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Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit

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Abstract

Cell death induced by agents that disrupt microtubules can kill cells by inducing a prolonged mitotic block. This mitotic block is dependent on the spindle assembly checkpoint, a surveillance system that ensures the bipolar attachment of chromosomes to the mitotic spindle before the onset of anaphase. Under some conditions, the spindle assembly checkpoint can become weakened, allowing cells to exit mitosis despite the presence of chromosomes that are not properly attached to the mitotic spindle. Here, we use an Aurora kinase inhibitor to drive mitotic exit and test the effect of mitotic arrest length on death in the subsequent interphase. Cells that are blocked in mitosis for >15 h die shortly after exiting from mitosis, whereas cells that exit after being blocked for <15 h show variable fates, with some living for days after exiting mitosis. Cells blocked in mitosis by either Taxol or epothilone B are acutely sensitive to the death ligand tumor necrosis factor-related apoptosis-inducing ligand, suggesting that prolonged mitosis allows the gradual accumulation of internal death signals, rendering cells hypersensitive to additional prodeath cues. Death under these conditions is initiated while cyclin B1 is still present, indicating that cells are in mitosis. Our experiments suggest that there is a point of no return during prolonged mitotic block after which mitotic exit can no longer block death. [Mol Cancer Ther 2009;8(6):1646–54]

Introduction

The microtubule-stabilizing drug Taxol has been used extensively for the treatment of diverse types of cancer. Despite its widespread use, it is not completely understood how this drug kills cancer cells. Disruption of the microtubule cytoskeleton by Taxol has multiple effects, including activation of the kinases extracellular signal-regulated kinase 1/2, c-Jun NH_2-terminal kinase 1, and p38, and the transcription factor NF-κB (1). In addition, changes in microtubule function in response to Taxol can activate the spindle assembly checkpoint (SAC), thereby blocking cells in mitosis (2). The normal function of the SAC is to ensure that cells do not enter anaphase until all chromosomes attain a bipolar attachment to the mitotic spindle. The SAC can be triggered by unattached chromosomes or inappropriate tension generated at chromosomes that are improperly attached to the spindle (3). There is evidence that chromosomal passenger proteins, found at the inner centromere, can sense tension defects and trigger the SAC (4). Cells exposed to Taxol can still assemble microtubules that are likely able to attach to kinetochores. For example, the recruitment of Mad2 to kinetochores, a marker of lack of attachment, is more robust in cells exposed to nocodazole than Taxol (5). However, the altered dynamics of tubulin polymerization induced by Taxol cause tension defects, which result in SAC-dependent arrest. Inhibiting the CPC protein Aurora B abrogates Taxol-induced mitotic arrest, consistent with a role in sensing tension defects (6, 7). Epothilones, a relatively new class of drugs, are currently under development for the treatment of cancer. The epothilones may be more effective than taxanes because they are more water-soluble and not subject to P-glycoprotein-mediated resistance (8). Epothilones share a binding site on β-tubulin with Taxol and may induce cell death by similar mechanisms (9). The role of Aurora B and the SAC in cell killing by epothilones has not been thoroughly investigated.

We have used the Aurora A/B inhibitor ZM447439 to investigate the relationship between the SAC, mitotic arrest, mitotic exit, and cell death induced by the drugs that trigger the SAC. We find that inducing mitotic exit by abrogating the SAC with ZM447439 can protect cells from death induced by Taxol or epothilone B. However, if cells are arrested for >15 h in mitosis and then are driven into G_1 with ZM447439, they still rapidly die. These observations suggest the existence of a point of no return during prolonged mitotic arrest. It has been postulated that proapoptotic signals slowly intensify during prolonged mitosis (10). This idea is consistent with the combined effects of Taxol and the proapoptotic ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). We find that Taxol or epothilone B sensitize cells to TRAIL and that this death occurs primarily in mitosis. Furthermore, abrogating Taxol-induced mitotic arrest using ZM447439 reduces killing by TRAIL, indicating a key role for prolonged mitosis in predisposing cells to TRAIL-induced killing.

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Materials and Methods

Cell Lines and Culture Conditions

All cell lines were grown at 37°C in a humidified atmosphere with 10% CO_2 in DMEM containing (10,000 units/mL), streptomycin (10,000 units/mL; Cambrex Biosciences), and 10% fetal bovine serum (Life Technologies). HeLaM, a subline of HeLa, were used for all experiments (11). Taxol, epothilone B, and nocodazole were from Sigma, TRAIL was from Peprotech, and ZM447439 was provided by AstraZeneca.

Time-Lapse Microscopy

Cells were maintained in a sealed flask containing growth medium preequilibrated to 10% CO_2. Flasks were placed on a 37°C heated stage on an inverted microscope. Images were captured using a ×40 microscope objective and an Olympus C740 digital camera controlled by Cam2Com software. Images were converted to stacks and navigated using ImageJ software. Alternatively, flasks were placed on a heated stage and images were captured using a ×10 objective of a Leitz Diavert microscope and a Spot camera. Between 50 and 100 cells were tracked for each experiment. Experiments were done at least twice and representative data are shown.

Proliferation and Cell Viability

Trypan blue exclusion: Adherent cells were collected by trypsinization and combined with floating cells. Trypan blue (MP Biomedicals) was added to a final concentration of 0.1% (w/v) and cells were counted with a hemocytometer. MTS viability assay: Cells were grown in DMEM lacking phenol red in 96-well plates and viable cells were detected using the CellTiter 96 kit (Promega). Briefly, 20 μL phenazine methosulfate/MTS solution was added per well of the 96-well plate, which was incubated at 37°C for color development. Absorbance was measured at 490 nm in a plate reader.

