5-Fluorouracil Interferes with Paclitaxel Cytotoxicity against Human Solid Tumor Cells¹

Korey R. Johnson, Liming Wang, Merrill C. Miller III, Mark C. Willingham, and Weimin Fan²

Department of Pathology and Laboratory Medicine [K. R. J., M. C. M., M. C. W., W. F.], Experimental Oncology [L. W.], and Medicine [W. F.], Medical University of South Carolina, Charleston, South Carolina 29425

ABSTRACT

Paclitaxel, a naturally occurring antimitotic agent, has shown efficacy in the treatment of certain solid tumors, particularly metastatic breast carcinoma and drug-refractory ovarian cancers. Recent studies have demonstrated that paclitaxel, in addition to its effects on microtubules and cell cycle arrest, possesses significant cell-killing activity in solid tumor cells by the induction of apoptosis. However, the mechanism by which paclitaxel leads to cell death and its relationship with paclitaxel-induced mitotic arrest is presently unclear. In this study, we attempted to determine whether pre-arresting tumor cells at other phases of the cell cycle could affect paclitaxel-induced apoptosis. We found that 5-fluorouracil (5-FU), another antineoplastic agent that usually arrests tumor cells at the G1-S phase of the cell cycle, could significantly repress the cell-killing activity of paclitaxel in solid tumor cells, even when it was added simultaneously with paclitaxel. Further studies indicated that 5-FU actually inhibits the cytotoxic effects of paclitaxel on both mitotic arrest and apoptotic cell death, suggesting that 5-FU might interfere with paclitaxel cytotoxicity at an early stage, probably by preventing tumor cells from entering G2-M phase. Because recent clinical trials have used a combination of paclitaxel and 5-FU in the treatment of metastatic breast cancers, our results also suggest that the combination of these two drugs might not be as valuable in clinical chemotherapy.

INTRODUCTION

Paclitaxel (Taxol®), a novel antineoplastic agent, was originally isolated from the bark of the Pacific yew, Taxus brevifolia (1). This naturally occurring antimitotic drug has shown great promise in the treatment of certain human solid tumors, particularly in metastatic breast cancer and drug-refractory ovarian cancer (2-4), although the exact mechanism of the cytotoxicity of paclitaxel against tumor cells is not entirely clear. Previous studies demonstrated that paclitaxel is a unique antimitotobule agent, and its antitumor effects result mainly from interference with the normal function of microtubules and the blockage of cell cycle progression in later G2-M phases via prevention of mitotic spindle formation (5, 6). However, recent studies have demonstrated that paclitaxel, at clinically relevant concentrations, was able to induce internucleosomal DNA fragmentation and the typical morphological features of apoptosis in a number of solid tumor cells (7-10). These results clearly indicate that taxol, in addition to its classical activity against microtubules and cell cycle arrest, also possesses cell-killing activity by induction of apoptosis.

Although it is well recognized that paclitaxel can cause both mitotic arrest and apoptotic cell death, it remains unclear whether paclitaxel-induced cell death is a secondary event resulting from mitotic arrest or represents a novel mechanism of action for paclitaxel against tumor cells. Morphologically, a sustained block of mitosis seems to be required for taxol-induced apoptosis in most solid tumor cells because, by using time-lapse video microscopy, most apoptotic events were observed to occur in cells showing prior mitotic arrest (10). However, this observation does not absolutely prove that paclitaxel-induced apoptosis is a secondary event resulting from mitotic arrest. In fact, several lines of evidence from our recent experiments and other laboratories have suggested that paclitaxel-induced apoptosis might take place via an independent pathway (10-12).

Received 3/28/97; revised 5/30/97; accepted 6/19/97.

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¹This work was supported by NIH Grants CA71851 and CA58846 (to W. F.) and The Health Science Foundation of the Medical University of South Carolina.
²To whom requests for reprints should be addressed, at Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425. Phone: (803) 972-5108; Fax: (803) 792-4157.

The abbreviations used are: 5-FU, 5-fluorouracil; MTT, thiazolyl blue.
tigations indicate that 5-FU can actually repress the cytotoxic effects of paclitaxel on both mitotic arrest and apoptosis. These findings suggest that 5-FU might interfere with the cytotoxicity of paclitaxel at an early stage. On the other hand, because recent clinical trials have used a combination of paclitaxel and 5-FU in the treatment of metastatic breast cancers (15-17), our results have also raised a question as to the value of this combination in clinical chemotherapy.

