate of gemcitabine was 15% and the acceptable response rate of combination chemotherapy was 30%, 10% statistical significance, and 80% power. It was concluded that the regimen would be worthy of further study if nine or more responses were observed from a total of 39 patients. Overall and progression-free survival were calculated from date of registration to date of death, and progression or death, respectively.

Translational research

Blood samples were taken from 12 patients before chemotherapy, immediately after carboplatin infusion, and immediately after gemcitabine on the first cycle of chemotherapy. This sequencing allowed measurement of DNA damage and repair of PBLs following carboplatin and after carboplatin and gemcitabine in the same patient. On day 8, blood samples were taken before and after gemcitabine infusion (Fig. 1). PBLs were isolated using the Vacutainer CPT system. Following centrifugation and removal of the mononuclear layer, cells were washed once and resuspended. PBLs were then incubated *ex vivo* for 0, 4, 24, and 48 hours, and carboplatin-induced DNA interstrand cross-link formation and repair (unhooking) were assessed using the single-cell gel electrophoresis (Comet) assay, as previously described (13, 14). Briefly, PBL samples were irradiated (12.5 Gy) to deliver a fixed number of random DNA strand breaks. After embedding cells in 1% low gelling temperature agarose on a precoated microscope slide, the cells were lysed (pH 10.5) for 1 hour,

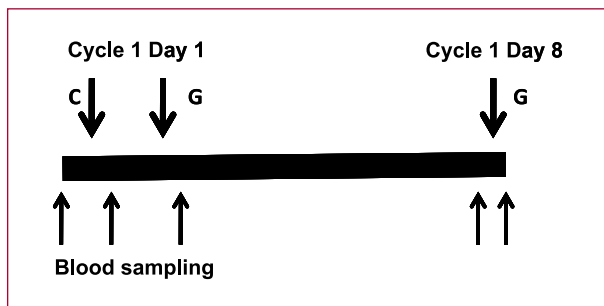


Fig. 1. Treatment and blood sampling schema. C, carboplatin; G, gemcitabine.

washed, and then incubated in alkali buffer (pH 12.5) for 45 minutes followed by electrophoresis. After drying, the slides were stained with propidium iodide. The images were visualized using an inverted fluorescent microscope and captured using a digital camera. For each duplicate slide, 25 cells were analyzed. The tail moment for each image was calculated using the Komet 5.5 Analysis software (Andor Technology, Kinetic Imaging) based on the definition of Olive et al. (15).

In patients receiving carboplatin and gemcitabine, DNA interstrand cross-linking was expressed as the percentage of decrease in tail moment compared with irradiated controls calculated by the formula below. This formula was used to compensate for any additional single-strand breaks induced by gemcitabine in addition to those produced by the irradiation step. The greater the percentage decrease in tail moment, the greater the level of DNA interstrand cross-linking.

$$\% \text{ Decrease in tail moment} = \left[1 - \left(\frac{\text{TMdi} - \text{TMcu}}{\text{TMci} - \text{TMcu} + (\text{TMdu} - \text{TMcu})} \right) \right] \times 100$$

where TMdi is the tail moment of drug-treated irradiated sample; TMdu is the tail moment of drug-treated unirradiated sample; TMcu is the tail moment of untreated, unirradiated control; and TMci is the tail moment of untreated, irradiated control.

Results

The baseline characteristics of patients are given in Table 1. All had developed progressive disease within 6 months of previous treatment; however, in some, the start of carboplatin and gemcitabine was delayed beyond this time, until there was a clinical indication to start treatment.

Toxicity

Twenty-one patients received six cycles of chemotherapy, and only six (15%) received less than three complete cycles. The main reason for early cessation of treatment was disease progression. Treatment administration is sum-

marized in Table 2. Nineteen patients had a dose delay due to toxicity. In 36%, this occurred at cycle 2. Three patients did not have a second cycle. Twenty-four percent of all chemotherapy cycles were delayed. Most of the toxicity was asymptomatic hematologic toxicity, and 30 patients required a dose reduction of gemcitabine or omission of the drug on day 8. Twenty-eight (15%) of all cycles were given without day 8 gemcitabine. Grade 3/4 neutropenia was seen in 15 (38%) patients, but there were only four cases of neutropenic fever. Grade 3/4 thrombocytopenia occurred in seven patients (18%). Other nonhematologic grade 3 and 4 toxicities, such as nausea, vomiting, or fatigue, each occurred in 5% or less of patients.

