Defective G₁-S Cell Cycle Checkpoint Function Sensitizes Cells to Microtubule Inhibitor-induced Apoptosis

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ABSTRACT

Defective cell cycle checkpoint function has been linked to enhanced sensitivity of tumor cells to certain genotoxic agents. To determine whether loss of the G₁-S checkpoint function would sensitize tumor cells to microtubule inhibitor (MTI)-induced apoptosis, we examined the effect of the MTIs Taxol and vincristine, on the cell cycle kinetics and survival of two isogenic cell lines, HCT116 p21+/+ and HCT116 p21−/−, which differ only at the p21 locus. p21-deficient cells displayed a dose-dependent, enhanced chemosensitivity to MTIs in both monolayer and soft agar assays as well as in mice xenograft tumors. The increased sensitivity of the p21-deficient cells to MTIs correlated with prolonged cyclin B1/Cdc2 activity and the occurrence of endoreduplication. Furthermore, sensitivity of p53-deficient cells to MTI-induced apoptosis was significantly reduced by induction of ectopic p21 protein. The results suggest that the status of G₁-S checkpoint function in tumor cells may be an important determinant in the efficacy of MTIs used clinically.

INTRODUCTION

Eukaryotic cells use precise biochemical pathways to coordinate cell cycle progression. During a normal cell cycle, entry into S-phase is dependent on completion of mitosis and subsequent activation of cyclin-dependent kinases in the G₁ phase. Cells have evolved checkpoint pathways to coordinate these events and halt cell cycle progression in response to cellular damage (1). The G₁-S checkpoint monitors DNA damage and replication (2), whereas the mitotic spindle checkpoint monitors spindle microtubule structure and chromosome alignment/kinetochore attachment (3, 4). Loss or attenuation of checkpoint function can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis (5).

Cell cycle checkpoint function is an important determinant of cellular sensitivity to various endogenous and exogenous agents, including chemotherapeutic drugs. After treatment with anticancer agents, cells may undergo an aberrant mitotic exit and reenter G₁ with a 4N DNA content (6–8). Although it is not understood how mitotic slippage occurs after treatment with select genotoxic agents or MTIs, cells lacking the gene products involved in G₁-S checkpoint function, including p53 (9–11), pRb (9, 12), p16 INK4A (12), or p21 Waf1/Cip1 (13, 14)−/−, have aneuploidy, all of which can contribute to tumorogenesis (5).

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Loss of p53 function has been shown to either reduce or increase cellular sensitivity to genotoxic agents, depending on the cell type and additional genetic alterations in the cell (14–18). Absence of p21, a downstream target of p53, results in enhanced chemosensitivity to DNA-damaging agents both in vitro (8, 19) and in vivo (20, 21). However, the effect of p53 or p21 status on the sensitivity of cells to MTIs remains less well defined. Several groups have reported that loss of p53 confers increased sensitivity to MTI treatment (22, 23), whereas others concluded that p53 or p21 status does not affect cellular sensitivity to MTIs (24, 25).

In the present study, we investigated whether cells lacking the G₁-S checkpoint, due to p21 deficiency, have increased sensitivity to chemotherapy used clinically. We found that p21-deficient cells had dose-dependent, enhanced chemosensitivity to MTIs in both in vitro and in vivo models. Moreover, we were able to reduce chemosensitivity to MTIs in p53-deficient cells by induction of ectopic p21 protein. The results suggest that the status of G₁-S checkpoint function in tumor cells may be an important determinant in the efficacy of MTIs used clinically.

MATERIALS AND METHODS

Cell Lines, Growth Conditions, and MTI Treatment. An HCT116 p21+/+ human colon carcinoma cell line and a derivative line, HCT116 p21−/−, in which both p21 Waf1/Cip1 alleles have been deleted through homologous recombination (26), were kindly provided by Dr. Bert Vogelstein (John Hopkins Oncology Center, Baltimore, MD). HCT116 cell lines were maintained in monolayer culture in McCoy’s 5A (HyClone) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. HIp21 human large cell lung carcinoma cells were derived as described previously (13) and maintained in monolayer culture in F-12 (HyClone) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml G418 sulfate, and 250 µg/ml zeocin at 37°C with 5% CO₂. Where indicated, cells were treated with 5–100 nM paclitaxel (Taxol; Sigma Chemical Co.) or 10–100 nM vincristine (Eli Lilly & Co.), resuspended in DMEM, and added directly to cell media.

