Induction of apoptosis by monastrol, an inhibitor of the mitotic kinesin Eg5, is independent of the spindle checkpoint

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Abstract

Spindle poisons such as paclitaxel are widely used as cancer therapeutics. By interfering with microtubule dynamics, paclitaxel induces mitotic arrest and apoptosis. Targeting the kinesin Eg5, which is required for the formation of a bipolar spindle, is a promising therapeutic alternative to drugs that interfere with microtubule dynamics. Recent data suggest that the spindle checkpoint can determine the response of tumor cells to microtubule poisons. The relationship between checkpoint function and Eg5 inhibition, however, has not yet been fully investigated. Here, we used time-lapse video microscopy and biochemical analysis to study the effect of spindle checkpoint abrogation on the response of HeLa cells to monastrol, a selective Eg5 inhibitor. In HeLa cells, monastrol activated the spindle checkpoint, leading to mitotic arrest and apoptosis. Small interfering RNA–mediated depletion of the spindle checkpoint proteins BubR1 or Mad2 significantly shortened drug-induced arrest, causing premature mitotic exit without cell division. Time-lapse microscopy as well as analysis of caspase activation shows that these checkpoint-deficient cells initiate apoptosis after mitotic exit in response to monastrol. Checkpoint-deficient cells treated with paclitaxel, on the other hand, yielded a higher frequency of cells with >4N DNA content and a decreased incidence of apoptotic events, particularly in Mad2-depleted cells. These results indicate that the immediate fate of post-mitotic cells is influenced by both the nature of the checkpoint defect and the type of drug used. Furthermore, these results show that inactivation of the kinesin Eg5 can induce apoptosis in tumor cells in the absence of critical spindle checkpoint components. [Mol Cancer Ther 2006;5(10):2580–91]

Introduction

Drugs that target the mitotic spindle are among the most effective cancer therapeutics currently in use. Vinca alkaloids, which promote microtubule depolymerization, and taxanes (paclitaxel and Taxotere), which stabilize microtubules, inhibit spindle function by disrupting microtubule dynamics, leading to mitotic arrest and apoptosis (1, 2). Mitotic arrest is mediated by the spindle checkpoint, which is activated by microtubule-targeted drugs. Recently, inhibiting the mitotic kinesin Eg5 [also known as kinesin spindle protein (KSP)], which is required for the formation of a bipolar spindle, has gained significant attention as an alternative strategy to interfere with spindle function (3–5). Blockage of Eg5 function with selective inhibitors, such as monastrol, results in the characteristic monoastral phenotype, mitotic arrest, and apoptosis in various tumor cell lines (6–8). Similar to microtubule poisons, inhibition of Eg5 leads to activation of the spindle checkpoint (9).

The spindle checkpoint prevents chromosome missegregation and aneuploidy by ensuring the accurate segregation of sister chromatids to the dividing daughter cells during mitosis (10–13). The spindle checkpoint remains active until all chromosome kinetochores are properly attached to the bipolar spindle and chromosomes are aligned at the metaphase plate. Proper function of the spindle checkpoint requires the concerted action of several checkpoint proteins, which include BubR1, Bub1, Bub3, Mad1, and Mad2. Several of these components have been shown to preferentially localize to unattached chromosomes. The active checkpoint generates a “wait anaphase signal” to inhibit the anaphase-promoting complex. Inhibition of the anaphase-promoting complex prevents the degradation of several key mitotic proteins, which must be degraded for anaphase initiation to occur. The presence of unattached chromosomes or a lack of spindle tension that is normally generated by bipolar chromosome attachment results in continued checkpoint activation, mitotic arrest, and eventually programmed cell death (14–17).

Recent studies have shown a correlation between defects in the spindle checkpoint and chromosomal instability, which is frequently observed in tumor cell lines (18–24). In addition to an association between defects in the spindle checkpoint and chromosomal instability, spindle checkpoint defects are also associated with the susceptibility of tumor cells to induction of mitotic arrest and apoptosis by microtubule-targeted agents, such as paclitaxel and nocodazole. Several studies have shown that impairment of spindle checkpoint function leads to a reduction in the level of mitotic arrest and apoptosis normally induced by antimicrotubule drugs (25–27). Other studies, however,
have concluded that inactivation of the spindle checkpoint sensitizes cells to apoptosis induced by antimicrotubule drugs (28, 29).

The relationship between the spindle assembly checkpoint and inhibition of the mitotic kinesin motor protein Eg5 is just beginning to be elucidated. In a recent study, Tao et al. (30) suggest that induction of apoptosis by an Eg5 inhibitor requires sustained mitotic arrest, followed by adaptation and slippage into the next G1 phase. In their study, cells refractory to slippage or cells with a weakened mitotic checkpoint showed a diminished apoptotic response. The authors suggest that the presence of activated checkpoint components, specifically BubR1, may be required for induction of apoptosis by Eg5-targeted drugs following exit from mitosis.