Immunofluorescence

Cells grown on coverslips were fixed with 2% formaldehyde in PBS for 10 min at room temperature (12). Fixed cells were permeabilized by adding 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.7), 0.1% (v/v) Triton X-100, and 0.1% (w/v) bovine serum albumin for a total of 9 min, with three changes of buffer. Cells were washed once with PBS

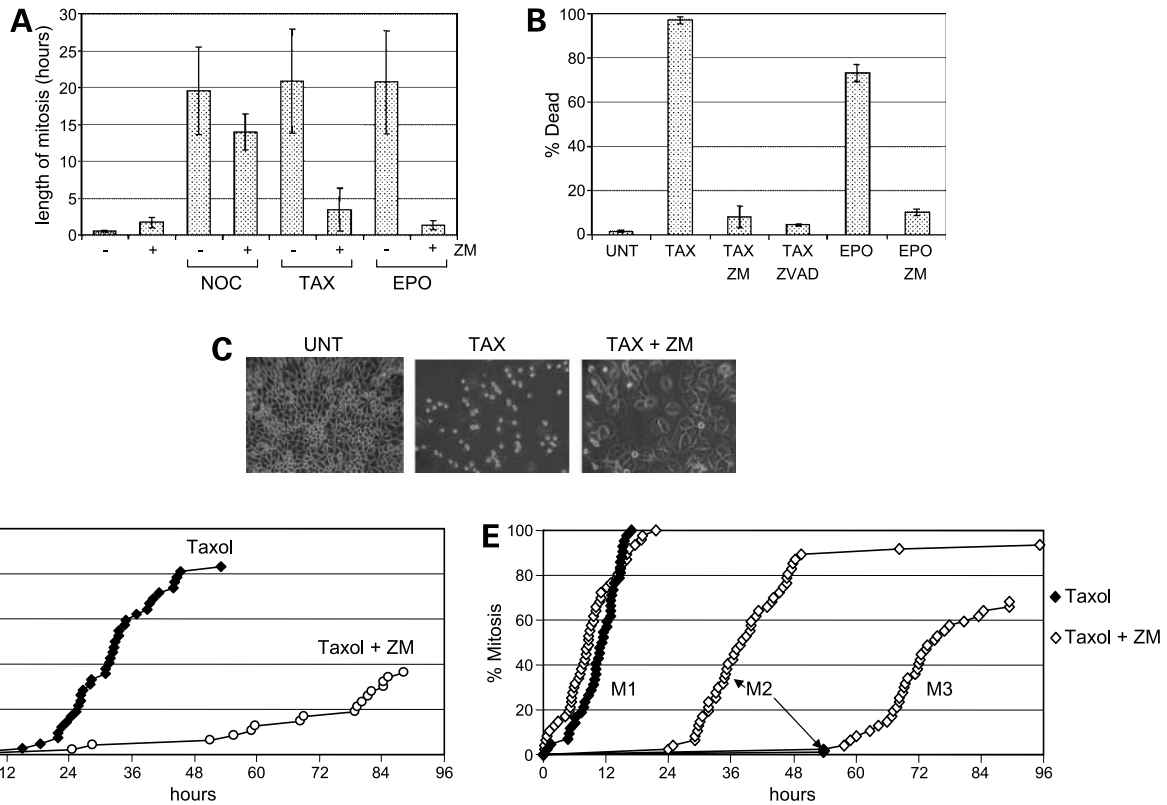

Figure 1. ZM447439 abrogates mitotic arrest and inhibits cell death by microtubule-disruptive drugs. HeLaM cells were exposed to 10 μmol/L Taxol (*TAX*), 20 nmol/L epothilone B (*EPO*), or 0.2 μg/mL nocodazole in the presence or absence of 2.5 μmol/L ZM447439 (*ZM*) or 40 μmol/L Z-VAD-FMK (*ZVAD*). **A,** length of mitosis as determined by time-lapse microscopy. In the case of cells treated with Taxol, nocodazole, or epothilone B alone, mitosis ended when the cells died. In other treatments, mitosis ended when the cells entered interphase. **B,** cell death as assessed by trypan blue exclusion. HeLaM cells were exposed to the indicated drugs for 48 h before staining with trypan blue. **C,** examples of cells exposed to Taxol for 48 h in the presence or absence of 2.5 μmol/L ZM447439. **D,** cell death as measured by time-lapse microscopy. HeLaM cells exposed to Taxol (10 μmol/L) in the presence or absence of ZM447439 (2.5 μmol/L) were tracked and cumulative events are shown. **E,** mitosis as assessed by time-lapse microscopy. HeLaM cells treated as in **D** attempted mitosis several times (M1, M2, and M3). Under these conditions, cells were unable to divide and those that attempted mitosis several times became giant and multinucleated.

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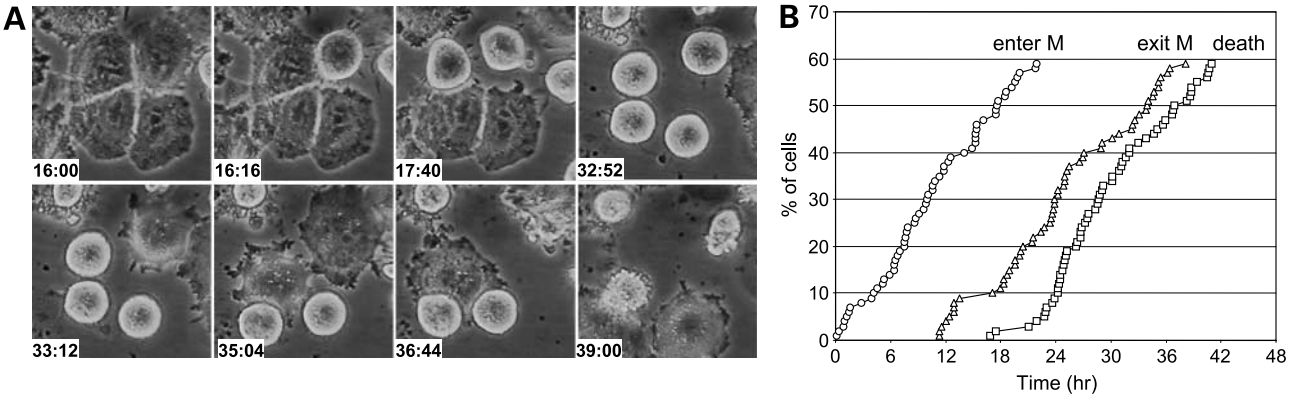

Figure 2. Mitotic exit followed by cell death after treatment with nocodazole and ZM447439. HeLaM cells simultaneously treated with 0.2 μg/mL nocodazole and 2.5 μmol/L ZM447439 were analyzed by time-lapse microscopy. **A,** examples of cells entering mitosis, exiting mitosis, and then dying. **B,** cumulative mitotic entry, mitotic exit, and death.

and blocked in PBS containing 0.1% bovine serum albumin for 1 h at room temperature. Cells were then stained with polyclonal antibodies against cleaved caspase-3 (ASP175; Cell Signaling) and monoclonal antibodies against either histone H3 phosphorylated at Ser^{10} (6G3; Cell Signaling) or cyclin B1 (GNS1; Santa Cruz). Antigens were detected using anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and anti-mouse antibodies conjugated to Alexa Fluor 568 (Invitrogen). Coverslips were mounted on microscope slides with 20 μL Vectashield Antifade agent (Vector Laboratories). Images of active caspase-3 were captured with the same exposure for all treated and untreated samples. Similarly, exposure times were constant between the cyclin B1 and the histone H3 groups. Exposures were varied (<10%) to obtain optimal images of DNA (stained with Hoechst 33342).

