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Gene Expression and Mitotic Exit Induced by Microtubule-Stabilizing Drugs

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ABSTRACT

To explore the molecular mechanisms underlying the actions of Taxol and the functionally related molecule epothilone B (EpoB), we have analyzed the gene expression profiles in A549 cells in response to increasing concentrations of these microtubule-stabilizing drugs. An almost identical expression pattern was observed in cells treated with either Taxol or EpoB. Low concentrations of the drugs induced aberrant mitosis including asymmetric and multipolar cell divisions. At drug concentrations that trigged G_2-M arrest, cells escaped from a prolonged mitotic arrest without cell division, resulting in tetraploid G_1 cells. This mitotic slippage is correlated with diminished expression of cdc2 kinase, topoisomerase IIα, BUB3, and BUB2-like protein 1, as well as with an increased expression of 14-3-3-σ. Poly(ADP-ribose) polymerase cleavage, an early indicator of apoptosis, occurred in cells undergoing mitotic slippage and in aneuploid cells resulting from aberrant mitosis. In contrast, cells arrested in mitosis demonstrated no signal for apoptosis but had an increased expression of survivin, an inhibitor of apoptosis. Induction of aneuploid or tetraploid G_1 cells was accompanied by increased expression of CD95, p21, and BTG2 that may contribute to cell death because their expression was diminished in an EpoB-resistant cell line. In contrast, expression of GADD45 and PTGF-β could promote cell survival. We conclude that abnormal mitotic exit is required for apoptotic cell death induced by microtubule-stabilizing drugs.

INTRODUCTION

Taxol has been approved by the United States Food and Drug Administration for treatment of ovarian, breast, and lung carcinomas and is used extensively for the treatment of human malignancies. The drug binds to β-tubulin and stabilizes microtubules (1, 2), thereby repressing dynamic instability of spindle microtubules (3) and inhibiting mitosis. Taxol-induced cytotoxicity correlates with selective perturbation of mitosis rather than gross changes in interphase microtubule arrays (4). At concentrations of drug less than micromolar, apoptosis, but not necrosis, is the mechanism responsible for cell death (5). Taxol-induced apoptosis has been associated with two different forms of cell cycle arrest (6, 7). Arrest in prometaphase was suggested to trigger a rapid cell death independent of p53. In contrast, those cells that pass through mitosis are arrested in the subsequent G_1 phase by a p53-dependent mechanism (8). It is not clear, however, whether mitotic arrest or a G_1 arrest is the major mechanism responsible for Taxol-induced apoptosis (9).

At concentrations less than that required for a G_2-M block, Taxol induced the formation of multipolar spindles and an aneuploid G_1 population of cells (10). The aneuploid G_1 cells may result from aberrant mitosis and die slowly of apoptosis. In human lung carcinoma A549 cells, inhibition of cell proliferation by low concentrations of Taxol is accompanied by an increase in p53 and p21 levels (11, 12). The tumor suppressor gene p53 restricts propagation of damaged cells by arresting the cell cycle and/or by induction of apoptosis (13, 14). In contrast, p53 could also contribute to the repair of genotoxic damage (15). p53 acts as a transcription factor and mediates its effect by modulating the expression of its downstream target genes. However, the nature of the p53 response in diverse mRNA species depends on the level of p53 protein, the type of inducing agent, and the cell type used (16, 17).

As the drug concentration is increased, cells become arrested at mitosis. However, mitotic slippage may occur when the mitotic spindle checkpoints fail to sustain mitotic arrest and the cells exit from mitosis in the absence of cytokinesis (18, 19). It is known that rodent cell lines escape mitosis easily when spindle assembly is disrupted. In contrast, human cell lines may remain permanently blocked in a mitotic state (20). Nevertheless, human cancer cell lines may have acquired the ability to escape from mitotic block that is induced by microtubule-stabilizing drugs. For example, in MCF-7 cells, the mitotic block induced by 100 nM Taxol is not sustained, and mitotic exit occurs without completion of late mitotic events, such as chromosome segregation and cytokinesis (21). Mitosis is controlled by cdc2 kinase that forms a complex with cyclin B1, thereby promoting chromosome condensation. Consequently, inactivation of cdc2 is responsible for mitotic exit including mitotic slippage (22). However, the detailed molecular mechanisms responsible for mitotic escape remain to be determined.

The low aqueous solubility of Taxol and the development of drug resistance, often by a P-glycoprotein mechanism, have led to a search for new compounds with Taxol-like activity. EpoB,[3] derived from a myxobacterium fermentation broth, is undergoing Phase I clinical trails as an anticancer agent. Like Taxol, it induces tubulin polymerization in the absence of GTP, and it causes microtubule stabilization and bundling (23, 24). However, epothilone has a distinct chemical structure that is less hydrophobic than Taxol. Epothilone is not a substrate for P-glycoprotein and there is evidence to suggest that it is active in Taxol-resistant cell lines and tumors (23, 25). To determine whether epothilone kills cells with a mechanism of action similar to that of Taxol, we compared the overall gene expression profiles induced by increasing concentrations of EpoB and Taxol. Analysis of expression profiles in a drug-resistant cell line demonstrated that microtubule stabilization induced both cell death genes whose expression was reduced and cell survival genes whose expression was elevated.

MATERIALS AND METHODS

DNA Microarray Screening. Human non-small cell lung carcinoma A549 cells were cultured in RPMI 1640 containing 1% penicillin-streptomycin and 10% fetal bovine serum. Subconfluent cells were treated with the indicated concentrations of Taxol and EpoB for 18 h. Total RNA was extracted from cells, and cDNA was synthesized. cRNA was then produced by *in vitro* transcription using Bioarray High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, Inc.). Fragmented cRNA, produced by heating at 94°C for 30 min, was hybridized to Affymetrix high-density U95A gene chips that contained approximately 12,000 probe sets, and signals were acquired from laser scanning. Average expression levels were calculated from two independ-

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[3] The abbreviations used are: PARP, poly(ADP-ribose) polymerase; EpoB, epothilone B; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole.

ent experiments. Genes with a >2-fold change after any drug treatment were selected and combined into a single table by Access's UNION function that removes duplicate genes automatically. A combined data query was exported to Gene Cluster (Stanford) to generate a tree view of the selected genes by the hierarchical clustering method.