Here, we have sought to explore the relationship between the spindle checkpoint and Eg5 inhibition in more detail. We used time-lapse video microscopy and time course fluorescence-activated cell sorting (FACS) analysis to monitor mitotic arrest and apoptosis of HeLa cells in response to the selective Eg5 inhibitor monastrol, as well as to the microtubule inhibitor paclitaxel. To analyze the effect of the spindle checkpoint on drug response, cells were rendered checkpoint deficient by small interfering RNA (siRNA)-mediated depletion of BubR1 or Mad2. Our results suggest that postmitotic events in checkpoint-deficient HeLa cells are dependent both on the type of drug used and the mode of checkpoint inhibition (BubR1 versus Mad2 depletion). Furthermore, our results show that a functional checkpoint is required for monastrol-induced mitotic arrest but not for apoptosis. In checkpoint-compromised HeLa cells, monastrol induced apoptosis following mitotic exit into the next G1 phase, showing that Eg5 inhibition can lead to caspase activation and apoptosis in the absence of critical checkpoint components, such as BubR1 or Mad2.

**Materials and Methods**

**Cell Culture**

HeLa cells and HeLa cells constitutively expressing a histone 2B-green fluorescent protein (31) were grown in DMEM supplemented with 10% fetal bovine serum and 0.01 mol/L HEPES.

**Drug Treatment**

Paclitaxel (Sigma, St. Louis, MO) and monastrol (Tocris Cookson, Inc., Ellisville, MS) were dissolved in DMSO and used at a final concentration of 100 nmol/L and 100 nmol/L, respectively. DMSO was added to mock-treated controls.

**siRNA**

siRNA duplexes were used to knock down gene expression in HeLa cells (32). All duplexes were synthesized by Dharmacon, Inc. (Lafayette, CO). The BubR1 target sequence was 5'-CUUCACUUCGGAGAACAU-3' and the Mad2 sequence was 5'-GAGUCGGGACCACAGUUUA-3' (33). A luciferase siRNA duplex (5'-CAUUCAUUCCUCAGGAGAUGG-3') was used as a control. Transfection of siRNA duplexes was done as described in Tanudji et al. (34) using LipofectAMINE 2000 (Invitrogen, Grand Island, NY) as the transfection reagent.

**Immunoblotting**

Both adherent and floating cells were collected in PBS and fixed in 4 mL of 70% ethanol overnight. Cells were washed once in PBS with 1% fetal bovine serum and 0.03 mg/mL saponin and resuspended in 100 μL of 10 μg/μL anti-MPM2-FSE diluted in wash buffer. After a 2-hour incubation on ice, cells were washed twice with wash buffer and resuspended in 0.4 mL of propidium iodide/RNase I solution. To quantitate the number of cells with activated caspase, a carboxyfluorescein caspase detection kit (Biocarta, San Diego, CA) was used according to the instructions of the manufacturer. Analysis was done on a FACScalibur (Becton Dickson, San Diego, CA) machine using CellQuest software.

**Time-Lapse Microscopy**

Time-lapse images were taken under a Zeiss Axiovert S-100 inverted microscope in an environmental chamber (temperature and CO2 controlled). Images for each condition were obtained in three separate fields of view with an image being taken every 10 to 15 minutes for 48 hours. Cells were pretreated with siRNA 24 hours before time-lapse analysis, and drug treatment was begun 1 to 2 hours before the first time-lapse image acquisition. Mitotic progression of individual cells was examined with Axiosvision V3.1 software (Zeiss, Thornwood, NY).

**Results**

**Mitotic Arrest and Apoptosis in Monastrol-Treated HeLa Cells**

The potential fates of cells treated with antimitotic drugs are either death in mitosis or adaptation and mitotic exit (slippage). Cells that manage to exit mitosis with 4N DNA content can either survive with continued rounds of DNA

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Mol Cancer Ther 2006;5(10). October 2006

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replication, arrest in G1, or die via apoptosis (35). The ultimate outcome may be influenced by the spindle checkpoint as well as other factors, such as p53. To investigate the effect of the spindle checkpoint on the response to Eg5 inhibition, we used HeLa cells that are frequently used for the study of mitosis as well for the study of antimitotic drugs. HeLa cells have a functional spindle checkpoint and show a durable mitotic arrest (no mitotic slippage) when treated with microtubule poisons at concentrations that fully block microtubule dynamics (35, 36). To inhibit Eg5 ATPase activity, we made use of monastrol, a selective allosteric inhibitor of Eg5 (6, 37, 38). The mitotic arrest induced by monastrol is dose dependent and reaches a maximum level of arrest at a concentration of 100 μmol/L in HeLa and other cell lines (9, 38, 39). At this concentration, centrosome separation is also maximally inhibited (9). Therefore, we used monastrol at a concentration of 100 μmol/L in all experiments. As a comparison, we also used the microtubule-stabilizing agent paclitaxel. In contrast to monastrol, paclitaxel shows a biphasic dose response. At low nanomolar concentrations, paclitaxel results in aberrant mitotic exit, micronucleation, and eventual cell death. At high concentrations, however, paclitaxel induces a durable mitotic arrest in many cell lines (40). Here, we used paclitaxel at a concentration of 100 nmol/L, which results in activation of the spindle checkpoint and maximal mitotic arrest (ref. 41; see also Fig. 1).