Results

Taxol and Epothilone B-Induced Mitotic Death Requires a Checkpoint Arrest

Tumor cells exposed to spindle disruptive agents are killed in multiple ways, including death in mitosis or death after aberrant mitosis and exit into G_1 (13–15). HeLa cells exposed to Taxol above ~20 nmol/L exhibit a prolonged mitotic block (2, 16). Micromolar serum concentrations of Taxol are not uncommon in treated patients, suggesting that the prolonged mitotic block is clinically relevant (17, 18). Our time-lapse observation of HeLaM cells exposed to 10 μmol/L Taxol indicated that 80% of them died in mitosis, whereas the remainder exited mitosis. Similar results were obtained when HeLaM cells were exposed to 0.5 μmol/L Taxol where 75% of cells died in mitosis after a prolonged block.[1] In cells exposed to 10 μmol/L Taxol, death occurred ~20 h after mitotic entry (Fig. 1A). As observed previously, Taxol-induced arrest was abrogated by inhibiting Aurora kinases with ZM447439 (Fig. 1A; ref. 19). In addition, ZM447439 dramatically reduced cell death in response to Taxol (Fig. 1B and C). The ability of ZM447439 to inhibit death was similar to the pan-caspase inhibitor ZVAD (Fig. 1B). Time-lapse microscopy indicated that whereas ~80% of cells were dead after 48 h of treatment with 10 μmol/L Taxol, only ~40% of cells treated with ZM447439 and Taxol were dead by 92 h of treatment (Fig. 1D). Time-lapse observation indicated that many cells treated with Taxol and ZM447439 were able to enter mitosis several times during the treatment period (Fig. 2E). The presence of both drugs blocked division leading to a gradual increase in cell size (Fig. 1C). Few cells treated with Taxol alone were able to attempt mitosis more than once as most died while in the first mitosis (Fig. 1D). Thus, Taxol does not block progression of HeLaM cells through the cell cycle when the SAC is abrogated.

Epothilones suppress microtubule dynamics and are able to trigger a mitotic arrest (8, 9). The roles of Aurora kinases in either mitotic arrest or cell death induced by epothilones have not been tested. Our time-lapse observations indicated that 20 nmol/L epothilone B was able to induce a prolonged mitotic block (~20 h) similar to Taxol (Fig. 1A). All of the cells treated with 20 nmol/L epothilone B died without exiting mitosis.[2] The addition of ZM447439 dramatically reduced the mitotic block (Fig. 1A) and enhanced cell survival (Fig. 1B). Thus, death induced by drugs that stabilize microtubules is correlated with a sustained mitotic block.

Length of Mitotic Arrest Determines Death after Exit into G_1

By abrogating the SAC, ZM447439 drives cells out of a checkpoint-induced mitotic arrest. This provided a means to correlate the length of mitotic arrest with cell death. Cotreatment of Taxol and ZM447439 resulted in a short arrest (3.5 ± 2.9 h), which mostly protected from acute death

[1] Our unpublished data.

[2] Our unpublished observations.

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(Fig. 1). ZM447439 more efficiently abrogates the mitotic arrest induced by drugs that stabilize microtubules than those that depolymerize microtubules such as nocodazole (20, 21). This effect is likely due to a requirement for Aurora kinase-independent pathways in the attachment-sensing arm of the SAC (triggered by loss of microtubules) versus the tension-sensing arm, which is highly dependent on Aurora kinases (3). To test whether the inhibition of death by ZM447439 was due to its ability to quickly abrogate the SAC, we exposed HeLaM cells to nocodazole and ZM447439. ZM447439 was able to partially shorten the nocodazole-induced mitotic arrest from 19.5 ± 5.9 h (nocodazole alone) to 14.0 ± 2.4 h (nocodazole + ZM447439; Fig. 1A). Interestingly, most (76%) of the cells that escaped from the nocodazole arrest in the presence of ZM447439 died 5.3 ± 3.7 h after exiting mitosis (Fig. 2A and B). Only 12% of the cells died in mitosis, whereas the remainder did not die during the duration of the experiment. Overall,

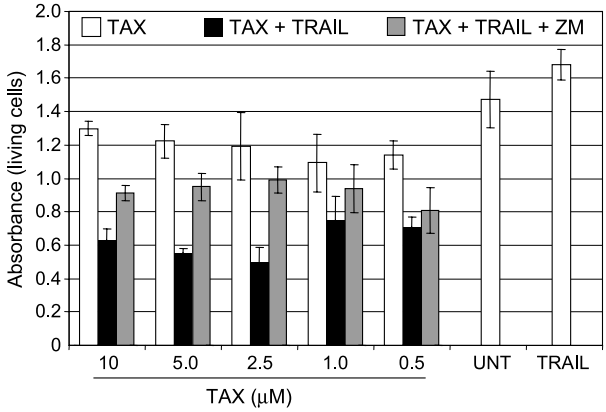

Figure 4. Inhibition of Taxol/TRAIL killing by ZM447439. HeLaM cells were exposed to the drugs indicated and cell viability determined 48 h later using the MTS assay as described in Materials and Methods. TRAIL was added at 10 ng/mL.