Real-Time RT-PCR. cDNA was synthesized from total RNA by the random primer method (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen). A control sample also was produced in the absence of reverse transcriptase. Gene-specific primers were designed and optimized by PrimerExpress (Applied Biosystems). The primer sequences and their locations in the gene can be found online.[4] Real-time RT-PCR was performed using ABI 7900 system (Applied Biosystems) and monitored based on SYBR Green I dye detection (26). After completion of the RT-PCR assay, reaction products were analyzed by gel electrophoresis to confirm the specific amplification of one single band with the predicted size. Gene expression levels, normalized to the reference (GAPDH) and relative to that in untreated cells, were calculated by comparative C_T method (26). C_T is the PCR cycle number at which the fluorescence signal grows beyond the value of the threshold setting.

PARP Cleavage, Protein Levels, and Cell Cycle Analysis. Cell lysates prepared from A549 cells were resolved by SDS-PAGE. Protein levels of p53 and the Ser-15-phosphorylated form of p53 were determined by Western blot analyses using antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Beverly, MA), respectively. Antibodies for cdc2 (Neo Markers, Fremont, CA), p21 (Santa Cruz Biotechnology), and survivin (NOVUS Biologicals, Littleton, CO) were used to determine the corresponding protein levels of these genes in total cell lysates. PARP cleavage was determined by using monoclonal anti-PARP antibody that recognized the NH_2-terminal 24-kDa cleavage product of PARP (Transduction Laboratories) or by using anti-PARP 85 fragment polyclonal antibody that only recognized the COOH-terminal 89-kDa cleavage product (Promega, Madison, WI). Cell cycle analysis was performed by flow cytometry using propidium iodide staining of DNA content as described previously (10).

Time-Lapse Video Microscopy. Subconfluent A549 cells were cultured in T75 flasks. Taxol, EpoB, or DMSO was added to the cells at the indicated final concentrations before recording. Cells in sealed flasks were imaged on an Olympus IX70 (Melville, NY) with a ×10 UplanFl objective with phase contrast. Temperature control at 37°C was provided by both a heated Plexiglas chamber around the microscope and a heated stage (Olympus). Images were collected with a Sensys cooled charge-coupled device camera (Roper, Tucson, AZ) controlled by IPLab Spectrum software (Scanalytics, Fairfax, VA) running on a Macintosh G3 (Apple, Cupertino, CA). Figures for publication were made with Adobe Photoshop 7.0 running on Windows XP on a Dell PC. Time-lapse movies for the published sequential images are available from the authors or can be found online.[4]

RESULTS

Gene Expression Profiles in Response to Taxol and EpoB. To explore the mechanism of cell death induced by low concentrations of microtubule-stabilizing drugs, we have used oligonucleotide microarrays (Affymetrix) to study mRNA profiles in A549 cells. Cells were treated for 18 h with Taxol (0, 4, 8, 16, and 45 nM) or EpoB (0, 2.4, 5, 10, and 40 nM). Based on observations from cell cycle analysis, the cells were treated first with a dose that induced minimal changes in the cell cycle. The concentration was increased to that which induced 80% of the cells to accumulate at G_2-M (4N DNA; Ref. 10). A total of 81 gene sets were selected whose expression, at least in one treatment, changed by >2-fold. Hierarchical clustering analysis found two major groups of genes that were either up- or down-regulated by the drugs. Although we did not intend to select commonly regulated genes by Taxol and EpoB, a marked similarity was found. A gene whose expression was inhibited by Taxol was also down-regulated by EpoB. Most of the up-regulated genes demonstrated a similar pattern of regulation by Taxol and EpoB (Fig. 1). This indicated that despite differences in the chemical structures of the two drugs, they have a similar mechanism of action in A549 cells. Because the microtubule is the common target of these two drugs, the results suggest that alterations in gene expression come mainly from perturbation of the microtubule network.

Within the up-regulated group, a subset of genes was increased by low concentrations of Taxol or EpoB, but not by 40–45 nM concentrations of the drugs that induced G_2-M arrest (Fig. 1). The genes that were up-regulated are known p53-targeted, stress response genes. The expression of these genes was validated by real-time RT-PCR (Fig. 2*A* and Table 1). Gene p21 is a direct target of p53 and an inhibitor of cyclin-dependent kinase 2 and cyclin-dependent kinase 4, which are required for G_1-S progression. In support of previous observations (11, 12, 27), p21 is increased significantly by 8 nM Taxol or 10 nM EpoB. These concentrations of drug also increased expression of BTG2 (Fig. 2*A*), an antiproliferation gene that inhibits cyclin D1 transcription (28). However, the expression of p21 and BTG2 in cells treated with 45 nM Taxol or 40 nM EpoB was only slightly higher than that in the untreated cells. The dose-response curves for activation of these G_1 checkpoint genes (Fig. 2*A*) support a proposal that cell cycle progression through mitosis is required for cells arrested at G_1 (6).

CD95 (Fas receptor) is an apoptosis-signaling molecule that is activated by p53 in response to DNA-damaging anticancer drugs (29, 30). Expression of CD95 was increased approximately 3-fold in cells treated with 8 nM Taxol or 10 nM EpoB, compared with the untreated control (Fig. 2*A*). CD95 expression was determined by two different sets of primers[4] that gave similar results. In human non-small cell lung cancer cells, exogenous Fas ligand greatly enhanced Taxol-induced apoptosis (31), although no significant Fas ligand up-regulation was observed (32). However, anticancer drugs may activate the Fas ligand-independent, Fas-associated death domain-mediated death pathway by activating downstream caspases (33). Alternatively, Taxol has been reported to induce slow (nonapoptotic) cell death (34). We have monitored PARP cleavage in drug-treated cells. PARP is one of the earliest proteins targeted for specific cleavage during apoptosis (35). PARP cleavage products (p24 and p85) increased in cells treated with 8 nM Taxol and 10 nM EpoB (Fig. 2*B*). Interestingly, PARP cleavage was reduced at 24 nM EpoB and increased again in cells treated with 40 nM EpoB (Fig. 2*C*). The two phases of increase in PARP cleavage indicate that apoptosis could be induced by different mechanisms, depending on drug concentration. Low concentrations of the drugs also caused an increase in p53 protein (Fig. 2*B*), as reported previously (11, 12).