MATERIALS AND METHODS

Drugs and Cell Culture. Paclitaxel was purchased from Calbiochem (La Jolla, CA) and dissolved in 100% DMSO to make a stock solution of 1.0 mM, which was then diluted in culture medium to obtain the desired concentration. 5-FU (Sigma Chemical Co., St. Louis, MO) was also dissolved in DMSO. The human breast tumor BCap37 (10) and KB human epidermoid carcinoma cell lines (American Type Culture Collection, Rockville, MD) were propagated in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT). As described previously (10, 12), paclitaxel and/or 5-FU were usually added when the cells reached approximately 60–70% confluence.

Determination of Internucleosomal DNA Cleavage. Internucleosomal DNA fragmentation was assayed by a modification of methods described previously (8). After treatment with 100 nM paclitaxel or 10 μM 5-FU, or their combination, cells were harvested and suspended in lysis solution containing 50 mM Tris-HCl, 10 mM EDTA, 0.5% N-lauroylsarcosine, and 0.5 mg/ml proteinase K for 1 h at 50°C. Crude DNA samples were extracted twice with equal volumes of phenol buffered with 0.1 M Tris-HCl (pH 7.4), followed by an equal volume extraction with chloroform:isoamyl alcohol (24:1). The remaining steps for DNA fragmentation analysis were performed exactly as described (8). DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2% ethidium bromide and visualized under UV illumination.

Flow Cytometry Analysis. Cell sample preparation and propidium iodide staining were performed according to the method described by Nicoletti et al. (18). Cells were treated with 10 μM 5-FU or 100 nM paclitaxel, or their combination, for 24 and 48 h. Cells were then harvested by trypsinization and washed twice with PBS. Cells were fixed in 1% formaldehyde in PBS and then dehydrated in 70% ethanol diluted in PBS. Approximately 1 h before flow cytometry analysis, cells were incubated in PBS containing 100 μg/ml RNase A and 40 μg/ml propidium iodide at 37°C. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp.), with an argon laser set to excite at 488 nm.

Cytospin Preparation. Cells treated with 10 μM 5-FU or 100 nM paclitaxel, and their combination, were harvested by trypsinization at the times indicated and washed twice with Opti-MEM reduced serum medium. Cell numbers were determined with a Coulter counter, and approximately 50,000–100,000 cells from each group were used for cytospin preparations. Slides were air dried and fixed in acetone prior to Giemsa staining and examined using bright-field microscopy (8).

Morphological Observations by Phase Contrast Microscopy. Cells in 35-mm dishes were placed on the stage of a warmed inverted microscope and examined using phase contrast microscopy as described previously (10). This microscope was equipped with a heater/recirculation device that maintains stage temperature at 37 ± 0.5°C. In addition, this system is supplied with a constant through-flow of 95% air/5% CO₂. Time lapse video recordings were prepared at a 1:720 time lapse ratio. Individual cells in each field were chosen to examine the frequency of mitotic arrest, apoptotic blebbing, and micronucleation events. Duplicated dishes treated with 5-FU, paclitaxel, or both or untreated were examined and photographed using a regular phase contrast microscope.

MTT Assay. BCap37 and KB cells were harvested with trypsin and resuspended to a final concentration of 2 × 10⁴ cells/ml in fresh medium with 10% FCS. Aliquots of 100 μl from cell suspension were distributed evenly into 96-well tissue culture plates with lids (Falcon, Oxnard, CA). Designated columns were treated with 10 μM 5-FU or 100 nM paclitaxel, or their combination. One column from each plate contained medium alone, and another contained cells without drug. Cell viability was quantified by the method of Carmichael et al. (19). After 24, 48, and 72 h, 100 μl of a 1 mg/ml MTT solution were added to each well, and the plate was incubated for 4 h, allowing viable cells to reduce the yellow MTT into dark-blue formazan crystals, which were dissolved in 100 μl of DMSO. The absorbance in individual wells was determined at 560 nm by a microplate reader (Molecular Devices, CA).