First, we compared the kinetics of monastrol-induced mitotic arrest with arrest induced by the microtubule-targeted drugs paclitaxel and nocodazole (Fig. 1A). In asynchronously growing HeLa cells, maximal arrest was observed at 18 hours of drug treatment. The accumulation of cells in the mitotic phase confirms that all three drugs used activate the spindle checkpoint in HeLa cells because checkpoint-defective tumor cells do not show this response to antimitotic drugs (42). Cells treated with monastrol arrested in mitosis with the characteristic rosette-like configuration of condensed chromosomes (monoaster) as a result of unseparated centrosomes (Fig. 1A; ref. 6). Phenotypic analysis of DNA-stained cells further suggested that the decrease in mitotic index at later time points is due to the induction of apoptosis (data not shown). Indeed, we found that cleavage of poly(ADP-ribose)polymerase, a key event in the apoptotic signaling cascade, coincided with a decrease in the mitotic marker phospho-histone H3 (Fig. 1B). To show on a molecular level that monastrol activates the spindle checkpoint in HeLa cells, we tested for a phosphorylation-induced band-shift of BubR1 protein. This band-shift has previously been correlated with activation of the spindle checkpoint (20, 24). Treatment of HeLa cells with monastrol, paclitaxel, or nocodazole resulted in hyperphosphorylation of BubR1, further confirming activation of the spindle checkpoint (Fig. 1C).

**Monastrol-Induced Mitotic Arrest Is Impaired in Spindle Checkpoint–Deficient Cells**

To ensure the fidelity of chromosome segregation, the spindle checkpoint monitors microtubule-kinetochore attachment, as well as tension generated by a bipolar spindle.
spindle. Mad2 and BubR1 are integral components of the checkpoint, which are required for checkpoint function (12). Because the mode of checkpoint inhibition may affect tumor cell response to antimitotic drugs, we established siRNA duplexes for both BubR1 and Mad2. As shown in Fig. 2A, transfection of HeLa cells with specific siRNA duplexes resulted in efficient depletion of Mad2 or BubR1. In repeated experiments, substantial knockdown of BubR1 and Mad2 was observed as early as 16 hours after transfection and lasted for up to 96 hours (Fig. 2A, and data not shown).

Using these siRNA duplexes, we tested the effect of spindle checkpoint abrogation on the mitotic arrest phenotype induced by either monastrol or paclitaxel treatment. Monastrol and paclitaxel both efficiently induce mitotic arrest in control luciferase-transfected HeLa cells as evidenced by the round, detached cells that accumulate following either drug treatment. In contrast, this response is noticeably absent in phase-contrast images of drug-treated HeLa cells that have been depleted of BubR1 protein (Fig. 2B). To better quantitate this difference, the accumulation of a mitotic marker, the MPM2 epitope found in many mitosis-specific proteins (43), was measured by FACS analysis. In response to monastrol and paclitaxel treatment, 24.0 ± 3.2% and 38.2 ± 4.5% of control luciferase-transfected HeLa cells accumulated in mitosis, respectively. In contrast to the robust mitotic arrest seen in control luciferase-transfected cells, only 1.5 ± 0.7% of monastrol-treated and 4.6 ± 0.3% of paclitaxel-treated BubR1-depleted HeLa cells were MPM2 positive. This failure to accumulate MPM2-positive cells following either drug treatment indicates that BubR1-depleted HeLa cells fail to maintain an efficient arrest in response to either monastrol or paclitaxel (Fig. 2C). Depletion of Mad2 similarly abrogated mitotic arrest in response to monastrol or paclitaxel. This mitotic arrest defect was also corroborated by immunoblot analysis of independent mitotic markers, phospho-histone H3 and cyclin B1 (data not shown).

To further explore the effect of spindle checkpoint abrogation on drug-induced mitotic arrest, we employed time-lapse video microscopy, which allowed us to follow the mitotic progression of individual cells. In luciferase siRNA–transfected control cells, an average mitosis (measured from the first sign of DNA condensation to the first sign of DNA decondensation) lasted 90 ± 19 minutes (Fig. 3A). This average correlated well with the duration of a normal HeLa cell mitosis described in previous reports (22, 36). Depletion of BubR1 resulted in a minor decrease in the length of mitosis whereas depletion of Mad2 had a more pronounced effect with cells exiting mitosis after 35.7 ± 10 minutes (Fig. 3A). This average correlated well with the duration of a normal HeLa cell mitosis described in previous reports (22, 36). Depletion of BubR1 resulted in a minor decrease in the length of mitosis whereas depletion of Mad2 had a more pronounced effect with cells exiting mitosis after 35.7 ± 10 minutes (Fig. 3A). Control luciferase-transfected HeLa cells treated with monastrol arrested in mitosis for 229 ± 19 minutes, which was followed by cell death as evidenced by hypercondensed and fragmented DNA indicative of apoptosis. In contrast, monastrol-induced arrest of BubR1- and Mad2-depleted HeLa cells was significantly shortened to 42 ± 5 and 22 ± 7 minutes, respectively (Fig. 3B). Paclitaxel treatment yielded similar results (Fig. 3C). In addition to the obvious decrease in the duration of mitotic arrest, the majority of spindle checkpoint–compromised cells treated with monastrol or paclitaxel also exited mitosis without division (see also below).

