Effects of Modulators of Protein Kinases on Taxol-induced Apoptosis of Human Leukemic Cells Possessing Disparate Levels of p26BCL-2 Protein

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ABSTRACT

Taxol-induced polymerization of tubulin into stable microtubules and cell cycle metaphase arrest have been demonstrated to result in internucleosomal DNA fragmentation and morphological features of apoptosis in human leukemia cells. Recent studies have also shown that Taxol-induced apoptosis, but not Taxol-induced microtubular bundling or mitotic arrest, is significantly inhibited in cells that overexpress the bcl-2 gene product p26BCL-2. In the present studies we examined the effects of several modulators of activities of protein kinases on Taxol-induced DNA fragmentation and apoptosis in human pre-B leukemia 697 cells transfected with the cDNA of the bcl-2 gene and expressing high intracellular levels of p26BCL-2. Treatment with 0.1–1.0 μg/ml Taxol for 24 h produced prolonged mitotic arrest of control 697/neo cells, which had been transfected with the neomycin resistance gene. This resulted in apoptosis-associated large DNA fragments ranging between 5 and 200 kb and internucleosomal DNA fragmentation. Cotreatment with the phorbol ester phorbol dibutyrate (PdBu) significantly reduced Taxol-induced internucleosomal and large DNA fragmentation and inhibited apoptosis of 697/neo cells. In contrast, a combined exposure to Taxol and staurosporine (ST; 5 or 50 ng/ml), a potent inhibitor of protein kinase C and other kinases, significantly increased DNA fragmentation and apoptosis of 697/neo cells. Additionally, in 697/BCL-2 cells, ST partially overcame the suppressive effects of high p26BCL-2 levels on Taxol-induced apoptosis. Cotreatment with the tyrosine kinase inhibitor Genistein (30 μM) markedly inhibited Taxol-induced DNA fragmentation and apoptosis of 697/neo cells. However, it is noteworthy that the modulations of Taxol-induced DNA fragmentation and apoptosis by PdBu, ST, and Genistein occurred without significant effects on Taxol-mediated mitotic arrest of 697/neo cells. These agents also did not affect intracellular p26BCL-2 levels in 697/neo or 697/BCL-2 cells. These findings indicate that Taxol-induced apoptosis can be modulated by agents that affect the activities of protein kinases, and these effects are not mediated by modulations of Taxol-induced mitotic arrest or by alterations of intracellular p26BCL-2 levels.

INTRODUCTION

Apoptosis or programmed cell death is an active and gene-directed form of cell death, which is fundamentally different from cell necrosis with respect to its morphology, biochemistry, and biological significance (1, 2). A variety of DNA-interactive anticancer drugs, including alkylating agents, topoisomerase inhibitors, and antimetabolites, have been shown to induce apoptotic cell death (3–5). These drugs affect a wide range of disparate intracellular molecular targets, which by an unclear mechanism engage the final common pathway of apoptosis associated with endonucleolytic DNA fragmentation (6). The bcl-2 proto-oncogene encodes for the p26BCL-2 protein, which is localized to the nuclear envelope, endoplasmic reticulum, and mitochondria (7, 8). High intracellular levels of p26BCL-2 may represent a mechanism of multidrug resistance, because it has been shown to retard apoptosis due to a variety of anticancer drugs (9, 10). Taxol is a prototype of a new class of antimitotubule agents, which have significant in vitro and in vivo activity against acute leukemias (11, 12). Intracellularly, Taxol enhances all aspects of tubulin polymerization and promotes microtubular assembly into stable but nonfunctional microtubular bundles (13). Taxol-induced intracellular microtubular bundling results in cell cycle G2-M arrest of AML cells, which has been correlated with the antileukemic efficacy of Taxol (14). Although Taxol is not a DNA-interactive agent, following Taxol-mediated cell cycle mitotic arrest, AML cells have been demonstrated to exhibit internucleosomal DNA fragmentation and the morphological features of apoptosis (15). It has been suggested that Taxol-induced prolonged cell cycle arrest in the G2-M phase may increase susceptibility to the DNA fragmentation associated with apoptosis (16). Recent reports have indicated that the formation of large-molecular weight fragments of DNA ranging between 5 and 200 kb in length may be the key committed step in drug-induced apoptosis of thymocytes (17). However, the generation of these DNA fragments has not been described in association with Taxol-induced apoptosis. In human pre-B leukemia 697 cells that have been retrovirally transfected with the cDNA of the bcl-2 gene and overexpress p26BCL-2 (697/BCL-2 cells), significant inhibitions of Taxol-induced internucleosomal DNA fragmentation and apoptosis were observed.

