In Vitro Evaluation of Schedule-dependent Interactions between Docetaxel and Doxorubicin against Human Breast and Ovarian Cancer Cells

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ABSTRACT

Docetaxel, a novel member of the taxoid family, has shown greater potency than paclitaxel in the treatment of advanced breast cancer and certain other solid tumors. The promising clinical activity of docetaxel has also promoted considerable interest in combining this drug with other antitumor agents. In this study, we assessed the cytotoxic interaction between docetaxel and doxorubicin administered at various schedules to human breast and ovarian cancer cells. Through a series of in vitro assays including DNA fragmentation analyses, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, and flow cytometric analyses, we found that the antagonistic interaction occurred when tumor cells were exposed to the two drugs simultaneously or exposed to doxorubicin before docetaxel. However, no antagonism was observed when docetaxel was added before doxorubicin. Further analyses demonstrated that doxorubicin could interfere with the cytotoxic effect of docetaxel on both mitotic arrest and apoptotic cell death. In addition, biochemical examinations revealed that docetaxel could induce phosphorylation of both Bcl-2 and Raf-1, but these changes were inhibited when tumor cells were pretreated or simultaneously treated with doxorubicin. These results indicate that the interaction between docetaxel and doxorubicin is highly schedule dependent. Exposure of tumor cells to doxorubicin before docetaxel could result in pronounced antagonism. The optimal schedule for this combination might be sequential exposure to docetaxel followed by doxorubicin.

INTRODUCTION

Docetaxel (Taxotere, N-debenzol-N-tert-butoxycarbonyl-10-deacetyltaxel; Rhone-Poulenc, Inc., Antony, France), a novel member of the taxoid family, is prepared by semisynthesis from 10-deacetyl baccatin III, an inactive taxoid precursor extracted from the needles of the European yew Taxus baccata (1). The cytotoxic effect of docetaxel is primarily due to its ability to promote tubulin assembly and inhibit microtubule depolymerization. Similar to paclitaxel, the first member of the taxoid family used in clinical studies, docetaxel also acts as a mitotic spindle poison and induces a mitotic block in proliferative cells (2, 3). In vitro studies have shown that docetaxel has a broad spectrum of activity against a variety of tumor types, including breast cancer, ovarian cancer, non-small cell lung cancer, head and neck cancer, colorectal cancer, and melanoma (4, 5). In vivo experiments in animal models and clinical trials have also shown that docetaxel is more potent than paclitaxel in the treatment of advanced breast cancer and other solid tumors (6–8).

Combination therapy with multiple drugs is a common practice in the treatment of cancer. The promising clinical activity of docetaxel has promoted considerable interest in combining this drug with other antitumor agents, such as etoposide, cyclophosphamide, 5-fluorouracil, and doxorubicin (4, 9). A number of these docetaxel-containing combinations are currently undergoing clinical evaluations, and preliminary results appear to be encouraging (9). Doxorubicin, a derivative of anthracyclines, is one of the most active agents with a broad spectrum of activity against solid tumors and hematological malignancies (10). The combination of docetaxel and doxorubicin has been proven effective in first-line treatment of metastatic breast cancer, with high response rates and acceptable toxicity (9, 11). In the present study, we conducted in vitro evaluations of the cytotoxic effects of docetaxel and doxorubicin against human breast and ovarian tumor cells in vitro. Our results demonstrated that pretreatment of tumor cell with doxorubicin or simultaneous exposure of tumor cell to doxorubicin could significantly repress the cell-killing activity as well as the general cytotoxic effect of docetaxel against tumor cells in vitro. These findings indicate that the interaction between docetaxel and doxorubicin is highly schedule dependent. The optimal schedule of this combination might be sequential exposure to docetaxel followed by doxorubicin.