**BubR1- and Mad2-Depleted Cells Abnormally Exit Drug-Induced Mitotic Arrest**

Monastrol- and paclitaxel-induced mitotic arrest in spindle checkpoint–competent HeLa cells leads to elimination by apoptosis. In contrast, we found that abrogation of the spindle checkpoint allowed cells to rapidly exit mitosis without cell division. To determine the fate of
postmitotic cells that lack either BubR1 or Mad2, we again employed time-lapse microscopy to monitor cell fate. Figure 4 shows representative still images from time-lapse movies of monastrol- and paclitaxel-treated HeLa cells expressing histone H2B-green fluorescent protein in the presence or absence of a functional spindle checkpoint (see also Supplementary movies). The effect of Mad2 depletion was more severe with cells undergoing a brief period of chromosome hypercondensation that was nevertheless followed by chromosome segregation and cell division (Fig. 4A). Aligned metaphase chromosomes were never observed in Mad2-depleted cells, and lagging chromosomes were identifiable in dividing cells. Using either duplex, only occasional apoptotic cells were observed within the 48-hour duration of time-lapse analysis. Thus, at least in the short term, loss of BubR1 or Mad2 function does not significantly affect the level of cell death.

In both BubR1- and Mad2-depleted cells, monastrol-induced mitotic arrest was followed by rapid chromosome decondensation and exit from mitosis (Fig. 4B; Supplementary movies). BubR1-depleted cells treated with monastrol (70.7%) exited without division into a pseudo-G1 state. Interestingly, 26.8% of BubR1-depleted cells treated with monastrol managed to exit with chromosome segregation and cell division despite the initial appearance of a monoastral spindle (Fig. 4A). Aligned metaphase chromosomes were never observed in Mad2-depleted cells; cell division was never observed (Fig. 4B). Following mitotic exit, we noticed that HeLa cells depleted of either BubR1 or Mad2 remained in interphase for varying lengths of time but nevertheless still initiated apoptosis in most cases. A measurement of the cumulative amount of cell death, which occurred during the 48 hours of monastrol treatment, revealed that 91.7% of BubR1-depleted and 80.4% of Mad2-depleted HeLa cells underwent apoptosis (Table 1).

Interestingly, treating checkpoint-deficient cells with paclitaxel elicited different postmitotic fates than monastrol (Fig. 4C; Table 1; Supplementary movies). First, cell division was never observed, among either BubR1- or Mad2-depleted cells. Second, at least within the 48-hour duration of time-lapse analysis, the frequency of postmitotic cell death was significantly reduced in paclitaxel-treated cells compared with monastrol-treated cells. The cumulative cell death among BubR1- and Mad2-depleted cells treated with paclitaxel was 60.7% and 20.5%,

Figure 3. Duration of mitotic arrest in drug-treated cells. Individual cells expressing a histone 2B-green fluorescent protein fusion protein were monitored by time-lapse microscopy (in a CO2- and temperature-controlled environment) to measure the duration of mitotic arrest. Images were acquired as described in Materials and Methods. The line represents the median duration of mitotic arrest (measured from the first sign of DNA condensation to first sign of decondensation) of at least 34 DMSO- or drug-treated cells. A, average duration of mitosis of DMSO-treated cells used as reference. B, duration of mitosis in monastrol-treated cells. C, duration of mitosis in paclitaxel-treated cells (Taxol). Note that for cells transfected with luciferase siRNA and treated with paclitaxel or monastrol, mitosis typically ends with the generation of apoptotic nuclei.

