Cisplatin Inhibits Paclitaxel-induced Apoptosis in Cisplatin-resistant Ovarian Cancer Cell Lines: Possible Explanation for Failure of Combination Therapy

Patricia L. Judson, Joanna M. Watson, Paola A. Gehrig, Wesley C. Fowler, Jr., and J. Stephen Haskill


ABSTRACT

Combination chemotherapy using paclitaxel with a platinum-based regimen is currently the standard first-line therapy for ovarian cancer after surgical cytoreduction. Whereas cisplatin-paclitaxel combination chemotherapy has shown significant efficacy over previous drug combinations in ovarian cancer, 20–30% of patients fail to respond to this combination. These patients are deemed cisplatin-paclitaxel resistant, although it is unclear whether the tumors are resistant to one or both drugs. Because the options available to ovarian cancer patients for second-line therapy are limited, and knowing that mechanistic differences exist between cisplatin and paclitaxel, we assessed the efficacy of combination drug therapy on cisplatin-resistant (cisplatinR) ovarian cancer cells. We found that paclitaxel induced apoptosis in cisplatinR cells as well as in the cisplatin-sensitive parental cell lines. In cisplatinR C-133 cells, the concomitant addition of cisplatin blocked paclitaxel-induced apoptosis as determined by DNA fragmentation assays, fluorescence microscopy, and flow cytometry. Paclitaxel-induced multinucleation was also inhibited when the cells were exposed sequentially to paclitaxel and then cisplatin. Cisplatin did not block paclitaxel-induced stabilization of microtubules or prevent paclitaxel-induced loss of Bcl-2 expression in cisplatinR cells. Conversely, paclitaxel did not inhibit p53 protein accumulation by cisplatin. These results suggest that cisplatin blocks paclitaxel-induced apoptosis at a point downstream of Bcl-2 degradation and independent of microtubule stabilization. Our research shows that cisplatin can inhibit the effectiveness of paclitaxel in cisplatinR cell lines. Therefore, the establishment of a clinical protocol to evaluate the efficacy of paclitaxel alone versus another second-line regimen in patients with cisplatin-paclitaxel-resistant ovarian cancer is warranted.

INTRODUCTION

Paclitaxel (Taxol) gained clinical attention as a chemotherapeutic agent for its activity against drug-resistant breast and ovarian cancers (1). Paclitaxel has shown promising clinical efficacy against such cancers in both primary and secondary treatment regimens. A Gynecological Oncology Group trial tested the effectiveness of paclitaxel as a second-line agent in patients with recurrent, persistent, or progressive ovarian cancer. All patients had previously been treated with a platinum-based chemotherapy. These patients had an overall response rate of 37% to paclitaxel (18% had a complete response and 19% had a partial response; Ref. 2). This was followed by a Gynecological Oncology Group trial comparing cisplatin and cyclophosphamide versus cisplatin and paclitaxel (3). The response rate for the cisplatin and paclitaxel combination was 77%, with a median survival that was 13.1 months longer than that of the cisplatin and cyclophosphamide-treated group. Based on this trial, cisplatin and paclitaxel became the standard first-line treatment regimen for patients with advanced ovarian cancer.

Whereas 77% of patients responded to cisplatin and paclitaxel chemotherapy, 23% of ovarian cancer patients had tumors that did not respond (3). These women are given various second-line regimens of chemotherapy in an attempt to prolong life and palliate symptoms. Second-line chemotherapeutic agents are rarely curative, with initial response rates ranging from 10–33%. However, the majority of patients with ovarian cancers experience a recurrence and fail further chemotherapeutic regimens. There is now clinical evidence that these patients may respond to subsequent single-agent therapy. In a study of patients with metastatic ovarian disease, 17 of 28 patients responded or had stable disease after treatment with paclitaxel alone (4). Furthermore, the authors found that 12 of 20 patients with cisplatinR ovarian tumors either responded or had stable disease (4, 5) after treatment with single-agent paclitaxel.