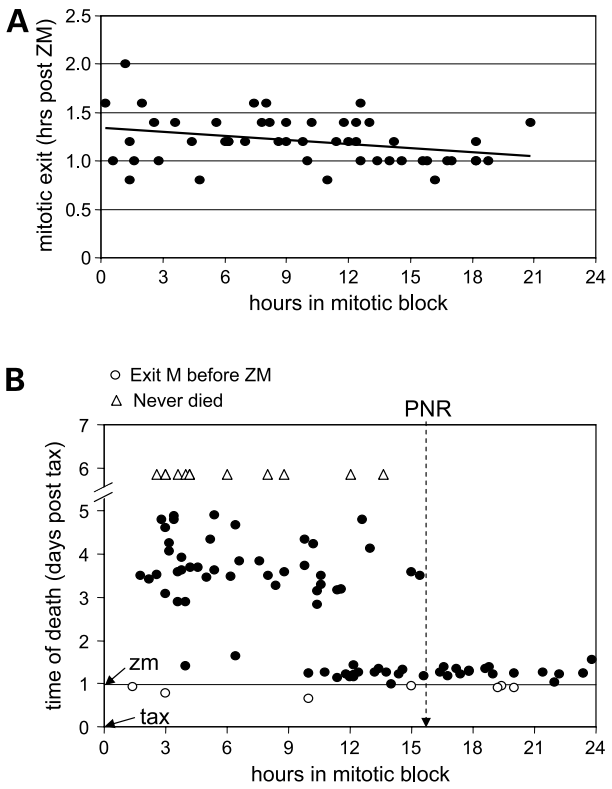

Figure 3. Length of mitotic arrest determines cell death on mitotic exit. HeLaM cells were exposed to 10 μmol/L Taxol for 24 h followed by 2.5 μmol/L ZM447439. Entry into mitosis during the Taxol treatment was tracked by time-lapse microscopy. Tracking was continued on the same field of cells after the addition of ZM447439 to determine mitotic exit and cell death. **A,** mitotic exit induced by ZM447439. The length of time for cells to exit mitosis after exposure to ZM447439 is compared with how long they had been previously blocked in mitosis in the presence of Taxol. **B,** fate of cells after mitotic exit as a function of the length of mitotic block. The time of cell death is compared with the length of the previous mitotic arrest. Some cells did not die during the entire length of the experiment (*white triangles*) and several exited mitosis before ZM447439 was added (*white circles*). All cells in the field did exit mitosis after treatment with ZM447439.

these results suggest that ZM447439 is not a general inhibitor of death in cells exposed to drugs that activate the SAC. In contrast, mitotic exit appears to end in death if the mitotic arrest is prolonged.

To further test the role of prolonged mitotic arrest in death after cells exit to G_1, we treated cells with Taxol and followed their entry into mitosis by time-lapse microscopy. In this way, we could obtain a record of how long each cell in the microscopic field had been blocked in mitosis (mitotic age). ZM447439 was then added and the same field of cells was followed to determine their fate. ZM447439 drove all cells out of mitosis within 0.5 to 2.0 h regardless of how long they were blocked in mitosis (Fig. 3A). In contrast, the fate of cells was critically influenced by their mitotic age. Cells that were blocked for ≥15 h died shortly (5.9 ± 2.7 h) after being driven out of mitosis by ZM447439 (Fig. 3B). Cells blocked for <15 h showed variable fates with some surviving for many days in the continued presence of ZM447439 and Taxol (Fig. 3B). The cells that lived continued to progress through the cell cycle entering mitosis several times and becoming large and multinucleated.[2] This experiment indicates that there is a point of no return during prolonged mitotic block. Cells that pass this point are destined to die even if they exit mitosis. Cells that exit mitosis before the point of no return may live for several days or may die earlier.

Taxol and Epothilone B Sensitize Mitotic Cells to TRAIL

One possible explanation for the effect of mitotic age on cell death is that proapoptotic signaling gradually becomes elevated during the mitotic arrest (10). If this were the case, we predict that cells arrested in mitosis would become sensitive to additional proapoptotic signals. TRAIL and Taxol can synergistically kill cells and this effect appears to be related to mitotic arrest (22). We also observed that Taxol or epothilone sensitized HeLaM cells to a dose of TRAIL that could not kill them on its own (Fig. 4). Next, we added Taxol and Trial together and assessed death in

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relation to mitotic entry using time-lapse microscopy. The majority of cells (~80%) died after entering mitosis. Furthermore, we observed a positive correlation when they entered mitosis and when they died (Fig. 5A). In a second type of experiment, we added Taxol and followed the entry of a field of cells into mitosis for 1 day. TRAIL was then added and the time it took to induce cell death was compared with how long each cell had previously spent in mitosis. Cell death was induced within a range of 2 to 8.2 h (Fig. 5B). There was a slight downward trend in the rapidity of death, as cells spent more time in mitosis before the addition of TRAIL. However, this effect was not statistically significant (Fig. 5C). Further, the ability of TRAIL to kill cells while blocked in mitosis was similar in two different doses of Taxol (0.5 and 10 μmol/L; Fig. 5A and B, respectively). Next, we repeated these experiments with epothilone B to determine whether the effect was specific to Taxol. Time-lapse analysis indicated that it only took ~6 h to kill ~80% of cells exposed to epothilone B when combined with TRAIL, whereas it took ~27 h for epothilone B on its own to kill a similar percentage of cells (Fig. 5D). Cells that died in mitosis in the presence of epothilone B and TRAIL did so ~5 h after entering mitosis (Fig. 5E).

Our observations suggested that Taxol and epothilone sensitize cells to TRAIL by blocking them in mitosis. For example, 90% of cells were dead within 8.5 h of entering mitosis in the presence of Taxol and TRAIL.[1] To test the idea that mitotic arrest sensitizes to TRAIL-induced killing, we abrogated the SAC using ZM447439. Exposing cells to ZM447439 reduced the killing of cells the TRAIL/Taxol combination over several different concentrations of Taxol (Fig. 4). Thus, the ability of Taxol to trigger a mitotic block is a key component of its ability to sensitize cells to TRAIL. The ability of TRAIL to kill cells arrested by treatment with Taxol was recently postulated to be due to the ability of TRAIL to drive cells out of mitosis (22). However, in our experiments, inducing mitotic exit with ZM447439 reduces death induced by the Taxol/TRAIL combination. To investigate this idea further, we triggered cell death by treating cells with Taxol, epothilone, or Taxol + TRAIL and analyzed mitotic and apoptotic markers simultaneously. Cells positive for an active form of caspase-3 (undergoing apoptosis) were often negative for histone H3 phosphorylated at Ser^{10} (Fig. 6A and B). However, some cells positive for active caspase-3 still expressed cyclin B1 (Fig. 6A and C). This indicates that apoptosis can occur while cells are still in mitosis.

