

In Vitro Evaluation of Schedule-dependent Interactions between Docetaxel and Doxorubicin against Human Breast and Ovarian Cancer Cells¹

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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 *c-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

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culture medium to obtain the desired concentrations. The human breast tumor cell line BCap37 (12) and the ovarian cancer cell line OV2008 (13) were propagated in RPMI 1640 supplemented with 10% FCS (Life Technologies Inc, Grand Island, NY) and 1% antibiotic-antimycotic (Life Technologies Inc.). The cells were usually treated with drugs when they reached approximately 60–70% confluence.

Determination of Internucleosomal DNA Cleavage.

Internucleosomal DNA fragmentation was assayed by a modification of previously described methods (12, 14). After treatment of cells with various concentrations of docetaxel, doxorubicin, and their combinations, cells were harvested, counted, and washed with PBS at 4°C. Cells were then suspended in lysis solution containing 20 mM Tris-HCl, 5 mM EDTA, and 5% (v/v) Triton X-100 for 30 min on ice. The remaining steps for DNA fragmentation were performed exactly as described previously (12). DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.2 µg/ml ethidium bromide and visualized under UV illumination.

Flow Cytometric Analysis. Cell sample preparation and PI³ staining were performed according to the method described by Nicoletti *et al.* (15). Briefly, cells treated with docetaxel, doxorubicin, or their combinations were harvested by trypsinization. After being washed twice with PBS, cells were fixed in 1% formaldehyde in PBS on ice and then dehydrated in 70% ethanol in PBS. Approximately 1 h before flow cytometry analysis, RNase A (1 mg/ml) and PI (10 µg/ml) were added to each sample. Samples were incubated at room temperature in complete darkness for 30 min. Cell cycle distribution was determined using a Coulter Epics V instrument (Coulter Corp.) with an argon laser and excitation at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA).

MTT Assays. BCap37 and OV2008 cells were harvested with trypsin and resuspended to a final concentration of 2×10^4 cells/ml in fresh medium with 10% FCS. Aliquots of 0.2 ml from each cell suspension were distributed evenly into 96-well tissue culture plates with lids (Falcon, Oxnard, CA). Designated columns were treated with 5 nM docetaxel, 100 nM doxorubicin, or their combinations. One column from each plate contained medium alone, and another column contained cells without drug as a blank control. After cells were incubated at 37°C for 24 h, 0.1 ml of MTT (1 mg/ml) solution was added to each well. After a 3-h incubation at 37°C to allow viable cells to reduce the yellow MTT into dark blue Formosan crystals, the resulting crystals were dissolved in 100 µl of DMSO. The absorbance in individual wells was determined at 562 nm by a microplate Reader (Molecular Devices). The fractional effect associated with a range of concentrations was determined for each drug alone and for various drug combinations. The data were analyzed by CalcuSyn version 1.1 software (Biosoft), assuming a mutually nonexclusive model. The combination index was used to signify antagonism (combination index > 1), additivity (combination index = 1), or synergism (combination index < 1).

Morphological Examination through Cytospin Preparation.

Cells treated with docetaxel and/or doxorubicin were harvested by trypsinization at the times indicated and washed twice with PBS. Cell numbers were determined with a hemocytometer, and approximately $0.5\text{--}1 \times 10^5$ cells were plated onto microscope slides using the Cytospin 3 cell preparation system (Shandon, Pittsburgh, PA). Slides were air dried and fixed in acetone before Wright-Giemsa staining and then examined using bright-field microscopy.

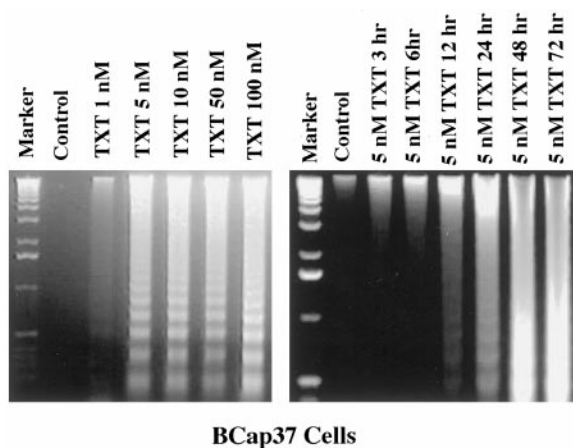
Western Blotting. Cells treated with docetaxel, doxorubicin, or their combinations at different schedules were harvested by trypsinization after a 24-h exposure. Protein extraction and immunoblot procedures were performed as described previously (16). Briefly, protein samples were loaded onto a 12% SDS polyacrylamide gel at equal protein concentrations. 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 1:1000 dilution (~0.3 µg/ml) in 3% BSA-PBS-T (PBS containing 0.5% Tween 20). 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 (Jackson ImmunoResearch). 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 predominate 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.