Aberrant Mitosis and Mitotic Slippage. To understand the different mechanisms that trigger apoptosis, we have monitored, at each concentration, approximately 100 A549 cells by time-lapse video microscopy. Control cells with no added drug rounded up at the beginning of mitosis and split into two symmetrical daughter cells as expected (Fig. 3*A*, *control*). In contrast, aberrant mitosis occurred in cells treated with low concentrations of drug. For example, approximately 25% of cells treated with 10 nM EpoB divided into three daughter cells. Even though bipolar cell division can be found in cells treated with 10 nM EpoB, many drug-treated cells result in unequal cell division (Fig. 3*A*, *EPO10*). When A549 cells were incubated with 40 nM EpoB, cells rounded up, entered mitosis, and remained there for more than 15 h without cell division or cell death taking place. Then the cells spread and appeared as large flattened interphase cells (Fig. 3*A*, *EPO40*). No obvious cell division occurred at this stage. Similar observations from time-lapse video were obtained for cells treated with 45 nM Taxol (Fig. 3*A*, *TX45*). Cells treated with 45 nM Taxol were stained with DAPI and examined by microscopy. Cells with strong DAPI staining were significantly increased after an 18-h incubation with 45 nM Taxol. However, 46% of the cells did not have

[4] Internet address: http://www.aecom.yu.edu/aif/users/sbh/jgchen/index.htm.

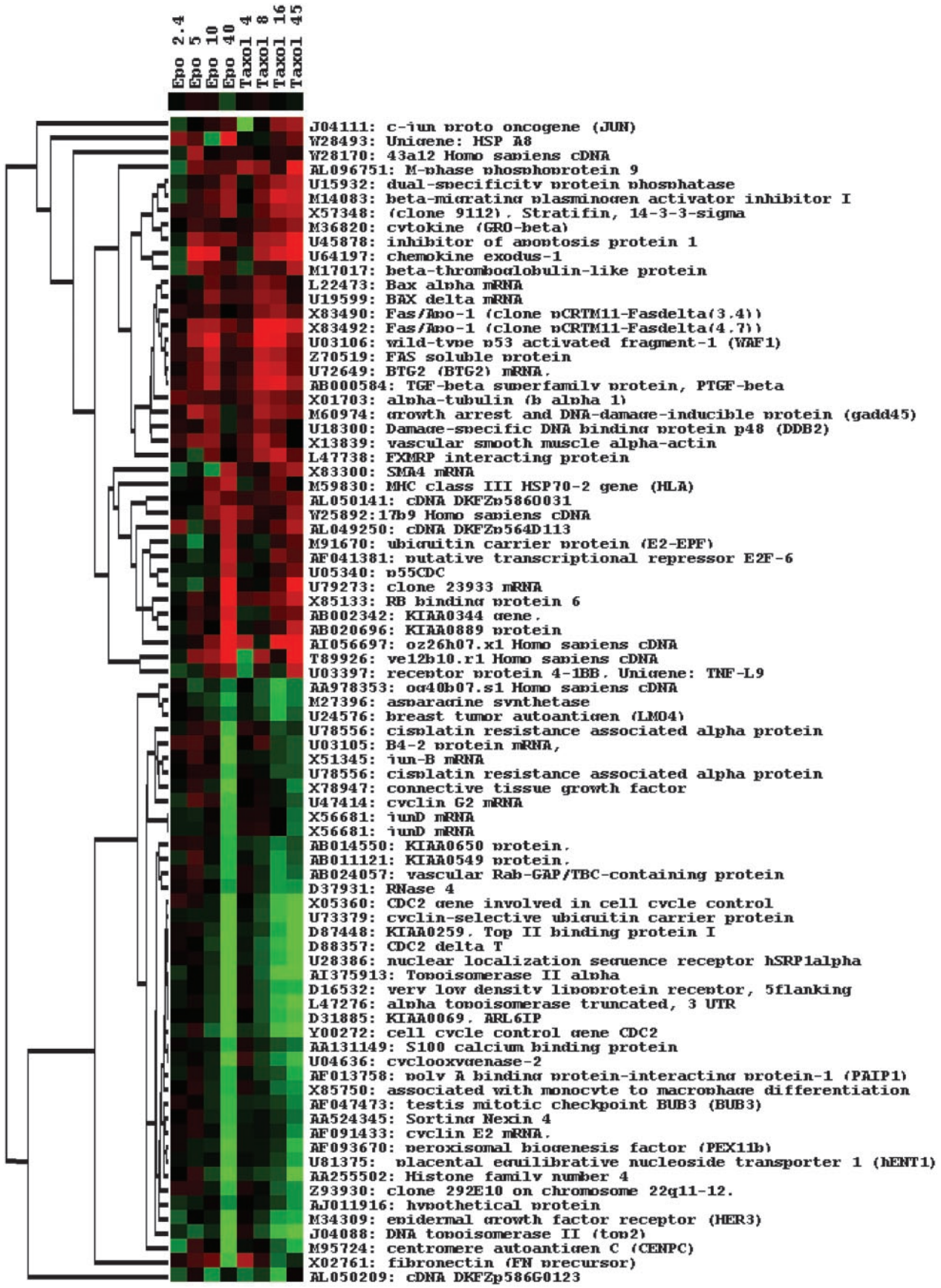

Fig. 1. Gene expression profiles of A549 cells treated with different nanomolar concentrations of Taxol and EpoB for 18 h. Figure shows hierarchical clustering analysis of 81 genes. Each *row* represents a single gene, and each *column* represents the average of two independent experiments. *Green squares* indicate underexpressed genes, and *red squares* represent overexpressed genes in drug-treated cells compared with untreated cells. *Black* indicates genes with equivalent expression levels in drug-treated and control cells. *Tree branches* represent the correlation between genes based on their expression data.

