Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells

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Abstract

Drugs that disrupt microtubule dynamics include some of the most important of cancer chemotherapies. While these drugs, which include paclitaxel (Taxol), are known to invoke the mitotic checkpoint, the factors that determine cancer cell killing remain incompletely characterized. Cells that are relatively resistant to killing by these drugs block robustly in mitosis, whereas cells sensitive to killing block only transiently in mitosis before undergoing nuclear fragmentation and death. Passage through mitosis was an absolute requirement of drug-induced death, because death was markedly reduced in cells blocked at both G_1-S and G_2. Cell killing was at least in part linked to the absence or inactivation of BubR1, a kinetochore-associated phosphoprotein that mediates the mitotic checkpoint. Sensitivity to paclitaxel correlated with decreased BubR1 protein expression in human cancer cell lines, including those derived from breast and ovarian cancers. Silencing of BubR1 via RNA interference inactivated the mitotic checkpoint in drug-resistant cells, and reversed resistance to paclitaxel and nocodazole. Together, these results suggest that the mitotic checkpoint is an important determinant of the efficacy of microtubule-targeting drugs in killing cancer cells, potentially providing novel targets for increasing treatment efficacy. [Mol Cancer Ther 2004;3(6):661–9]

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Introduction

Microtubule dynamics is targeted by several drugs useful in the clinic and in biomedical research, such as the taxanes [paclitaxel (Taxol), docetaxel (Taxotere)], the benzimidazole derivatives (nocodazole), and vinca alkaloids (vincristine, vinblastine). The taxanes bind to a subunit of the tubulin heterodimers that form cellular microtubules; the binding of the taxanes accelerates the polymerization of tubulin, effectively stabilizing and inhibiting the depolymerization of the microtubules (1). Nocodazole and the vinca alkaloids in contrast inhibit the polymerization of the tubulin heterodimers, in turn preventing the formation of microtubules (2-4). By disrupting microtubule dynamics, these drugs cause the accumulation of cells in mitosis (5-10).

The mitotic checkpoint is thought to be invoked by these microtubule-disrupting drugs through mechanisms that monitor correct spindle formation and tension, and which in normal cells help ensure that equal numbers of chromosomes are distributed to daughter cells and, therefore, avoid missegregation and the potentially catastrophic consequences of aneuploidy (for two recent reviews, please see refs. 11, 12). The mitotic checkpoint blocks progression into anaphase until all chromosomes have completely aligned at the metaphase plate. Current models propose that the mitotic checkpoint proteins—BubR1, Bub1, Bub3, Mad1, and Mad2—sense lack of tension or attachment between the kinetochore and microtubules of the mitotic spindle, and transmit a ''wait signal'' to inhibit the anaphase promoting complex. This in turn inhibits the degradation of proteins, such as cyclin B1, to mediate the onset of anaphase. The precise nature of the ''wait signal'' is not entirely clear, but unaligned chromosomes preferentially accumulate BubR1, BUB1, and MAD1 at the kinetochore; once the chromosomes become properly aligned, the kinetochore localization of these proteins diminishes. It is likely that these mitotic checkpoint proteins participate in other pathways to effect orderly mitotic progression. For example, depletion of the essential mitotic kinesin protein CENP-E results in decreased BubR1 activity and recruitment to kinetochores during mitosis, and results *in vitro* and *in vivo* in increased aneuploidy (13).

We investigated whether resistance to the lethal effects of microtubule-disrupting drugs might be mediated at least in part through cell cycle effects, and in particular, the mitotic checkpoint. We found that nuclear fragmentation and rapid killing induced by microtubule-disrupting drugs at nanomolar concentrations correlated with progression of cells into mitosis, coupled with inactivation of the mitotic checkpoint. Cell lines sensitive to killing by paclitaxel and nocodazole were inactivated for BubR1, consistent with a poorly

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active mitotic checkpoint. However, in these cells, inhibition of cell cycle progression reduced the lethal effects of the drugs. In contrast, resistant cell lines showed a robust mitotic checkpoint, including strong expression of BubR1 protein, and which localized to kinetochores in cells blocked in mitosis by drugs. The drug resistance of these cells could, however, be reversed by silencing of BubR1, which abrogated the mitotic checkpoint. These results together suggest that the integrity of the mitotic checkpoint is an important determinant of sensitivity of cancer cells to microtubule-disrupting chemotherapy.

Materials and Methods

All cell lines cells were obtained from the American Type Culture Collection (Manassas, VA), and grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 15% fetal bovine serum at 37°C in 5% CO_2. Nocodazole, aphidicolin, alsterpaullone, and trichostatin A were obtained from Sigma Chemical Co. (St. Louis, MO), and prepared as 1,000× stock in DMSO. For experiments, these were diluted in media, and used at concentrations and conditions as previously described or noted in the figure legends (14, 15). Specifically, final concentration of nocodazole was 0.4 μg/mL, aphidicolin was 1 μg/mL, and alsterpaullone was used at 10 μmol/L. Mock-treated controls were handled in an identical manner to experimental samples, with an identical amount of media added (without drug). In experiments in which cells were synchronized, this was accomplished via aphidicolin double synchrony as previously described (16). In experiments involving pulse treatment with microtubule-disrupting drugs, exponentially growing cells were exposed to the paclitaxel or nocodazole for 4 hours, washed three times with liberal amounts of PBS, and replated into fresh drug-free media. Colony survival assays were done by plating cells in triplicate at serial dilutions following the pulse drug treatment, and left to grow undisturbed for 10 days in the incubator. Colonies were simultaneously fixed and stained with crystal violet in methanol, and scored as those containing 50 or more cells. For each cell line, colony counts were corrected for plating efficiency.