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(18). However, in these cells high intracellular p26BCL-2 levels neither affected Taxol-mediated microtubular bundling nor cell cycle G2-M arrest (18), thereby suggesting that p26BCL-2 blocks only the terminal events associated with Taxol-induced apoptosis.

Modulation of the activities of PKC and tyrosine kinases have been reported to affect internucleosomal DNA fragmentation and apoptosis (19, 20). For example, treatment of lymphocytes with phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate and PdBu, which activate PKC, have been demonstrated to block apoptosis induced by glucocorticoids or calcium ionophores (21). Also, the isoflavone Genistein, which is known to inhibit tyrosine kinase and topoisomerase II, arrests cell cycle progression in the G1 and S-G2 border and may prevent apoptosis (20, 22, 23). In contrast, inhibition of PKC activity has been shown to stimulate drug-induced apoptosis (19). ST, which is a strong inhibitor of several protein kinases, including the cyclin-dependent kinases and PKC, has been shown to arrest the cell cycle in the G2 and to enhance the chemosensitivity of leukemic cells (24–26). The aim of the present studies was to examine the effects of modulators of various protein kinase activities including PdBu, ST, and Genistein on Taxol-induced DNA fragmentation and apoptosis of human leukemia cells. An additional aim was to determine whether the treatment with a modulator that enhances Taxol-induced apoptosis in 697/neo cells would also result in the reversal of p26BCL-2-mediated resistance against Taxol-induced apoptosis in 697/BCL-2 cells.

**MATERIALS AND METHODS**

**Cells.** Human pre-B leukemia 697/neo cells and 697/BCL-2 cells were maintained in culture in RPMI 1640 medium with 10% fetal bovine serum, as previously described (9). Logarithmically growing cells were used for all experiments.

**Drugs and Chemicals.** Taxol was kindly provided free by Dr. Patricia A. Pilia (NaPro Bio Therapeutics, Inc., Boulder, CO). Taxol was made fresh for each experiment. It was dissolved in 100% DMSO to make a stock solution of 10.0 mM, which was diluted with medium to obtain the desired concentrations for the experiments. In no condition did the DMSO concentration exceed 0.1%. All other chemicals were reagent grade and were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Springfield, NJ). Drugs were stored as dry powders at −20°C and reconstituted in sterile medium prior to use.

**Quantitative and Qualitative Analyses of Internucleosomal DNA Fragmentation.** The internucleosomal DNA fragmentation was assayed by a modification of previously described methods (5, 15). Following treatment with the designated concentrations and schedules of Taxol and/or the protein kinase modulators, cells were washed and incubated in drug-free medium for an additional 4 h. At the end of this incubation, cells were pelleted and washed with PBS at 4°C and disrupted by suspension for 20 min at 4°C in 5 mM Tris-HCl buffer containing 0.5% (v/v) Triton X-100 and 20 mM EDTA. The cellular lysates were centrifuged at 27,000 × g for 20 min to separate low-molecular-weight DNA from intact chromatin. The pellet was resuspended in the lysis buffer (0.5 ml), and 50 μM BSA (2 mg/ml) were added to this as well as to the supernatant. After adding 1 ml 10% trichloroacetic acid, the microfuge tubes were centrifuged for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 ml 100% ethanol at 4°C and incubated at −20°C. Following another centrifugation, the pellet was treated with 500 μl 1 n sodium hydroxide to hydrolyze RNA and incubated at 37°C for 1 h or until it was resuspended. The reaction mixture was incubated for 15 min on ice, treated with 1.5 ml 20% trichloroacetic acid for 30 min at 4°C, and centrifuged at 4°C for 10 min at 1500 rpm. The pellet was vortexed or sonicated, and DNA was hydrolyzed with 1 ml 5% perchloric acid for 15 min at 90°C with frequent vortexing to break up the pellet and release DNA from the pellet. Subsequently, the reaction mixture was centrifuged at 3000 rpm, and the supernatant was treated overnight at 37°C with 2 ml Burton’s reagent, which uses a diphenylamine reaction. The absorbance at 600 nm was compared with that of 0–250 μg/ml DNA as standard, using linear regression. The quantity of fragmented DNA in the supernatants was expressed as percentage of total DNA from 2 × 10⁶ cells.