3 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
Figure 4. Analysis of mitotic and postmitotic events by time-lapse video microscopy. Drug-treated and control HeLa cells expressing histone H2B-green fluorescent protein were monitored by time-lapse video microscopy in a CO2- and temperature-controlled environment. Still images of green fluorescent protein–labeled chromosomes and phase-contrast cell morphology for a single cell are representative of several independent experiments. The number of minutes that have elapsed at the time of image capture is indicated. A, images of controls treated with drugs or siRNA only. B and C, images of checkpoint-deficient cells treated with monastrol or paclitaxel (Taxol), respectively. Cells with hypercondensed and fragmented DNA were considered apoptotic. To confirm cell death, cells were monitored for the full duration of the movie (images representing apoptotic cells are indicated with “+”). A, DMSO-treated control cells show normal mitotic progression. Monastrol treatment elicits mitotic arrest, followed by apoptosis. Paclitaxel-treated cells undergo programmed cell death following a prolonged mitotic arrest. Bar, 10 µm. B, BubR1 and Mad2 depletion affects mitotic progression. Mitotic chromosome congression is abbreviated in BubR1-depleted cells. Unaligned chromosomes and lagging chromosomes are sometimes observed. Mad2-depleted cells rapidly initiate anaphase and cell division following chromatin condensation, typically without a detectable metaphase alignment. Cells transfected with luciferase siRNA and not drug treated showed normal mitotic progression similar to DMSO-treated cells described above (not shown). Bar, 10 µm. B, loss of BubR1 or Mad2 function bypasses monastrol-induced mitotic arrest. BubR1- and Mad2-depleted cells predominantly escape mitotic arrest and exit without cell division (pictured). Apoptosis of cells depleted of either protein occurs after escape from mitotic arrest (+; final image of the sequence). Bar, 10 µm. C, BubR1- and Mad2-depleted cells respond differently to paclitaxel treatment. BubR1-depleted cells treated with paclitaxel undergo abbreviated mitotic arrest and exit mitosis with micronucleation (image at 90 min). For the cell shown, apoptosis was observed at 40 h (+; last image in sequence). In contrast, most MAD2-depleted cells treated with paclitaxel were still viable at the end of the 48-h movies. Mad2-depleted cells treated with paclitaxel often show repeated cycles of chromosome condensation and decondensation with progressive enlargement of the nuclear and cell body. Bar, 10 µm.
respectively (Table 1). Furthermore, examination of fluorescent and phase-contrast images of Mad2-depleted cells treated with paclitaxel revealed successive rounds of DNA condensation and cell body enlargement, which could indicate DNA replication without subsequent cell division. Together, these results show that abrogation of the spindle checkpoint allows cells to avoid monastrol- or paclitaxel-induced mitotic arrest. They also show that most of checkpoint-deficient cells treated with monastrol trigger cell death following exit from mitosis. The differences observed between the type of drug treatment and the type of checkpoint defect emphasize the idea that the ultimate fate of cells exiting mitosis is dependent on both the drug used and the underlying checkpoint defect.

DNA Synthesis in Paclitaxel- and Monastrol-Treated Cells That Are Spindle Checkpoint Compromised

The results presented above indicate that Mad2-depleted cells continue to synthesize DNA following exit from mitosis without cell division, particularly when treated with paclitaxel. Similarly, the majority of BubR1-compromised cells that have been drug treated both escape apoptosis and fail to undergo nuclear division. Consequently, a high fraction of these cells would be expected to have abnormal DNA content. To test this directly, we quantitated the percentage of cells with a >4N DNA content by FACS analysis of propidium iodide–stained cells. Figure 5A shows representative FACS profiles of checkpoint-competent and checkpoint-deficient cells following 24 hours of drug treatment. Compared with monastrol treatment, paclitaxel treatment resulted in a greater fraction of cells with >4N DNA content. This observation was further bolstered by monitoring the increase of cells with >4N DNA content at multiple time points (Fig. 5B). A distinct peak of cells with 8N DNA content, however, was not observed at the time points analyzed, which is likely due to the eventual initiation of apoptosis following exit of checkpoint-deficient cells into the next G1 phase. Consistent with data shown in Fig. 4C and Table 1, DNA content of >4N was most prominent in Mad2-depleted cells treated with Taxol. Cells treated with monastrol showed the lowest increase in the >4N population during the time course. In Mad2-depleted cells, 56.2 ± 3.3% and 18.8 ± 1.7% of cells had >4N DNA content following 40 hours of treatment with paclitaxel or monastrol, respectively. The lower percentage of cells with >4N DNA content in monastrol-treated cells is likely related to the higher incidence of apoptosis following exit from mitosis. This FACS data confirms that spindle checkpoint-deficient HeLa cells treated with either monastrol or paclitaxel are competent to exit mitotic arrest and enter another cell cycle, including a second round of DNA synthesis. In monastrol-treated cells, however, apoptosis was the most frequent cell fate following mitotic exit.

Abrogation of the Spindle Checkpoint Delays Monastrol- and Paclitaxel-Induced Caspase Activation

Our time-lapse experiments showed that, in the absence of a functional spindle checkpoint, cells fail to trigger apoptosis within the mitotic phase in response to drug treatment. However, cell death was still observed at later time points, particularly in monastrol-treated cells. These observations suggest that activation of the apoptotic machinery is not eliminated in the absence of an active spindle checkpoint but is merely delayed. Caspases play a central role in apoptotic cell death and are activated in response to a variety of anticancer agents, including microtubule-targeted drugs (46). Using a quantitative FACS-based assay, we monitored caspase activation at several time points in control and BubR1-depleted cells treated with either monastrol or paclitaxel. As shown in Fig. 6A, treatment of luciferase-transfected control cells with monastrol or paclitaxel resulted in a significant time-dependent increase in the caspase-positive population. Caspase was also activated in BubR1-depleted cells by monastrol and paclitaxel, albeit to a lesser degree. After 24 hours of monastrol or paclitaxel treatment, activated caspases were detected in 35% and 30% of BubR1-depleted cells, respectively. In comparison, caspases were active in 45% of control-transfected cells at this time point. After 40 hours of drug treatment, the caspase-positive population increased to ~65% (monastrol and paclitaxel) in checkpoint-deficient cells and to 75% (monastrol) and 80% (paclitaxel) in luciferase-transfected cells.