Western Blots. Cells treated with 1 μM paclitaxel and/or 10 μM 5-FU were harvested by trypsinization at the times indicated. Extraction of cellular proteins and Western blots of bcl-2 were performed as described previously (13). The reactive bcl-2 band was identified using a chemiluminescent substrate to horseradish peroxidase (Amersham Corp.).

RESULTS

5-FU Inhibits Paclitaxel-induced Apoptotic Cell Death. An important hallmark of apoptotic cell death is the fragmentation of genomic DNA into integer multiples of 180-bp units, producing a characteristic ladder on agarose gel electrophoresis (20). To investigate the possible influence of 5-FU on paclitaxel-induced apoptotic cell death, we first examined whether paclitaxel-induced DNA fragmentation would be affected by cotreatment with 5-FU. Fig. 1 shows the DNA fragmentation of BCap37 and KB cells exposed to 100 nM paclitaxel or 10 μM, or their combination, for 48 or 72 h. As reported previously (10, 12), the characteristic DNA fragmentation ladders were observed in both BCap37 and KB cells following treatment with 100 nM paclitaxel alone for 72 h (Fig. 1, Lane 4). When those cells were treated with 10 μM 5-FU alone, essentially no clear DNA fragmentation was observed (Fig. 2, Lane 6), although morphological observation by video microscopy indicated that a small number of cells may actually undergo apoptosis (see below). However, when those cells were treated with both 5-FU and paclitaxel simultaneously, taxol-induced DNA fragmentation was inhibited dramatically (Fig. 2, Lanes 7 and 8), suggesting that 5-FU could repress paclitaxel-induced apoptotic cell death in both cell lines. Furthermore, this 5-FU-mediated inhi-
The Antagonistic Effect of 5-FU on Paclitaxel Cytotoxicity Is Schedule Dependent. In clinical trials, 5-FU has been widely used in combination therapy with paclitaxel to treat metastatic breast carcinomas (15-17) and other solid tumors (22, 23). Thus, this inhibitory action of 5-FU on paclitaxel-induced mitotic arrest and apoptosis has raised a clinically relevant question as to whether the combination of 5-FU with paclitaxel might actually produce an antagonistic effect. To evaluate further the potential antagonistic effects between paclitaxel and 5-FU in human solid tumor cells in vitro, we have used the MTT assays to assess the cytotoxic interaction of these two drugs administered at various schedules in both BCap37 and KB cells. This assay indicated that 5-FU could inhibit cell growth, although it had little effect on inducing apoptosis (Figs. 1 and 3). In addition, results shown in Fig. 4 also indicated that pretreatment or simultaneous exposure of tumor cells with 5-FU could significantly antagonize paclitaxel cytotoxic effects against solid tumor cells in vitro. However, when these tumor cells were pretreated with paclitaxel for 12 h, 5-FU-mediated inhibition of paclitaxel cytotoxicity was clearly attenuated, although an effect as great as paclitaxel alone was not achieved. Furthermore, if tumor cells were exposed to paclitaxel for 24 h prior to 5-FU, the antagonistic effects of 5-FU on paclitaxel cytotoxicity were no longer observed (Fig. 4). We have also performed the clonogenic survival assay; pretreatment with 5-FU could clearly repress the cell-killing activity of paclitaxel in both BCap37 and KB cells (data not shown).

5-FU Prevents Paclitaxel-induced bcl-2 Hyperphosphorylation. Recent studies have demonstrated that paclitaxel could induce bcl-2 hyperphosphorylation (13, 24). This modification was believed to cause attenuation of bcl-2 antiapoptotic activity (25). To determine whether this paclitaxel-induced bcl-2 hyperphosphorylation is also affected by 5-FU, we have used Western blotting to examine the expression of bcl-2 in both BCap37 and KB cells treated with the combination of paclitaxel and 5-FU. In Fig. 5, it can be seen that paclitaxel induced a slower mobility form of bcl-2 protein, which was demonstrated to be a hyperphosphorylated form of bcl-2 (24). However, this bcl-2 hyperphosphorylation caused by paclitaxel was clearly blocked when the cells were cotreated with 10 µM 5-FU.