For qualitative DNA fragmentation analysis, 1 × 10⁶ drug-treated cells were washed in PBS and resuspended in the lysis buffer, as above. The suspension was centrifuged, and DNA was extracted from the supernatant by treatment with phenol:chloroform:isoamyl alcohol (25:24:1; Ref. 15). To the upper aqueous layer placed in a fresh tube, 50 μl 3 M sodium acetate and 1 ml 100% ethanol at 4°C were added to precipitate the DNA. The mixture was vortexed and incubated for 30–60 min at −70°C. Following a cold centrifugation for 30 min at 10,000 rpm, the supernatant was discarded, and the pellet was resuspended in 0.5 ml TE buffer at pH 8.0. After adding 10 μl RNase-free RNase A to the suspension, it was vortexed and incubated for 30 min at 37°C. To this, 50 μl 5 M NaCl were added, and the phenol:chloroform:isoamyl alcohol extraction was repeated. DNA was precipitated with ethanol as above and centrifuged, and the pellet was dried. DNA samples (5 × 10⁶ cells) were mixed with 16 μl TE (pH 8.0) loading buffer and 2 μl of 5× tracking dye containing glycerol, bromophenol blue, and xylene cyanol. The reaction mix was loaded into wells of a 1% agarose gel and electrophoresed in a 1 X Tris-acetate-EDTA running buffer containing ethidium bromide. DNA was separated over 90 min at 60 V, and the bands were visualized by UV illumination. Alternatively, an improved method for the isolation of pure genomic DNA and the detection of internucleosomal DNA fragmentation in <1.0 μg DNA sample by agarose gel electrophoresis was also used to estimate DNA fragmentation in cells treated with Taxol and/or the modulators of protein kinases (4).

**Tdt Assay for Labeling DNA Strand Breaks.** After incubations with 0.1 μM Taxol and/or 30 μM Genistein for 24 h, a previously described Tdt assay for labeling DNA strand breaks in individual apoptotic cells and visualization under a fluorescent microscope were performed (27).
Preparation of DNA Plugs and Field Inversion Gel Electrophoresis. Formation of large DNA fragments was determined by a modification of previously described methods (17, 28). Briefly, following treatment with the designated concentrations and schedules of the drugs, the cells were washed and suspended, and an equal volume of 1.5% InCert agarose was added to the cell suspension and mixed gently. Immediately, this mixture was used to prepare 10-μl plugs on a prelabeled Petri dish (35 mm) on ice and allowed to solidify over 30 minutes. The agarose-embedded cells (2 × 10^5 cells/plug) were treated with a lysis solution (lysis buffer containing 0.2% sarkosyl and 0.5/ml proteinase K) at 42°C with slow shaking for 2 days. The lysis solution was removed, and plugs were rinsed with TE buffer and inserted into the wells of a 1% (w/v) agarose gel (pulsed field certified; Bio-Rad, Hercules, CA) and sealed with the molten agarose. Field inversion gel electrophoresis was carried out in a horizontal apparatus. Two sets of marker DNA were used: λ ladder and λ digested with HindIII. After electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide, destained, and photographed on an UV transilluminator. Subsequently, the negative was developed for the DNA profile.