MATERIALS AND METHODS

Drugs and Cell Culture. Docetaxel was obtained from Rhone-Poulenc Inc. and dissolved in DMSO to make a 1 mM stock solution. Doxorubicin hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO to make a 1 mM stock solution. These drugs were then diluted in
crystals were dissolved in 100 μl of DMSO. The absorbance in each well was determined using a flat-bottomed microplate reader (Molecular Devices). The immunoreactive bands were visualized using a chemiluminescent substrate to horseradish peroxidase. After electrophoresis, samples were transferred to a nitrocellulose membrane according to the Bio-Rad protocol. Primary antibodies against p53, bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), c-raf, and p21 (Transduction Laboratories) were used at a 1:1000 dilution (37°C). The secondary antibody, goat antimouse IgG conjugated to horseradish peroxidase, was used at a concentration of 0.1 μg/ml in 3% BSA-PBS-T (PBS containing 0.5% Tween 20). The immunoreactive bands were visualized using a chemiluminescent substrate to horseradish peroxidase (Amersham) and exposure to Kodak X-OMAT film.

RESULTS

Characterization of Docetaxel-induced Apoptosis in Solid Tumor Cells. An important feature 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. To determine whether docetaxel can cause tumor cell apoptosis, DNA fragmentation was analyzed after tumor cells were exposed to different concentrations of docetaxel over various time courses. Fig. 1A shows the DNA fragmentation after BCap37 cells were treated with different concentrations of docetaxel (1–100 nM) for 48 h. The characteristic DNA fragmentation ladder was observed in the cells treated with ≥5 nm docetaxel. This indicates that docetaxel is about 10 times stronger than paclitaxel in the induction of apoptotic cell death (12). In addition, we also examined the cells treated with 5 nm docetaxel over different time courses, and we determined that, at this concentration, DNA fragmentation was detectable at 24 h of drug exposure, but more fragmented DNA was observed by 48 h of drug treatment (see Fig. 1B). Next, flow cytometric assays were performed to analyze the kinetic changes of cell cycle distribution and apoptotic cell death in docetaxel-treated BCap37 cells. The results depicted in Fig. 2 indicate that docetaxel first causes cell cycle arrest at the G2–M phase. This was observed 3 h after the addition of docetaxel and continued to increase through 24 h of incubation. Subsequently, a distinct peak representing an apoptotic cell population (Fig. 2, Ap) was detected. This apoptotic peak continuously increased and became predominant after 48 h of drug treatment. These results indicate that docetaxel, similar to paclitaxel, can cause both mitotic arrest and apoptotic cell death, and apoptotic events seem to occur after mitotic arrest.
Schedule-dependent Antagonistic Effect of Doxorubicin on the Overall Cytotoxicity of Docetaxel. To investigate the possible influence of doxorubicin on the antitumor activity of docetaxel, we first used MTT assays to assess the cytotoxic interaction of these two drugs in both BCap37 and OV2008 cells. As described in “Materials and Methods,” the dose-effect curves of docetaxel and doxorubicin were determined by exposing tumor cells to various concentrations of these drugs individually for 48 h. The results indicated that IC_{50} concentrations for docetaxel and doxorubicin were around 5 and 100 nM, respectively (Fig. 3). Next, the sequential administration of these two drugs was examined by treatment of tumor cells with 5nM docetaxel, 100 nM doxorubicin, or their combinations at various schedules for 24 and 48 h. The experimental results, which are summarized in Fig. 4, show that pretreatment or simultaneous treatment of tumor cells with 100 nM doxorubicin produced less cytotoxic effects than treatment with docetaxel alone. However, an increased overall cytotoxicity was observed when these tumor cells were exposed to docetaxel for 12 h before the addition of doxorubicin (Fig. 4). These results suggested that pretreatment or simultaneous treatment with doxorubicin might antagonize the cytotoxic effect of docetaxel. Furthermore, we performed the median effect analysis with a fixed concentration ratio of 1:20 (docetaxel:doxorubicin). The fractional inhibition for each drug in combination was analyzed in relation to the fractional inhibition observed with each drug alone using CalcuSyn software with a mutually nonexclusive model. The results indicated that simultaneous treatment or sequential doxorubicin followed by docetaxel produced less cytotoxic effects than treatment with docetaxel alone. However, an increased overall cytotoxicity was observed when these tumor cells were exposed to docetaxel for 12 h before the addition of doxorubicin (Fig. 4). These results suggested that pretreatment or simultaneous treatment with doxorubicin might antagonize the cytotoxic effect of docetaxel. Furthermore, we performed the median effect analysis with a fixed concentration ratio of 1:20 (docetaxel:doxorubicin). The fractional inhibition for each drug in combination was analyzed in relation to the fractional inhibition observed with each drug alone using CalcuSyn software with a mutually nonexclusive model. The results indicated that simultaneous treatment or sequential doxorubicin followed by docetaxel produced significant antagonistic effects. However, when BCap37 cells were exposed to docetaxel for 12 h before the addition of doxorubicin, the antagonistic effect gradually disappeared. Instead, a marginal additive effect was observed with an increase in drug concentrations. These findings indicate that doxorubicin possesses schedule-dependent antagonism on the antitumor activity of docetaxel in vitro.