For immunoblotting, cells were harvested, pelleted and resuspended, sonicated, and resuspended in Laemmli buffer, followed by boiling for 5 minutes, before separation via SDS-PAGE (10 μg protein/lane) and transfer to nitrocellulose membranes. After transfer, the membranes were blocked with 5% non-fat milk in PBS, and then probed with the indicated primary antibodies, followed by the appropriate secondary antibodies conjugated with horseradish peroxidase. Washes were done with PBS with 0.1% Tween. Finally, membranes were exposed to film after enhanced chemiluminescence [enhanced chemiluminescence membranes were processed with enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ)]. Antibodies used were as follows: anti-Bub1 and anti-BubR1 antibodies were prepared and used as described (11); anti-human centromeric and anti-CENP-F antibodies were generous gifts of Dr. Tim J. Yen and used at 1:1,000; anti-topoisomerase antibody (Kamiya Biochemical, Thousand Oaks, CA), anti-α-tubulin, and anti-β-actin (Sigma) were all used at 1:1,000 for immunoblotting.

Immunofluorescence and fluorescence-activated cell sorting (FACS) analysis of DNA content was done as previously described (14-16). During the FACS, no gating was done on the propidium iodide–stained nuclei, in order that all cells were included in the analysis, including those with sub-G_1 content representative of nuclear fragmentation. Before staining of the nuclei, cells with sub-G_1 content were confirmed to be nonviable through Trypan blue and propidium iodide exclusion assays. For immunofluorescence, cells grown on coverslips were fixed in ice-cold acetone-methanol (50:50), washed in PBS and KB buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% bovine serum albumin], before labeling with specific antibodies. The respective primary antibody was then detected via the species-specific secondary antibody conjugated to Alexa Fluor 594 or 488 (Molecular Probes, Eugene, OR). DNA was stained with 0.1 mg/mL of 4′,6-diamidino-2-phenylindole (Sigma). The coverslips were then mounted in 0.1% *para*-phenylenediamine in glycerol. Stained cells were examined with a 100× PlanNeofluor objective mounted on a Nikon TE-200 microscope equipped with epifluorescence optics. Images were captured with a Hammamatsu CCD camera that was controlled with IP LabSpectrum v2.0.1 (Scanalytics Inc., Fairfax, VA).

RNA interference was done with short interfering RNA (siRNA) obtained from Dharmacon (Lafayette, CO), and used as described in protocols provided by the manufacturer. Cells were treated with siRNA to a final concentration of 10 μmol/L. The BubR1 siRNA target sequence was GCTCCAATCATCCGTGTAG. Control siRNA consisted of the unannealed single-strand RNA and siRNA targeted against luciferase (both of which did not affect levels of endogenous proteins).

Results

Cell Line-Specific Differences in Sensitivity to Nocodazole and Paclitaxel

We first defined the response of the breast cancer cell line SkBr3 and cervical cancer cell line HeLa to a range of paclitaxel and nocodazole treatment concentrations and exposure times.[3] While fragmentation of nuclei generally increases with dose and duration of exposure for these and other human cell lines tested, SkBr3 cells were consistently more sensitive than HeLa cells at each level (Figs. 1-3, and data not shown). At low nanomolar concentrations, paclitaxel induced the mitotic checkpoint in HeLa cells. At high concentrations (μmol/L), paclitaxel likely has lethal effects not clearly related to the stabilization of microtubules, such as lipopolysaccharide or tumor necrosis

[3] Supplemental material for this article is available at MCT online (http://mct.aacrjournals.org).

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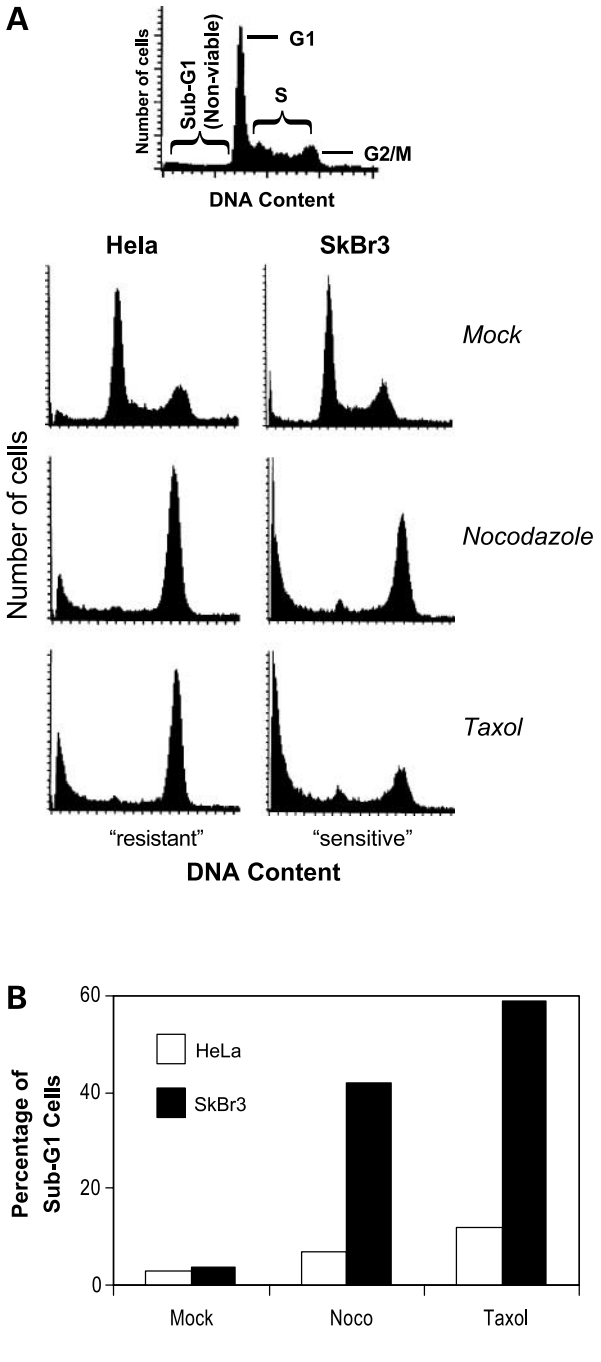