Western Blot Analysis of p26BCL-2 Oncoprotein and P-Glycoprotein Expression. The expression of p26BCL-2 oncprotein in untreated and Taxol-treated 697/neo or 697/BCL-2 cells was determined by Western blot analyses by a previously described method using a monoclonal anti-BCL-2 antibody (18, 29). For immunoblot analyses, protein was extracted from cells with 100 mm Tris-HCl (pH 6.8) buffer containing 2% SDS, 5 mm β-mercaptoethanol, and 10% glycerin. Appropriate protein amounts were subjected to SDS-PAGE (10% gel). After electrophoresis, proteins were transferred to nitrocellulose sheets (0.5 A at 100 V at 4°C) for 1 to 3 h. The blots were blocked in nonfat milk, PBS, and 0.02% sodium azide, pH 7.4 for 3 h at room temperature with gentle shaking. This was followed by incubation with mouse anti-BCL-2 antibody (1:1000 dilution) at room temperature and then with antimouse peroxidase-conjugated secondary IgG antibodies. Immune complexes were detected with an enhanced chemiluminescence detection method by immersing the blot for 1 min in a 1:1 mixture of chemiluminescence reagents A and B (Amersham, United Kingdom) and then exposing it to Kodak XCL film for a few seconds.

Flow Cytometric Cell Cycle Analyses. Flow cytometric analysis of cellular DNA content by propidium iodide staining was performed according to a previously described technique (18, 30). Following drug treatment of 697/neo or 697/BCL-2 cells, aliquots of cells were withdrawn, stained with propidium iodide, and analyzed for DNA content by a Coulter Elite flow cytometer. The percentage of cells in each of the G1, S-phase, and G2-M was calculated using Multicycle Software (Phoenix Flow Systems, San Diego, CA).

Assessment of the Morphology of Apoptosis of Leukemic Cells. Briefly, following incubations with the designated concentrations and schedules of drugs, cells were cytopspun onto glass slides and stained with Wright stain. Cell morphology was determined by light microscopy. Five different fields were randomly selected for counting at least 500 cells. The percentage of apoptotic cells was calculated for each experiment. Cells designated apoptotic were those that displayed the characteristic morphological features of apoptosis, including cell volume shrinkage, chromatin condensation, and the presence of membrane-bound apoptotic bodies (1, 4). The assessment of the percentage of apoptotic cells was confirmed by an additional independent observer who was blinded to the results of the first observer.

Assessment of Cytotoxicity by the MTT Assay. The MTT assay for cell cytotoxicity was used as previously described (31, 32). The assay is based on the conversion of the yellow tetrazolium salt MTT to a colored formazan product by mitochondrial enzymes in the viable cells. The 697/neo and 697/BCL-2 cells were incubated with the designated concentrations and schedules of the protein kinase modulators and/or Taxol for 24 h at 37°C. At the end of 24 h, the drug-treated cells were washed and resuspended in drug-free media. One hundred-μl aliquots of 40,000 cells/condition were dispensed into 96-well flat-bottom microtiter plates (Costar) and incubated at 37°C for an additional 24 h. At the end of this incubation, 50 μl 5 mg/ml solution of MTT (Sigma) were added to each well, and the plates were incubated for another 5 h at 37°C. Next, the plates were centrifuged for 10 min at 500 × g. After removing the supernatants, the formazan crystals were dissolved with 150 μl 1:1 DMSO:ethanol solution. The absorbance of the wells was measured with an Anthos plate reader (Anthos Labtec Instruments, Salzburg, Austria) at 540 nm. The percentage of cell survival was defined as: mean A of treated wells/A of untreated control wells ×100%.

Statistical Analysis. Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined using paired t test analyses. A one-way ANOVA was also applied to the results of the various treatment groups, and post hoc analysis was performed using the Bonferroni correction method.