Response

Response assessments were made after three cycles in 34 patients with measurable disease, and after six cycles in 19 patients. Five patients had no target lesions measured, and one patient had missing RECIST response data. Five of 34 patients died or were too ill to be assessed by the end of cycle 3 and were considered to have progressive disease by cycle 3. Ten of 34 (29%) patients had a measurable response (one complete response and nine partial responses) by the end of chemotherapy. Seventeen of 39 patients (44%) had progressive disease while on treatment.

Table 1. Patient baseline characteristics

| | No. of patients (%) |
|--|---------------------|
| ECOG performance status | |
| 0 | 16 (40) |
| 1 | 18 (45) |
| 2 | 6 (15) |
| Primary site | |
| Ovary | 35 (88) |
| Primary peritoneal | 4 (10) |
| Missing | 1 (3) |
| Age at registration (y) | |
| <60 | 10 (25) |
| 60-69.9 | 23 (58) |
| 70+ | 7 (18) |
| Median age, 64.3 y (range 47.5-80.1 y) | |
| Measurable disease, according to RECIST criteria | |
| Yes | 35 (88) |
| No (no measured target lesions) | 5 (13) |
| Previous anticancer therapy | |
| No. of prior platinum-contained regimens | |
| 1 | 27 (68) |
| 2 | 10 (25) |
| 3+ | 2 (5) |
| Missing | 1 (3) |

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

Table 2. Treatment summary

| Compliance with chemotherapy* | No. of patients (%) |
|-------------------------------------|---------------------|
| No. of chemotherapy cycles received | |
| ≤2 | 6 (15) |
| 3 | 6 (15) |
| 4 | 5 (13) |
| 5 | 2 (5) |
| 6 | 21 (53) |
| Chemotherapy dose reductions† | |
| Cycle 1 | 9/40 (23) |
| Cycle 2 | 16/36 (44) |
| Cycle 3 | 14/34 (41) |
| Cycle 4 | 16/28 (57) |
| Cycle 5 | 15/23 (65) |
| Cycle 6 | 14/20 (70) |
| Any cycle | 30/40 (75) |

*Not all patient records are complete with respect to chemotherapy dates and dosages. Therefore, the number of patients at risk of having a reduction may not match the number of cycles received.

†Chemotherapy reductions are calculated as dose reductions >10% compared with a day 1, cycle 1, or day 8 omission.

Response was assessed by CA125 measurement in 27 of the 40 patients. Five patients were not assessable because of an initially low CA125 level; seven patients had less than three CA125 measurements; and one patient did not have a CA125 assessment within the specified time from the start of chemotherapy. Overall, 17 of 27 (63%) patients had a 50% or greater fall in CA125. The global response rate based on either RECIST or CA125 was 47% (18 of 38).

Survival

At the time of analysis, 25 of 40 (63%) patients had died (Fig. 2A). Median survival was 11.7 months (95% confidence interval, 9.0-18.4 months). Thirty-one of the 40 (78%) patients had progressive disease or died (Fig. 2B). The median progression-free survival was 6.9 months (95% confidence interval, 3.7-8.8 months). When measured by CA125, progression-free survival was 8.1 months (95% confidence interval, 6.2-10.1 months).

Effect of gemcitabine on repair of carboplatin-induced DNA damage

The cross-link data for the 12 patients, from whom PBL samples were taken, are shown in Fig. 3. Carboplatin-induced DNA interstrand cross-link formation was observed to increase over 24 hours postcarboplatin (Fig. 3A) and in the sample taken after carboplatin and gemcitabine (Fig. 3B). In the majority of patients (75%), complete repair (unhooking) of DNA interstrand cross-links was observed at 48 hours posttreatment following carboplatin alone

(Fig. 3A). However, in the same patients following treatment in combination with gemcitabine, the level of repair of carboplatin-induced interstrand cross-links at 48 hours was significantly reduced. The Wilcoxon signed rank test indicated no treatment difference at 0 and 4 hours; however, at 24 and 48 hours, evidence showed that after the addition of gemcitabine, there is a larger decrease in tail moment ($P = 0.006$ and 0.002 , respectively), with no patient sample showing complete repair (Fig. 3B).