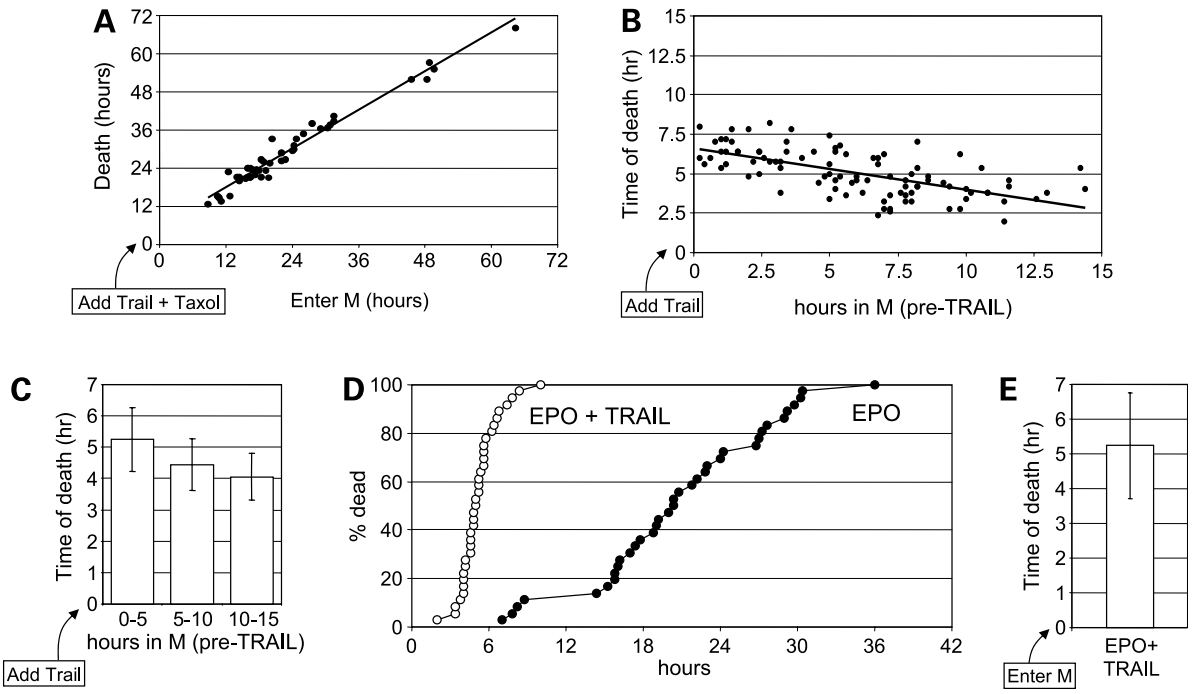

Figure 5. Cell death in response to TRAIL in combination with Taxol or epothilone B. HeLaM cells were exposed to the indicated drugs and cell fates were determined by time-lapse microscopy. **A,** simultaneous exposure to Taxol and TRAIL. HeLaM cells were exposed to 0.5 μmol/L Taxol and 10 ng/mL TRAIL and then immediately tracked by time-lapse microscopy to correlate mitosis with cell death. Eighty-nine percent of cells died during filming, with the majority dying during mitosis (79% of total). Time of death is compared with time of entry into mitosis. **B,** addition of TRAIL after Taxol. HeLaM cells were exposed to 10 μmol/L Taxol for 18 h after which 10 ng/mL TRAIL was added. Entry into mitosis during the Taxol treatment was tracked by time-lapse microscopy after which the same field of cells was tracked after adding TRAIL. The time of death relative to the addition of TRAIL is compared with the length of time each cell had been blocked in mitosis before the addition of TRAIL. **C,** TRAIL kills mitotic cells in 4 to 5 h. The length of time needed for TRAIL to kill mitotic cells is compared among groups of cells that had spent the indicated amounts of time in mitosis in the presence of Taxol. Data from **B** were compiled for these measurements. **D,** acceleration of cell death by TRAIL and epothilone B. Time-lapse analysis of cell death in cultures treated with 20 nmol/L epothilone B alone or in combination with 10 ng/mL TRAIL. **E,** time of death induced by epothilone B and TRAIL. HeLaM cells were exposed to 20 nmol/L epothilone B and 10 ng/mL TRAIL and tracked by time-lapse microscopy. The average time between entering mitosis and death in the presence of both drugs is shown.

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Discussion

Despite its widespread use in chemotherapy, the mechanism by which Taxol kills cells is not completely understood. One of the most obvious effects of Taxol is its ability to trigger mitotic arrest due to its effects on the mitotic spindle. Mitotic arrest may be lethal in several ways. Higher doses of Taxol cause a prolonged mitotic arrest followed by death, which appears to be initiated while still in mitosis (this study; ref. 13). Short-term arrest that occurs at lower doses of the drug can allow mitotic exit with subsequent death during interphase (13). The mechanisms responsible for this type of death are not well known. Alternatively, cells with an intact p53 signaling pathway may become blocked in G_1 following a defective mitosis in the presence of low doses of Taxol (23–25).