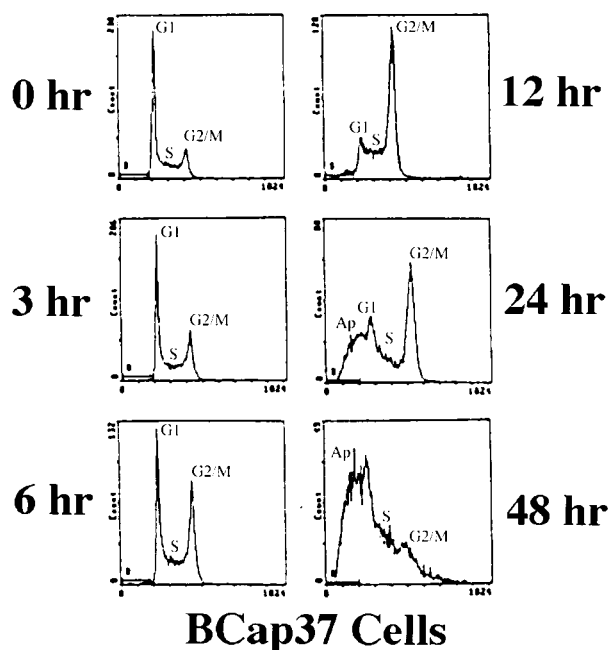
³ The abbreviations used are: PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.



BCap37 Cells

Fig. 1 Characterization of docetaxel-induced DNA fragmentation. BCap37 cells treated with different concentrations of docetaxel (A) or 5 nM docetaxel at the indicated times (B) were harvested for DNA extraction as described in "Materials and Methods." Fragmented DNAs were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.1% ethidium bromide. *TXT*, docetaxel.

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 exposure of 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 5 nM 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 (see Fig. 5). 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*.



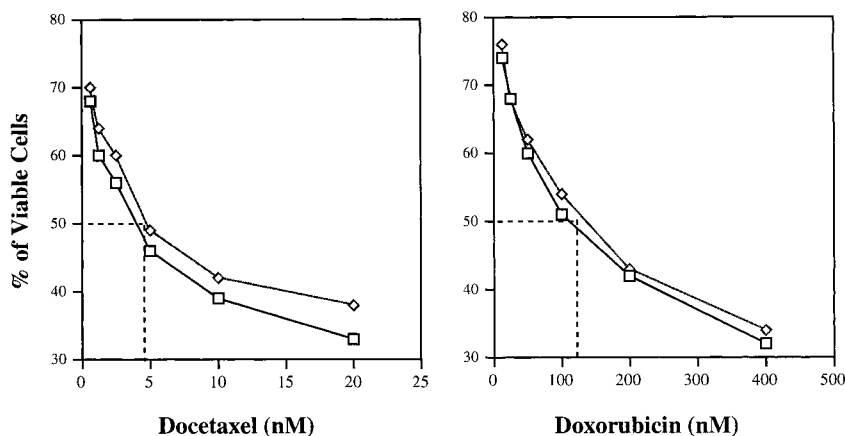
BCap37 Cells

Fig. 2 Flow cytometric analyses. Bcap37 cells treated with 5 nM docetaxel at different time points were harvested, and DNA was stained with PI and analyzed by flow cytometry as described in "Materials and Methods." The distribution of cells in G₁, S phase, and G₂-M phase of the cell cycle and of apoptotic cells (Ap) is indicated above each corresponding peak.

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.