Figure 1. SkBr3 cells are more sensitive than HeLa cells to nocodazole and Taxol-induced nuclear fragmentation and death. HeLa and SkBr3 cells were mock treated or treated with nocodazole (0.4 μg/mL) or paclitaxel (5 nmol/L) and harvested 8 hours later for analysis of DNA content. Cells at the respective phases of the cell cycle and with sub-G_1 content (indicative of nonviable cells) are, as indicated in the histogram, representative of untreated cells at the *top* of the figure. **A,** FACS histograms displaying number of cells and DNA content, demonstrating that SkBr3 showed greater proportions of nuclear fragmentation than HeLa cells after treatment with nocodazole and paclitaxel (*Taxol*) under identical treatment conditions, whereas mock-treated cells did not. **B,** bar graphs showing the proportions of HeLa and SkBr3 cells with sub-G_1 DNA content in histograms shown in **A,** with each treatment (*Noco* = nocodazole). This experiment was repeated three times with similar results.

factor-like effects (17). Pharmacokinetic studies of plasma concentrations of paclitaxel administered intravenously to patients indicated that the drug is rapidly metabolized, such that only low nanomolar concentrations are sustained hours after administration (18). For these reasons, and to focus our studies on the potential effects of paclitaxel treatment on the mitotic checkpoint, we standardized our subsequent studies at a paclitaxel concentration of 5 nmol/L.

To formally confirm that the nuclear fragmentation detectable soon after nocodazole or paclitaxel treatment reflected sustained loss of cellular viability, we did standard colony survival assays after pulse treatment. HeLa and SkBr3 cells were mock treated or briefly exposed to nocodazole or paclitaxel. Under these experimental conditions, HeLa cells block transiently in mitosis, before completing cell division and reentering the cell cycle.[3] The transient block in mitosis was by itself not lethal, because this led to only modest reduction in colony formation in HeLa cells. However, under the same treatment conditions, fewer colonies of SkBr3 were formed (Fig. 2A and B). The

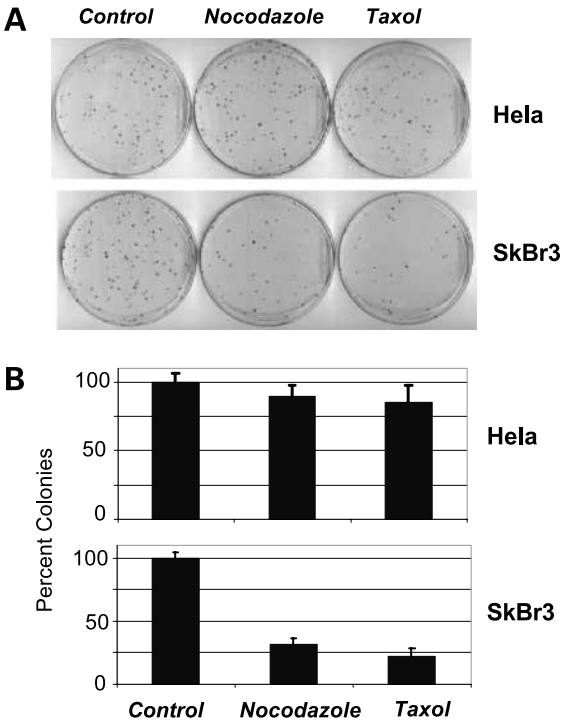

Figure 2. Nocodazole and paclitaxel treatment leads to decreased survival of SkBr3 cells. The sensitivity of HeLa and SkBr3 cells to nocodazole and paclitaxel (*Taxol*) was assessed via colony formation assays. Cells were treated, exposed to each drug for 4 hours at concentrations as in Fig. 1, followed by replating into new plates with fresh media to grow undisturbed for an additional 10 days. All plates were then stained, and counted for colonies of 50 cells or more. The assay was done in triplicate and at four different cell densities of plating. **A,** representative plates showing colonies after mock treatment (*Control*) or treatment with nocodazole or paclitaxel. **B,** histograms showing colony survival after treatment. Colony counts are expressed as a percentage normalized to the control untreated cells; *error bars*, SD.