With carboplatin alone, median repair of DNA interstrand cross-links at 48 hours was 100% (Fig. 4). Eight of 12 patients showed 100% repair at 48 hours following carboplatin alone, with the remaining four patients demonstrating repair ranging from 0% to 75%. However, following combination with gemcitabine in the same patients, the repair of carboplatin-induced DNA interstrand cross-links was significantly reduced (nonparametric Wilcoxon signed rank test; $P = 0.003$) with a median repair of 17.5% (Fig. 4). Five patients showed no repair at 48 hours. Seven patients showed repair ranging from 7% to 60%. No single-strand breaks were seen in any patient following gemcitabine alone. These data show that the addition of gemcitabine significantly inhibits the repair

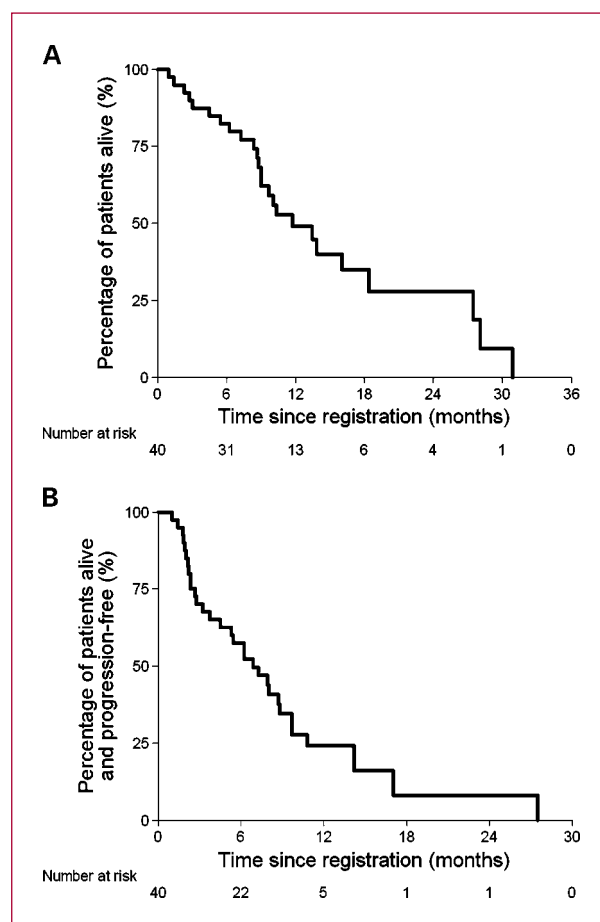
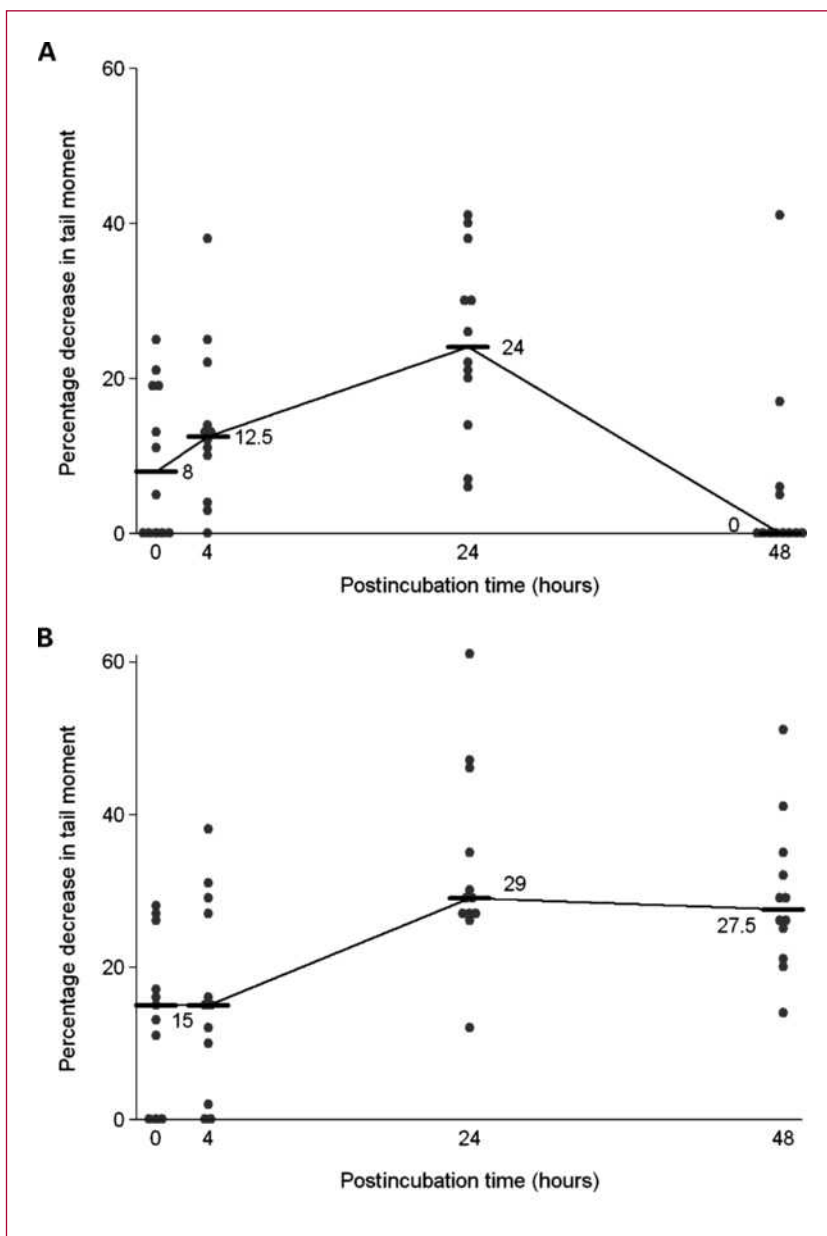