Doxorubicin Inhibits Docetaxel-induced DNA Fragmentation. Because docetaxel can cause tumor cell apoptosis, we next used DNA fragmentation assays to assess whether the cell-killing activity of docetaxel would also be affected by doxorubicin. Fig. 4 shows the DNA fragmentation analyses of BCap37 and OV2008 cells exposed to docetaxel, doxorubicin, or their combinations. As described above, the characteristic DNA fragmentation ladders were observed in both BCap37 and OV2008 cells exposed to 5 nM docetaxel for 48 h, but no DNA fragmentation was detected when these cells were treated with 100 nM doxorubicin alone. When these cells were exposed to both drugs simultaneously or pretreated with doxorubicin for 12 h, docetaxel-induced DNA fragmentation was dramatically inhibited, particularly in the group pretreated with doxorubicin (see Fig. 6). However, when cells were exposed to docetaxel first for 12 h, doxorubicin was unable to inhibit docetaxel-induced DNA fragmentation (Fig. 6). Furthermore, the inhibitory effect of doxorubicin on docetaxel-induced cell death was investigated by flow cytometric analyses. From the results depicted in Fig. 7, we can clearly see that apoptotic cell death induced by docetaxel (Fig. 7, Ap) was significantly repressed in the cells treated simultaneously or pretreated with doxorubicin. These findings further confirmed that doxorubicin could antagonize the cytotoxic effects of docetaxel against solid tumor cells.
with doxorubicin alone or treated with doxorubicin and docetaxel simultaneously. This phenomenon raised our concern because such doxorubicin-induced G2-M-phase arrest was not observed in cultured tumor cells. In fact, through phase-contrast microscopy, we found that the number of cells arrested at the G2-M phase (rounded and detached from the dish) was significantly less in the groups cotreated with doxorubicin and docetaxel than in the cells treated with docetaxel alone (data not shown). This phenomenon implies that doxorubicin might actually interfere with the effect of docetaxel on mitotic arrest. To determine whether doxorubicin does indeed affect the cytotoxic effect of docetaxel on cell cycle arrest, cytospin slides were prepared on which mitotically arrested cells were easily identified by their morphological features, e.g., condensed chromosomes. Through bright-field microscopy, we observed that the number of mitotic cells was significantly decreased when BCap37 cells were cotreated or pretreated with doxorubicin (see Fig. 8). Furthermore, we counted those cells that appeared to contain condensed chromosomes. The results summarized in Table 1 indicate that more than 50% of cells in the groups treated with docetaxel alone or pretreated with docetaxel before doxorubicin were arrested at M phase after 48 h of drug exposure. However, the percentage of mitotic cells was dramatically decreased when cells were cotreated or pretreated with doxorubicin. These results indicated that doxorubicin alone obviously caused predominantly G2 arrest rather than mitotic arrest. Pretreatment or simultaneous treatment of tumor cells with doxorubicin prevented mitotic arrest caused by docetaxel (Fig. 8; Table 1).

