Cell Type Variation in Responses to Antimitotic Drugs that Target Microtubules and Kinesin-5

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Abstract

To improve cancer chemotherapy, we need to understand the mechanisms that determine drug sensitivity in cancer and normal cells. Here, we investigate this question across a panel of 11 cell lines at a phenotypic and molecular level for three antimitotic drugs: paclitaxel, nocodazole, and an inhibitor of kinesin-5 (also known as KSP, Eg5, Kif11). Using automated microscopy with markers for mitosis and apoptosis (high content screening), we find that the mitotic arrest response shows relatively little variation between cell types, whereas the tendency to undergo apoptosis shows large variation. We found no correlation between levels of mitotic arrest and apoptosis. Apoptosis depended on entry into mitosis and occurred both from within mitosis and after exit. Response to the three drugs strongly correlated, although paclitaxel caused more apoptosis in some cell lines at similar levels of mitotic arrest. Molecular investigations showed that sensitivity to apoptosis correlated with loss of an antiapoptotic protein, XIAP, during the drug response, but not its preresponse levels, and to some extent also correlated with activation of the p38 and c-Jun NH2 kinase pathways. We conclude that variation in sensitivity to antimitotic drugs in drug-naive cell lines is governed more by differences in apoptotic signaling than by differences in mitotic spindle or spindle assembly checkpoint proteins and that antimitotics with different mechanisms trigger very similar, but not identical, responses. [Cancer Res 2008;68(9):3269–76]

Introduction

Genetic and phenotypic heterogeneity of cancer presents a large challenge to effective chemotherapy. Drug responses vary greatly, not only between disease types, but also between individual patients with the same disease. For certain inhibitors of kinase oncogenes, variation in drug sensitivity can be caused by mutations in the drug binding site (1), but for most anticancer drugs, the mechanistic basis of drug sensitivity is poorly understood. This question has been investigated in several ways, including sequencing target protein genes, selection of drug-resistant cell lines, and molecular profiling. However, for most commonly used anticancer drugs, including antimitotics, this problem is far from solved (2).

Established antimitotic drugs are mostly from the taxane and Vinca alkaloid classes, which bind to microtubules and inhibit their polymerization dynamics (3). Here, we use two antimicrotubule drugs: paclitaxel, which stabilizes microtubules and is used clinically, and nocodazole, which destabilizes microtubules and is only used as a laboratory tool. Experimental antimitotics include novel microtubule inhibitors, such as the epothilones and dolastatins (4), as well as compounds that inhibit nonmicrotubule proteins in the mitotic spindle. The latter class include inhibitors of the motor protein kinesin-5 (KSI; also known as KSP, Eg5, and Kif11) and inhibitors of three mitotic kinases (Aurora-A, Aurora-B, and Plk-1; ref. 5). Here, we use a KSI, EMD534085, that is similar in mechanism, but not structure, to recently described compounds (6, 7). Like other K5Is, it prevents bipolar spindle assembly and causes arrest in mitosis with monopolar spindles. All the antimitotics mentioned above activate the spindle assembly checkpoint (except Aurora-B inhibitors, which block checkpoint activation). Checkpoint activation sequesters Cdc20, inhibiting cyclin-B proteolysis and the metaphase-to-anaphase transition (8). Drug-treated cells eventually slip out of mitotic arrest without assembling a normal spindle (9). This abnormal mitotic exit pathway involves ubiquitination of Cdc20 (10) and gradual proteolysis of cyclin B (11). After exit without cytokinesis, the now tetraploid cells enter an abnormal G1 phase of the cell cycle and may arrest or proliferate, depending in part on their p53 status (12).

Depending on the cell type and drug, cells treated with antimitotics may also undergo programmed cell death, either directly from the abnormal mitosis or after exit (9, 13). This death is central to both the therapeutic and toxic effects of antimitotics, but its mechanism is poorly understood. Most studies reported that cell death triggered by antimitotics was classic apoptosis, with caspase activation and poly(ADP-ribose) polymerase 1 (PARP1) cleavage (6, 14–16). However, a few studies reported death by poorly understood caspase-independent mechanisms (17, 18). Inactivation of antiapoptotic proteins Bcl2 and Bcl-xL by phosphorylation was implicated in death triggered by paclitaxel (19, 20), as were several kinases, including c-Jun NH2 terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 mitogen activated protein (MAP) kinase, AKT, and protein kinase A (21–26). There are contradictory reports regarding the role of the spindle assembly checkpoint in mediating cell death triggered by antimitotics. Most studies reported that checkpoint activation was required for death triggered by paclitaxel or KSI (6, 27–29), but some suggested death was checkpoint independent (30, 31).