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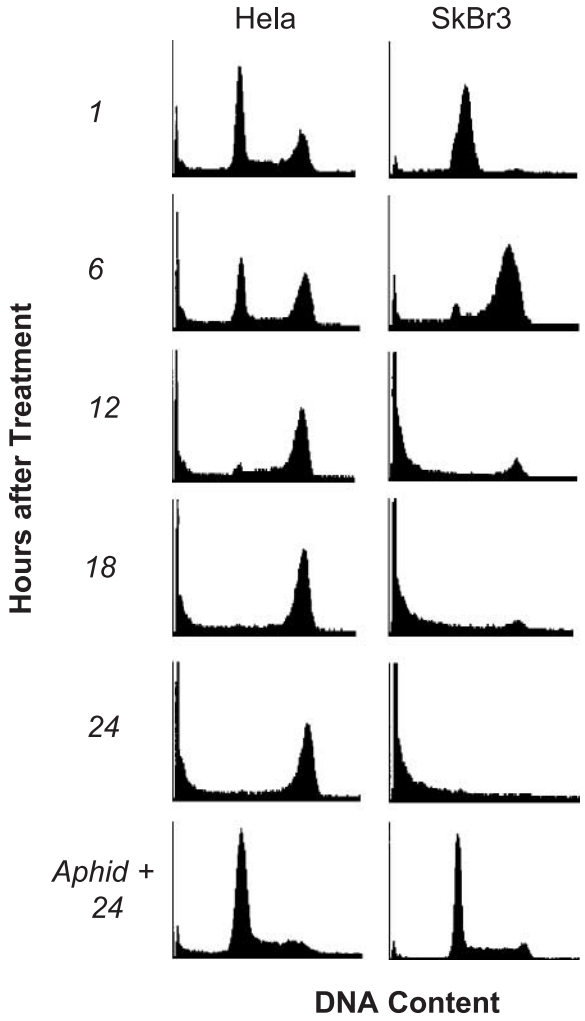

Figure 3. SkBr3 cells show only transient mitotic delay after drug treatment. HeLa and SkBr3 cells were treated with nocodazole and harvested at the serial time points indicated for analysis of DNA content. The resulting FACS histograms indicate that HeLa cells show a robust mitotic block after drug treatment that persists for the entirety of the experiment. SkBr3 cells, in contrast, block transiently, but then rapidly undergo nuclear fragmentation and death. As an additional control, parallel plates of HeLa and SkBr3 cells were treated for 24 hours with aphidicolin but otherwise handled under identical conditions. The aphidicolin results in both types of cells blocking in G_1-S (*bottommost histograms*), indicating that other cell cycle checkpoints are intact.

differences in colony formation between these cell lines were not due to a transient growth delay, because the colony counts in the SkBr3 cells did not recover even with the additional passage of time (data not shown). These results together indicate that SkBr3 are more sensitive than HeLa cells to killing by these drugs.

Nocodazole and Paclitaxel Do Not Impede Progression of Cells through Interphase, but Cause Death in Mitosis

To further investigate the time course of the killing of SkBr3 cells by paclitaxel and nocodazole, we harvested cells at serial time points after addition of drug. Six hours after the addition of nocodazole, most SkBr3 cells were in G_2-M, compared with approximately half of HeLa cells, reflecting the slightly faster doubling time of SkBr3 (15 hours) than HeLa cells (17 hours). However, by 12 hours after drug treatment, most of the SkBr3 cells had already undergone nuclear fragmentation, whereas most of the HeLa cells were now blocked in mitosis (Fig. 3). A high proportion of HeLa cells remained blocked in mitosis even at 18 and 24 hours after drug treatment (albeit a small proportion of cell death was discernable, increasing with the passage of time). The response of SkBr3 and HeLa cells to paclitaxel treatment paralleled that of nocodazole (data not shown). The inability of the SkBr3 cells to maintain a durable mitotic checkpoint was not due to a global inactivation of cell cycle checkpoints, because these cells robustly blocked in G_1-S after aphidicolin treatment, with little discernable cell death (bottom FACS histogram in Fig. 3). These data indicate that after nocodazole and paclitaxel treatment, SkBr3 cells accumulate transiently in mitosis, but the block is not maintained. In contrast, under identical treatment conditions, HeLa cells show a robust mitotic checkpoint that persists far longer than that of the SkBr3 cells. Concomitant with the inability to maintain a robust mitotic checkpoint, the SkBr3 cells rapidly undergo cell death after treatment. The lack of a robust mitotic checkpoint in SkBr3 cells, therefore, coincided with heightened sensitivity to the lethal effects of the drugs.

The results described above suggested that cell death after drug treatment in SkBr3 cells was a consequence of entering mitosis, because substantial nuclear fragmentation was noted only after the induction of a transient G_2-M delay. However, to formally exclude the possibility that cell death may result earlier from effects on other phases of the cell cycle, we synchronized SkBr3 cells in G_1-early S phase, and followed their progression through the cell cycle following release and drug treatment. The addition of nocodazole and paclitaxel soon (2 hours) after release did not have a discernable effect on progression into either S or G_2 phase compared with control cells (Fig. 4). By 12 hours after release, however, at a time when control cells had largely completed mitosis and returned back into G_1, cells treated with nocodazole and paclitaxel showed substantial nuclear fragmentation and death. These results together suggest that nocodazole and paclitaxel do not impede cell cycle progression through interphase, but death caused by these drugs occurs after progression into mitosis.

Nocodazole- and Paclitaxel-Induced Cell Death Is Reduced by Inhibition of Cyclin-Dependent Kinase 1 Activity

Cell cycle progression into mitosis is driven by the cyclin-dependent kinase 1 (cdk1), which is thought to phosphorylate nuclear substrates critical for this transition. Because of the critical role of cdk1 as the main mitotic kinase, inhibition of cdk1 activity interferes with progression into mitosis (reviewed in ref. 19). We reasoned that if progression into mitosis was necessary for cell killing by nocodazole and paclitaxel, blocking cdk1 activity should