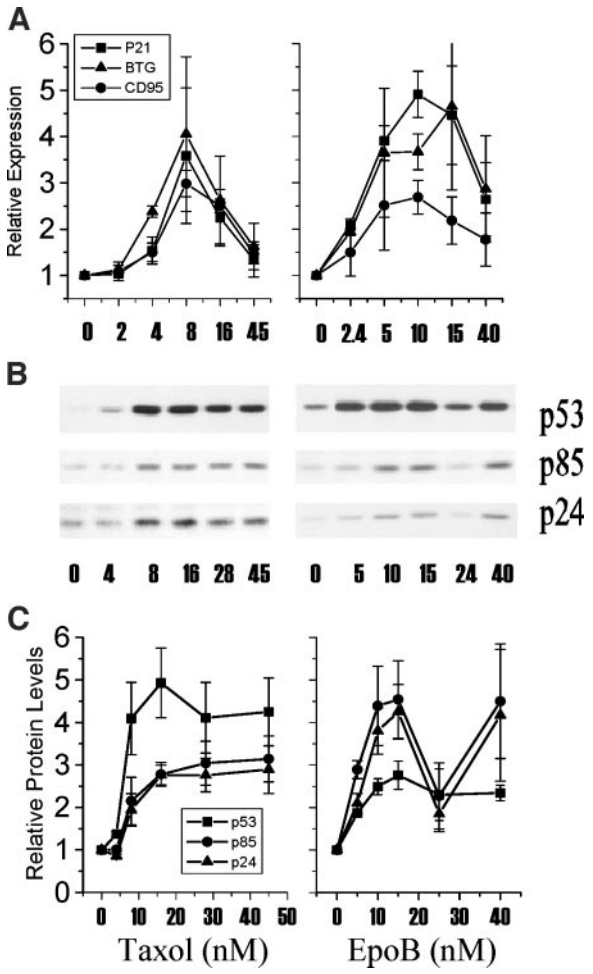

Fig. 2. *A*, regulation of p21, BTG2, and CD95. A549 cells were treated with the indicated nanomolar concentrations of Taxol or EpoB for 18 h. Expression of p21, BTG2, and CD95 was determined by real-time RT-PCR as described in "Materials and Methods." The expression level, normalized to GAPDH, was calculated as the fold change in expression compared with untreated cells. Results are mean ± SD from three experiments. *B*, PARP cleavage products in Taxol- and EpoB-treated cells. Total cell lysates from drug-treated cells were separated by electrophoresis and transferred to nitrocellulose membranes. PARP cleavage, identified by its NH_2-terminal 24-kDa and COOH-terminal 89-kDa cleavage products, and p53 levels were determined by Western blot analyses as described in "Materials and Methods." The data shown are from a representative experiment. *C*, p53, p85, and p24 were image quantitated and normalized to untreated cells. Data are expressed as relative levels with SDs ($n = 2$–4).

condensed chromosomes (Fig. 3*B*), despite the fact that close to 80% of cells had 4N DNA content as measured by flow cytometry (data not shown). This is identical with a previous observation that most A549 cells accumulated at G_2-M in response to 100 nM Taxol, but only 48% of cells displayed condensed chromosomes (12). This suggests that not all cells with 4N DNA were mitotic cells. DAPI staining in most cells had a reduced intensity after a prolonged (30 h) incubation (Fig. 3*B*). These studies established that A549 cells eventually escaped from drug-induced mitotic arrest, in the absence of chromosome separation and cell division.

Mitotic Exit and Apoptotic Cell Death. To determine the importance of mitotic exit for drug-induced apoptosis, we compared PARP cleavage in A549 cells treated with EpoB in three different ways. First, treatment with 10 nM EpoB for 18 h induced aberrant mitosis that resulted in a large population of aneuploid G_1 cells (Fig. 4*A*, *b*). Second, cells were treated with 40 nM EpoB for 18 h, and mitotic cells were collected by shaking off the unattached, rounded up cells (Fig. 4*A*, *c*). Third, after a 30-h incubation with 40 nM drug, most cells had escaped from mitotic arrest. After washing off the small percentage of the remaining rounded-up cells in the medium, the adherent cells were collected for cell cycle analysis. As expected, these cells had a 4N DNA content but had a reduced cyclin B1 level compared with the control, indicating that these were pseudo-G_1 cells arising from mitotic slippage (Fig. 4*A*, *d*; Fig. 4*B*, *d*). PARP cleavage products, p24 and p85, were markedly increased in the pseudo-G_1 cells compared with the control (Fig. 4*B*, *a* and *d*). Cells with an aneuploid population showed limited PARP cleavage (Fig. 4*B*, *b*). Surprisingly, mitotic cells demonstrated little if any PARP cleavage (Fig. 4*B*, *c*), despite the fact that they were elicited with a higher concentration of drug, compared with aneuploid cells that resulted from aberrant mitosis. Analysis of DNA isolated from the above four sets of cells indicated that the pseudo-G_1 cells produced significantly more internucleosomal DNA fragments of approximately 200-bp multiples than the control cells, aneuploid cells, or mitotic cells (data not shown). Consistent with the absence of apoptosis in mitotic cells, survivin was dramatically increased in these cells (Fig. 4*B*). Survivin functions as a regulator of spindle microtubules and is an inhibitor of apoptosis protein. The protein is phosphorylated at Thr-34 by mitotic kinase cdc2, leading to stabilization of survivin (36). cdc2 was increased in mitotic cells but diminished in cells that had undergone mitotic slippage (Fig. 4*B*). Mitotic cells induced by 45 nM Taxol also demonstrated increased levels of survivin and cdc2 and little PARP cleavage (data not shown). In addition, the number of adherent cells after a 30-h incubation with 40 nM EpoB or Taxol was similar to the number of cells before drug treatment. When treated with 400 nM EpoB for 30 h, attached cells still accounted for 80% of the original cell population. This further suggests that minimal cell death occurred in cells temporarily arrested in mitosis.