Over the last 35 years, cisplatin has been one of the few clinically beneficial chemotherapeutic drugs used for the treatment of ovarian cancer. Cisplatin belongs to the alkylating agent group of chemotherapies. Cisplatin binds to DNA base pairs, creating adducts, cross-links, and strand breaks that inhibit DNA replication. Detection of damaged DNA leads to the activation of cyclin-dependent kinase inhibitors such as p21 and wee1/mik1, which subsequently arrest cells in either G1 or G2. Unrepairable DNA damage often results in activation of the apoptotic pathway. p53 plays a significant role in DNA repair, proliferative arrest, and apoptosis (6) and has led to a correlation between p53 and cisplatin sensitivity (7–9). Resistance to cisplatin has been associated with increased glutathione levels, increased metallothioneins, decreased drug uptake, increased DNA repair, and the tolerance of the formation of platinum-DNA adducts (10).

Paclitaxel, a mitotic inhibitor, promotes the formation and stabilization of microtubules, and this results in a cell cycle block at the metaphase to anaphase transition (11). The arrest in mitosis correlates with paclitaxel-induced cytotoxicity (12). Paclitaxel-arrested cells often undergo apoptosis that is mediated at least in part through the degradation of Bcl-2 (13). Some cells escape from mitosis and arrest with interphase-like multinuclei (14). In contrast to cisplatin, sensitivity to paclitaxel is independent of p53 status (12, 15, 16). Recently, alterations in β-tubulin isoforms have been associated with resistance to paclitaxel in vitro (17–20).

There is increasing evidence that both cisplatin and paclitaxel can elicit multiple molecular responses in cells. These responses include the rapid activation of c-Jun-NH2-terminal kinase/stress-activated protein kinase and modulation of the extracellular signal-stimulated mitogen-activated protein kinase (21, 22).5 Initiation of these signaling pathways can result in the activation and translocation of the
transcription factors AP-1, nuclear factor κB, and SP-1 (23–27). Consequently, transcription increases for numerous genes including those encoding DNA damage response proteins, cytokines, and proteins involved in proliferation, apoptosis, and inflammation (28–33). These responses often precede the activation of cell death pathways. We have recently shown that paclitaxel-induced gene induction occurs within minutes of paclitaxel exposure and correlates with paclitaxel-induced cytotoxicity, a late event that requires days (30). The induction or modulation of a variety of genes from either the tumor cells (28–30), immune cells (31–35), or supporting fibroblasts (36) may account for the enhanced efficacy of paclitaxel in vivo over that of other antimitotics.

Cisplatin and paclitaxel are highly suited for combination chemotherapy because they have distinct mechanisms of action. However, synergy between cisplatin and paclitaxel is highly schedule dependent (37, 38), suggesting a competition between the mechanisms of action of both drugs. In vitro, we found that cisplatin exerts mechanistic dominance over paclitaxel when ovarian cancer cells are exposed to both drugs simultaneously. Cisplatin blocked the effects of paclitaxel, even when paclitaxel exposure preceded cisplatin exposure by 3 h. Whereas cisplatin ovarian cancer cell lines retain sensitivity to paclitaxel, concomitant exposure with cisplatin blocks paclitaxel-induced apoptosis. Cisplatin does not interfere with paclitaxel-induced stabilization of microtubules or Bcl-2 degradation, suggesting an action downstream of these events. These data provide a possible explanation for why some patients whose disease progressed with combination therapy are still sensitive to single-agent paclitaxel. A detailed assessment of the biological effects of cisplatin and paclitaxel on the induction of apoptosis may thus directly influence the treatment of women with ovarian cancer.

MATERIALS AND METHODS

Reagents. Cisplatin [cis-diaminedichloroplatinum(II)] was purchased from Sigma Chemical Co. and stored at a concentration of 3 mm. Paclitaxel from Taxus Brevifolia was purchased from Sigma Chemical Co. and suspended in 100% DMSO at a concentration of 20 mm. Drugs were stored at 4°C and diluted in warmed media before use. Antibodies to p53 (Calbiochem; Ab6) and diluted in warmed media before use. Antibodies to p53 (Calbiochem; Ab6) and monoclonal anti-tubulin antibody (Clone Tub 2.1; 1:200; Sigma) followed by a tetramethylrhodamine isothiocyanate-labeled secondary goat antimouse antibody. Cells were counterstained with Hoechst (0.2 g/ml) to visualize nuclear morphology.