Table 1. Loss of BubR1 or Mad2 function alters the post-arrest fate of monastrol- and paclitaxel-treated HeLa cells

<table>
<thead>
<tr>
<th>Spindle checkpoint siRNAi, drug treatment</th>
<th>Cell division (% total)*</th>
<th>G1 exit, no division (% total)*</th>
<th>Mitotic arrest and apoptosis (% total)*</th>
<th>Cumulative cell death (% total)*</th>
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<td>Control siRNAi, monastrol</td>
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<td>0</td>
<td>60.7</td>
</tr>
<tr>
<td>Mad2 siRNAi, paclitaxel</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>20.5</td>
</tr>
</tbody>
</table>

*Cells were transfected with siRNA, drug treated as indicated, and monitored by time-lapse video microscopy for 48 hours. Individual cells were then monitored for the duration of the movie to score their fate. Each value represents the percent of the total number of cells scored that fell into each category (N of at least 33).

†Cells that underwent apoptosis at any point during the time-lapse movie were identified, and a cumulative total of cell death was scored. The value takes into account the change in cell number in a given field due to cell division. Each value represents the percent of cells that died in the time-lapse field (N of at least 33).
To confirm activation of apoptotic effector caspases, we monitored a caspase-3-dependent event, the cleavage of poly(ADP-ribose)polymerase from its 116-kDa pro-form to its 85-kDa active form (Fig. 6B). Compared with luciferase-transfected cells, poly(ADP-ribose)polymerase cleavage elicited by monastrol in BubR1-depleted cells was significantly reduced. However, during the time course, a gradual increase in the cleaved p85 form was nevertheless observed. Similarly, the appearance of the cleaved p85 form of poly(ADP-ribose)polymerase in BubR1-depleted cells treated with paclitaxel was delayed in comparison with control cells. Similar results were observed in Mad2-depleted cells (data not shown). Together with the data shown in Figs. 1 and 4, these results suggest that monastrol as well as paclitaxel leads to caspase activation during the arrest stage of spindle checkpoint–competent HeLa cells. In addition, the absence of a functional checkpoint delays, but does not prevent, the activation of the apoptotic caspase pathway.

Discussion

Disrupting the mitotic spindle is an effective strategy for cancer therapy. Microtubule-targeted drugs, which directly interfere with spindle dynamics, are widely used in the clinic. Other aspects of spindle function may also be targeted to interfere with mitosis. For example, histone deacetylase inhibitors were recently shown to interfere with assembly of kinetochores, leading to activation of the spindle checkpoint and arrest of tumor cells at the prometaphase stage (47). Monastrol is the first example of a new generation of antimitotic compounds that block mitosis by inhibiting the kinesin motor protein Eg5. There is great interest in developing more potent Eg5 inhibitors for clinical applications due to the potential for improved side effect profiles over taxanes. Recently, Muller et al. (48) described new analogues of monastrol with significantly improved activity. Structurally distinct Eg5 inhibitors are S-trityl-L-cystein and representatives of the dihydropyrazole and quinazolinone compounds, which were also shown to mediate significant tumor growth inhibition in...
animal models (8, 49). Interestingly, these more potent structures seem to target the same binding site in Eg5 as monastrol (49–51). Several of these structures may provide clinical leads, and the first quinazoline-based Eg5 inhibitors have entered clinical trials (52).

The effects of microtubule poisons on tumor cells have been studied in considerable detail. In light of potential clinical implications, the relationship between the spindle checkpoint and the response of tumor cells to microtubule-targeted drugs has been of significant interest in recent years. However, the effect of Eg5 inhibitors on tumor cells and the possible implications of the spindle checkpoint in mitotic progression and induction of apoptosis have not yet been studied in detail. Using spindle checkpoint–competent and checkpoint-deficient HeLa cells, we show here that a functional checkpoint is required for Eg5 inhibitor–induced mitotic arrest but is not required for induction of apoptosis.

Several studies have previously argued in favor of the need for an active spindle checkpoint in achieving efficient arrest and cell death by microtubule-targeted drugs. Masuda et al. (27) found a correlation between human cancer cell lines that are spindle checkpoint impaired and resistant to apoptosis induced by antimicrotubule agents. In that study, cell lines that failed to proficiently induce mitotic arrest when challenged with an antimicrotubule agent, such as nocodazole, produced a much lower level of apoptosis compared with spindle checkpoint–competent cells (27). Similarly, another study showed that MCF-7 cells depleted of either BubR1 or Mad2 showed an increased resistance to paclitaxel-induced apoptosis (26).