RESULTS
We have previously reported that a 24-h exposure to 0.05–1.0 μM Taxol results in internucleosomal DNA fragmentation and morphological features of apoptosis in human leukemia cells, which is significantly inhibited in 697/BCL-2 cells (4, 18). The effect of treatment with PdBU (12.5 nM) and/or staurosporine (5 ng/ml) alone, or in combination with Taxol (0.1 μM) for 24 h, was determined on internucleosomal DNA fragmentation and apoptosis of 697/neo versus 697/BCL-2 cells (Fig. 1 and Table 1). These concentrations of PdBU and ST are known to affect the activities of protein kinases without inducing DNA fragmentation or apoptosis (19, 24). In addition, the modulation of Taxol-induced DNA fragmentation and apoptosis was correlated with the intracellular p26BCL-2 levels in the two cell types. Agarose gel electrophoresis of fragmented DNA purified from the supernatant of pelleted cells treated with different conditions is depicted in Fig. 1. Fig. 1A, left, shows that the exposure of 697/neo cells to PdBU (Lane 2) or ST (Lane 3) alone did not have a significant effect on internucleosomal DNA fragmentation. However, compared with Taxol alone (Lane 4), cotreatment with PdBU inhibited (Lane 5), whereas cotreatment with ST increased (Lane 6) the intensity of the ladder of Taxol-induced internucleosomal DNA fragmentation. In the 697/neo cells, the addition of PdBU (12.5 nm) to the culture conditions...
Fig. 1  697/neo cells (A and B, left, Lanes 1–6) and 697/BCL-2 cells (A and B, right, Lanes 1–6) were exposed to Taxol, PdBU, or ST alone or Taxol plus PdBU or ST. Following these treatments, the purified DNA fragments from the supernatant of lysed cells were electrophoresed on agarose gels and detected by staining with ethidium bromide (A). Alternatively, 20 μg cytosolic protein extracted from these cells was probed with a monoclonal anti-BCL-2 antibody (B). DNA (A) or protein (B) loaded in each lane is from cells treated as: Lane 1, untreated cells; Lane 2, 12.5 nM PdBU for 24 h; Lane 3, 5 ng/ml ST for 24 h; Lane 4, 0.1 μM Taxol for 24 h; Lane 5, cotreatment with Taxol and PdBU; and Lane 6, cotreatment with Taxol and ST. M, molecular weight marker.

Table 1  Effect of modulators of protein kinase activities on Taxol-induced internucleosomal DNA fragmentation and apoptosis in 697/neo and 697/BCL-2 cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% DNA fragmentation</th>
<th>% Apoptosis</th>
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<tbody>
<tr>
<td></td>
<td>697/neo</td>
<td>697/BCL-2</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>PdBU (12.5 nM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ST (5 ng/ml)</td>
<td>5.5 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>Genistein (30 μM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Taxol (0.1 μM)</td>
<td>26.2 ± 0.8</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Taxol + PdBU</td>
<td>10.0 ± 0.3*</td>
<td>0</td>
</tr>
<tr>
<td>Taxol + ST</td>
<td>35.9 ± 1.9*</td>
<td>6.5 ± 1.6*</td>
</tr>
<tr>
<td>Taxol + Genistein</td>
<td>5.6 ± 0.8*</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were incubated with Taxol and/or designated concentrations and schedules of the modulators of protein kinase activities, as described in the text. Following these treatments, cytosup and Wright-stained preparations of cells were examined by light microscopy to determine the percentage of cells displaying the morphological features of apoptosis. Alternatively, cells were washed, pelleted, and lysed. Purified DNA in the supernatant and pellet of the cells was quantitated, and DNA fragmentation was expressed as percentage of total starting DNA.

* Mean ± SE of three separate experiments.

* Values significantly different (P < 0.05) from those seen in cells treated with Taxol alone.