Cytotoxicity Assay. Cytotoxicity and cell survival were determined by the MTT assay (39). In brief, cells were plated at 5000 cells/well in 96-well microtiter plates. After an overnight adherence, cells were treated for 3 h with clinically relevant doses of cisplatin (30 μm) and/or paclitaxel (2 μm), rinsed, and incubated for 48 h in drug-free medium. During the final 2 h of incubation, MTT (0.5 mg/ml) was added to each well. At the termination of the experiment, the culture media were removed, and the precipitated dye was solubilized in 100% DMSO. The absorbance of each well was determined using the microplate reader at 595 nm. The percentage of cell survival was defined as the relative absorbance of control versus treated cells. All assays were performed in triplicate and repeated at least twice. The LD50 for each drug and each cell line is shown in Table 1. The drug concentrations used in all assays represent the near Cmax values that are clinically achievable for a 3-h infusion (paclitaxel, 2 μm; cisplatin, 30 μm).

Flow Cytometry. After exposure to chemotherapy as described above and incubation for 48 h in drug-free media, cells were rinsed, trypsinized, pelleted, and resuspended in cold 1× PBS. They were then fixed dropwise with ice-cold 100% ethanol to yield a final concentration of 70%. Nuclei were isolated and stained with propidium iodide as described previously (40). Flow cytometric analysis was carried out on a Becton Dickinson FACSscan, and data were analyzed using the Cicer Cell Cycle Analysis program after collecting 30,000 events per sample (Cytometry, Fort Collins, CO).

Apoptosis by DNA Fragmentation. Treated cells were harvested after 48 h. Approximately 2 × 106 cells were lysed in 500 μl of ice-cold lysis buffer containing 100 μl Tris (pH 7.4), 2 mm EDTA, and 0.2% Triton X-100. After incubation for 10 min on ice, cell debris was removed by centrifugation, and the supernatant was incubated at 35°C for 18 h in the presence of 10 μg/ml proteinase K. The supernatant was then treated with RNase A (10 μg/ml) for 2 h at 37°C. Fragmented DNA was isolated after the supernatants were extracted twice with phenol:chloroform:isoamyl alcohol, and the DNA was precipitated in 100% ethanol in the presence of 2 μg/ml oyster glycogen. DNA was pelleted, washed, dried, and resuspended in 20 μl of Tris-EDTA buffer (20 mm Tris (pH 7.4) and 10 mm EDTA). DNA fragments were separated by electrophoresis through a 2% agarose gel and visualized by staining with ethidium bromide.

Western Blot Analysis. Cells were treated with chemotherapy for 3 h and harvested 24 h later for Western blot analysis by direct lysis into 2× Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transblotted to nitrocellulose, and blocked for 1 h in TBS-T. All primary antibodies were incubated overnight at room temperature in TBS-T. Horseradish peroxidase-conjugated secondary antibodies were added for 1 h after the blots were washed three times in TBS-T. Proteins were visualized by enhanced chemiluminescence (Amersham).

Immunohistochemistry. Cells (approximately 1–5 × 106) were plated onto acid-washed 12-mm coverslips, adhered overnight, and treated with 30 μm cisplatin, 2 μm paclitaxel, or a combination of cisplatin and paclitaxel for 3 h. The slides were subsequently washed and maintained in drug-free complete media. After treatment with chemotherapy, cells were fixed at various time intervals (4, 8, 24, and 48 h) in a solution of 3% paraformaldehyde and 1% gluteraldehyde in PBS and stored at 4°C for up to 2 weeks. Cells were permeabilized for 5 min on ice in a buffer consisting of 20 mm HEPES (pH 7.4), 300 mm sucrose, 50 mm NaCl, 3 mm MgCl2, and 0.5% Triton X-100 before blocking in 25% normal goat serum. Cells were stained for tubulin using a mouse monoclonal anti-tubulin antibody (Clone Tub 2.1; 1:200; Sigma) followed by a tetramethylrhodamine isothiocyanate-labeled secondary goat antitubulin. Cells were counterstained with Hoechst (0.2 μg/ml) to visualize nuclear morphology.