In our experiments, cells were driven out of a checkpoint-induced mitotic arrest using a drug that inactivates Aurora B, a proximal sensor in the checkpoint pathway. This mitotic

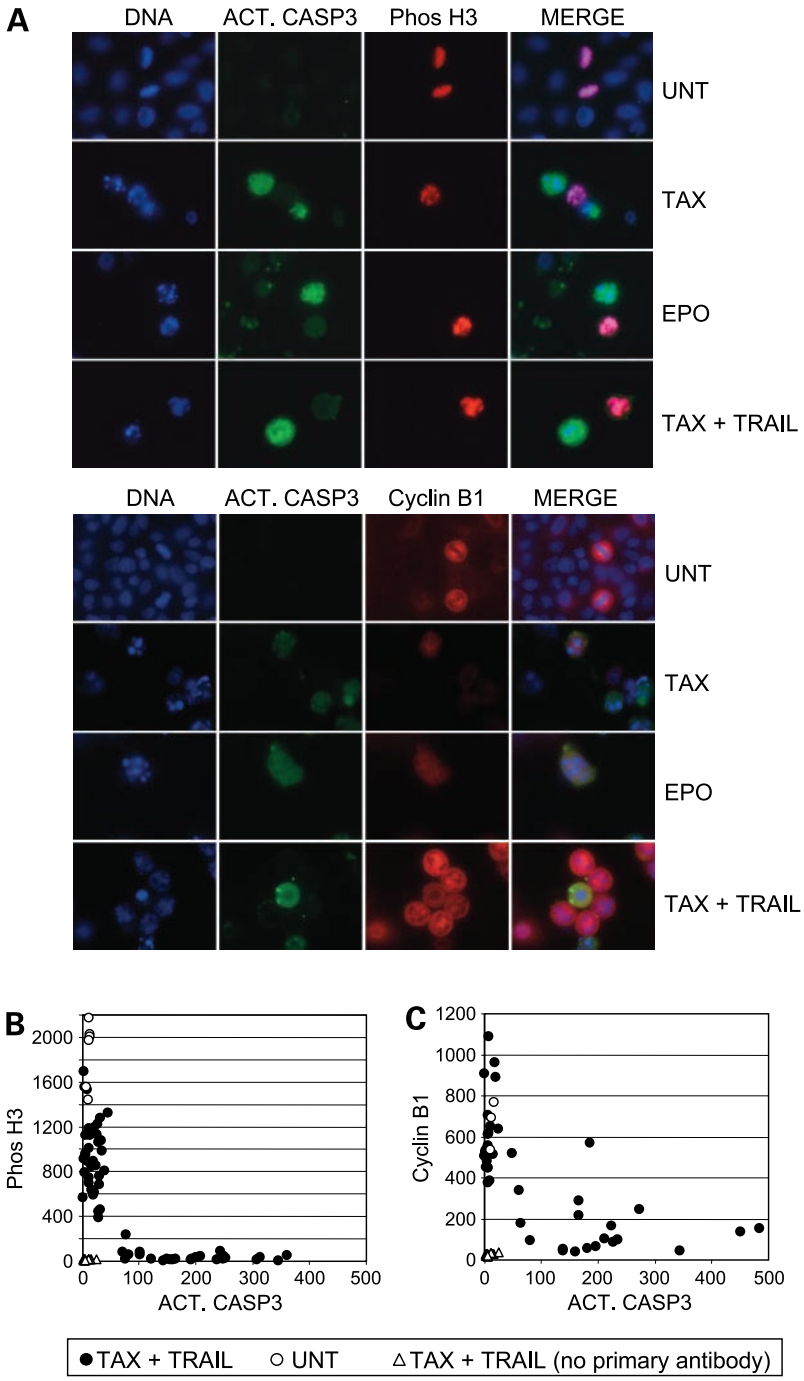

Figure 6. Presence of active caspase-3 and mitotic markers in cells exposed to microtubule-disruptive agents. HeLaM cells were exposed to the drugs indicated. **A,** examples of cells containing active caspase-3 (*ACT. CASP3*) and either histone H3 phosphorylated at Ser^{10} (*Phos H3*) or cyclin B1. Treatments with 10 μmol/L Taxol or 20 nmol/L epothilone were for 48 h. For "Tax + TRAIL," cells were exposed to Taxol for 24 h followed by 10 ng/mL TRAIL for an additional 24 h before being stained. Antigens were detected by immunofluorescence. **B,** levels of caspase-3 and phosphorylated histone H3 in single cells. HeLaM cells were exposed to 10 μmol/L Taxol and 10 ng/mL TRAIL for 24 h and analyzed by immunofluorescence and pixel intensities were measured using ImageJ software. **C,** levels of caspase-3 and cyclin B1. Cells were treated as described in **B** but stained with antibodies to cyclin B1 in place of histone H3. If we define a positive signal as one five times higher than the average staining in a negative control, we find that 22% of caspase-positive cells are positive for phosphorylated histone H3 and 50% are positive for cyclin B1.

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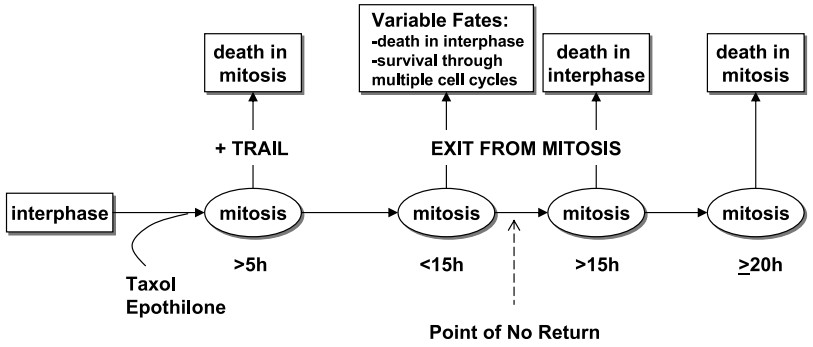

Figure 7. Model for events during prolonged mitotic block. Early during the block, cells become sensitized to TRAIL. Mitotic exit can protect from death as long as the mitotic block is <15 h. After 15 h blocked in mitosis, cells are destined to die. If they exit mitosis after this point, they die in their first interphase. If they remain in mitosis they die by a caspase-dependent mechanism that is initiated while still in mitosis.

exit occurs despite the presence of the initiating signal for the checkpoint (spindle damage) and has some similarity to checkpoint adaptation. Checkpoint adaptation, originally described in budding yeast, describes a process in which checkpoint-induced cell cycle arrest eventually is released in the presence of unrepaired DNA damage (26). Orthologues of PLK1 contribute to the inactivation of the checkpoint pathway to bring about adaptation (27, 28). Our experiments and those involving checkpoint adaptation involve processes, which are different from checkpoint recovery in which the checkpoint is inactivated due to repair of the initial damage. The mitotic arrest induced by spindle disruptive agents also can be released by the gradual loss of the checkpoint effector, cyclin B, despite the continued presence of spindle damage and the activation of upstream checkpoint proteins (29, 30).