In contrast to the differential effects on PARP cleavage, all three groups of cells have increased expression of p53 compared with the control. Phosphorylation of Ser-15 is partly responsible for the stabilization and therefore the increase in p53 protein because p53 and phospho-p53 (Ser-15) antibodies both detected an increase in protein (Fig. 4*B*). Preincubation with a peptide containing Ser-15 of p53 blocked all signals from the Ser-15 antibody (data not shown), indicating a specific detection of the Ser-15 phosphorylation by the antibody. The results support a previous observation that Taxol at 100 nM induced p53 phosphorylation at Ser-15 and Thr-18 (37). At low concentrations (10 nM), the drugs suppressed microtubule dynamics and enhanced microtubule-dependent trafficking that brought about a fast equilibrium of p53 between nuclei and cytosol (38). Phosphorylation of p53 may also cause the protein to be trapped in the nuclei (39). An increase in p53 protein in nuclei will activate p53-targeted genes, including G_1 checkpoint genes. For example, p21 is slightly increased in aneuploid cells and markedly induced in cells having undergone mitotic slippage (Fig. 4*B*). However, no p21 is induced in mitotic cells despite an elevated level of p53. Although it is not clear

Table 1 *Alterations (fold change) in gene expression in response to EpoB*

Expression of p53-targeted genes was measured by real time RT-PCR using SYBR green I. Relative gene expression, compared to the A549 control, was determined in cells treated with 40 nM EpoB for 30 h (1), 10 nM EpoB for 18 h (2), or in EpoB480 cells that are resistant to 480 nM EpoB (3). The suggested gene function is shown in 4.

Genes	1 EpoB40/A549	2 EpoB10/A549	3 EpoB480/A549	4 Function
p21	4.19 ± 0.27	4.91 ± 0.50	0.22 ± 0.01	Death
BTG2	3.35 ± 0.29	3.67 ± 0.39	0.11 ± 0.01	Death
CD95	4.33 ± 0.12	3.69 ± 0.36	0.04 ± 0.01	Death
PTGF-β	3.64 ± 0.99	3.69 ± 0.13	3.23 ± 0.33	Survival
GADD45	2.98 ± 0.13	4.36 ± 0.52	6.56 ± 4.41	Survival
Bax	1.39 ± 0.17	1.57 ± 0.08	0.63 ± 0.25	
DDB2	1.13 ± 0.33	2.94 ± 1.65	0.22 ± 0.03	
p53	0.35 ± 0.02	1.36 ± 0.68	0.02 ± 0.02	

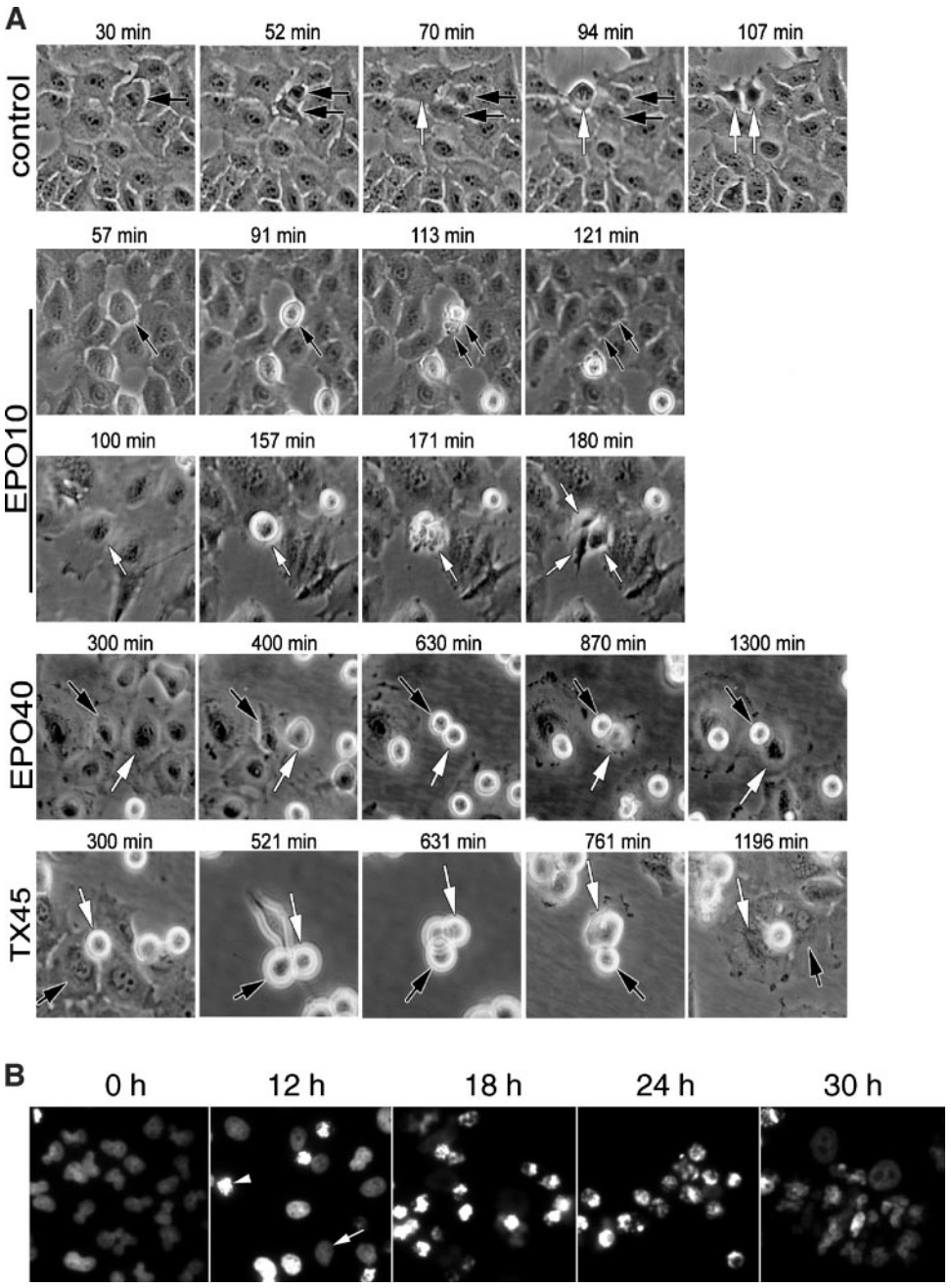

Fig. 3. *A*, time-lapse microscopy. Subconfluent cells incubated with 0, 10, or 40 nM EpoB or 45 nM Taxol were examined by phase-contrast microscopy (see "Materials and Methods"). Sequential imagines from *left* to *right* were taken at the indicated times after drug treatment. Parental cells and their corresponding offspring are marked by the same *arrow*. Cells treated with 10 nM EpoB underwent asymmetric (*Epo10 top row*) and tripolar cell division (*Epo10 bottom row*). Cells treated with 40 nM EpoB or 45 nM Taxol did not divide but escaped from a prolonged mitotic arrest. *B*, DAPI staining of Taxol-treated cells. Cells were treated with 45 nM Taxol for 0, 12, 18, 24, or 30 h and then fixed with 3.7% formaldehyde added directly to the culture medium. After fixing for 15 min, cells were stained with DAPI for 30 min and examined by microscopy. The *arrowhead* points to a highly stained mitotic cell, and the *arrow* depicts an interphase cell with much less staining.

why p53 does not induce p21 in mitotic cells, the result is consistent with the function of p21 as a G_1 checkpoint gene.