RESULTS

Cisplatin6 Cells Retain Sensitivity to Paclitaxel. We compared the sensitivity and responsiveness of paired cisplatin6 and cisplatin8 ovarian cancer cell lines to paclitaxel, cisplatin, and the combination of both drugs in cytotoxicity assays. As shown in Fig. 1, the parental cell lines (2008 and A2780) showed sensitivity to both cisplatin and paclitaxel.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CDDP (μm)</th>
<th>Ptx (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>14.7 ± 9.8</td>
<td>0.30 ± 0.2</td>
</tr>
<tr>
<td>C-13</td>
<td>&gt;75 ± 10</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>A2780</td>
<td>27.0 ± 5.4</td>
<td>1.27 ± 0.4</td>
</tr>
<tr>
<td>A2780CP</td>
<td>12.9 ± 2</td>
<td>0.12 ± 0.1</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>33.7 ± 11</td>
<td>0.29 ± 0.4</td>
</tr>
<tr>
<td>OVCA 420</td>
<td>30.2 ± 7.3</td>
<td>0.26 ± 0.16</td>
</tr>
<tr>
<td>OVCA 429</td>
<td>74.8 ± 10</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>OC-194</td>
<td>53.6 ± 16</td>
<td>0.31 ± 0.2</td>
</tr>
</tbody>
</table>


paclitaxel. None of the cell lines examined showed a significantly enhanced response when the two drugs were combined simultaneously. The cisplatinR cell line C-13 showed decreased sensitivity to cisplatin compared to its respective parental cell line, 2008. The cisplatinR cell line A2780CP showed a sensitivity to cisplatin that was almost comparable to that of its parental A2780 line, suggesting that resistance to cisplatin may have been lost. Both cisplatinR cell lines C-13 and A2780CP demonstrated an increased sensitivity to paclitaxel when used as a single therapy. However, when these cisplatinR cell lines were exposed to paclitaxel in combination with cisplatin, this increased cytotoxicity was diminished. The survival for C-13 was 6.5% when treated with paclitaxel alone, 24.2% when treated with a combination of cisplatin and paclitaxel, and 78.6% when treated with cisplatin alone. A2780CP had an 8.5% survival when treated with paclitaxel alone, a 40.7% survival when treated with a combination of cisplatin and paclitaxel, and a 38.9% survival when treated with cisplatin alone.

We observed similar results with additional human ovarian cancer cell lines. Paclitaxel-induced cytotoxicity was often reduced when used in combination with cisplatin (Fig. 1), particularly in cell lines that showed sensitivity to paclitaxel but poor responsiveness to cisplatin in vitro such as OVCA 420 or OVCA 429.

Cisplatin and Paclitaxel Combination Results in Cell Cycle Changes That Are Most Consistent with Exposure to Cisplatin Alone. To begin to assess why paclitaxel-induced cytotoxicity was diminished by the concomitant presence of cisplatin, we performed flow cytometry. After 48 h, both cisplatinR and cisplatinS ovarian cancer cells treated with cisplatin undergo an S-phase to early G2-phase arrest (Fig. 2). Both the A2780CP and C-13 cell lines showed an accumulation in G2. The parental cisplatinS cell lines, A2780 and 2008, showed less G2 accumulation, a greater accumulation of cells in or transiting through S phase, and the presence of hypodiploid nuclei (Fig. 2, A and B). The presence of hypodiploid DNA is often associated with cells undergoing apoptosis. All cell lines treated with paclitaxel alone show a dramatic increase in the percentage of cells in G2-M phase and a loss of cells in G1 (Fig. 2B). The percentage of cells with hypodiploid DNA content ranged from 7–12%, depending on the cell line.