**Doxorubicin Inhibits Docetaxel-induced bcl-2 and c-raf-1 Phosphorylation.** In the last few years, a number of apoptosis-associated genes or regulatory proteins including bcl-2, c-raf-1, p21Waf1, and p53 were reported to be activated or regulated by paclitaxel or other antimitotic agents, such as colchicine and vinblastine (17–21). Moreover, our recent studies indicated that some G2-S-phase arresting agents, such as 5-fluorouracil and hydroxyurea, could prevent paclitaxel-induced phosphorylation of bcl-2 and c-raf-1 (16, 22). To investigate whether docetaxel can modulate these apoptosis- or cell cycle-regulatory proteins and the possible influence of doxorubicin, Western blotting was performed to analyze the possible alterations of these proteins in BCap37 cells treated with docetaxel, doxorubicin, or their combinations in different schedules. The results depicted in Fig. 7 indicate that docetaxel and doxorubicin have little effect on either p21Waf1 or p53 protein, although a slight increase of p21Waf1 protein was observed in the cells treated with docetaxel alone. However, docetaxel was found to induce phosphorylation of both bcl-2 and c-raf-1 (Fig. 9). Doxorubicin alone did not alter either bcl-2 or c-raf-1 protein (Fig. 9, Lane 3), but when BCap37 cells were exposed to docetaxel and doxorubicin simultaneously or exposed to doxorubicin first for 12 h, the ability of docetaxel to induce bcl-2 or c-raf-1 phosphorylation was completely inhibited (Fig. 6, Lanes 4 and 6). These findings suggest that doxorubicin can prevent docetaxel-induced phosphorylation of bcl-2 and c-raf-1.

**DISCUSSION**

Chemotherapy plays a critical role in virtually every phase of cancer treatment. Clinical protocols for cancer chemotherapy rarely use a single drug but usually combine two or more drugs with different mechanisms of action. The purpose of using drugs in combinations is to achieve therapeutic effects greater than those provided by a single drug alone. An optimal protocol of combination chemotherapy may increase the therapeutic efficacy, decrease toxicity toward the host or non-target tissues, and minimize or delay the development of drug resistance (22, 23). However, when anticancer agents with similar or different modes of actions are combined, the outcome can be synergistic, additive, or antagonistic. Synergism implies that two drugs may produce greater therapeutic efficacy than the expected additive effect, whereas antagonism implies that the actual therapeutic activity produced by two drugs may be smaller than the expected additive effect (24–26). Docetaxel is a novel member of the taxane family. The promising clinical activity of docetaxel has promoted considerable interest in combining this drug with other antimitotic agents. In clinical trials, doxorubicin is one of these agents used with docetaxel in first-line treatment of advanced breast cancer and certain other solid tumors (4, 9). In this study, we investigated the cytotoxic interaction between docetaxel and doxorubicin against human breast and ovarian cancer cells in vitro.
observed that simultaneous exposure to docetaxel and doxorubicin produced antagonistic effects in these tumor cells. Sequential exposure of tumor cells to doxorubicin followed by docetaxel also produced significant antagonistic effects. However, no antagonistic effect was observed when tumor cells were treated with docetaxel before being treated with doxorubicin. In fact, the overall cytotoxicity produced by such a sequential combination was even slightly higher than the cytotoxicity produced by exposure to docetaxel alone (see Figs. 4 and 5).