Fig. 2. Overall survival (A) and progression-free survival (B).

Fig. 3. Formation and repair of carboplatin-induced DNA interstrand cross-links in PBLs taken from 12 patients following treatment with carboplatin (A) and then in a sample taken after the administration of gemcitabine following carboplatin (B). Results are expressed as percentage decrease in tail moment and the horizontal bars and numbers indicate the median values.



(unhooking) of carboplatin-induced DNA interstrand cross-links *in vivo*.

Discussion

This study further supports the view that in patients, gemcitabine has a synergistic effect on platinum therapy. First, in patients with platinum resistance, the global response rate was 45% (18 of 40). Platinum resistance is not absolute; some tumors respond and others are truly resistant. Historic data suggest that the response rate in platinum-resistant tumors is less than 20% (1, 2). Thus, some caution is needed in interpreting the results of a nonrandomized study due to the heterogeneity of plati-

num resistance. A similar reasoning applies to single-agent gemcitabine for which the response rate in this population was reported to be 17% (16).

Nevertheless, our results using carboplatin and gemcitabine are in keeping with other reported studies of cisplatin and gemcitabine, all of which used cisplatin in a day 1 and day 8 schedule (5, 6, 17–19). Dividing the cisplatin schedule into a day 1 and day 8 administration exploits the knowledge from *in vitro* studies that have suggested the effect of the two drugs is synergistic. The initial preclinical report by Peters et al. (20) did not identify a mechanism but suggested that the effects on platinum-sensitive and platinum-resistant cell lines were schedule dependent. It was not due to accumulation of gemcitabine triphosphate

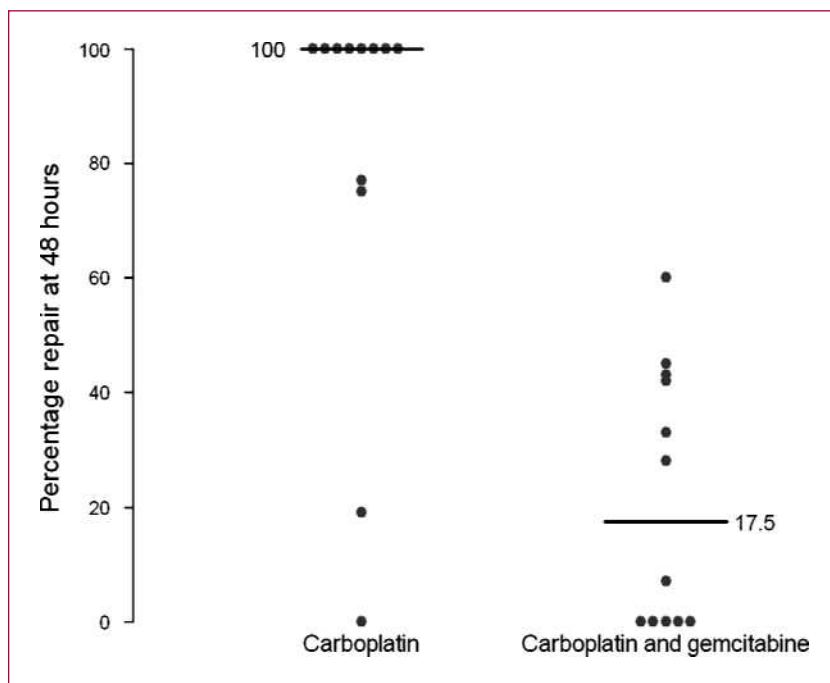


Fig. 4. Percentage repair of carboplatin-induced DNA interstrand cross-links in patient PBLs at 48 hours in the blood samples taken after treatment with carboplatin alone and after treatment with the combination of carboplatin and gemcitabine. Horizontal bars indicate median values.