Flow Cytometry. Control and treated cells were trypsinized, and 1.0 × 10⁶ cells were analyzed by flow cytometry. The remaining cells were processed for protein analysis (see below). Cells were incubated with 50 µg/ml propidium iodide (Sigma), and DNA content was measured using a FACSCaliber (Becton-Dickson). Data were analyzed using Cell Quest software (Becton-Dickson; 15,000 events were analyzed for each sample).

Xenograft Growth Assay. HCT116 p21+/+ and p21−/− cells were plated at 1 × 10⁶ cells/35-mm dish in McCoy’s 5A medium supplemented with 10% fetal bovine serum, 0.4% agar, and the indicated concentrations of MTIs. Colonies were counted after 7 days using an Omnicon FACS III image analyzer (Bausch & Lomb). Values are representative of two independent experiments carried out in triplicate.

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The abbreviations used are: MTI, microtubule inhibitor; DAPI, 4',6-diamidino-2-phenylindole.

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Western Analysis. Protein was prepared as described previously (13). Total cell protein extracts were normalized for concentration by the Bradford assay (Bio-Rad), and 25 μg of protein were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were incubated with antibodies against p53 (clone PAb1801; Calbiochem, Oncogene Research Products), pRb (clone LM95.1; Calbiochem, Oncogene Research Products), p21 (clone EA10; Calbiochem, Oncogene Research Products), cyclin B1 (clone GNS1; Santa Cruz Biotechnology), Bcl-2 (clone 100; Santa Cruz Biotechnology), cyclin E (clone HE12; Santa Cruz Biotechnology), MPM-2 (mitotic protein monoclonal 2; Upstate Biotechnology), and Bcl-xL. Primary antibodies were detected using goat α-mouse horseradish peroxidase-conjugated secondary antibody (Pierce) and enhanced chemiluminescence detection.

Kinase Assays. For each sample, 200 μg of total cell protein extract were precleared for 2 h at 4°C with 5 μg of mouse IgG prebound to protein A-Sepharose (Pharmacia Biotech). Precleared lysates were transferred to new microfuge tubes and incubated with α-cyclin B1 with mixing for 2 h at 4°C. Protein A-Sepharose was added, and the samples were mixed for 2 h at 4°C. The immunoprecipitates were washed twice with kinase lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton-X, 0.1% NP40, and 4 mM EDTA] and twice with kinase buffer [100 mM Tris (pH 7.4), 20 mM MgCl2, and 2 mM DTT] prior to incubation with 5 μg of histone H1 (Boehringer Mannheim) and 15 mM ATP for 10 min at 25°C. Samples were incubated with 2 μCi of [γ-32P]ATP at 30°C for 10 min; reactions were stopped by the addition of 2× Laemmli sample buffer and resolved by SDS-PAGE. Kinase assays were quantified using an Instant Imager (Packard Instrument) prior to autoradiography.

Centrifugal Elutriation. Two days before elutriation, HCT116 p21+/+ and p21+/− cells were transferred to 850 cm² roller bottles and grown in L-15 (Hyclone) supplemented with 10% fetal bovine serum. Centrifugal elutriation was performed using a Beckman JE-5.0 preparative scale rotor and model J6-MC centrifuge (Beckman Instruments), as described previously (28). The initial 1-liter
fraction was collected at a flow rate of 88 ml/min with an increase of 10 ml/min for each successive fraction. After the fraction collection, cells were pelleted at 1500 rpm using a JS-4.2 rotor in a J6-HC centrifuge (Beckman Instruments) at 4°C. The pellets were resuspended in PBS, and 1 × 10⁶ cells were aliquoted for flow cytometry. The remaining cells were processed for protein analyses.