To analyze the possible mechanism by which doxorubicin interferes with the cytotoxic effects of docetaxel, we performed a series of experiments including DNA fragmentation, flow cytometry, and cytospin analyses. Our results demonstrated that doxorubicin affected the cytotoxic effects of docetaxel on both mitotic arrest and apoptosis (Figs. 6–8; Table 1). Docetaxel, like paclitaxel, is an antimitotic agent. Antimitotic agents largely disrupt the dynamic tubulin network in individual cells, resulting in aberrant aster formation. Thus, affected cells are unable to transverse successfully from metaphase to anaphase (1, 2). Ultimately, the prolonged mitotic arrest in most of these cells may lead to apoptotic cell death. For example, paclitaxel has proven especially effective in its cell killing, apparently through disruption of the microtubule network. By morphological observation and flow cytometric analyses, we also noted...
that the majority of tumor cells treated with docetaxel were arrested at G2-M phase and subsequently underwent apoptosis (Figs. 2 and 7). These results suggest a possible correlation between mitotic arrest and apoptosis, although these data cannot exclude the possibility that docetaxel, like paclitaxel, may also cause apoptotic cell death via a signal pathway independent of mitotic arrest (27). Indeed, our recent studies have demonstrated that baccatin III, a precursor of paclitaxel and docetaxel, could cause tumor cell death without mitotic arrest (28). In the present study, doxorubicin was found to repress the cytotoxic effect of docetaxel in both mitotic arrest and apoptosis unless docetaxel was administered before doxorubicin. Interestingly, however, flow cytometric assays showed that doxorubicin alone caused G2-M-phase arrest (Fig. 7), although cytospin and morphological examinations clearly indicated that doxorubicin did not cause mitotic arrest in either BCap37 or OV2008 cells. Instead, pretreatment with doxorubicin could significantly block docetaxel-induced mitotic arrest (Fig. 8; Table 1). These results implied that doxorubicin may actually cause predominantly G2 arrest rather than mitotic arrest. In flow cytometry, both G2 and mitotic arrest are included in the G2-M-phase peak, and it is hard to distinguish them (Fig. 7), but they can be easily distinguished by cytospin assay and morphological examination. Thus, although the G2-M-phase arrest by docetaxel was exclusively mitotic, the G2-M-phase arrest by doxorubicin was exclusively G2 arrest, which in turn inhibited the mitotic arrest caused by docetaxel. A recent study conducted by Blagosklonny et al. (29) also reported that doxorubicin caused predominantly G2 arrest and prevented mitotic arrest induced by paclitaxel in HCT116 cells. Therefore, it appears that the mechanism by which doxorubicin antagonizes the cytotoxic effects of docetaxel is, in part, by preventing the mitotic arrest of tumor cells.

Previous studies in several laboratories including ours have
demonstrated that paclitaxel and several other antimitotic agents could induce bcl-2 hyperphosphorylation (17, 30). Due to this posttranslational modification, bcl-2 has been postulated to lose its ability to block apoptosis (17). More recently, the phosphorylation of bcl-2 has also been suggested to reflect the damage of microtubules because the modification is commonly induced by antimicrotubule agents and always occurs in the G2-M phase of the cell cycle (21). Moreover, Schandl et al. (31, 32) have reported recently that this phosphorylation even occurs normally as part of mitosis, suggesting that the phosphorylation of bcl-2 was more likely an implication of G2-M-phase arrest. In this study, we performed Western blot analysis and determined that docetaxel can also induce bcl-2 phosphorylation. Interestingly, the combination of docetaxel with doxorubicin blocked the phosphorylation of bcl-2. Coincidentally, c-raf-1 phosphorylation was observed in BCap37 cells after exposure to docetaxel (Fig. 9). c-raf-1 activation through phosphorylation has been demonstrated after mitotic agent treatment and has been linked to bcl-2 phosphorylation and the subsequent induction of apoptosis (33). These findings may provide another piece of evidence that doxorubicin antagonizes the cytotoxic effects of docetaxel by preventing the mitotic arrest of tumor cells.

In summary, this study investigates the possible influence of doxorubicin on the cytotoxic effect of docetaxel against human breast and ovarian cancer cells in vitro. Our results demonstrate that antagonistic interaction occurred when tumor cells were exposed to two drugs simultaneously or exposed to doxorubicin before docetaxel. Further analyses demonstrated that doxorubicin could interfere with the cytotoxic effect of docetaxel on both mitotic arrest and apoptotic cell death. These findings suggest that doxorubicin might antagonize the cyto-

Table 1: Effect of docetaxel (TXT) and doxorubicin (Dox) on mitotic arrest

<table>
<thead>
<tr>
<th>Drug exposure</th>
<th>Percentage of cells at G2-M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>TXT</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>Dox</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>TXT + Dox</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Pre-TXT + Dox</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>Pre-Dox + TXT</td>
<td>9 ± 4</td>
</tr>
</tbody>
</table>

*a This table is based on three separate experiments, and data are presented as the mean ± SE.
*b Cytospin slides were stained with Giemsa. A total of 300 cells were counted from each slide, and only those cells with typical morphological features of condensed chromosomes were counted as mitotically arrested cells.
toxic effects of docetaxel against tumor cells by preventing tumor cells from progressing to the M phase of the cell cycle. On the other hand, the combination of doxorubicin and docetaxel is commonly used in the treatment of certain human solid tumors. Our results also suggest that careful consideration or further experimental evaluation using animal models might be necessary for the combination of these two drugs.

REFERENCES
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