When cells were treated with a combination of cisplatin and paclitaxel, the resultant cell cycle profiles resembled those of cells treated with cisplatin alone (Fig. 2; Table 3). Both cisplatinS and cisplatinR cell lines showed increases in the percentage of cells in S phase and in G2-M phase (Fig. 2B). In cisplatinR cell lines, the combination treatment resulted in fewer cells with hypodiploid DNA (Fig. 2B; Table 2). When the cisplatinR cell line C-13 was treated with a combination of cisplatin and paclitaxel, there was a loss of cells in G1 and an apparent decrease in the percentage of hypodiploid cells. However, overall, the cell cycle profile resembled that of cells treated with paclitaxel more than that of cells treated with cisplatin. When
Cisplatin and paclitaxel induced DNA fragmentation in the parental cisplatin<sup>a</sup> cell lines. A2780CP cells show some high molecular weight DNA fragmentation after exposure to cisplatin, suggesting that these cells may have lost their resistance to the drug.

When cells were treated with a combination of cisplatin and paclitaxel, the DNA fragmentation profile again resembled that of cells treated with cisplatin alone (Fig. 3; Table 3). Thus, C-13 cells that are resistant to cisplatin have little or no apoptosis when exposed to a combination of cisplatin and paclitaxel (Fig. 3; Table 3). In contrast, for A2780CP cells, the DNA fragmentation profiles obtained with combination treatment resembled that of single treatment paclitaxel more than that of cisplatin. The cisplatin<sup>b</sup> cells A2780 and 2008 have similar or increased apoptosis when exposed to a combination of cisplatin and paclitaxel. This suggests that cisplatin may have a dominant effect over paclitaxel when used in combination for the majority of cell lines.

Cisplatin Inhibition of Paclitaxel-induced Apoptosis Occurs Downstream of Bcl-2 Degradation and Independent of Microtubule Stabilization. To understand the mechanism by which cisplatin could prevent paclitaxel-induced apoptosis, we assessed the expression of Bcl-2 in the ovarian cancer cell lines. Paclitaxel has previously been shown to induce apoptosis through a Bcl-2-dependent pathway in several cancer cell lines (13, 41). Cisplatin<sup>a</sup> cells were treated with cisplatin, paclitaxel, or a combination of cisplatin and paclitaxel for 3 h and then cultured in the absence of any drug for an additional 24 h. Western blot analysis revealed that both cisplatin<sup>b</sup> cell lines had detectable levels of Bcl-2 (Fig. 4). Bcl-2 protein could not be detected in the parental cell lines (data not shown). Treatment with cisplatin did not alter the level of protein expression. In contrast, treatment with paclitaxel or a combination of cisplatin and paclitaxel readily induced a loss of Bcl-2 expression.

The tumor suppressor protein p53 is important in apoptosis and in sensitivity to cisplatin (6–9), therefore we examined the effects of paclitaxel, cisplatin, and combination therapy on p53 protein expression by Western blot analysis. In agreement with the findings of Siddik et al. (42) cisplatin up-regulated expression of p53 in A2780CP cells (Fig. 4). Exposure to paclitaxel did not alter the basal level of p53 seen in these cells but partially modulated that induced by cisplatin when both drugs were given in combination. p53 was neither detected in C-13 cells nor induced by cisplatin, paclitaxel, or both drugs in combination.

The results of the cell cycle analysis revealed that when cells were treated with a combination of cisplatin and paclitaxel, the majority exhibited a cell cycle profile analogous to that of cells treated with cisplatin alone. To eliminate the possibility that cisplatin in combina-

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**Table 2** Comparison of the percentage of cells with less than 2N DNA content

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>CDDP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ptx</th>
<th>CDDP/Ptx</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>2.3 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.6 ± 4</td>
<td>15.4 ± 7</td>
<td>8.5 ± 3</td>
</tr>
<tr>
<td>A2780CP</td>
<td>2.1 ± 0.6</td>
<td>5.0 ± 2.3</td>
<td>20 ± 8</td>
<td>8.7 ± 5</td>
</tr>
<tr>
<td>2008</td>
<td>2.1 ± 0.3</td>
<td>4.0 ± 1</td>
<td>17.2 ± 8</td>
<td>7.6 ± 2</td>
</tr>
<tr>
<td>C-13</td>
<td>2.6 ± 1</td>
<td>2.9 ± 0.6</td>
<td>17.1 ± 11</td>
<td>12.3 ± 8</td>
</tr>
</tbody>
</table>

<sup>a</sup> CDDP, cisplatin; Ptx, paclitaxel.