or increase in DNA strand breaks. *In vitro* studies have shown that gemcitabine impairs repair of platinum-induced cross-links (10). The initial clinical reports of activity of the combination of cisplatin and gemcitabine in a heterogeneous group of patients, including those with clear platinum-resistant disease, supported this hypothesis and included some patients who failed to respond to single-agent gemcitabine but responded when cisplatin was added (6).

Second, the key finding in our study is that we have shown a mechanism of synergy between carboplatin and gemcitabine in patients, supporting the observations with cisplatin and gemcitabine. Although we were not able to study the effect of the drugs on tumor tissue, the interaction of carboplatin and gemcitabine was shown *in vivo* by studying the real-time effect of DNA cross-link formation and repair in PBLs removed from patients at varying time points. The maximum formation of interstrand cross-links was seen 24 hours after carboplatin administration. Following incubation of cells removed after carboplatin was administered, the repair of cross-links was efficient in the majority of patients, with 8 of 12 showing complete repair at 48 hours. We have previously shown significant repair of cross-links in ovarian cancer cells from patients who had received platinum-based therapy (9). In contrast, in samples taken after gemcitabine administration, repair of the cross-links produced by carboplatin was significantly impaired at 48 hours (Fig. 4). The slightly higher DNA cross-link formation at 24 hours after carboplatin and gemcitabine compared with carboplatin alone (Fig. 3) is likely to be due to the balancing effects of cross-link formation and repair. It has been shown in a lung cancer cell line that there were more platinum-DNA adducts in the presence of gemcitabine (21). No strand breaks were observed following gemcitabine

alone in PBLs in the present study under the assay conditions used, although a previous *in vitro* study has shown the formation of DNA strand breaks following gemcitabine (22).

Gemcitabine is believed to inhibit nucleotide excision repair by incorporation into repair patches, thereby causing chain termination (10). Repair of interstrand cross-links is complex and is thought to involve components of the nucleotide excision repair pathway and recombination (8). A modified comet assay was used in the current study to measure interstrand cross-linking. The disappearance of cross-links measured using this assay reflects an early step in the repair process, the unhooking of the cross-link from one strand. This study shows that this step is clearly inhibited *in vivo* for carboplatin-induced cross-links by gemcitabine. One possible mechanism for this is that the nucleotide excision repair of carboplatin-induced intrastrand adducts is inhibited by incorporation of gemcitabine into repair patches resulting in sequestering of repair proteins, including those required for the initial unhooking step of DNA interstrand cross-links.

This study supports the notion that platinum resistance is usually not absolute and that patients in this group may continue to benefit from platinum-based therapy. First-line studies have not shown that carboplatin-gemcitabine combinations are superior to standard chemotherapy with carboplatin and paclitaxel (23), but progression-free survival is prolonged when gemcitabine is added to carboplatin in patients with platinum-sensitive relapse (24). This may be because in this group of patients, there is a reduction in platinum sensitivity that is partially overcome through the synergistic effect of gemcitabine. In the standard three-weekly schedule of carboplatin and gemcitabine, used frequently in lung and ovarian cancer,

carboplatin is given only on day 1 of each cycle and gemcitabine on days 1 and 8. In recurrent ovarian cancer, we and others (24) have found that this regimen has significant but asymptomatic hematologic toxicity, frequently requiring a dose reduction or omission of day 8 gemcitabine.

If the improved antitumor effect results from synergy between the drugs as shown in PBLs, there is a strong rationale for changing the schedule to combine each dose of gemcitabine with carboplatin, as is the case with cisplatin and gemcitabine (5, 6, 17–19). Our *in vivo* data in PBLs support this alternative approach to therapy, dividing the dose of carboplatin between day 1 and day 8, always giving carboplatin with gemcitabine. Reports of using dose-fractionated therapies with, for example, carboplatin and paclitaxel in both platinum-resistant and newly diagnosed ovarian cancer have been encouraging (25, 26). Regimens of dose-fractionated carboplatin with gemcitabine are now being explored (27), and these should be more extensively studied in ovar-

ian cancer. This will make best use of synergy between the drugs, and this schedule may be less myelosuppressive.

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