Doxorubicin Prevents Docetaxel-induced Mitotic Arrest. In the above-mentioned flow cytometric assays, we noted that many cells seemed to be arrested at the S phase and G₂-M phase of the cell cycle when BCap37 cells were treated

Fig. 3 Dose-response cytotoxicity of docetaxel and doxorubicin given individually. BCap37 and OV2008 cells were exposed to the indicated concentrations of docetaxel or doxorubicin for 48 h. The data are compiled from three separate MTT experiments, each done in duplicate. □, OV2008 cells; ◇, BCap37 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, p21^{Waf1}, and p53 were reported to be activated or regulated by paclitaxel or other antimetabolic agents, such as colchicine and vinblastine (17–21). Moreover, our recent studies indicated that some G₁-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 alter-

ations 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 p21^{Waf1} or p53 protein, although a slight increase of p21^{Waf1} 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 antimetabolic 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*. We

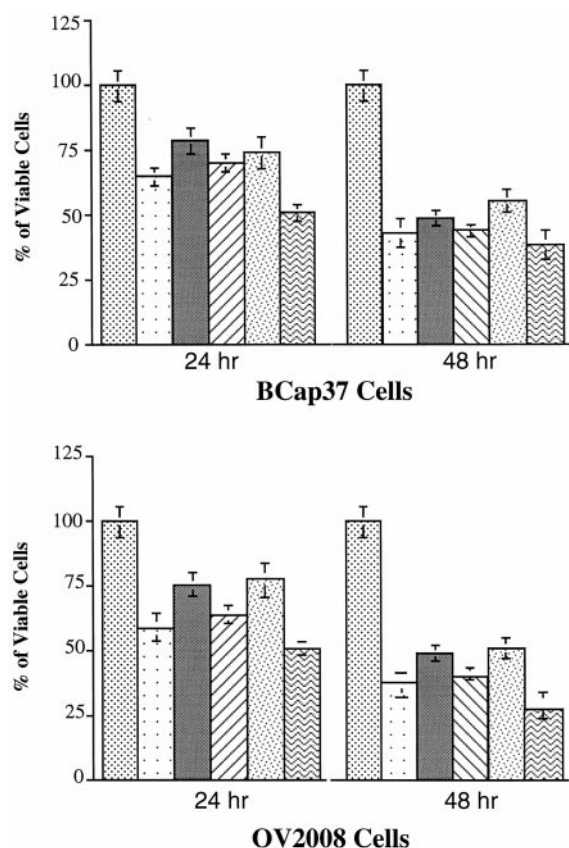


Fig. 4 Cytotoxic effects of sequential exposure to docetaxel and doxorubicin in BCap37 and OV2008 cells. Approximately 2×10^3 tumor cells were cultured in 96-well microplates. After 24 h, cells were exposed to 5 nM docetaxel (TXT), 100 nM doxorubicin (Dox), or their combinations at different schedules. All groups were exposed to docetaxel for the exact same time (24 or 48 h), but the exposure time for doxorubicin was 12 h shorter in the *pre-TXT+Dox* group and 12 h longer in the *pre-Dox+TXT* group. MTT assays were performed as described as "Materials and Methods." Data are presented as the mean \pm SE for three separate experiments. □, control; ▨, docetaxel; shaded box, doxorubicin; hatched box, docetaxel + doxorubicin; ▩, doxorubicin was added 12 h before docetaxel; ▤, docetaxel was added 12 h before doxorubicin.

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). These findings suggest that simultaneous exposure or treatment with doxorubicin before docetaxel is inadequate for the combination of these two drugs. The optimal schedule for this combination may be the sequential administration of docetaxel followed by doxorubicin.

To analyze the possible mechanism by which doxorubicin interferes with the cytotoxic effects of docetaxel, we performed