<sup>b</sup> Percentage of cells with <2N DNA content determined from two experiments (mean ± SD).

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**Table 3** Paclitaxel-induced changes in cell cycle, apoptosis, and nuclear morphology are blocked by cisplatin in human ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell cycle</th>
<th>Apoptosis</th>
<th>Nuclear morphology&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>C-13</td>
<td>P</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>A2780</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>A2780CP</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>C</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
<td>OVCA 420</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>OVCA 429</td>
<td>P</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
<td>OC-194</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>OC-494</td>
<td>C</td>
<td>ND</td>
<td>C</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nuclear morphology includes abnormal mitoses and multinucleinucleation.

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**Table 3** Paclitaxel-induced changes in cell cycle, apoptosis, and nuclear morphology

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**Table 3** Paclitaxel-induced changes in cell cycle, apoptosis, and nuclear morphology are blocked by cisplatin in human ovarian cancer cell lines

**Fig. 3.** Cisplatin blocks paclitaxel-induced DNA fragmentation in the cisplatin<sup>b</sup> cell line C-13. Ovarian cancer cells were exposed to cisplatin (CDDP), paclitaxel (Ptx), or a combination of cisplatin and paclitaxel (CDDP/Ptx) for 3 h. DNA was isolated from cells after 48 h in drug-free medium and separated in a 2% agarose gel. Fragmented DNA was visualized by staining with ethidium bromide. Representative gels are shown.

**Fig. 4.** Effects of cisplatin and paclitaxel on mediators of apoptosis, p53 or Bcl-2.
tion with paclitaxel was preventing the uptake and function of paclitaxel, we assessed whether cisplatin interfered with paclitaxel-induced stabilization of microtubules. Fig. 5 shows that paclitaxel-induced microtubule stabilization is partially blocked in the presence of cisplatin. Control cells for both 2008 and C-13 show diffuse microtubules throughout the cell. This pattern is not affected after treatment with cisplatin. However, treatment with paclitaxel results in the formation of stable aster-like microtubule structures that are detected after 4 h and continue to accumulate over time. In 2008 cells, paclitaxel induced approximately 57.1 ± 4.9% microtubule asters, and in C-13 cells, paclitaxel induced approximately 94.4 ± 2.5% microtubule asters. The combination of cisplatin and paclitaxel resulted in a diminished number of aster-like structures in C-13 cells (38.3 ± 6.6%) and an almost complete ablation of the structures from 2008 cells (13.9 ± 1.4%). Whereas microtubule aster formation could be induced in both cell lines with nanomolar paclitaxel concentrations, it was only blocked with 30 μM cisplatin. Lower concentrations of cisplatin had little effect (data not shown).

In evaluating the microtubule status of cells exposed to a combination of cisplatin and paclitaxel, we noted an absence of both abnormal mitoses and multimininucleation, which are often seen in cells treated with paclitaxel alone (Fig. 6A). When cells were treated with a combination of cisplatin and paclitaxel, the majority (>70%) of nuclei retained an interphase-like morphology, resembling that of both control or cisplatin-treated cells. This dominance was observed with both cisplatin\(^\text{S}\) and cisplatin\(^\text{R}\) cells (Fig. 6A), showing again that cisplatin exerts a dominant effect over paclitaxel.

We used these nuclear changes to assess the temporal requirements for cisplatin inhibition of paclitaxel-induced multimininucleation. Sequential administration of either cisplatin for 3 h followed by paclitaxel or paclitaxel for 3 h followed by cisplatin resulted in an inhibition of paclitaxel-induced multimininucleation comparable to that seen after exposure to both drugs simultaneously. In all sequences tested of combination cisplatin and paclitaxel, cisplatin blocked paclitaxel-induced multimininucleation by 85–90% (Fig. 6B).