containing ST and Taxol did not impair the ST-mediated enhancement of Taxol-induced apoptosis (data not shown). Fig. 1A, right, shows the absence of internucleosomal DNA fragmentation in 697/BCL-2 cells treated with Taxol, PdBU, or ST alone, or Taxol plus ST or PdBU (Lanes 2–5). In contrast, 697/BCL-2 cells exposed to Taxol plus ST showed a slight but detectable presence of internucleosomal DNA fragmentation (Lane 6). Although not shown, similar effects of PdBU and ST on Taxol-induced DNA fragmentation were detected by the TdT assay for labeling apoptosis-associated DNA strand breaks. In
addition to their effects on Taxol-induced DNA fragmentation and apoptosis, the modulatory effect of ST or PdBU on Taxol cytotoxicity was determined by the MTT assay and represented as the mean percentage of control viability (mean of two experiments performed in duplicate). These studies showed that the loss of cell viability due to 0.1 μM Taxol for 24 h (52.0% of control) was increased by cotreatment with ST (32.2% of control) and decreased by cotreatment with PdBU (80.1% of control). Fig. 1B demonstrates the results of the Western blot analyses of p26BCL-2 levels in 697/neo and 697/BCL-2 cells treated with PdBU (Lane 2), ST (Lane 3), or Taxol alone (Lane 4), or Taxol plus PdBU (Lane 5) or ST (Lane 6). Fig. 1B shows that compared with 697/neo, 697/BCL-2 cells contain significantly higher levels of p26BCL-2. Neither the treatment with Taxol, PdBU, or ST alone nor the cotreatment of Taxol with PdBU or ST significantly altered the intracellular p26BCL-2 levels in 697/neo (Fig. 1B, right, Lanes 1–6) or 697/BCL-2 cells (Fig. 1B, left).

The effect of Genistein on Taxol-induced DNA fragmentation was examined by agarose gel electrophoresis (Fig. 2A) and by the TdT assay for labeling DNA strand breaks (Fig. 2C). Fig. 2A shows that the exposure to 30 μM Genistein alone for 24 h did not induce internucleosomal DNA fragmentation (Lane 2), but cotreatment with Genistein significantly inhibited Taxol-induced DNA fragmentation (Lane 4 versus Lane 3). Fig. 2C demonstrates that the untreated (a) or Genistein-treated (b) cells did not demonstrate DNA strand breaks. Exposure to 0.1 μM Taxol for 24 h markedly increased the percentage of cells exhibiting DNA strand breaks (c), which was significantly inhibited by cotreatment with Genistein (d). With the MTT assay a protective effect of Genistein on the cytotoxic effect of Taxol was also observed (Taxol, 52.0% of control viability; Genistein plus Taxol, 82.0% of control viability; mean of two experiments performed in duplicate). Fig. 2B shows the intracellular p26BCL-2 levels in 697/neo cells treated with Genistein (Lane 2), Taxol (Lane 3), or Genistein plus Taxol (Lane 4), as determined by Western analyses using an anti-p26BCL-2 monoclonal antibody. There was no significant effect of these treatments on the intracellular p26BCL-2 levels in 697/neo cells.

Following treatments with Taxol and/or the modulators of protein kinases, the quantitative estimation of the fragmented DNA purified from the supernatant of pelleted cells, expressed as the mean percentage of total starting DNA, and the percentage of cells exhibiting the morphological features of apoptosis were determined and are shown in Table 1. Treatment of 697/neo cells with 0.1 μM Taxol increased DNA fragmentation and the percentage of apoptotic cells. A combined treatment with Taxol and ST increased, whereas cotreatment with PdBU or Genistein significantly inhibited, Taxol-induced DNA fragmentation and apoptosis in 697/neo cells (Table 1). In 697/BCL-2 cells, only the combined treatment of Taxol and ST produced internucleosomal DNA fragmentation or apoptosis compared with the untreated cells or those cells treated with Taxol and/or Genistein or PdBU (Table 1).

Recent reports have indicated that the cleavage of DNA into kilobase-size fragments followed by internucleosomal DNA fragmentation may be distinct steps in the degradation of DNA during drug-induced apoptosis (17, 28). The effects of the modulators of protein kinases on Taxol-induced 5- to 200-kb DNA fragments, as determined by field inversion gel electrophoresis, corresponded to their effects on Taxol-induced internucleosomal DNA fragmentation of the genomic DNA extracted from the 697/neo cells (data not shown). Taken together, these results indicate that the commitment to apoptosis following Taxol-treatment involves the generation of 5- to 200-kb
DNA fragmentation as well as internucleosomal DNA fragmentation. In addition, cotreatment with PdBU, ST, or Genistein has modulatory effects on both types of Taxol-induced DNA fragmentation associated with apoptosis.