Despite the high degree of evolutionary conservation of spindle and checkpoint proteins, different cell types can respond very differently to antimitotics, and the mechanistic basis of this variation remains poorly understood. For example, variation in clinical response to taxanes does not seem to correlate with proliferation rate, drug pump expression, tubulin alterations, or p53 status (2). Here, we investigate variation in antimitotic drug responses in cell culture. This question has been extensively investigated before for paclitaxel, but we departed from previous studies in using information-rich readouts, including high content
screening and quantitative time-lapse microscopy, to quantify different aspects of drug responses, and in systematically comparing paclitaxel to K5I, which arrests cells in mitosis by a different mechanism.

Materials and Methods

Cell culture. Solid tumor lines were purchased from American Type Culture Collection. OVCAR-5 was kindly given by Dr. T. Gulob (Broad Institute). All cell lines were cultured under 37°C and 5% CO₂ in appropriate medium supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Specifically, RPE was maintained in DMEM/F12K 50:50; HeLa and MDAMB435S in DMEM; A549 and PC3 in F-12K; MCF7, OVCAR5, and H460 in RPMI; U2OS, H116, and HT29 in McCoy’s.

Chemicals and antibodies for immunostaining. Paclitaxel, nocodazole, staurosporine, aphidicolin, propidium iodide, neocarzinostatin, and Hoechst were purchased from Sigma. Caspase inhibitor zVAD-FMK was purchased from Calbiochem. The K5I (EMD534085) was provided by Merck-Serono. Details of its structure and pharmacology will be described elsewhere.1 Anti-α-tubulin antibody (Sigma) was used for tubulin staining; anti–phosphorylated histone 3 (Upstate) or anti–phosphorylated Ser/Thr (MPM-2; Upstate) antibody was used for staining mitotic cells; anti–cleaved PARP1 (Epitomics) or anti–cleaved caspase-8 (Cell signaling) antibody was used to stain apoptotic cells. Secondary antibodies conjugated with different AlexaFluors were purchased from Invitrogen.

Flow cytometric analysis of cell cycle. After 24-h drug treatment, cells were harvested and fixed in 80% ethanol and 20% PBS. After at least 2 h of fixation at −20°C, cells were washed by pelleting and resuspending in PBS.
Data for NCI60 cell line panel and the COMPARE algorithm can be accessed at the NCI database website: http://dtp.nci.nih.gov/docs/dtp_search.html.

and then stained with 25 μg/mL propidium iodide, 0.2 mg/mL Ruase A, and 0.1% Triton in PBS at 37°C for 20 min. Flow cytometric measurement of cellular DNA content was conducted with a FACSCalibur cytometer (Becton Dickinson). Percentages of cells in different cell cycle stages were estimated by the observed distribution of DNA content, i.e., sub-G₀ (DNA < 2N), G₁-G₂ (DNA = 2N), S (2N < DNA < 4N), G₂-M (DNA = 4N), and polyploid (DNA > 4N). EC₅₀ and Fₚ₅ₐₓ values were obtained from standard sigmoidal fits using the data analysis software Origin (OriginLab Corporation).

### Phenotypic Profiling by Automated Microscopy
Cells were plated in 384-well imaging plates (Corning) at an initial density of 3,500 per well. Drugs were added 24 h later. Cells were fixed and permeabilized at different time points using 3.7% formaldehyde, 100 mM PIPES, 10 mM EGTA, 1 mM MgCl₂, 0.2% Triton X-100 (pH 6.8). Before fixation, plates were spun at 1,500 rpm for 5 min to minimize loss of floating cells. Then cells were stained with antibody to phosphorylated histone 3 and cleaved caspase-9, XIAP, p38, Bid, phosphorylated p38, JNK, phosphorylated JNK, phosphorylated Chk1, phosphorylated Chk2, and phosphorylated PERK (Thr980) from Cell Signaling; Bcl2 from Biosource. Anti–α-tubulin (Sigma) was used as a loading control.

#### Image Analysis
Cells (100–200 per well) were quantified, and eight replicate wells were averaged for each measurement. Using Metamorph software (Molecular Devices), nuclei were segmented in the Hoechst 33258. Plates were imaged with an ImageXpress automated microscope (Molecular Devices) using a 20× objective lens.