**DISCUSSION**

Combination cisplatin and paclitaxel therapy is currently the first-line therapy for women with ovarian cancer after surgical cytoreduction. This treatment regimen was found to be more efficacious than previous combination therapies, specifically cisplatin and cyclophosphamide (3). However, approximately 30% of women on this regimen will suffer from progression of ovarian cancer. Patients with progression are treated with second-line chemotherapy, which is rarely curative. When patients progress on cisplatin and paclitaxel chemotherapy, it is not known whether they have developed resistance to one or both of the drugs, although they are deemed cisplatin/paclitaxel resistant. In this study, we have used paired cisplatin\(^\text{S}\) and cisplatin\(^\text{R}\) human ovarian cancer cell lines and found that cisplatin\(^\text{R}\) cells retain sensitivity to clinically relevant doses of paclitaxel. However, when exposed to a sequential or simultaneous combination of cisplatin and paclitaxel, ovarian cancer cells responded in a fashion analogous to that seen in cells treated with cisplatin alone. This suggests that cisplatin has mechanistic dominance over paclitaxel at a cellular level.

Several studies have previously shown that cisplatin\(^\text{R}\) cells retain sensitivity to single-agent paclitaxel (4, 5, 10, 43–45). We show here that human ovarian cancer cells that are resistant to cisplatin are highly sensitive to paclitaxel, perhaps even more so than their parental counterparts. Our results support those of Motzer et al. (43) and Woo et al. (44), who demonstrated that platinum-resistant gynecological cancers show marked cytotoxicity to paclitaxel. This increased paclitaxel sensitivity in cisplatin\(^\text{R}\) tumors is not limited to gynecological neoplasms but is also reflected in various other cancers in which paclitaxel has been shown to be effective (45, 46). Our results also provide a mechanistic basis for the clinical observations seen in patients found to have cisplatin\(^\text{R}\) cancers who continue to respond to single-agent paclitaxel (4, 5).

Several mechanisms may explain the observed dominance of cisplatin over paclitaxel. One mechanism may simply involve a cisplatin-induced cell cycle blockade analogous to that recently described with 5-fluorouracil (47). By arresting cells in the G1-S phase of the cell cycle, 5-fluorouracil inhibited both paclitaxel-induced mitotic arrest and apoptotic death. Similarly, we found that by primarily arresting cells in late S phase to early G2, cisplatin can block paclitaxel-induced activities that occur at several other phases of the cell cycle, particularly mitotic arrest (M phase) and apoptosis (G1).

An alternative mechanism for the observed dominance of cisplatin over paclitaxel may reflect initial mechanisms of resistance to cisplatin. One proposed mechanism of resistance to cisplatin is an increased cellular tolerance of platinum DNA damage that provides a broad
resistance to other drugs, including paclitaxel (48). Our results would partially support this hypothesis, with the addition that resistance to other drugs requires the presence of cisplatin or at least recent cisplatin exposure. Consequently, tolerance of platinum DNA damage may result in failure to activate the apoptotic pathway. This was shown to be true of cisplatinR HeLa cells that had a reduced expression of interleukin 1β-converting enzyme-related proteases (49).

The cisplatin R C-13 ovarian cancer cells readily undergo apoptosis after exposure to paclitaxel (Fig. 3), which is suggestive of at least one intact apoptotic pathway. Because apoptosis is reduced or blocked when cells are exposed simultaneously to both cisplatin and paclitaxel, we cannot rule out the possibility that cisplatin modulates one or several of the specific proteases that are important effectors of apoptosis. Both individually and together, cisplatin and paclitaxel induced apoptosis in the parental 2008 cells. However, as denoted by tubulin staining and flow cytometry, in the presence of paclitaxel, cisplatin completely blocked the appearance of paclitaxel-induced aster-like microtubule structures, prolonged the transit of cells through S phase, and increased the percentage of cells in G2 in the 2008 cells. This implies that cisplatin also exerts dominance in this particular cell line and other cisplatinR cells (data not shown). The difference between the parental and cisplatinR cell lines is the ability to induce apoptosis in response to cisplatin.