Because previous work from our laboratory had indicated that Taxol-mediated mitotic arrest may be an important determinant of Taxol-induced DNA fragmentation and apoptosis (15, 16), we examined the effect of PdBU, ST, or Genistein on the percentage of cells that accumulate in the G2-M following exposure to 0.1 μM Taxol for 24 h. Fig. 3 and Table 2 demonstrate that PdBU or ST alone did not significantly alter the percentage of 697/neo cells in the S-phase or G2-M, whereas Genistein increased the percentage of cells in the G2-M of the cell cycle from 11.0 to 20.0% (Table 2). Taxol-induced accu-

**DISCUSSION**

Taxol has been an important addition to the list of clinically active drugs used against a variety of human cancers (11). Previous published reports have demonstrated that Taxol-induced internucleosomal DNA fragmentation and apoptosis as well as the Taxol-mediated loss of cell viability (by MTT assay) are significantly regulated by the levels of either the intracellular p26BCL-2 or the mdr-1 gene-encoded membrane P-glycoprotein (18, 29). In this report, evidence is presented for the first time that cotreatments with PdBU, ST, or Genistein, known to be modulators of protein kinases, significantly affect Taxol-induced DNA fragmentation, cytotoxicity, and apoptosis of human leukemic cells. However, these effects were not associated with any significant alterations in the intracellular p26BCL-2 levels or Taxol-mediated mitotic arrest of leukemic cells.

The mechanism by which treatment with Taxol, a non-DNA-interactive drug, results in DNA fragmentation and apoptosis is not entirely clear. Recently, it has been suggested that because Taxol-induced prolonged metaphase arrest is associated with a prolonged mixing of the nuclear and cytoplasmic compartments, it may create the permissive conditions for the endonucleolytic DNA fragmentation triggering apoptosis (16). Although the precise nature of this trigger is not known, the presence of high intracellular p26BCL-2 levels protects against this fate (18). As noted above, the formation of large DNA fragments has been regarded as the key committed step of apoptosis (17). This is supported by our data demonstrating that with the onset of Taxol-induced apoptosis, large DNA fragments along with the characteristic DNA fragmentation ladder are produced. In 697/neo cells, the modulations of Taxol-induced apoptosis produced by cotreatment with PdBU, ST, or Genistein were also associated with similar alterations in the

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**Table 2** Effect of modulators of protein kinase activities on Taxol-induced cell cycle perturbations in 697/neo cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>46.0</td>
<td>43.0</td>
<td>11.0</td>
</tr>
<tr>
<td>PdBU (12.5 nM for 24 h)</td>
<td>51.5</td>
<td>40.5</td>
<td>8.0</td>
</tr>
<tr>
<td>ST (5 ng/ml for 24 h)</td>
<td>44.5</td>
<td>50.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Genistein (30 μM for 24 h)</td>
<td>46.5</td>
<td>33.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Taxol (0.1 μM for 24 h)</td>
<td>1.5</td>
<td>32.0</td>
<td>66.5</td>
</tr>
<tr>
<td>Taxol + PdBU</td>
<td>1.0</td>
<td>31.0</td>
<td>68.0</td>
</tr>
<tr>
<td>Taxol + ST</td>
<td>3.5</td>
<td>38.0</td>
<td>58.5</td>
</tr>
<tr>
<td>Taxol + Genistein</td>
<td>1.0</td>
<td>36.0</td>
<td>63.0</td>
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*Values represent mean percentage of cells in the different phases of cell cycle obtained from two separate experiments performed in duplicate.*
amount of large DNA fragments, the intensity of the internucleaseosomal DNA fragmentation ladder, and Taxol-induced cytotoxic effects (by MTT assay). Because these alterations occurred without any significant effects on Taxol-induced cell cycle mitotic arrest, our findings also indicate that the DNA fragmentation and apoptosis due to Taxol occur downstream of the molecular signals initiated by its antimitotubule and cell cycle effects. Recently, it has been shown that the premature activation of p34cdc2 kinase is required for apoptosis, and p34cdc2 kinase is activated coincidentally with Taxol-induced apoptosis (33, 34). In addition, Taxol has been demonstrated to induce p21 (WAF-1/CIP-1) expression in conjunction with apoptosis of p53 positive or negative cells (35). These findings suggest that the conflicting signals represented by the induction of cell cycle-dependent kinases as well as their inhibitors, following Taxol-induced prolonged metaphase arrest, may be an integral component of the molecular signaling leading to apoptosis.