Death and Survival Genes. The molecular basis for the induction of apoptosis in aneuploid cells and in cells that have undergone mitotic slippage is not clear. Activation of p53 modulates the expression of multiple genes, and some may participate in different cellular processes (40). From gene expression profiles, we found a group of p53-targeted genes that were increased in cells treated with low concentrations of EpoB and Taxol for 18 h. The expression of p53-targeted genes was confirmed by real-time RT-PCR in cells treated with 10 nM EpoB for 18 h (Table 1). This group of genes is also up-regulated in cells treated with 40 nM EpoB for 30 h (Table 1), suggesting that tetraploid G_1 cells derived from mitotic slippage have at least a partial molecular fingerprint that is similar to that of aneuploid G_1 populations resulting from aberrant mitosis.

Not all of the differentially expressed genes induced by the drugs function as death machinery. To evaluate functional significance of the induced genes, we have compared their expression in sensitive cells treated with 10 nM EpoB with that in the highly EpoB-resistant cell line A549.EpoB480 that was created by stepwise selection in EpoB. At each step, the cells were challenged with the drug at a concentration far below that needed for G_2-M arrest. Genetic alterations in the cells must provide them with the necessary growth advantage against aberrant mitosis induced by low concentrations of the drug. Nonessential alterations, or ones that would lead to cell death, would be lost during the development of the resistant cells. The cell line is cultured routinely in the presence of 480 nM EpoB and is 900-fold resistant to the drug compared with the parental cells. Expression of p21, BTG2, and CD95 in parental A549 cells was increased by treatment with 10 nM EpoB for 18 h. In contrast, these genes were markedly reduced in the resistant cells compared with the sensitive cells (Table 1). Genes that are depressed in drug-resistant

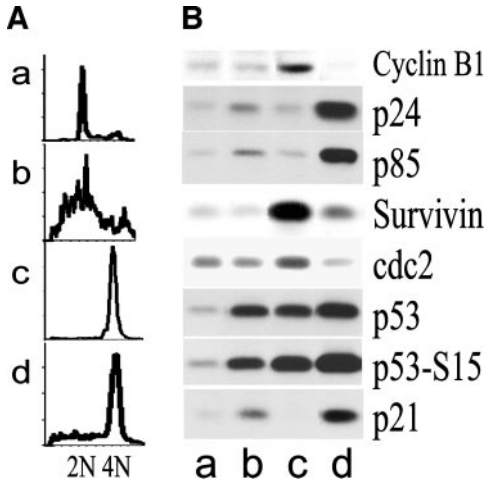

Fig. 4. *A*, cell cycle analysis. A549 cells were treated with no drug (*a*) or 10 nM EpoB for 18 h (*b*). Detached cells were collected from the cell culture medium after treatment with 40 nM EpoB for 18 h and designated as mitotic cells (*c*). Adherent cells were obtained after treatment with 40 nM EpoB for 30 h (*d*). The cells were fixed in 75% ethanol and stained with propidium iodide, and cell cycle profiles were determined by flow cytometry. *B*, comparison of protein levels after different drug treatments. Populations containing aneuploid cells (*b*), mitotic cells (*c*), and pseudo-G_1 cells (*d*) were compared with the untreated control (*a*). Cyclin B1, PARP cleavage products p24 and p85, survivin, cdc2, p53, phospho-p53 (Ser-15), and p21 were determined by Western blot analysis.

cells but increased by the drugs in the sensitive cells should contribute to drug-induced cell death. Therefore, activation of G_1 checkpoint genes and the death receptor may limit cell proliferation and contribute to apoptotic cell death in the aneuploid cells.

On the contrary, adaptive changes may occur that provide cells with resistance to the drugs, and these changes may persist in the resistant cell line. The genes that are activated both in drug-treated cells and in resistant cells can be considered as survival mechanisms. PTGF-β and GADD45 were increased in the sensitive cells after drug treatment, as well as in the resistant cells, when compared with the untreated sensitive cells (Table 1). Both genes could be activated by p53 (17, 41). However, p53 protein was diminished in the resistant cells (data not shown), suggesting that in these cells, the genes may be activated by means other than p53. PTGF-β, a new member of type β transforming growth factor superfamily, is a potent growth inhibitor with tumor suppressing activity (42). Consistent with its protective role, it was found recently that both apoptosis and inhibition of proliferation in response to DNA damage decreased as a function of transforming growth factor β1 gene dosage in embryonic epithelial tissues (43). GADD45 is a G_2 checkpoint gene and also assists in DNA repair processes (44). EpoB may be accumulated in nuclei (45), suggesting a possible DNA-damaging effect of the drug. GADD45 activation would help to protect the genetic integrity of the drug-treated cells.