The role of the SAC in cell death in response to spindle disruption is complicated. For example, a dominant-negative Bub1 reduced acute cell death in HeLa cells exposed to 0.2 μg/mL nocodazole (6). Suppressing Mad2 or Bub1 using small interfering RNA also reduced the short-term survival of MCF7 cells exposed to 100 nmol/L Taxol (31). In contrast, the long-term survival of HeLa cells exposed to 10 nmol/L Taxol was greater when Mad2 or Bub1 were suppressed with small interfering RNA (32). Experiments using ZM447439 suggest that the SAC is required for the maximal short-term lethality of spindle-disruptive agents (this study; ref. 10). Therefore, the SAC may have distinct roles in determining the short- and long-term viability of cells in response to drugs such as Taxol. Cell death is much more variable among different tumor cell types than mitotic arrest on spindle disruption (33). This suggests that cell type differences in apoptotic signaling pathways may play a large role in the cell death response and may explain some differences in the role of the SAC in cell death in different cell types.

Taxol and epothilone B do not cause massive depolymerization of microtubules as do the spindle poisons nocodazole, vincristine, and vinblastine (34). Because microtubules are present in cells exposed to Taxol and epothilone B, there is an opportunity for kinetochores to be captured. However, these drugs cause tension defects, which are sensed by an Aurora kinase-dependent mechanism to trigger the SAC. Spindle poisons, such as nocodazole, depolymerize microtubules, thereby disrupting both the attachment of kinetochores to microtubules and the tension between kinetochores. These two signals trigger the SAC via Aurora-dependent and Aurora-independent pathways. Consequently, Aurora inhibitors (ZM447439) can efficiently abrogate the mitotic arrest by Taxol and epothilone but not nocodazole. We took advantage of the ability of ZM447439 to abrogate the SAC to trigger mitotic exit at defined time points relative to mitotic arrest. These experiments show that short mitotic arrest followed by ZM447439-induced exit into G_1 does not result in immediate death, whereas longer arrest does. By comparing cells that have been arrested for various lengths of time, we find that there is a point of no return during prolonged mitotic arrest. Cells blocked for ≥15 h in Taxol do not survive long on exiting from mitosis. Cells arrested for shorter times in mitosis show variable fates, with some surviving for days (Fig. 7).

Ultimately, many of the cells that escape mitosis before the point of no return do eventually die. Thus, the length of mitotic arrest has less effect on the long-term fate of cells than it does on their short-term fate. We believe that the effects of mitotic age on short-term fate provide important clues about events that transpire during the mitotic block that affect cell killing. The fact that it takes ~20 h for Taxol to induce death in mitosis suggests that the mechanism of death is linked to gradual changes in cellular biochemistry. This time frame is less consistent with rapid signal transduction events and more consistent with gradual accumulation of prodeath signals (10, 35). Given that most transcription factors are displaced from mitotic chromatin, it has been postulated that the decay of mRNAs may influence cell fates during prolonged mitotic block (35, 36). We predict that the differential decay of mRNAs encoding proteins that influence apoptotic cell death during mitosis leads to a gradual inclination toward death. By 20 h in HeLaM cells, cell death ensues. By 15 h, enough prodeath signal has accumulated that reversal is not possible even in cells that exit mitosis and resume transcription. Cells blocked in mitosis for <15 h may be rescued by exiting mitosis, because there is enough time to resume transcription of antiapoptotic proteins during G_1 to counter the prodeath program (Fig. 7). The variability in the fate of cells blocked for <15 h may be related to

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differences in their original levels of proapoptotic and antiapoptotic mRNAs and proteins when they arrive in mitosis. Variability may also arise from differences in the ability of cells to resume transcription after mitotic exit.

The idea that a prodeath signal gradually accumulates during mitotic arrest has been previously suggested (10). This idea is consistent with the effects of the death ligand TRAIL. Taxol can sensitize cells to sublethal doses of TRAIL and this effect is cell cycle dependent, with most cells dying in mitosis (this study; ref. 22). A normal, short mitosis likely does not allow the accumulation of prodeath signals sufficient to sensitize to sublethal concentrations of TRAIL. Blocking cells in mitosis with Taxol leads to death, on average, 5 h after adding TRAIL (Fig. 7). This was observed even in cells that had been prearrested for ≥10 h in mitosis before adding TRAIL. This suggests that the combined effects of TRAIL and Taxol are more complicated than a simple gradual elevation of proapoptotic signals. If that were the case, one might expect old mitotic cells to be killed more quickly. The ability of epothilone B to sensitize HeLaM cells to TRAIL indicates that the sensitizing effect is not specific to Taxol. To test the role of the mitotic block in the sensitizing effect of Taxol, we abrogated the SAC with ZM447439. Under these conditions, fewer cells were killed by the TRAIL/Taxol combination, providing evidence that the mitotic block plays a key role in the effect. Although ZM447439 could protect cells treated with Taxol and TRAIL, cell survival was not returned to levels observed with TRAIL alone. This effect may indicate that there is a mitosis-independent sensitizing effect of Taxol perhaps via its ability to activate stress pathways. Alternatively, although ZM447439 abrogates the prolonged Taxol-induced mitotic block, cells exposed to Taxol and ZM447439 still have a longer than normal mitosis (~3.5 versus 0.5 h; Fig. 1A). This extended mitosis may allow the accumulation of prodeath signals sufficient to sensitize cells to TRAIL.