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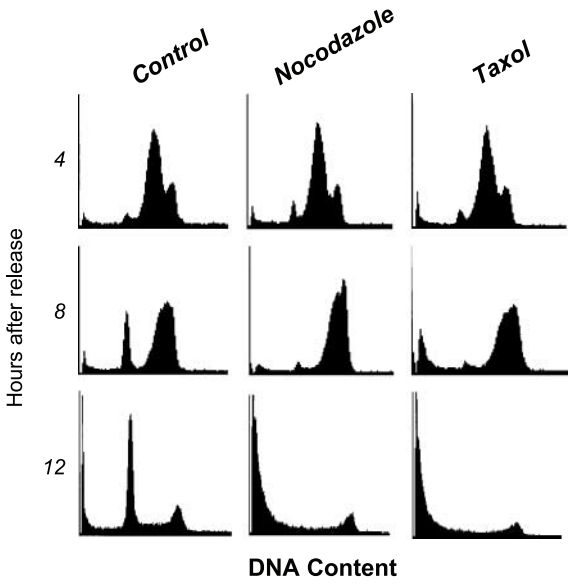

Figure 4. SkBr3 cells treated with nocodazole and paclitaxel die predominantly after entry into mitosis. SkBr3 cells synchronized in G_1-S with aphidicolin were treated 2 hours after release with nocodazole or paclitaxel, and then harvested at the indicated time points for analysis of DNA content. DNA histograms showing that in the presence of the drugs, cells readily enter S (*top panels*, 4 hours after release) and G_2 (*middle panels*, 8 hours). By 12 hours after release, the most of the mock-treated control cells have completed mitosis and reentered G_1 (*left bottom panel*); in contrast, nocodazole- and paclitaxel (*Taxol*)-treated cells have largely undergone nuclear fragmentation and death, and do not show emergence of the expected G_1 peak (*bottom panels*, *middle* and *right*).

likewise reduce the lethal effects of these drugs. We treated synchronized SkBr3 cells in S phase with nocodazole and paclitaxel, alone or in combination with alsterpaullone, a specific cdk1 inhibitor (20). As expected, control cells treated with nocodazole or paclitaxel had largely undergone nuclear fragmentation by 12 hours after release. In contrast, cells that were treated with alsterpaullone concomitant with nocodazole or paclitaxel treatment showed levels of cell death comparable with alsterpaullone alone (Fig. 5A). Consistent with its effect in blocking the cdk1 activity necessary for progression into mitosis, cells were blocked with G_2 DNA content and virtually no mitotic cells could be discerned by microscopy after alsterpaullone with or without nocodazole and paclitaxel (Fig. 5A and data not shown). To better define the stage in the cell cycle at which alsterpaullone blocked cells, we assessed for expression of CENP-F, a nuclear protein maximally expressed in late G_2-mitosis, and which during mitosis associates with the kinetochore (21). In control HeLa cells, CENP-F expression markedly increases in cells treated with and blocked in mitosis with nocodazole. SkBr3 cells treated with nocodazole and paclitaxel also strongly expressed CENP-F, consistent with entry into mitosis (Fig. 5B). In contrast, cells treated with alsterpaullone with or without nocodazole and paclitaxel showed little detectable CENP-F expression. These results together indicate that blocking cdk1 activity blocks cells at a point in the cell cycle before expression of CENP-F. This in turn prevents the cell death that ensues when sensitive cells progress into mitosis in the presence of nocodazole or paclitaxel.

The Mitotic Checkpoint Protein BubR1 and Drug Sensitivity

The death of sensitive cells after progression into mitosis in the presence of microtubule-disrupting drugs suggested a defect in the mitotic checkpoint. By disrupting microtubule dynamics, these drugs impede formation of the mitotic spindle that is necessary for proper chromosomal congression and segregation, events that ensure successful cell division and equal chromosomal distribution to the daughter cells (9). To begin to assess the integrity of the mitotic checkpoint, we measured expression of BubR1, a key component of the mitotic checkpoint that becomes phosphorylated and accumulates at kinetochores during mitosis (22-24). To our surprise, little BubR1 protein was detectable in the drug-sensitive SkBr3 cells via immunoblotting or immunofluorescence (Figs. 5B and 6A and B). In contrast, HeLa cells showed the expected slower migrating phosphorylated form of BubR1 protein on Western analysis (Figs. 5B and 6A), and localization of BubR1 to the paired kinetochores in cells treated with paclitaxel (Fig. 6B). The lack of BubR1 recruitment to the kinetochore was not due to gross alterations in centromeric structure, because anti-human centromere antibodies (ACA) readily labeled paired kinetochores in both HeLa and SkBr3 cells (Fig. 6C).

To more directly examine the role of BubR1 in mediating resistance to microtubule-disrupting drugs, we silenced the expression of BubR1 via RNA interference with siRNA in HeLa cells. Treatment with siRNA targeting BubR1 led to a marked decrease in protein levels, and increased sensitivity to paclitaxel treatment (Fig. 6D and E). These results are consistent with previous reports of increased drug sensitivity after knockdown of BubR1 (25).

To further investigate the association of BubR1 and other checkpoint-related proteins with susceptibility to paclitaxel, we assessed BubR1 protein expression and drug sensitivity in a variety of human cancer cells lines. In addition to confirming that SkBr3 breast cancer cells showed minimal BubR1 protein expression, decreased levels were also found in the breast cancer line HCC-1433, and the ovarian cancer lines A2780 and OVCAR, whereas the colorectal cancer HT29 and fibrosarcoma HCT116 cell lines showed robust expression, as did the renal cancer cell line 786-0 (Fig. 7). SkBr3 and HCC-1433 cells also showed little detectable BUB1 proteins as well, but levels appeared intact in the A2780 and OVCAR cells. All cell lines expressed near-equivalent levels of β-actin, which serve as loading controls.