DISCUSSION

The initial purpose of this study was to investigate the possible relationship between paclitaxel-induced apoptotic cell death and mitotic arrest. If we assume that paclitaxel-induced apoptotic cell death is a secondary event resulting from mitotic arrest, then cell death should be blocked once tumor cells are prevented from entering cell cycle arrest at the G2-M phase. A feasible approach to test this hypothesis was to synchronize or pre-arrest tumor cells at other phases of the cell cycle by using...
other drugs or agents and then examine whether paclitaxel still causes cell death. Because of the ability of 5-FU to arrest cells in G1-S phase (14), 5-FU was selected for this study. As shown in our results, paclitaxel-induced apoptosis was indeed inhibited by pretreatment of tumor cells with 5-FU. Furthermore, we found that this antagonistic effect was still observed, although 5-FU was added simultaneously with paclitaxel (Fig. 1). In addition, through cytospin and flow cytometric analyses, it was demonstrated that 5-FU could predominantly arrest the tumor cells at G1-S phase and nearly completely prevent the cells from entering G2-M phase, unless paclitaxel was added prior to 5-FU (Table 1 and Fig. 3). Therefore, the mechanism by which 5-FU represses paclitaxel-induced apoptosis was mainly through the prevention of tumor cells from entering G2-M phase. Based on these findings, it may be presumed that paclitaxel-induced apoptotic cell death is cell cycle dependent.

Combination therapy with multiple drugs is a common practice in the treatment of cancer. When anticancer agents with similar or different modes of action are combined, the outcome can be synergistic, additive, or antagonistic. Synergism implies that two drugs may produce greater therapeutic efficacy than an expected additive effect, whereas antagonism implies that the actual therapeutic activity produced by two drugs may be smaller than their additive effect. The promising clinical activity of paclitaxel has promoted considerable interest in combining this drug with other antitumor agents. In clinical trials, 5-FU is one of these agents widely used with paclitaxel in the treatment of metastatic breast carcinomas (15–17) and other solid tumors, including tumors of the head and neck (22) and gastrointestinal tract (23). Thus, the finding of the antagonistic effect of 5-FU on paclitaxel cytotoxicity has also raised a clinically relevant question as to whether the combination of 5-FU with paclitaxel is really beneficial in clinical chemotherapy. By using MTT assays, we have further evaluated the in vitro cytotoxic interaction and potential antagonistic effects of these two drugs administered at different schedules in both BCap37 and KB cells. Data generated by this assay indicated that, unless paclitaxel was added 24 h prior to 5-FU, the cytotoxic effect of this combination of 5-FU and paclitaxel is not as effective as paclitaxel alone (Fig. 4). These results suggest that the combination of paclitaxel and 5-FU actually produces subadditive effects against those solid tumor cells in vitro. In a recent report, the possible antagonistic effect between 5-FU and paclitaxel has also been addressed by Kano et al. (26). In their study, the subadditive cytotoxic effect was also observed when MCF-7 breast cancer cells were pretreated with 5-FU or 5-FU was administered simultaneously with paclitaxel. Thus, pretreatment with 5-FU or coadministration of 5-FU and paclitaxel essentially produces no synergistic effect against tumor cells in vitro. However, it remains uncertain whether an additive effect is generated when tumor cells are pretreated with paclitaxel prior to 5-FU. Kano et al. (26) reported that an additive effect occurred with this

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Table 1  Effect of 5-FU and paclitaxel on mitotic arrest

<table>
<thead>
<tr>
<th>Drug exposure</th>
<th>Percentage of cells at G2-M phasea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCap37</td>
</tr>
<tr>
<td>Control</td>
<td>24 h</td>
</tr>
<tr>
<td>5-FU</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>5-FU + paclitaxel</td>
<td>5 ± 3</td>
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</tbody>
</table>

a Based on three separate experiments and presented as mean ± SE.

b Cytospin slides were stained with Geimsa. Three hundred cells were counted from each slide, and only those cells with typical morphological features of condensed chromosomes were counted as mitotically arrested cells.
Fig. 3 Flow cytometric analysis of cell cycle distribution. BCap37 cells, treated with 100 nm paclitaxel or 10 μM 5-FU, both drugs simultaneously, 6-h preincubation with 5-FU followed by paclitaxel, or 6-h preincubation with paclitaxel followed by 5-FU, for the indicated times, were harvested and stained for DNA with propidium iodide and analyzed by flow cytometry as described in “Materials and Methods.” The distribution of cells in G0-G1, S, and G2-M phases of the cell cycle and apoptotic cells (Ap) are indicated above each corresponding peak.
### BCap37 Cells