Phase-contrast microscopy suggests that cells exposed to 10 μmol/L Taxol as well as the Taxol/TRAIL combination die while they are still in mitosis. To test this idea, we analyzed markers of apoptosis (active caspase-3) and mitosis (S10-phosphorylated histone H3 and cyclin B1). Because some apoptotic cells still express cyclin B1, this suggests that apoptosis induced by either Taxol, epothilone, or Taxol plus TRAIL can be initiated while cells are in mitosis. However, the presence of active caspase-3 and S10-phosphorylated histone H3 appears to be mutually exclusive. It is possible that caspases inactivate the H3 kinase, activate the H3 phosphatase, or directly degrade histone H3 during apoptosis. We also observed cells with active caspase-3 and low levels of cyclin B1, indicating that these dying cells eventually do exit mitosis. However, the fact that driving cells out of mitosis with ZM447439 reduced cell death by Taxol, epothilone B, or Taxol in combination with TRAIL suggests that mitotic exit is not a major prodeath event.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta 2008;1785:96–132.
2. Jordan MA, Toso RJ, Thrower D, Wilson L. Mechanism of mitotic block and inhibition of cell proliferation by Taxol at low concentrations. Proc Natl Acad Sci U S A 1993;90:9552–6.
3. Pinsky BA, Biggins S. The spindle checkpoint: tension versus attachment. Trends Cell Biol 2005;15:486–93.
4. Sandall S, Severin F, McLeod IX, et al. A Bir1-15 complex connects centromeres to microtubules and is required to sense kinetochore tension. Cell 2006;127:1179–91.
5. Lens SM, Wolthuis RM, Klompmaker R, et al. Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. EMBO J 2003;22:2934–47.
6. Taylor SS, McKeon F. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell 1997;89:727–35.
7. Lens SM, Medema RH. The survivin/Aurora B complex: its role in coordinating tension and attachment. Cell Cycle 2003;2:507–10.
8. Goodin S, Kane MP, Rubin EH. Epothilones: mechanism of action and biologic activity. J Clin Oncol 2004;22:2015–25.
9. Kamath K, Jordan MA. Suppression of microtubule dynamics by epothilone B is associated with mitotic arrest. Cancer Res 2003;63:6026–31.
10. Gascoigne KE, Taylor SS. Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. Cancer Cell 2008;14:111–22.
11. Tiwari RK, Kusari J, Sen GC. Functional equivalents of interferon-mediated signals needed for induction of an mRNA can be generated by double-stranded RNA and growth factors. EMBO J 1987;6:3373–8.
12. Kaur H, Stiff AC, Date DA, Taylor WR. Analysis of mitotic phosphorylation of borealin. BMC Cell Biol 2007;8:5.
13. Chen JG, Horwitz SB. Differential mitotic responses to microtubule-stabilizing and -destabilizing drugs. Cancer Res 2002;62:1935–8.
14. Torres K, Horwitz SB. Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res 1998;58:3620–6.
15. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. Cancer Cell 2005;8:7–12.
16. Ikui AE, Yang CP, Matsumoto T, Horwitz SB. Low concentrations of Taxol cause mitotic delay followed by premature dissociation of p55CDC from Mad2 and BubR1 and abrogation of the spindle checkpoint, leading to aneuploidy. Cell Cycle 2005;4:1385–8.
17. Ohtsu T, Sasaki Y, Tamura T, et al. Clinical pharmacokinetics and pharmacodynamics of paclitaxel: a 3-hour infusion versus a 24-hour infusion. Clin Cancer Res 1995;1:599–606.
18. Nakajima M, Fujiki Y, Kyo S, et al. Pharmacokinetics of paclitaxel in ovarian cancer patients and genetic polymorphisms of CYP2C8, CYP3A4, and MDR1. J Clin Pharmacol 2005;45:674–82.
19. Ditchfield C, Johnson VL, Tighe A, et al. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol 2003;161:267–80.
20. Hauf S, Cole RW, LaTerra S, et al. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J Cell Biol 2003;161:281–94.
21. Morrow CJ, Tighe A, Johnson VL, Scott MI, Ditchfield C, Taylor SS. Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20. J Cell Sci 2005;118:3639–52.
22. Kim M, Liao J, Dowling ML, et al. TRAIL inactivates the mitotic checkpoint and potentiates death induced by microtubule-targeting agents in human cancer cells. Cancer Res 2008;68:3440–9.
23. Blagosklonny MV. Prolonged mitosis versus tetraploid checkpoint: how p53 measures the duration of mitosis. Cell Cycle 2006;5:971–5.
24. Blagosklonny MV, Demidenko ZN, Giovino M, et al. Cytostatic activity of paclitaxel in coronary artery smooth muscle cells is mediated through transient mitotic arrest followed by permanent post-mitotic arrest: comparison with cancer cells. Cell Cycle 2006;5:1574–9.

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25. Demidenko ZN, Kalurupalle S, Hanko C, Lim CU, Broude E, Blagosklonny MV. Mechanism of G_1-like arrest by low concentrations of paclitaxel: next cell cycle p53-dependent arrest with sub G_1 DNA content mediated by prolonged mitosis. Oncogene 2008; 27:4402–10.

26. Toczyski DP, Galgoczy DJ, Hartwell LH. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. Cell 1997;90: 1097–106.

27. Pellicioli A, Lee SE, Lucca C, Foiani M, Haber JE. Regulation of *Saccharomyces* Rad53 checkpoint kinase during adaptation from DNA damage-induced G_2/M arrest. Mol Cell 2001;7:293–300.

28. Yoo HY, Kumagai A, Shevchenko A, Dunphy WG. Adaptation of a DNA replication checkpoint response depends upon inactivation of claspin by the Polo-like kinase. Cell 2004;117:575–88.

29. Brito DA, Yang Z, Rieder CL. Microtubules do not promote mitotic slippage when the spindle assembly checkpoint cannot be satisfied. J Cell Biol 2008;182:623–9.

30. Brito DA, Rieder CL. Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. Curr Biol 2006; 16:1194–200.

31. Sudo T, Nitta M, Saya H, Ueno NT. Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. Cancer Res 2004;64: 2502–8.

32. Niikura Y, Dixit A, Scott R, Perkins G, Kitagawa K. BUB1 mediation of caspase-independent mitotic death determines cell fate. J Cell Biol 2007; 178:283–96.

33. Shi J, Orth JD, Mitchison T. Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. Cancer Res 2008; 68:3269–76.

34. Sudakin V, Yen TJ. Targeting mitosis for anti-cancer therapy. BioDrugs 2007;21:225–33.

35. Blagosklonny MV. Mitotic arrest and cell fate: why and how mitotic inhibition of transcription drives mutually exclusive events. Cell Cycle 2007;6:70–4.

36. Delcuve GP, He S, Davie JR. Mitotic partitioning of transcription factors. J Cell Biochem 2008;105:1–8.

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Length of mitotic arrest induced by microtubule-stabilizing drugs determines cell death after mitotic exit

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