Previous studies have demonstrated that high concentrations of Genistein and ST alone are growth inhibitory and may induce apoptosis in leukemic cells (23, 25). Bruno et al. (25) had demonstrated that the treatment of MOLT-4 cells with 5 ng/ml ST for 24 h resulted in the accumulation of 28% of the cells in the G2-M. In contrast, we found that this concentration and schedule of ST did not affect the cell cycle distribution of 697/neo cells. This difference in response to ST may be due to the different lymphocytic leukemia cells used in the two studies. These cells may differ with respect to their growth fractions, cell cycle times, and the levels and activities of the cell cycle-dependent kinases. Indeed, other investigators failed to notice any significant effect of low concentrations of ST (21 nm) on the cell cycle distribution of FM3A mammary carcinoma cells (24). Therefore, in the present studies, we used lower but active concentrations of these agents that, when used alone, did not induce DNA fragmentation, apoptosis, or cytotoxicity in 697/neo or 697/BCL-2 cells. The observation that PdBU, a known PKC activator, and Genistein, an inhibitor of tyrosine kinase activity, reduce Taxol-induced apoptosis in 697/neo cells suggests that the serine-threonine kinase activity of PKC retards, whereas tyrosine kinases promote, Taxol-mediated apoptosis. This is supported by the previous reports in which PKC activators and tyrosine kinase inhibitors were shown to inhibit apoptosis due to other agents (19, 22). ST is a pleiotropic inhibitor of a variety of serine-threonine kinases, and it has also been shown to enhance drug-induced apoptosis (24, 36). However, we were unable to demonstrate that cotreatment with PdBU significantly affected the ST-mediated enhancement of Taxol-induced apoptosis. This suggests that ST potentiates Taxol-induced apoptosis by a mechanism involving inhibition of protein kinases in addition to and/or other than the PKC. In a recent report the indolocarbazole serine-threonine kinase inhibitor K252a and its analogues were also found to act synergistically with Taxol to promote toxicity in Chinese hamster ovary cells (37). Results presented in this report demonstrate that ST significantly enhances Taxol-induced DNA fragmentation, apoptosis, and cytotoxicity of human leukemic cells exhibiting relatively low p26BCL-2 levels. In addition, cotreatment with ST may modestly overcome the suppressive effect of high p26BCL-2 levels on Taxol-induced apoptosis. Taken together with the effects of PdBU and Genistein, our results also indicate that these agents modulate the signals initiated by Taxol-mediated metaphase arrest, which culminate in DNA fragmentation and apoptosis. It is noteworthy that, as reported by Jarvis et al. (38), our data also indicate that the modulators of protein kinases, either alone or in combination with Taxol, do not significantly affect p26BCL-2 levels, although our studies do not rule out the possibility that the alterations in Taxol-induced apoptosis due to treatment with the modulators of protein kinase may be due to the modification of the phosphorylation status and activity of p26BCL-2.

Our results indicate that the activities of protein kinases may influence an important step(s) in the molecular mechanism initiated by Taxol-induced mitotic arrest, leading to DNA fragmentation and apoptosis of leukemic cells. Although high intracellular p26BCL-2 levels block the final step of DNA fragmentation associated with apoptosis, cotreatment with ST may partially overcome this effect of p26BCL-2. This highlights a potential strategy to enhance Taxol-induced apoptosis. Recent evidence suggests that ST may also inhibit the function of P-glycoprotein and reverse the multidrug-resistant phenotype (39). Taken together with our findings, these data raise the possibility that cotreatment with ST, or drugs with similar actions, may improve the activity of Taxol against multidrug-resistant cells due to different mechanisms of resistance.

REFERENCES


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Effects of modulators of protein kinases on taxol-induced apoptosis of human leukemic cells possessing disparate levels of p26BCL-2 protein.

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