Simultaneous exposure to both cisplatin and paclitaxel did not appear to modulate early effectors of apoptosis in any of the cell lines examined. In one cell line, A2780CP, paclitaxel did partially block p53 up-regulation by cisplatin. However, the significance of this observation is unclear because apoptosis was not blocked by the concomitant exposure to both drugs in A2780CP cells. Conversely, although cisplatin can modulate the expression of Bcl-2 family members in melanoma cells (50), cisplatin had no effect on Bcl-2 expression in any of the ovarian cancer cell lines and did not block Bcl-2 degradation by paclitaxel. This would imply that these pathways are distinct for each drug and that cisplatin inhibition of paclitaxel-induced apoptosis occurs downstream of Bcl-2 degradation.

An additional mechanism by which cisplatin may exert dominance over paclitaxel centers on the ability of each drug to modulate transcription. Cisplatin intercalates into DNA, forming adducts, and has been shown to both activate and block transcription (28, 51–53). Paclitaxel also transcriptionally activates a variety of genes, some of which are likely to directly influence cytotoxicity. We have recently shown that there is a direct correlation between cytotoxicity and paclitaxel-induced transcriptional activation, leading us to postulate that at least part of the mechanism of action for paclitaxel involves direct gene activation (29, 30). Moos and Fitzpatrick (33) have further shown that paclitaxel-mediated gene induction of modulators of apoptosis is independent of microtubule stabilization and is specifically attributable to paclitaxel. One possible explanation for cisplatin blockage of paclitaxel-induced cytotoxicity may center around the ability of cisplatin to induce specific genes that counteract those induced by paclitaxel, or cisplatin may block the transcription of specific paclitaxel-related cytotoxicity genes.
We observed that close sequential administration of cisplatin and paclitaxel was insufficient to overcome the mechanistic dominance of cisplatin. Even when paclitaxel was administered alone and before cisplatin, cisplatin blocked multiple effects of paclitaxel. This raises the question of whether a 3-h in vivo infusion of paclitaxel immediately followed by cisplatin or carboplatin will ultimately prove to be less efficacious in patients than a 24-h infusion of paclitaxel. Using an in vivo mouse tumor model, Milross et al. (37) found that only the sequential administration of cisplatin and paclitaxel separated by 24 or 48 h resulted in an additive cytotoxic effect. The sequence combination of paclitaxel administration before cisplatin administration yielded significantly less morbidity and mortality than the administration of cisplatin before or simultaneously with paclitaxel. Similarly, in vitro synergistic interactions could only be reproducibly achieved by sequentially exposing cells to paclitaxel for 24 h before a 4-h exposure to cisplatin (54), and there was a significant and prolonged antagonism between cisplatin and paclitaxel when cisplatin administration preceded that of paclitaxel (38). Neither cisplatin nor paclitaxel had an effect on the uptake of the other drug, negating this as a possible explanation for the antagonism of sequential administration (10, 38). Based on our findings, we would propose that cisplatin exerts a prolonged mechanistic dominance over paclitaxel, although both cisplatin and paclitaxel induce apoptosis through the activation of drug-specific pathways. In cells with low sensitivity to cisplatin, paclitaxel-induced apoptosis is readily blocked by the concomitant or close administration of cisplatin. These observations are not limited to the combination of paclitaxel and cisplatin; Kano et al. found that sequential (but not simultaneous) administration of paclitaxel and the active metabolite of irinotecan, SN-38, was more cytotoxic against a variety of human cancer cell lines (55). These studies all demonstrate that sequential administration separated by long periods of time may be the most beneficial treatment for patients.

In conclusion, we have demonstrated that cisplatin exerts mechanistic dominance over paclitaxel when human ovarian cancer cells are simultaneously exposed to cisplatin and paclitaxel in vitro. Whereas this does not influence the cisplatin-sensitive cells, it may be detrimental to cisplatin-resistant cells in that it blocks paclitaxel-induced apoptosis. Based on these findings, we propose that patients may significantly benefit from a trial of paclitaxel alone as a second-line regimen when first-line cisplatin/paclitaxel treatment has failed.

ACKNOWLEDGMENTS

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