RESULTS

Taxol and Vincristine Induce Endoreduplication in p21-deficient Cells. To determine whether the loss of G₁-S checkpoint function in p21-deficient cells would result in increased sensitivity to chemotherapeutic MTIs, we investigated the effect of Taxol and vincristine on HCT116 p21+/+ and HCT116 p21−/− cells, an isogenic pair of cell lines which differ only at the p21 locus (26). After 24 h of Taxol treatment, the HCT116 p21+/+ cells maintained a 4N DNA content, as assessed by flow cytometric analysis (Fig. 1A; only cells with ≥2N DNA content are shown). The accumulation of cells with a 4N DNA content was accompanied by significant elevations in both p53 and p21 protein levels by 18 h of treatment (Fig. 1B). In contrast, HCT116 p21−/− cells acquired a distinct 8N population by 48 h (Fig. 1A). Similar flow cytometric (Fig. 1A) and Western results (data not shown) were obtained when the cells were treated with vincristine.

p21-deficient Cells Exhibit Enhanced Sensitivity to MTIs. p21-deficient cells have enhanced chemosensitivity to DNA-damaging agents (8, 19, 20, 26), but sensitization to MTIs has not been observed (19). To further evaluate whether p21 status modulates cellular sensitivity to MTIs, we treated HCT116 p21+/+ and p21−/− cells with 10, 25, or 100 nm of Taxol or vincristine. The cells were analyzed by flow cytometry, and the percentage of cells with a subdiploid DNA content was used as an indicator of apoptosis (Fig. 1C). The increase in the percentage of the population with a subdiploid DNA content was accompanied by the appearance of cells with nuclear blebbing and chromatin condensation, as visualized by staining with 5 μm DAPI (data not shown). The HCT116 p21−/− cells had enhanced sensitivity to Taxol- and vincristine-induced apoptosis as compared with HCT116 p21+/+ cells at all doses evaluated (Fig. 1C).

To further examine the role of p21 in MTI chemosensitivity and to extend the studies performed in monolayer, the effect of MTI treatment on anchorage-independent growth of the HCT116 p21+/+ and p21−/− cells was assayed. In one set of experiments, HCT116 p21+/+ and p21−/− cells were grown in soft agar in the presence of increasing concentrations of Taxol or vincristine. As compared with the p21-containing cells, the p21-deficient cells had increased chemosensitivity to both Taxol and vincristine (Table 1). HCT116 p21−/− cells had a 11-fold reduction in colony formation at 5 nm Taxol and at least a 20-fold reduction in colony formation at doses ≥10 nm Taxol as compared with the HCT116 p21+/+ cells (Table 1). Similar results were observed with vincristine treatment, because p21-deficient cells had at least a 20-fold reduction in colony formation at all doses of vincristine evaluated as compared with the parental cell line (Table 1).

Table 1  Effect of MTIs on anchorage-independent growth of HCT116 p21+/+ and p21−/− cells

<table>
<thead>
<tr>
<th></th>
<th>% of control colony number&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>p21+/+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p21−/−&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Taxol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>5 nm</td>
<td>42.5 ± 4.4</td>
<td>4.0 ± 0.4</td>
<td></td>
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<tr>
<td>10 nm</td>
<td>9.0 ± 0.3</td>
<td>0.4 ± 0.2</td>
<td></td>
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<tr>
<td>25 nm</td>
<td>7.9 ± 1.3</td>
<td>0.4 ± 0.3</td>
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<tr>
<td>50 nm</td>
<td>8.0 ± 1.3</td>
<td>0.4 ± 0.1</td>
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<tr>
<td><strong>Vincristine</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
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<tr>
<td>10 nm</td>
<td>67.2 ± 9.6</td>
<td>3.0 ± 0.6</td>
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<tr>
<td>25 nm</td>
<td>24.6 ± 5.1</td>
<td>1.1 ± 0.4</td>
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<tr>
<td>50 nm</td>
<td>9.4 ± 3.1</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>100 nm</td>
<td>6.2 ± 2.0</td>
<td>0.8 ± 0.7</td>
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<sup>a</sup> Colonies were counted after 7 days.
<sup>b</sup> Values shown represent the mean ± SD of three dishes.