Next, we assessed the sensitivity of these cell lines to paclitaxel by determining the proportion of cells that form colonies after pulse treatment. As expected, considerably fewer SkBr3 and HCC-1433 cells formed colonies after treatment than HeLa or HT29 cells. A2780 and OVCAR cells were also readily killed by paclitaxel, albeit to a

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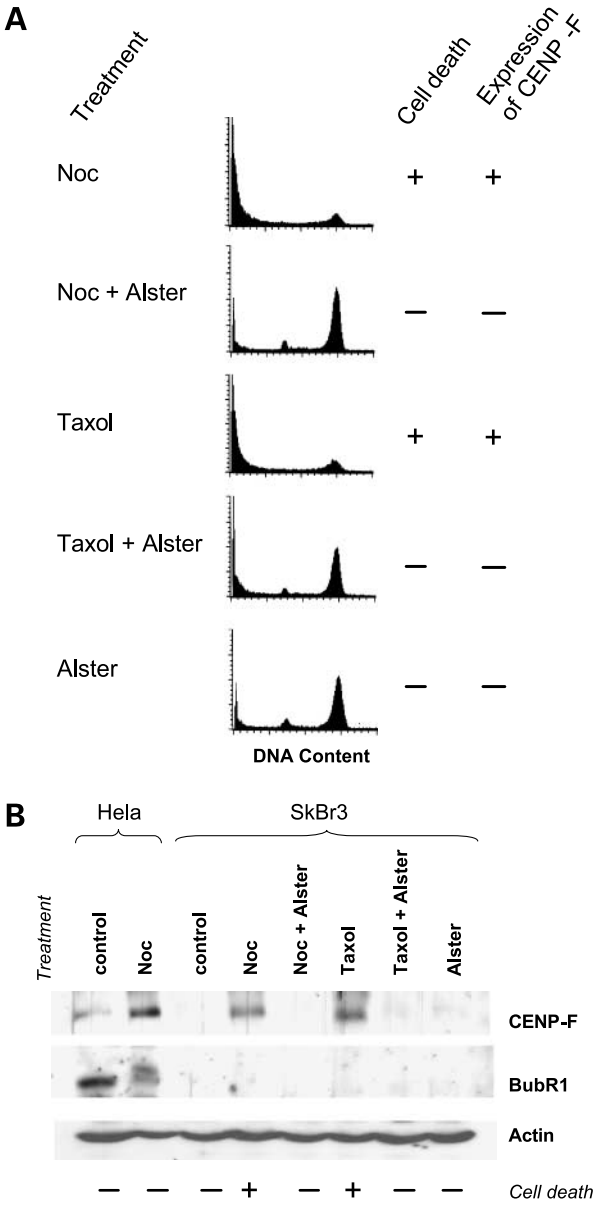

Figure 5. Nocodazole and paclitaxel-induced cell death is reduced by inhibition of cdk1 activity. SkBr3 cells synchronized and treated with nocodazole and paclitaxel as in Fig. 4 were treated in the presence or absence of alsterpaullone, a specific cdk1 inhibitor. Twelve hours after release (10 hours after treatment), the cells were harvested for analysis of DNA content (**A**) or protein via SDS-PAGE and immunoblotting (**B**). Substantial cell death (*+ Cell death*) was defined as greater than 20% of cells with sub-G_1 DNA content as defined in the FACS histograms. *Noc*, nocodazole; *Alster*, alsterpaullone; *Taxol*, paclitaxel. Alsterpaullone blocks the nuclear fragmentation induced by nocodazole and paclitaxel to levels associated with alsterpaullone alone, with either nocodazole or paclitaxel. CENP-F protein expression is strongly increased in mitosis, as indicated in the HeLa cells blocked by nocodazole (*Noc*) relative to untreated cells (*control*) shown in **B**. SkBr3 cells treated with nocodazole and paclitaxel also strongly express CENP-F, indicating entry into mitosis; both CENP-F expression and entry into mitosis are blocked by alsterpaullone. β-Actin protein (*Actin*) serves as a loading control. SkBr3 cells enter mitosis but the mitotic checkpoint is defective, as reflected in lack of BubR1 protein expression (BubR1 protein is hyperphosphorylated in mitosis, as shown in the slower migrating form in HeLa cells treated with nocodazole).

slightly lesser degree than SkBr3 cells. HCT116 and 786-0 cells were resistant to killing by paclitaxel to a degree that matched or was even superior to HeLa cells. The ability to form colonies after paclitaxel treatment, therefore, intriguingly correlated with the lack of expression of BubR1 protein. Because several of these sensitive cell lines also showed decreased levels of BUB1 protein, it remains possible that other mitotic checkpoint proteins also contribute to paclitaxel resistance, along with other mutations that may have arisen in the context of a ''leaky'' checkpoint. In support of these possibilities, forced expression of BubR1 in SkBr3 cells was not successful in restoring the mitotic checkpoint (data not shown). Together with the sensitizing effects of knockdown of BubR1 protein in cells resistant to killing by paclitaxel, these results strongly suggest that mechanisms mediated by mitotic checkpoint proteins contribute to resisting the lethal effects of drugs that impair microtubule dynamics.