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>% of Viable Cells</th>
</tr>
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<tbody>
<tr>
<td>24</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

- Control
- 5-FU
- Taxol
- 5-FU+TX
- Pre 5-FU6+Taxol
- Pre TX6+5-FU
- Pre TX24-5-FU

### KB Cells

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>% of Viable Cells</th>
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<tr>
<td>24</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
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<tr>
<td>72</td>
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</tbody>
</table>

- Control
- 5-FU
- Taxol
- 5-FU+Tx
- Pre 5-FU6+Tx
- Pre TX6+5-FU
- PreTX24-5-FU

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Sequential combination, but such a synergistic effect was not confirmed in our experiments. As we can see from Figs. 3 and 4, 5-FU was still able to interfere with the activity of paclitaxel in both mitotic arrest and apoptotic cell death, although 5-FU was added 6 or 12 h later. However, when tumor cells were pretreated with paclitaxel for 24 h, the cytotoxic effect of paclitaxel was indeed no longer affected by 5-FU, but the expected additive effect was still not observed. Basically, the combined cytotoxic effect of these two drugs in this schedule was not greater than when treated with paclitaxel alone (Fig. 4). Therefore, more studies, particularly in vivo experiments with animal models, are necessary to address this clinically important issue.

In addition, our recent studies have demonstrated that paclitaxel could induce bcl-2 hyperphosphorylation in both BCap37 and KB cells (13). Due to this posttranslational modification, bcl-2 has been postulated to lose its ability to block apoptosis (25). More recently, the phosphorylation of bcl-2 has been also suggested to reflect the damage of microtubules, because this modification is commonly induced by antimicrotubule agents and always occurs in the G2-M phase of the cell cycle (27). Moreover, Schandl et al. (28) have reported recently that this phosphorylation event occurs normally as part of mi-

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**Fig. 4** Cytotoxic effects of 5-FU and paclitaxel against BCap37 and KB cells. Approximately $2 \times 10^6$ BCap37 or KB cells were cultured in 96-well microculture plates. After 24 h, cells were exposed to 100 nM paclitaxel or 10 μM 5-FU, or both drugs in different sequences. After the indicated time, MTT assays were performed as described in “Materials and Methods.” Bars, SD.

**Fig. 5** Western analysis of bcl-2 protein content. Cellular proteins were extracted from BCap37 and KB cells treated with 1 μM paclitaxel or 10 μM 5-FU, or their combination. Equal amounts (100 μg/lane) of cellular protein were fractionated on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was then immunoblotted with anti-bcl-2 antibody as described in “Materials and Methods.”
tosis, suggesting that it may have no relevance to the antiapoptotic effects of bcl-2. Therefore, it was necessary to examine the possible influence of 5-FU on paclitaxel-induced bcl-2 phosphorylation. Our results show that the hyperphosphorylation of bcl-2 was, indeed, repressed when those tumor cells were treated simultaneously with 5-FU (Fig. 5). This finding may provide another piece of evidence that 5-FU antagonizes the cytotoxicity of paclitaxel by preventing tumor cells from entering the G2-M phase of cell cycle.

In summary, this study has investigated the possible influence of 5-FU on the cytotoxic effect of paclitaxel against human solid tumor cells in vitro. Our results demonstrated that 5-FU could repress paclitaxel cytotoxicity in both apoptotic cell death and mitotic arrest. Moreover, 5-FU was found to arrest tumor cells at the G1-S phase and inhibit paclitaxel-induced bcl-2 hyperphosphorylation. These findings suggest that 5-FU antagonizes the cytotoxic effects of paclitaxel against tumor cells by preventing the cells from entering the G2-M phase of the cell cycle. On the other hand, because the combination of 5-FU and paclitaxel is commonly used in the treatment of certain human solid tumors, our results also suggest that the combination therapy with these two drugs might need to be further evaluated.

ACKNOWLEDGMENTS

Special thanks to Kristy Young and Cynthia Schandl for their critical review of the manuscript.

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