To test whether the enhanced MTI chemosensitivity of p21-deficient cells occurred in vivo, xenograft tumors comprised of the HCT116 p21+/+ and p21−/− cell lines were established in athymic nude mice.
When the tumors reached a mean size of 200 mm\(^3\), the mice received either vehicle (PBS), 5 mg/kg Taxol, or 10 mg/kg Taxol by i.p. injection on 5 consecutive days/week for 3 weeks. Consistent with the results of the soft agar assays, the p21 status of the tumors affected sensitivity to Taxol. After 28 days of treatment with the lower dose of Taxol (5 mg/kg), there was a 3-fold reduction in the HCT116 p21\(^{-/-}\) tumor volume as compared with the HCT116 p21\(^{+/+}\) tumors (Fig. 2). However, both the HCT116 p21\(^{+/+}\) and p21\(^{-/-}\) xenograft tumors displayed similar sensitivity to the higher dose of Taxol (10 mg/kg) over the time course (Fig. 2). The combined results of the soft agar and xenograft tumor analyses further demonstrate that HCT116 p21\(^{+/+}\) cells had enhanced chemosensitivity to low-dose treatments of MTIs.

**Molecular Determinants of Enhanced Sensitivity in p21\(^{-/-}\) Cells.** To gain insight to the differences in MTI sensitivity displayed by the HCT116 p21\(^{+/+}\) and p21\(^{-/-}\) cell lines, we examined the levels, phosphorylation status, and activity of proteins implicated in cell cycle checkpoint control and apoptosis after treatment with 25 or 100 nM Taxol. The HCT116 p21\(^{+/+}\) cells accumulated a persistent 4N DNA content, as assessed by flow cytometric analysis, after treatment with both doses of Taxol (25 nM, Fig. 1A; 100 nM, data not shown). Initially, 6–18 h after Taxol exposure, the p21-containing cells were in mitosis as assessed by a 5–7-fold increase in cyclin B1/Cdc2 kinase activity and elevation in MPM-2 epitope positivity (a marker of mitosis; Ref. 29; Fig. 3). After 18 h, there was a decrease in cyclin B1 protein levels, accompanied by a loss of cyclin B1/Cdc2 activity as cells exited mitosis and entered a G1 state. Entry into G1 was verified by loss of MPM-2 phosphoepitopes, modulation of cyclin E protein levels, and dephosphorylation of pRb at both doses of Taxol (Fig. 3).

The HCT116 p21\(^{-/-}\) cells accumulated a 4N DNA content by 24 h of Taxol treatment (25 nM, Fig. 1A; 100 nM, data not shown) and peak levels of cyclin B1/Cdc2 kinase activity between 6 and 18 h, similar to the HCT116 p21\(^{+/+}\) cells after 25 or 100 nM Taxol treatment (Fig. 3). However, between 28 and 72 h, a distinct 8N population accumulated in the p21\(^{-/-}\) cells (25 nM, Fig. 1A; 100 nM, data not shown). This endoreduplication correlated with biochemical reentry into G1, as assessed by modulation of cyclin E protein levels and loss of MPM-2 positivity at 32 h (25 nM) or 48 h (100 nM). pRb did not become dephosphorylated in the p21-null cells, a result consistent with a non-functional G1-S checkpoint. Furthermore, the continued cell cycle progression of the p21-deficient cells was consistent with the maintenance of cyclin B1 protein levels as the cells endoreduplicated (Fig. 3). Of note, it appeared that the length of the Taxol-mediated mitotic arrest was prolonged in the HCT116 p21\(^{-/-}\) cells, as assessed by MPM-2 positivity and elevated cyclin B1/Cdc2 activity (Fig. 3).

Previously, sensitivity to anticancer agents has been shown to be influenced by alterations in protein levels or posttranslational modifications of Bcl-2 family members, including the proapoptotic protein Bax and the antiapoptotic proteins Bcl-2 and Bcl-xL (30–34). In the HCT116 p21\(^{+/+}\) and p21\(^{-/-}\) cells, Bax protein levels did not change after exposure to either dose of Taxol (data not shown). After Taxol treatment, Bcl-2 and Bcl-xL phosphorylation were evident in both cell lines at 6 h, when cells first began to accumulate in mitosis.