Discussion

Recent progress in identifying mechanisms mediating mitotic progression, including those that mediate the mitotic checkpoint, has made available reagents specific for key components of thee mechanisms. The development of protocols using RNA interference to specifically silence expression has further provided additional tools with which to test the potential function of these specific components. We have used these new tools to reexamine the response of human cancer cells to drugs that disrupt the spindle. We first found that these drugs induce nuclear fragmentation mainly after cells had progressed into mitosis; conversely, the lethal effects of these drugs were significantly lessened when cells were prevented from progressing into mitosis. Once cells had progressed into mitosis in the presence of these drugs, whether or not nuclear fragmentation and death quickly ensues depends on the integrity of the mitotic checkpoint. Cells resistant to rapid killing were able to block for prolonged periods in mitosis, whereas sensitive cells were not and quickly underwent nuclear fragmentation. We found next a correlation between the integrity of the mitotic checkpoint and expression of the key mitotic checkpoint components BubR1, and possibly Bub1. Drug-sensitive cells were found to lack one or more components, whereas resistant cells could be rendered sensitive by RNA interference–mediated knockdown of BubR1. These results together suggest that the efficacy of paclitaxel and other drugs that disrupt microtubule dynamics may hinge in part on active cell cycling that allows cancer cells to progress into mitosis. Once cells are in mitosis, the integrity of the mitotic checkpoint then determines whether the cells rapidly undergo nuclear fragmentation, or are able to temporarily block in mitosis, and then resume cell division once the microtubule-disrupting drug is removed.

Previous studies have found variable degrees of mitotic checkpoint defects in human cancer cell lines which are

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consistent with the results reported here (26-28). Our results extend these studies by showing that progression into mitosis is, in fact, a prime determinant of nuclear fragmentation, that perturbation of specific protein components of the checkpoint apparatus can inactivate the mitotic checkpoint, and that this results in measurably decreased survival as shown by reduced colony formation after drug treatment. Why have certain cancer lines evolved a less robust mitotic checkpoint? It has been proposed that defects in the mitotic checkpoint may confer a growth advantage, such as by enabling cells to tolerate chromosomal instability or aneuploidy (29, 30) or by allowing faster proliferation. In support of the latter, Shichiri et al. (31) found few mutations of BubR1 or Bub1 in a series of surgically resected colorectal tumors, but the subset that showed low mRNA expression (presumably due to epigenetic silencing) was associated with a significantly higher recurrence rate.

By highlighting checkpoint integrity as a contributing factor, this work adds to the understanding of mechanisms that mediate resistance to these important classes of chemotherapy. Following the pioneering work of Schiff and Horwitz that established tubulin as the target of paclitaxel, mutations of the protein were found to confer drug resistance (32-34). Specific isotypes of β-tubulin were found to vary in stability, which may contribute to drug sensitivity (35). The emergence of the multidrug resistance (MDR) phenotype, associated with increased expression of