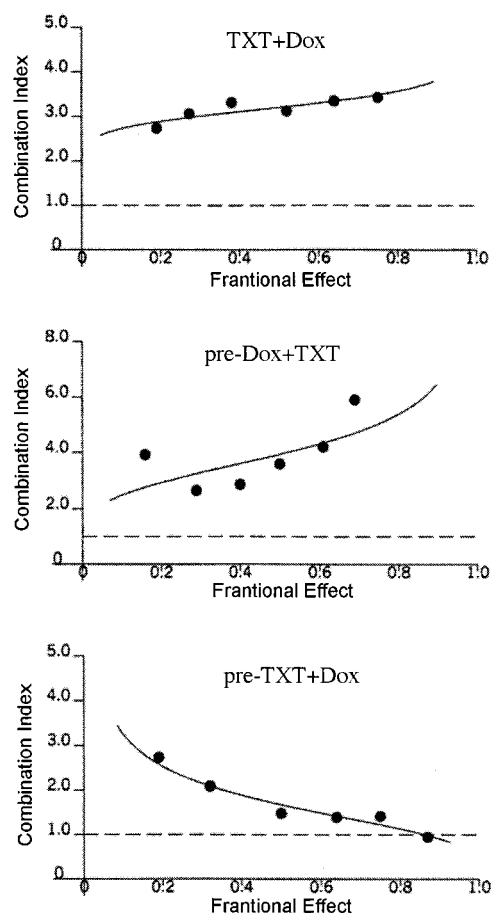


Fig. 5 Combination index of sequential exposure to docetaxel (TXT) and doxorubicin (Dox). BCap37 cells were treated with six concentrations at a 1:20 fixed ratio of docetaxel (0.625–20 nM) and doxorubicin (12.5–400 nM). *TXT+DOX*, simultaneous treatment with both drugs for 48 h; *pre-Dox+TXT*, cells were pretreated with doxorubicin for 12 h and then exposed to both drugs for 48 h; *pre-TXT+Dox*, cells were exposed to docetaxel for 12 h, and then doxorubicin was added and incubated for additional 36 h. The data represent a combination of three separate experiments, each done in duplicate. The fractional effect of each drug in combination was analyzed by CalcuSyn software with a mutually nonexclusive model. The solid line reflects the combination index plot predicted by the software. A combination index of 1.0 (shown by the dashed line) reflects additive effects, whereas values above and below 1.0 indicate antagonism and synergy, respectively.

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 antimetabolic agent. Antimetabolic 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

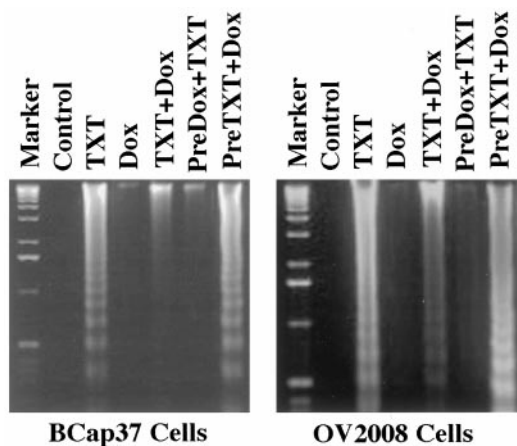


Fig. 6 Effect of doxorubicin on docetaxel-induced DNA fragmentation in BCap37 and OV2008 cells. Tumor cells were treated with 5 nM docetaxel, 100 nM doxorubicin, or their combinations at different schedules. After 48 h of drug exposure, cells were harvested for DNA extraction. Fragmented DNAs were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.1% ethidium bromide. *TXT*, docetaxel; *Dox*, doxorubicin; *PreDox+TXT*, doxorubicin was added 12 h before docetaxel; *PreTXT+Dox*, docetaxel was added 12 h before doxorubicin.