However, in contrast to the aforementioned reports, recently a few studies have provided evidence that inactivation of the spindle checkpoint sensitizes cells to the effects of antimicrotubule drugs. Sihn et al. (29) showed that a truncated form of hCDC20, the anaphase-promoting complex target of BubR1 and Mad2, could bypass microtubule drug-induced mitotic arrest and induce an increase in apoptosis when compared with similarly treated control cells. Another study has shown that depletion of BubR1 protein in HeLa cells by RNA interference increased HeLa cell sensitivity to both paclitaxel and nocodazole (28). Finally, Kienitz et al. (53) have shown that partial down-regulation of Mad1 inactivated the spindle checkpoint but did not confer resistance to either paclitaxel- or monastrol-induced apoptosis.

There are several factors that may have contributed to the alternative results produced by these two sets of studies. One major difference between them is the duration of drug treatment that occurred before apoptosis was measured. As cells need to complete a cell cycle and traverse from G2 into M phase to be inhibited by antimitotic drugs, the cell cycle length or doubling time of the chosen cell system also needs to be considered. For example, the doubling time of MCF-7 cells used by Sudo et al. (26) is significantly longer than that of HeLa cells (54). Here, a time point beyond 48 hours may have revealed more substantial (or delayed) apoptosis in checkpoint compromised cells. In addition, drug concentration and the type of drug used varied between studies and could have affected the outcome because microtubule-stabilizing and microtubule-destabilizing drugs can produce different mitotic and postmitotic effects in tumor cells (55). Paclitaxel is known to activate a variety of signaling pathways, including the mitogen-activated protein kinase pathway, in various cancer cell lines. These effects have also been linked to the mitotic and apoptotic effects of paclitaxel (refs. 56, 57 and references therein). Interestingly, monastrol, which does not directly interfere with microtubules, does not stimulate activity of extracellular signal–regulated kinase or p38 kinases in several cell lines tested.4 The ability or inability of antimitotic drugs to modulate certain signaling pathways may also account for some of the differences noted in this study between paclitaxel and monastrol. Differences in the expression of proapoptotic and antiapoptotic factors, such as Bcl2 family members, also need to be considered when comparing different cell lines (see also below). For example, MCF-7 cells do not express caspase-3 and contain high levels of antiapoptotic Bcl2 (refs. 58, 59 and references therein). Whereas MCF-7 cells respond to paclitaxel (26), we found these cells relatively resistant to Eg5 inhibitor–induced cell death.

Finally, as noted by Kienitz et al. (53) and our study, altering the functional status of different spindle checkpoint components may produce varying effects on the sensitivity of a cell to antimitotic drugs (see below). Thus, at least in the case of antimicrotubule drugs, the requirement of a functional spindle checkpoint for drug-induced apoptosis may be dependent on a variety of different factors.

The relationship between mitotic arrest and apoptosis induced by Eg5 inhibition and the spindle checkpoint is just beginning to be elucidated. Using the Eg5 inhibitor KSP-IA, Tao et al. (30) observed that abrogation of the spindle checkpoint significantly reduced the apoptotic response. These results led them to propose that induction of apoptosis in response to Eg5 inhibition requires mitotic slippage into the next G1 phase in the presence of an activated spindle checkpoint. The authors concluded that activated checkpoint components like BubR1, together with the inactivation of antiapoptotic factors like survivin, are likely required for initiation of cell death following mitotic slippage.