Mechanism of Mitotic Slippage. Multiple mitosis-related genes, including genes involved in the G_2-M transition and spindle checkpoint, were altered by 40 nM EpoB and 45 nM Taxol after an 18-h incubation (Fig. 1). Considering that a prolonged mitotic arrest occurred before mitotic slippage (Fig. 3, *EPO40* and *TX45*), we have determined the time course of gene expression by real-time RT-PCR (Fig. 5). Expression of cyclin B-dependent kinase cdc2, whose activation is essential for mitosis, was increased after a 12-h incubation with 40 nM EpoB, at a time when mitotic cells were accumulating. cdc2 mRNA had decreased by 18 h of drug treatment and was $<$20% of its control level after 35 h (Fig. 5). cdc2 protein level followed a similar pattern and decreased dramatically after 35 h of incubation with the drug (data not shown), at a time when most cells escaped from mitotic arrest. The same drug treatment caused a decrease in the mRNA level of topoisomerase IIα but an increase in 14-3-3-σ. Topoisomerase II helps to bring about a high order of compaction of chromatin to form condensed mitotic chromosomes, whereas 14-3-3-σ anchors cdc2 in the cytoplasm, where it cannot induce mitosis (46). Therefore, reduction of topoisomerase II and cdc2, as well as increased expression of 14-3-3-σ, supports mitotic exit. It must be emphasized, however, that gene expression was compared with that in untreated controls, where the majority of cells were in a normal G_1 phase. Therefore, these changes in gene expression were not due to a normal mitotic exit but reflect mitotic slippage. In addition, spindle checkpoint genes BUB3 and BUB2-like protein 1 were reduced by the drug treatment (Fig. 5). BUB3 localizes to unattached kinetochores in lagging chromosomes and delays the onset of anaphase in cells treated with Taxol (47). Checkpoint genes are required to delay cell division in response to spindle damage in the yeast *Saccharomyces cerevisiae* (48). Down-regulation of spindle checkpoint genes would make it possible for mitotic cells with damaged spindles to escape from mitosis.

DISCUSSION

Previous studies have found that Taxol induced mitotic arrest and also caused a G_1 block in those cells that passed through mitosis (6, 7). However, no attempt was made to dissect the pathway leading to G_1 arrest. In addition, aberrant mitosis can be confused with mitotic slippage (8). In this study, we have directly observed two different forms of mitotic exit in the same cell line, depending on the concentration of drug. Aberrant mitosis, as we discuss here, represents abnormal mitosis due to formation of multipolar spindles that result in unequal distribution of DNA into daughter cells (10, 11, 49). Aberrant mitosis leads to aneuploid G_1 cells with loss or gain of individual chromosomes. This may also result from incomplete congression of the chromosomes. As the drug concentration increases, cells may be arrested at mitosis. However, mitotic slippage occurs after a prolonged arrest in mitosis and results in tetraploid G_1 cells in the absence of cell division.

The relationship between mitotic arrest and cell death induced by microtubule-stabilizing drugs remains controversial. It was suggested previously that activation of the mitotic kinase cdc2 correlates with Taxol-induced apoptosis (7). Inhibition of cdc2 by a chemical inhibitor or by a dominant negative mutant of cdc2 blocked Taxol-induced apoptosis in MDA-MB-435 breast cancer cells (50). By preventing

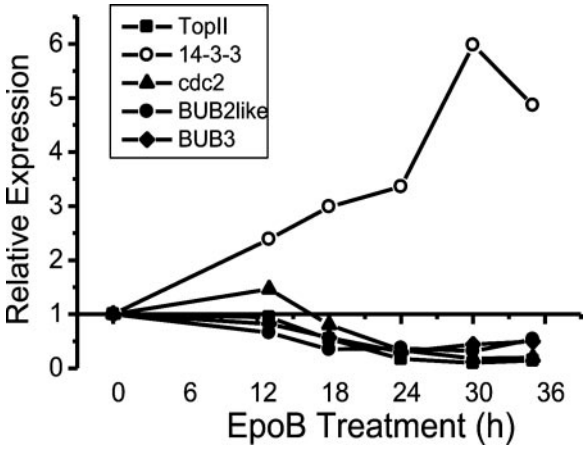

Fig. 5. Expression of mitosis-related genes. A549 cells were treated with 40 nM EpoB for the indicated times. Expression of cdc2, topoisomerase IIα, 14-3-3-σ, BUB3, and BUB2-like protein 1 was determined by real-time RT-PCR as described in "Materials and Methods." BUB2-like protein 1 is vascular Rab-GAP/TBC-containing protein in Fig. 1. The expression levels, normalized to GAPDH, were calculated as the fold change compared with untreated cells. Results are from a representative experiment.

cells from entering mitosis, pretreatment with drugs that induce G_2 arrest reduced the cytotoxicity elicited by microtubule-interacting drugs (9, 51). Conversely, loss of normal p53 function in fibroblast cells conferred sensitization to Taxol by increasing G_2-M arrest and apoptosis (52). However, most of these studies did not determine whether mitotic slippage had occurred after mitotic arrest. Although inhibition of cdc2 kinase activity blocked Taxol-induced apoptosis in breast carcinoma MCF-7 cells, activation of proteases as well as the cleavage of their substrate PARP occurred later than the peak activation of cdc2 (53). A recent study found that elevated cdc2 kinase activity in HeLa cells during spindle checkpoint activation resulted in increased survivin expression and cancer cell viability. Abolishing cdc2 activity with inhibitors after microtubule stabilization resulted in escape from mitotic arrest and massive apoptosis. In contrast, when the cdc2 inhibitor was used before Taxol treatment, apoptosis was not promoted, but tumor growth *in vivo* was enhanced (36). The study suggests that cdc2 activation or mitotic entry is a prerequisite for Taxol-induced apoptosis. In this study, we found that A549 cells that have undergone mitotic slippage demonstrate a remarkable increase in PARP cleavage. In contrast, no PARP cleavage was observed in detached mitotic cells induced by treatment with 40 nM EpoB or Taxol, despite the fact that incubation with a lower concentration (10 nM) of EpoB elicits a small amount of PARP cleavage (Fig. 4*B*). The results indicate that mitotic slippage, but not mitotic arrest, is responsible for apoptotic cell death induced by nanomolar concentrations of the drug.

Mitotic slippage results in significant apoptosis in A549 cells that have a functional p53 (Fig. 4*B*). In the absence of p53 protein, cells having undergone mitotic slippage will move into the cell cycle and become hyperploid cells (>4N) without immediate cell death (8, 54). Otherwise, the pseudo-G_1 cells will be prevented from entry into S phase by the activation of p21 (19, 55). In the human prostate cancer cell line DU145 treated with Taxol, the tetraploid cells resulting from mitotic slippage did not progress into the cell cycle but efficiently underwent apoptosis by 24 h (56). The mechanism for p53 activation in A549 cells is not clear, but it could involve DNA damage. Abnormal mitosis might create chromosomal breakage and interchromosomal concatenation (57), and the resulting DNA damage may activate DNA surveillance programs that converge on p53. Ser-15 phosphorylation in p53 (Fig. 4) is consistent with the possibility of DNA damage (39, 58). A DNA repair process has been found in cells from mitotic slippage before apoptotic cell death (59). In addition, polyploid cells induced by a spindle inhibitor demonstrated partial DNA fragmentation, possibly due to the lack of synchrony in the nuclear cycle among multiple nuclei (60). On the other hand, prolonged activation of cdc2 kinase during mitotic arrest may prime the cells for apoptosis, for example, by induction of morphological characteristics of apoptosis such as chromatin condensation and lamina disassembly. Because of the activation of survivin by cdc2 (36), apoptosis is inhibited until mitotic slippage occurs, at which time cdc2 and survivin are diminished (Fig. 4*B*).