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

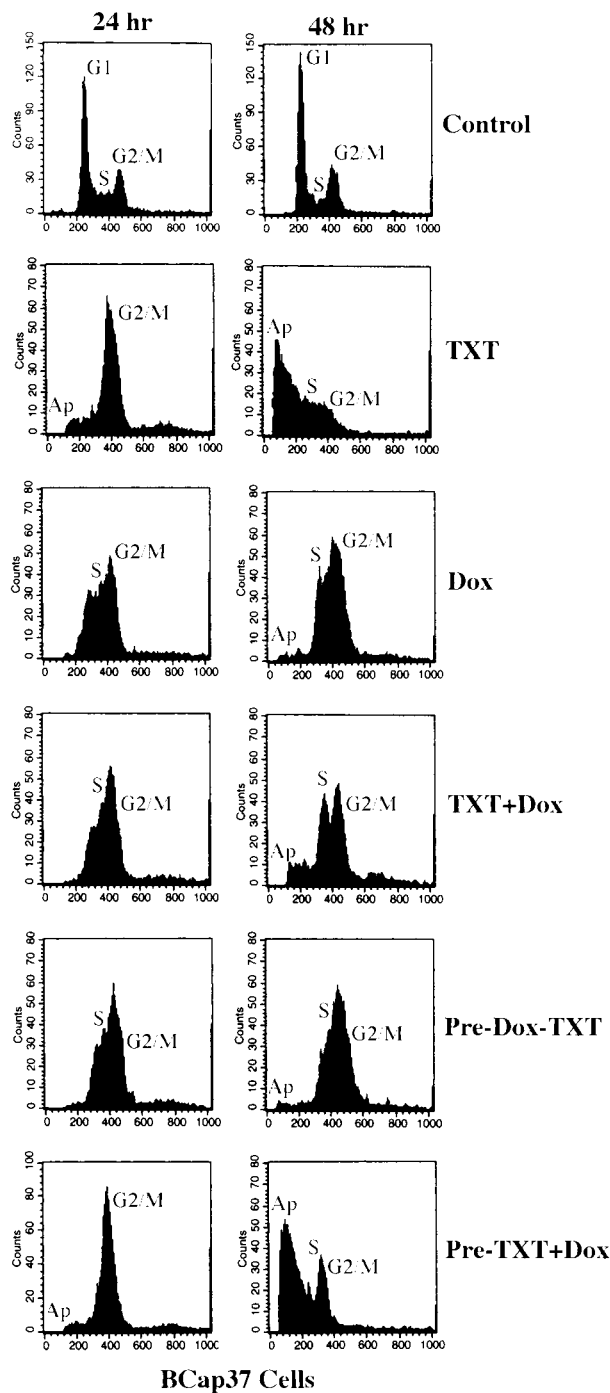


Fig. 7 Flow cytometric analyses of BCap37 cells treated with 5 nM docetaxel, 100 nM doxorubicin, or their combinations for 24 or 48 h. Where applicable, pretreatments were performed for 6 h. Samples were analyzed by flow cytometry as described in "Materials and Methods." The distribution of cells in G₁, S phase, and G₂-M phase of the cell cycle and of apoptotic cells (*Ap* peak) is indicated *above* each corresponding peak. *TXT*, docetaxel; *Dox*, doxorubicin; *Pre-Dox-TXT*, doxorubicin was added 12 h before docetaxel; *PreTXT+Dox*, docetaxel was added 12 h before doxorubicin.

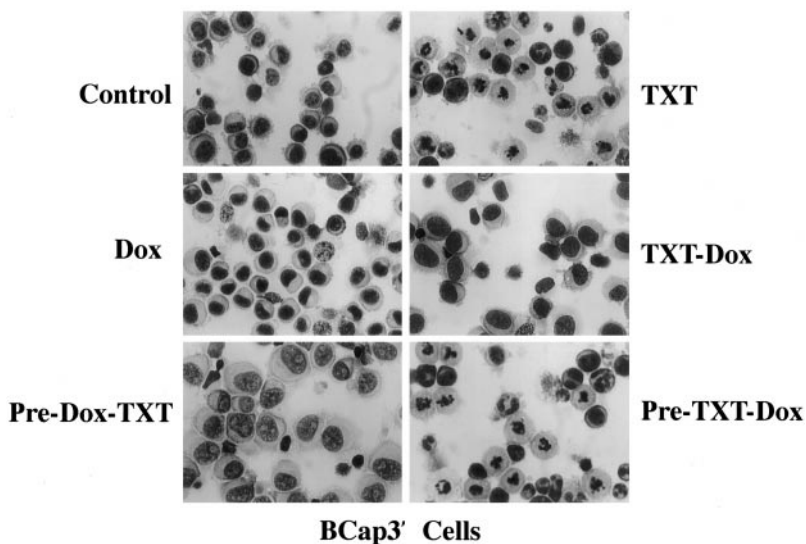


Fig. 8 Morphological examination of BCap37 cells treated with docetaxel, doxorubicin, or their combinations. Cells treated with 5 nM docetaxel, 100 nM doxorubicin, or their combinations at different schedules for 48 h were harvested, and 50,000–100,000 cells were used for cytospin preparation. Slides were air dried and fixed in acetone before Giemsa staining and photographed using bright-field microscopy. *TXT*, docetaxel; *Dox*, doxorubicin; *PreDox-TXT*, doxorubicin was added 12 h before docetaxel; *PreTXT-Dox*, docetaxel was added 12 h before doxorubicin.