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Fig. 3. Molecular determinants of MTI chemosensitivity. Asynchronous cultures of HCT116 p21\(^{+/+}\) and p21\(^{-/-}\) cells were treated with either 25 nM (A) or 100 nM (B) Taxol for the indicated times, and protein was harvested. Western analyses of cyclin B1, MPM-2, Bcl-2, Bcl-xL, cyclin E, and pRb were performed. Cyclin B1/Cdc2 kinase activity was also evaluated. Results are representative of three independent experiments.
and acquire cyclin B1/Cdc2 activity (Fig. 3). When peak levels of cyclin B1/Cdc2 activity were apparent at either 6 h (25 nM) or 18 h (100 nM), there was a significant conversion of Bcl-2 and Bcl-xL to the phosphorylated forms (Fig. 3). The protracted mitosis in the p21-deficient cells was accompanied by a longer duration of Bcl-2 and Bcl-xL phosphorylation. Subsequent loss of the phosphorylated forms of these proteins coincided with the mitotic exit and biochemical reentry of cells into G1, as evidenced by the loss of cyclin B1/Cdc2 activity and MPM-2 positivity (Fig. 3).

To further verify that the alterations observed in Bcl-2 phosphorylation were a reflection of cell cycle position and not directly causative in the increased chemosensitivity of the p21-deficient cells, HCT116 p21−/− cells were size fractionated by centrifugal elutriation. Relatively pure populations of untreated, rapidly dividing G1 and G2-M cells were obtained and analyzed for cyclin B1 protein levels, cyclin B1/Cdc2 activity, MPM-2 positivity, and alterations in Bcl-2 phosphorylation (Fig. 4). Fractions 1 and 2 from p21−/− cells contained a nearly pure 2N population of cells with little, if any, detectable cyclin B1 protein, cyclin B1/Cdc2 activity, or phosphorylated Bcl-2 (Fig. 4). Fractions 5–7 were comprised of cells with a predominant 4N DNA content, elevated cyclin B1 protein levels, and cyclin B1/Cdc2 activity (Fig. 4). The enhanced cyclin B1/Cdc2 activity correlated with cells that had entered mitosis, as evidenced by the increase in MPM-2 positivity (Fig. 4). Phosphorylated Bcl-2 was readily detectable in fractions 5–7 in both cell lines, indicating that Bcl-2 phosphorylation was present normally in mitosis during peak cyclin B1/Cdc2 activity (Fig. 4).

Ectopic Expression of p21 Protein in p53-deficient Cells Reduces Sensitivity to MTIs. We hypothesized that if the increased chemosensitivity to MTIs was a direct consequence of p21 loss, then ectopic expression of p21 protein in deficient cells should attenuate sensitivity. To test this hypothesis, we used the previously described HIp21 cell line (13). The HCT116 p21−/− cell line was not used for this experiment because of difficulties encountered in generating stable cell lines containing four selectable markers required to maintain both the p21-null phenotype and the two vectors necessary for inducible p21 expression. The HIp21 cell line was derived from the p53-deficient H1299 cell line and contains an ecdysone-inducible p21 expression vector. After treatment of HIp21 cells with the ecdysone analogue ponasterone, p21 protein levels increased (Fig. 5A), resulting in a G1-S arrest by 12 h that was maintained through 72 h of ponasterone treatment (Fig. 5B). HIp21 cells were treated with Taxol (100 nM) and vincristine (100 nM), both in the presence and absence of ponasterone, and evaluated by flow cytometric (Fig. 6A) and Western analyses (Fig. 6C). Ponasterone induction of p21 resulted in reduced sensitivity to both Taxol and vincristine, as demonstrated by maintenance of cells with a 4N DNA content and reduction in the number of cells with a subdiploid DNA content (Fig. 6A). Also, cells were stained with DAPI, and the percentage of apoptotic cells in the treated cultures was determined at 24 and 48 h (Fig. 6B). Ectopic expression of p21 resulted in a significant reduction of the percentage of apoptotic cells after treatment with both MTIs. Western analysis verified that ponasterone-induced p21 expression over basal levels correlated with the reduction in MTI-mediated cell death in these p53-deficient cells (Fig. 6C). As Blagosklonny et al. (35) have reported previously, there was a p53-independent increase in p21 protein levels after Taxol or vincristine treatment of the HIp21 cells in the absence of ponasterone (Fig. 6C), although this elevation in endogenous p21 protein was below a threshold level required to reduce MTI-induced apoptosis in our system (Fig. 6, A and B). However, elevation of p21 above basal levels, through ectopic expression, resulted in partial restoration of G1-S checkpoint function and reduced sensitivity of the cells to
MTI-induced apoptosis as compared with the noninduced cells (Fig. 6, A and B). Similar to the results obtained with the HCT116 p21+/+ and p21−/− cells, Bcl-2 phosphorylation status was not a determinant of differential sensitivity in the HIp21 cells (Fig. 6C).