Mitotic slippage has been reported previously, based on time-dependent changes in the mitotic index, such as cdc2 kinase activity (19, 61). In this study, we have used time-lapse microscopy to monitor mitotic slippage, and the possible mechanisms involved have been suggested from analysis of gene expression. Accumulation of cells in mitotic slippage is accompanied by decreased expression of cdc2 and topoisomerase II and by an induction of 14-3-3-σ, three genes involved in the G_2-M transition (62). Our results suggest that the transcriptional regulation of mitotic genes may contribute to mitotic slippage. This is in contrast to normal mitosis, where the exit is controlled by ubiquitin-dependent proteolysis of cyclin B that leads to a decrease in cdc2 kinase activity, but not to a decrease of cdc2 mRNA and protein levels. Transcriptional suppression of cdc2 by p53 may be partly responsible for the down-regulation of cdc2 (62) because p53 protein is increased in mitotic cells (Fig. 4*B*). However, cells lacking wild-type p53 are capable of undergoing mitotic slippage (56, 63), suggesting that other mechanisms could be involved in depression of cdc2. In addition, reduced expression of BUB3 and BUB2-like protein 1, spindle checkpoint genes, may explain why the cells cannot remain in mitosis for longer times. BUB2-like protein is a negative regulator of mitotic progression in *S. cerevisiae* (64). Mitotic exit requires the activation of Tem1, which is inhibited by a GTPase-activating complex composed of Bub2 and Bfa. Phosphorylation of Bub2 relieves the inhibition and allows normal exit from mitosis. Spindle damage or disorientation prevents Bub2 phosphorylation and inhibits mitotic exit (64). However, a reduction of Bub2 mRNA may eventually mitigate its inhibition and permit mitotic slippage to occur (Fig. 5). Our results suggest that inhibition of mitotic gene expression may be responsible for the prolonged process (>15 h) of mitotic slippage. In contrast, normal mitosis takes approximately 30 min (Fig. 3) when epigenetic events such as proteolysis and phosphorylation/dephosphorylation are required.

In this study, microarray technology has been used to explore gene expression induced by both Taxol and EpoB at nanomolar concentrations. Common genes related to microtubule stabilization were identified. In addition to the well-known genes such as p21 that are induced by Taxol, multiple genes that reveal novel effects due to microtubule stabilization were discovered for the first time. For example, EpoB increased expression of the antiproliferative genes BTG2 and PTGF-β in G_1-arrested cells (Table 1).

A traditional way to determine gene function is to overexpress or knock out genes and then study the cellular consequences. However, a gene may have different functions, depending on the intrinsic milieu of cells, that may change. As we become increasingly knowledgeable about multifactorial actions of antimitotic drugs, it is important to study gene function in the presence of other genetic and epigenetic modulations resulting from drug treatment. By comparing gene expression elicited by drug treatment and by drug resistance, we have assigned death or survival functions to several p53-targeted genes (Table 1). The method successfully identified CD95 as one prominent death mechanism induced by microtubule-stabilizing drugs, as is true for other anticancer drugs (29, 30). Our study also suggests that p21 and BTG2, induced by low concentrations of microtubule stabilizing drugs, may assist in cell death (Table 1). The increased expression of p21, BTG2, and CD95 may contribute to G_1 arrest and limited apoptosis of the aneuploid population resulting from aberrant mitosis [Fig. 2*A* (11, 65)]. Consistent with the death function for G_1 checkpoint genes, sequential treatment with EpoB followed by flavopiridol, which enhances G_1 arrest, induced significantly more apoptosis of MB-468 cells than treatment with the reverse sequence of drugs or treatment with either agent alone (66).

Previous studies have suggested that p21 contributes to resistance of Taxol-induced apoptosis (67, 68). The discrepancy may be rooted in the different methods used to evaluate gene expression. Modulation of p21 levels by transfection, before drug treatment, may interfere with cdc2 kinase and mitotic entry (50, 55, 68). By emphasizing mitotic entry, which is required for cell death, p21 behaves more like a survival gene (67). As discussed earlier for cdc2, ascertaining the functions of a gene by expression or inhibition that prematurely arrest mitotic progression could obscure its role during mitotic exit. In contrast, our method compares the regulation of gene expression by the drug in sensitive and resistant cell lines, thereby summing up multiple roles played by the gene. Because p21 contributes differently to mitotic entry and exit, its contribution to cell death may be compromised, and its expression in resistant cells remains relatively

greater than that for CD95, which is only a death signal (Table 1). However, the resistant cell line that we have used for evaluating gene function is generated from multiple challenges with low doses of EpoB; thereby the death and survival genes are more likely to recognize their functions related to aberrant mitosis. It remains to be proven whether p21 might also function as a death mechanism in cells undergoing mitotic slippage. Currently we are in the process of generating new resistant cell lines that are being produced by challenging cells with 40 nM EpoB, a concentration that is capable of arresting cells in mitosis. Resistant cells may arise due to genetic alterations that help them survive under adverse conditions. By comparing the two sets of resistant cells, we hope to determine whether p21 and other death or survival genes can function similarly in the cells treated with different concentrations of drug.

In summary, we have determined gene expression profiles in a lung carcinoma cell line in response to the microtubule-stabilizing drugs Taxol and EpoB. Depending on the drug concentration used, cells treated with microtubule-stabilizing drugs may undergo aberrant mitosis or mitotic slippage before apoptotic cell death. Mitotic slippage may result from altered expression of G_2M and spindle checkpoint genes. The induction of multiple genes, including checkpoint genes and death receptors, may contribute differently to cell death and survival.

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