Here, we have shown that the predominant outcome of treating HeLa cells with monastrol is apoptosis, regardless of checkpoint function. HeLa cells show a durable arrest and do not undergo mitotic slippage in response to moderate concentrations of paclitaxel and other mitotic drugs (35). In the present study, we used monastrol and paclitaxel at concentrations that trigger a durable mitotic arrest (Figs. 1A and 2C). The absence of mitotic slippage is...
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further confirmed by our time-lapse analysis of monastrol- and paclitaxel-treated HeLa cells (Table 1). Our time-lapse analysis also shows that drug-treated HeLa cells can efficiently undergo apoptosis during the mitotic arrest phase. Chromosome decondensation, which would indicate mitotic slippage into the next G1 phase, was never observed before the appearance of apoptotic nuclei. These time-lapse observations are also supported by an increase in caspase activation and poly(ADP-ribose)polymerase cleavage, which coincides with a drop in mitotic index in drug-treated cells (Fig. 1). HeLa cells with an intact spindle checkpoint showed these responses whether they were treated with the Eg5 inhibitor monastrol or the microtubule poison paclitaxel. Although checkpoint-compromised HeLa cells were able to avoid drug-induced cell death during mitosis, apoptosis nevertheless occurred following mitotic exit in most cases. Careful observation of individual HeLa cells by time-lapse video microscopy not only confirmed that the apoptotic pathway was, in fact, still capable of activation but also revealed that death occurred in a postmitotic stage. Although both paclitaxel and monastrol elicited postmitotic cell death, we found that Eg5 inhibition induced a higher level of apoptosis in checkpoint-deficient cells. Quantitative time-lapse analysis also showed that, relative to controls, the duration of the mitotic phase in checkpoint-deficient cells is significantly shortened, especially in Mad2-depleted cells. However, despite the decreased mitotic phase, we found that checkpoint-compromised cells initiated cell death later than their checkpoint-competent counterparts. These time-lapse observations correlate with a measurable delay in caspase activation and poly(ADP-ribose)polymerase cleavage in checkpoint-compromised cells treated with either monastrol or paclitaxel. Interestingly, the results reported by Tao et al. (30) may also indicate a delayed apoptotic response in checkpoint-compromised cells, although the overall level of cell death achieved after 48 hours of drug treatment is significantly lower than reported here. This may, at least in part, be due to the different methods used to assess apoptosis and/or the different approaches to override the spindle checkpoint. The results presented here show that, following exit from mitosis, apoptosis can be induced by Eg5 inhibitors, even in the absence of critical spindle checkpoint components, such as BubR1 and Mad2. Whereas paclitaxel can trigger cell death through multiple mechanisms (40), the intrinsic mitochondrial death pathway seems to be critical for Eg5 inhibitor–induced apoptosis (60). Thus, the integrity of the mitochondrial apoptotic pathway, rather than the integrity of the spindle checkpoint, may be a major requirement for Eg5 inhibitor–induced apoptosis.

The results obtained with paclitaxel further emphasize the importance of individual checkpoint components in the fate of drug-treated cells. Similar to monastrol, the majority of BubR1-depleted cells treated with paclitaxel undergo apoptosis within 48 hours (the duration of our time-lapse analysis). In contrast, most Mad2-depleted cells treated with paclitaxel remained viable at this point. Cumulative cell death amounted to 60.7% and 20.5% of the BubR1- and Mad2-depleted cell populations, respectively. This discrepancy in apoptotic response may be explained by the increased level of cells with >4N DNA content observed among Mad2-depleted cells.

Thus, whereas depletion of either BubR1 or Mad2 will abrogate spindle checkpoint function, these approaches may not be functionally equivalent (61). In accordance with several studies (22, 62–64), we found that the time spanning the first sign of chromatin condensation at the onset of mitosis to the first sign of chromatin decondensation during telophase is significantly reduced in cells depleted of either BubR1 or Mad2 protein. Meraldi et al. (63) proposed that BubR1 and Mad2 not associated with the kinetochore may be responsible for the control of mitotic timing occurring between the checkpoint function of Emi1 in early prometaphase and the kinetochore spindle checkpoint function during mitosis. The significant differences in timing of the two checkpoint-depleted cell types observed in our studies further suggest that Mad2 and BubR1 may monitor different aspects of mitotic progression. The prometaphase chromatin morphology of Mad2-defective cells appeared disorganized, and condensed metaphase and anaphase chromosomes were never observed. Instead, the chromatin seemed to decondense rapidly following an abnormal prometaphase. In contrast, DNA condensation occurred normally in BubR1-depleted HeLa cells despite a decrease in mitotic duration. In addition to the requirement for Mad2 in checkpoint function, these observations may suggest additional roles in the regulation of mitotic events, such as chromatin condensation, and could account for the different outcomes induced by paclitaxel in BubR1- and Mad2-depleted cells. These observations may also help to reconcile at least some of the differences in the earlier studies addressing the effect of the spindle checkpoint on the response to antimitotubule drugs.

Inhibitors of the mitotic kinesin Eg5 are promising new alternatives to antimicrotubule drugs, such as the taxanes and Vinca alkaloids. Similar to microtubule-targeted drugs, Eg5 inhibition leads to mitotic arrest and apoptosis. However, due to the specific requirement of this kinesin for mitosis, this approach is expected to have a better side effect profile in the clinic (5, 7, 8). Furthermore, Eg5 inhibitors have proved to be effective in paclitaxel-resistant tumor cells (65). The results presented here have expanded our understanding of the antiproliferative and proapoptotic effects of monastrol and also revealed both similarities and significant differences between Eg5 inhibition and the microtubule-targeted drug paclitaxel. Finally, our results suggest that Eg5-targeted therapeutics should be effective in spindle checkpoint–compromised tumor cells, irrespective of the underlying checkpoint defect.

Acknowledgments

We thank Emma Lees, Wolfgang Seghezzi, Xiaomin Schebye, Marcel Tanudji, Jerelyn Wong, Ulka Vijaipurkar, and Wei Wang for helpful discussions and critical reading of the manuscript. DNAX Research Institute of Molecular and Cellular Biology Research is a subsidiary of the Schering-Plough Corporation.

Mol Cancer Ther 2006;5(10). October 2006
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Mol Cancer Ther 2006;5:2580-2591.

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