#### Time-Lapse Phase-Contrast Microscopy
Cells were plated in 24-well glass-bottomed plates (MatTek) in CO₂-independent medium (Invitrogen), supplemented with 10% FCS, 100 units/mL penicillin, and 100 μL streptomycin and observed with an inverted microscope using a 20× objective (numerical aperture, 0.95; Nikon) enclosed in a humidified chamber maintained at 37°C. Each well was imaged every 10 min using a motorized stage.

### Western Blot Analysis
Drug-treated cells grown in six-well plates were lysed at 12 time points after drug addition using LDS sample buffer (NuPAGE, Invitrogen). Proteins were resolved on 10% or 12% Tricine gels and transferred onto nitrocellulose membranes. Blots were probed with commercial primary antibodies and chemiluminescent detection using enhanced chemiluminescence (Amersham, ECL-plus (Amersham), or SuperSignal Femto (Pierce), depending on the strength of the signal. Antibodies used were as follows: phosphorylated histone H3 and phosphorylated histone H2AX purchased from Upstate; PARP1, caspase-8, caspase-9, XIAP, p38, Bid, phosphorylated p38, JNK, phosphorylated JNK, phosphorylated Akt (Thr308), phosphorylated Akt (Ser437), phosphorylated Chk1, phosphorylated Chk2, and phosphorylated PERK (Thr980) from Cell Signaling; Bcl2, Bcl-xl, Akt, p53, BIP (GRP78), and CHOP (GADD 153) from Santa Cruz; Bc2 from Biosource. Anti–α-tubulin (Sigma) was used as a loading control.

### Results

#### Dose-Response of Human Cell Lines Treated with Antimitotic Drugs
Eleven human cell lines were chosen on the basis of approximately similar doubling times (~18–26 hours) and possible variation in antimitotic response from the National Cancer Institute–Developmental Therapeutics Program database.² They were derived from cancers of the lung (A549, H460), colon (HCT116, HT29), breast (MCF7, MDAMB435S), ovary (OVCAR3), cervix (HeLa), prostate (PC3), and bone (U2OS). RPE, a telomerase immortalized line derived from retinal pigment epithelium, was included as a noncancer control. Preliminary time-lapse imaging and fixed time point immunofluorescence revealed that mitotic arrest peaked at ~24 hours and cell death at ~48 hours, after drug addition for the majority of cell lines.

To obtain preliminary data on drug sensitivity, we performed dose titrations using flow cytometry analysis of DNA content to measure mitotic arrest at 24 hours (Fig. 1A and Supplementary Fig. S1). EC₅₀ (half maximal effective concentration) and Fₚ₅ₐₓ

### Table 1. Summary of dose titration data from measurements of mitotic arrest by flow cytometry

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Paclitaxel</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀ (nmol/L)</td>
</tr>
<tr>
<td>HeLa</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>HCT116</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>MDAMB435S</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>U2OS</td>
<td>45 ± 7</td>
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<tr>
<td>H460</td>
<td>113 ± 50</td>
</tr>
<tr>
<td>HT29</td>
<td>63.6 ± 0.6</td>
</tr>
<tr>
<td>OVCAR5</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>RPE</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>A549</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>MCF7</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>PC3</td>
<td>30 ± 6</td>
</tr>
<tr>
<td></td>
<td>EC₅₀ (nmol/L)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>167 ± 8</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>301 ± 18</td>
</tr>
<tr>
<td>K5I</td>
<td>113 ± 9</td>
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<tr>
<td></td>
<td>191 ± 21</td>
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<td></td>
<td>232 ± 28</td>
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<td></td>
<td>102 ± 6</td>
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<tr>
<td></td>
<td>254 ± 5</td>
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<tr>
<td></td>
<td>258 ± 26</td>
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<tr>
<td>HT29</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>OVCAR5</td>
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<tr>
<td>PC3</td>
<td>144 ± 19</td>
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<table>
<thead>
<tr>
<th>Nocodazole K5I</th>
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<tbody>
<tr>
<td>EC₅₀ (nmol/L)</td>
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<tr>
<td>Eₚ₅ₐₓ (%)</td>
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<tr>
<td>K5I</td>
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<tr>
<td>EC₅₀ (nmol/L)</td>
</tr>
<tr>
<td>Eₚ₅ₐₓ (%)</td>
</tr>
</tbody>
</table>

### Table 2. Pearson correlation coefficients for between-drug comparison for the data in Table 1

<table>
<thead>
<tr>
<th>Paclitaxel nocodazole</th>
<th>Paclitaxel K5I</th>
<th>Nocodazole K5I</th>
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</thead>
<tbody>
<tr>
<td>EC₅₀</td>
<td>0.42</td>
<td>0.78</td>
</tr>
<tr>
<td>Eₚ₅ₐₓ (%)</td>
<td>0.34</td>
<td>0.74</td>
</tr>
</tbody>
</table>

<!>EC₅₀ is the half maximal effective concentration, at which the percentage of cells in mitotic arrest is 50% of the maximal level (Eₚ₅ₐₓ). Eₚ₅ₐₓ is the maximal percentage of cells in mitotic arrest at saturating concentration of a given drug.