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

| Drug exposure | Percentage of cells at G ₂ -M phase ^b | |
|---------------|---|---------|
| | 24 h | 48 h |
| Control | 4 ± 1 | 4 ± 2 |
| TXT | 36 ± 5 | 60 ± 11 |
| Dox | 4 ± 2 | 5 ± 3 |
| TXT + Dox | 6 ± 3 | 8 ± 4 |
| Pre-TXT + Dox | 38 ± 7 | 56 ± 10 |
| Pre-Dox + TXT | 9 ± 4 | 15 ± 5 |

^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.

demonstrated that paclitaxel and several other antimetabolic 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 apo-

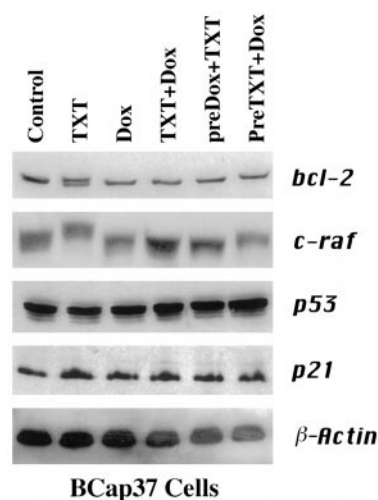


Fig. 9 Western blot analysis for bcl-2, c-raf-1, p21, and p53 proteins. Total proteins were extracted from BCap37 cells treated for 24 h with 5 nM docetaxel, 100 nM doxorubicin, or their combinations. Equal amounts (100 µg/lane) of cellular proteins were fractionated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were immunoblotted with different monoclonal antibodies as described as in “Materials and Methods.”