DISCUSSION

In the present study, we demonstrated that loss of p21 resulted in increased sensitivity of tumor cells, both in vitro and in vivo, to the cytotoxic effects of MTIs commonly used in cancer therapy. Furthermore, we were able to show that induction of ectopic p21 protein in p53-deficient cells significantly reduced MTI-induced apoptosis. Our findings are consistent with several recent studies that support a role for p21 as an important determinant of anticancer drug sensitivity.

Taxol treatment of MCF-7 breast carcinoma cells resulted in abnormal mitotic exit and subsequent p21 elevation (36). Moreover, reduction of p21 levels through the use of p21 antisense oligonucleotides in MCF-7 cells resulted in enhanced apoptosis after Taxol exposure (36). MDA-MB-435 breast carcinoma cells overexpressing ErbB2 were found to be resistant to Taxol-induced apoptosis, and the resistance was correlated with ErbB2 up-regulation of p21 (37). Also, ErbB2-overexpressing MDA-MB-435 cells expressing antisense p21 were sensitized to Taxol-induced apoptosis (37), although the authors did not examine whether mitotic slippage and endoreduplication occurred under these conditions. Alternatively, our results differ from a previous study reporting that p21 deficiency in HCT116 cells and mouse embryo fibroblasts did not alter chemosensitivity to MTIs (19). In this latter study, chemosensitivity was determined using a monolayer clonogenic survival assay. Subsequent studies have shown that anchorage-dependent assays may fail to predict chemosensitivity of p21-deficient cell lines in vivo (20, 21).

The levels and phosphorylation status of several proteins involved in cell cycle regulation and apoptosis were examined in the present study, with the intent of identifying a molecular determinant(s) that correlated with sensitization to MTIs. It has been proposed that Taxol-induced Bcl-2 or Bcl-xL phosphorylation may inactivate these antiapoptotic proteins and thus mediate apoptosis (31–34, 38). However, we found that Bcl-2 and Bcl-xL phosphorylations were modulated similarly in the p21-containing and p21-deficient cells and that Bcl-2 phosphorylation occurred during mitosis. Consistent with these results, it has been shown previously that Bcl-2 phosphorylation was not a determinant of Taxol-induced apoptosis in either MDA-MB-435 cells overexpressing ErbB2 (37) or HeLa cells (39); rather, Bcl-2 phosphorylation reflected cell cycle arrest in mitosis (28, 39, 40).

MTI clinical efficacy has been correlated with alterations of tubulin dynamics and cell cycle position (41–44). However, it remains unclear how the MTI-induced changes in microtubule dynamics result in cell death. Several studies suggest that the mitotic arrest mediated by MTIs results in cytotoxicity through alteration of normal mitotic signal transduction pathways, including prolonged cyclin B1/Cdc2 kinase activity (28, 45), and there are numerous studies that suggest a link between Cdc2 activation and apoptosis (46–50). In the present study, both the p21+/+ and p21−/− cells had prolonged mitotic arrest and cyclin B1/Cdc2 activity after 100 nM Taxol treatment. Thus, the longer duration of cyclin B1/Cdc2 activity in the p21-deficient cells may be an important determinant in the observed dose-dependent sensitivity to MTI-induced apoptosis.

The majority of human solid tumors are genetically unstable (51) and have defects in their cell cycle checkpoint control mechanisms (52). Proteins that function at the interface between cell cycle pro-
gression and control of apoptosis likely modulate the sensitivity of cells to chemotoxic agents. Several studies, including the present one, suggest that the proteins that control G1-S checkpoint are important determinants of chemosensitivity. Our observation that G1-S checkpoint-deficient cells are sensitized to lower doses of MTIs may explain some of the present success of MTIs in the treatment of a wide range of solid tumors (53), as well as provide the basis for further design of chemotherapeutic strategies that exploit the alterations in cell cycle checkpoint pathways present in tumor cells. Finally, our results suggest that therapeutic interventions aimed at restoring G1-S checkpoint activity in tumor cells may, in parallel, reduce sensitivity to subsequent anticancer agents.

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REFERENCES

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