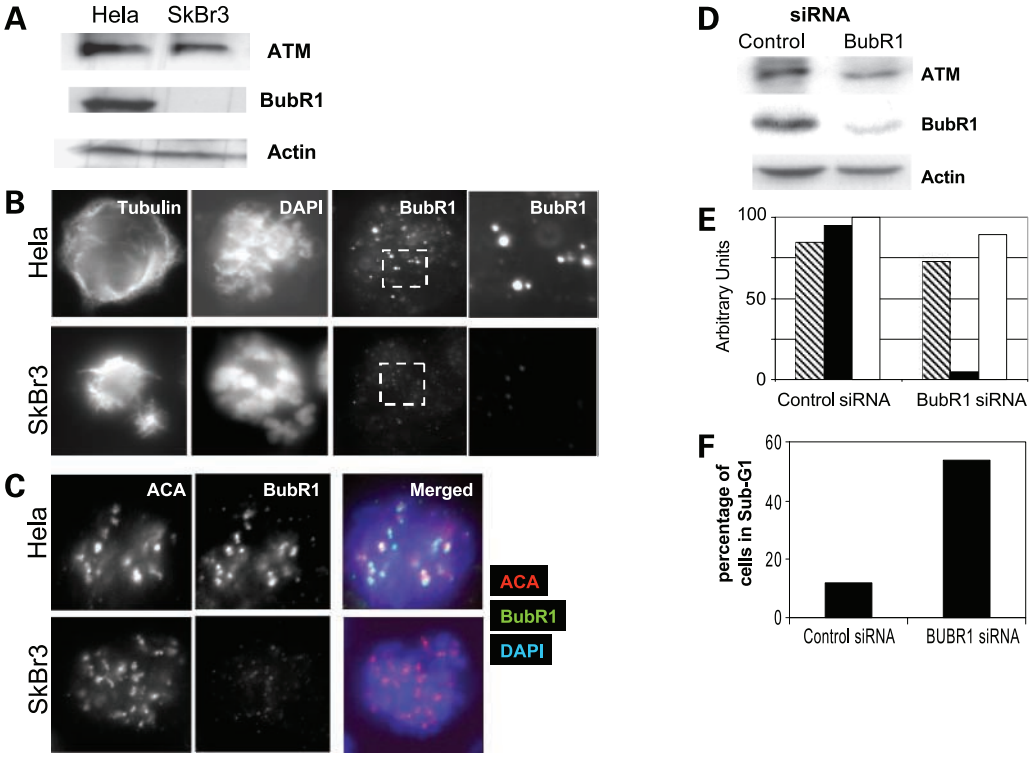

Figure 6. Lack of BubR1 is associated with sensitivity to disruption of microtubule disruption. **A,** lack of the mitotic checkpoint protein BubR1 in SkBr3 cells. Western blot showing decreased BubR1 protein in SkBr3 cells. Cell lysates from asynchronous HeLa and SkBr3 cells were separated via SDS-PAGE and immunoblotting for the indicated proteins. The ataxia-telangiectasia gene product (*ATM*) and β-actin (*Actin*) serve as loading controls. **B,** lack of BubR1 localization to kinetochores in Taxol-treated SkBr3 cells. HeLa and SkBr3 cells were grown on coverslips, treated with paclitaxel, and stained for α-tubulin (*Tubulin*), DNA [4′,6-diamidino-2-phenylindole (*DAPI*)], and BubR1. *Rightmost panel*, inset area in the image of BubR1 enlarged. Tubulin staining shows aggregation characteristic of the effects of paclitaxel. The respective images of HeLa and SkBr3 cells were obtained with identical exposures, and are characteristic of at least 50 cells. **C,** paclitaxel treatment does not disrupt centromeric structure. Cells treated with paclitaxel as in **B** were stained for centromeres and BubR1. *Leftmost panels*, HeLa and SkBr3 cells probed with anti-human centromere antibody (*ACA*); *middle panels*, BubR1 staining; *rightmost panels*, results of ACA and BubR1 staining merged with DAPI staining of the same cells (*Merged*). HeLa cells show the characteristic localization of BubR1 to the paired kinetochores, and which overlaps with that of the stained centromeres (which are likewise paired in these mitotic cells). Staining of the paired centromeres is also seen in the SkBr3 cells, but as in **B** show less staining of BubR1 than HeLa cells. **D,** silencing of BubR1 by RNA interference in drug-resistant cells leads to sensitization to paclitaxel. Lysates from HeLa cells treated with control or BubR1 siRNA were separated via SDS-PAGE and immunoblotted for the indicated proteins, showing specific knockdown of BubR1. The ataxia-telangiectasia gene product (*ATM*) and β-actin (*Actin*) serve as loading controls (the slightly decreased levels of both control proteins are likely due to increased cell death after silencing of BubR1). The experiment was repeated three times with similar results. **E,** densitometry after knockdown of BubR1. ▨, ATM; ■, BubR1; □, tubulin. The immunoblot shown in **D** was quantitated with NIH Image, and the results plotted as shown. The values were normalized to the tubulin in the control siRNA-treated cells (which was set at 100 arbitrary densitometric units). **F,** histograms showing the results of FACS analysis of HeLa cells treated with control or BubR1 siRNA and then exposed to paclitaxel, showing increased nuclear fragmentation with silencing of BubR1.

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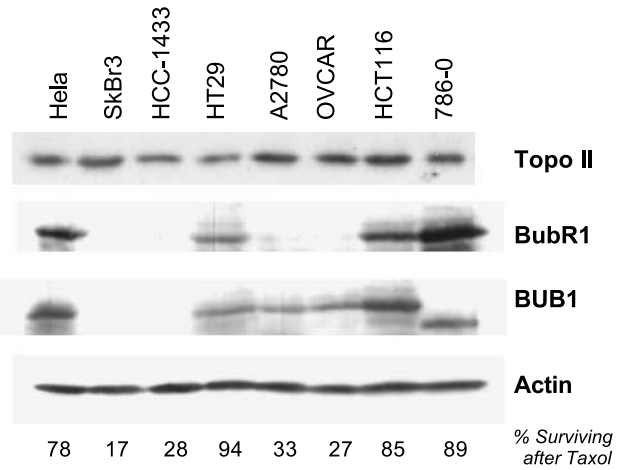

Figure 7. Expression of mitotic checkpoint proteins in human cancer cell lines correlates with sensitivity to paclitaxel. Whole-cell extracts of the human cancer cell lines were separated via SDS-PAGE and immunoblotted for the indicated proteins. Some cell lines show decreased expression of BubR1 and Bub1 protein. Probing for topoisomerase-II and actin serve as loading controls for each cell line assessed. Parallel cultures of the indicated cell lines were exposed to 5 nmol/L paclitaxel for 4 hours, washed, replated, and allowed to grow undisturbed. The percentage of surviving colonies relative to mock-treated controls were counted and recorded (*% Surviving after Taxol*) for each cell line. The origin of each cancer cell line is as follows: *Hela*, cervical; *SkBr3* and *HCC-1433*, breast; *HT29*, colorectal; *A2780* and *OVCAR*, ovarian; *HCT116*, fibrosarcoma; *786-0*, renal.

P-glycoprotein, has been linked to resistance to paclitaxel (36, 37), but contradictory data have also been reported (38). More recently, the Horwitz group reported increased levels of the anti-apoptotic protein survivin in A549 lung cancer cells blocked in mitosis by stabilization of microtubules, whereas increased survivin expression was not seen in cells unable to maintain the block (39).

The results reported here may also have potential clinical ramifications. First, it is tempting to speculate that the clinical response of certain tumors to microtubule-disrupting drugs may be at least partially reflected in the intrinsic sensitivity of cell lines derived from these tumors. For example, the efficacy of paclitaxel in treating patients with breast and ovarian cancer is well established, whereas results in treating cervical, colorectal, and renal cancer have been disappointing (40-42); it is intriguing that this experience is reflected in the cell lines we have studied here. Second, the observation that entry into mitosis seems to be a requirement for rapid killing after microtubule disruption in drug-sensitive cells has implications on the sequencing of these drugs and other forms of anticancer treatment. For example, it may be preferable to administer radiation therapy *after* paclitaxel treatment, rather than the converse; radiation induces a G_1 or G_2 checkpoint in most cancer cells by inhibiting cdk1 activation or its nuclear translocation, but this in turn might reduce the lethal effects of microtubule-disrupting drugs. Conversely, cells blocked in mitosis by microtubule-disrupting drugs might be preferentially sensitive to radiation. Indeed, the sequence of taxane treatment followed by radiation has been found to be superior in tissue culture and mouse models of human cancers (43, 44). Third, the efficacy of microtubule-disrupting drugs would likely be improved by agents or strategies that disrupt mitotic checkpoints (45). This could be accomplished by targeting specific components of the mitotic checkpoint, either pharmacologically or through vector-based strategies.

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