ptosis (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-

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

- Gelmon, K. The taxoids: paclitaxel and docetaxel. *Lancet*, 344: 1267–1272, 1994.
- Von Hoff, D. D. The taxoids: same roots, different drugs. *Semin. Oncol.*, 24 (Suppl. 13): 3–10, 1997.
- Fulton, B., and Spencer, C. M. Docetaxel. *Drugs*, 51: 1075–1092, 1996.
- D'Andrea, G. M., and Seidman, A. D. Docetaxel and paclitaxel in breast cancer therapy: present status and future prospects. *Semin. Oncol.*, 24 (Suppl. 13): 27–44, 1997.
- Hennequin, C., Giocanti, N., and Favaudon, V. S-phase specificity of cell killing by docetaxel (Taxotere) in synchronized HeLa cells. *Br. J. Cancer*, 71: 1194–1198, 1998.
- Lavelle, F., Bissery, M. C., Combeau, C., Riou, J. F., Virgnaud, P., and Andre, S. Preclinical evaluation of docetaxel (Taxotere). *Semin. Oncol.*, 22: 2–16, 1995.
- Dieras, V., Chevallier, B., Fumoleau, P., Kerbrat, P. A multicentre Phase II study of docetaxel 75 mg/m² as first-line chemotherapy for patients with advanced breast cancer. Report of clinical screening group of EROTC. *Br. J. Cancer*, 74: 650–656, 1996.
- Fumoleau, P., Chevallier, B., and Kerbrat, A. Multicentre Phase II study of the efficacy and safety of docetaxel as first line treatment of advanced breast cancer: Report of clinical screening group of EROTC. *Ann. Oncol.*, 14: 58–65, 1996.
- Khayat, D., and Antoine, D. Docetaxel in combination chemotherapy for metastatic breast cancer. *Semin. Oncol.*, 24 (Suppl. 13): 13–19, 1999.
- Henderson, I. C. Chemotherapy for metastatic diseases. *In: J. R. Harris, S. Hellman, and I. C. Henderson (eds.), Breast Disease*, 2nd ed., pp. 504–625. Philadelphia, PA: Lippincott, 1991.
- Dieras, V. Review of docetaxel/doxorubicin combination in metastatic breast cancer. *Oncology (Basel)*, 11 (Suppl. 8): 31–39, 1997.
- Cheng, L., Zheng, S., Raghunathan, K., Priest, D. G., Willingham, M. C., Norris, J. S., and Fan, W. Characterization of Taxol-induced apoptosis and altered gene expression in human breast cancer cells. *Cell. Pharmacol.*, 2: 249–257, 1995.
- Liu, Y., Bhalla K., Hill, C., and Priest, D. G. Evidence for involvement of tyrosine phosphorylation in Taxol-induced apoptosis in a human ovarian tumor cell line. *Biochem. Pharmacol.*, 48: 1265–1272, 1995.
- Fan, W., Everett, E. T., Tang, C., Cooper, T., Bhalla, K., and Norris, J. S. Glucocorticoid-mediated inhibition of Taxol-induced apoptosis in leiomyosarcoma cells. *Cell. Pharmacol.*, 1: 205–212, 1994.
- Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, 139: 271–279, 1991.
- Johnson, K. R., Wang, L., Miller, M. C., III, Willingham, M. C., and Fan, W. 5-Fluorouracil interferes with paclitaxel cytotoxicity against human solid tumor cells. *Clin. Cancer Res.*, 3: 1739–1745, 1997.
- Haldar, S., Chintapalli, J., and Croce, C. M. Taxol-induced bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res.*, 56: 1253–1255, 1996.
- Schandl, C. A., Priest, D. G., and Willingham, M. C. Phosphorylation of bcl-2 is a cell cycle-regulated event. *Cell. Pharmacol.*, 3: 367–372, 1996.
- Lanni, J. S., Lowe, S. W., Licitra, E. J., Liu, J. O., and Jack, T. p53-independent apoptosis induced by paclitaxel through an indirect mechanism. *Proc. Natl. Acad. Sci. USA*, 94: 9679–9683, 1997.
- Blagosklonny, M. V., Schulte, T. W., Nguyen, P., Mimnaugh, E. G., Trepel, J., and Neckers, L. Paclitaxel induction of p21^{Waf1} and p53 requires c-raf-1. *Cancer Res.*, 55: 4623–4626, 1995.
- Haldar, S., Basu, A., and Croce, C. M. Bcl-2 is the guardian of microtubule integrity. *Cancer Res.*, 57: 229–233, 1997.
- Johnson, K. R., Young, K. K., and Fan, W. Antagonistic interplay between antimetabolic and G₁-S arresting agents observed in experimental combination therapy. *Clin. Cancer Res.*, 5: 2559–2565, 1999.
- Frei, E., III. Clinical studies of combination chemotherapy for cancer. *In: T.-C. Chou and D. C. Rideout (eds.), Synergism and Antagonism in Chemotherapy*, pp. 103–108. San Diego, CA: Academic Press, 1991.
- Fan, W., Johnson, K. R., and Miller, M. C. *In vitro* evaluation of combination therapy against human tumor cells (Review). *Oncol. Report*, 5: 1035–1042, 1998.
- Rideout, D. C., and Chou, T.-C. Synergism, antagonism, and potentiation in chemotherapy: an overview. *In: T.-C. Chou and D. C. Rideout (eds.), Synergism and Antagonism in Chemotherapy*, pp. 3–60. San Diego, CA: Academic Press, 1991.
- Bertino, J. R., and Chou, T.-C. Chemotherapy: synergism and antagonism. *In: J. R. Bertino (ed.), Encyclopedia of Cancer*, Vol. 1, pp. 368–379. San Diego, CA: Academic Press, 1997.
- Fan, W. Possible mechanisms of paclitaxel-induced apoptosis. *Biochem. Pharmacol.*, 57: 1215–1221, 1999.
- Miller, C. M., Johnson, K. R., Willingham, M. C., and Fan, W. Apoptotic cell death induced by baccatin III, a precursor of Taxol, occurs without G₂/M arrest. *Cancer Chemother. Pharmacol.*, 44: 444–452, 1999.
- Blagosklonny, M. V., Robey, R., Bate, S., and Fojo, T. Pretreatment with DNA-damaging agents permits selective killing of checkpoint-deficient cells by microtubule-active drugs. *J. Clin. Invest.*, 105: 533–539, 2000.
- Fan, W., Schandl, C. A., Cheng, L., Norris, J. S., and Willingham, M. C. Glucocorticoids modulate paclitaxel cytotoxicity in human solid tumor cells. *Cell. Pharmacol.*, 3: 343–348, 1996.
- Schandl, A. S., Li, S., Re, G. G., Fan, W., and Willingham, M. C. Mitotic chromosomal bcl-2. I. Stable expression throughout the cell cycle and association with isolated chromosomes. *J. Histochem. Cytochem.*, 47: 139–149, 1999.
- Schandl, A. S., Li, S., Re, G. G., Fan, W., and Willingham, M. C. Mitotic chromosomal bcl-2. II. Localization to interphase nuclei. *J. Histochem. Cytochem.*, 47: 151–158, 1999.
- Blagosklonny, M. V., Schulte, T., Nguyen, P., Trepel, J., and Neckers, L. M. Taxol-induced apoptosis and phosphorylation of bcl-2 protein involves c-raf-1 signal transduction pathway. *Cancer Res.*, 56: 1851–1854, 1996.