² Data for NCI60 cell line panel and the COMPARE algorithm can be accessed at the NCI database website: https://dtp.nci.nih.gov/docs/dtp_search.html.

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(saturating mitotic response) values are reported in Table 1. Correlations between drugs are shown in Table 2 and discussed below. Paclitaxel exhibited a noticeably more variable EC_{50} (~22-fold range) than the other two drugs (~6-fold range). We used these data to determine the lowest drug concentration that gave a saturating response (E_{max}) for the least sensitive cell line and conducted all following experiments at that single concentration (paclitaxel 150 nmol/L; nocodazole 500 nmol/L; K5I 500 nmol/L).

**Apoptosis varies more than mitotic arrest.** To quantify mitotic arrest and cell death at the single cell level, we used automated microscopy assay with molecular markers, sometimes called high content screening (32, 33). Cells were cultured in 384-well plates, treated with drugs at the saturating concentration, and fixed at appropriate time points. They were then stained with a fluorescent DNA probe to measure cell number, antibody to phosphorylated histone 3 to measure mitosis, and antibody to cleaved PARP1 to measure apoptosis. Mitotic index at 24 hours and apoptotic index at 48 hours were calculated by dividing the number of cells positive for that marker by the total number of cells in each microscopy field. Very similar results were obtained with different markers for mitosis (MPM2 antibody that recognizes phosphorylated Ser/Thr-Pro epitopes) or apoptosis (antibody to cleaved caspase-8) (data not shown). Cell survival ratio was calculated by measuring total cells remaining per field at 72 hours and divided by the number of cells at time 0 (before drug addition).

Figure 1B shows the paclitaxel response across the 11 cell lines as an example. Cell lines are ordered on the x axis according to their sensitivity to paclitaxel as determined by the 72-hour cell survival ratio. For ease of comparison, we retain this order for all the figures. Two points are immediately clear: the apoptotic index at 48 hours varies widely between the cell lines (>20-fold range), and this parameter correlates well with cell survival ratio at 72 hours. The mitotic index at 24 hours, in contrast, varies less (~3-fold range) and correlates poorly with cell survival.

Figure 1C and D reports mitotic arrest and apoptotic indices for all the drugs and cell lines, and Table 3 reports correlations between these responses and cell survival ratio. These data generalize the result stated for paclitaxel above: for each of the drugs, the apoptotic index was highly variable across the cell panel (ranges >20-fold) and correlated well with the cell survival ratio (coefficients between ~0.77 and ~0.86). For each of the drugs, the mitotic index was much less variable (ranges ~4-fold), and it correlated poorly with cell survival (coefficients between ~0.1 and ~0.31).

**Responses to the different antimitotics correlate.** The three drugs act in different ways, but all trigger the same spindle checkpoint, and thus, we might expect correlated responses across the cell line panel. Such correlation is evident from inspection of Fig. 1C and D, wherein the three colored bars for each cell line are similar in height. We quantified this using between-drug correlation coefficients for both the bulk mitotic arrest assay, where we measured both EC_{50} and E_{max} values (Table 2), and the microscopy data, which are only E_{max} values (Table 3). Between-drug comparison of E_{max} values showed strong correlation in both assays (coefficients, 0.72–0.86). In contrast, between-drug comparisons of EC_{50} values in the bulk assay gave weaker correlations (~0.21 to 0.42). E_{max} values are, in principle, insensitive to expression levels of drug efflux pumps and to other factors that cause variation in drug sensitivity in a manner that depends on the chemical structure of a drug rather than its biological activity, whereas EC_{50} values are sensitive to these factors. This may explain why drug sensitivity measured by E_{max} values give stronger between-drug correlations for a set of drugs that differ greatly in structure but little in activity.

**Paclitaxel is more toxic than the other antimitotics.** Although responses to the three antimitotics correlated strongly across the cell lines for all measured E_{max} variables, we did notice one interesting difference. Paclitaxel was consistently more effective in causing apoptosis than the other two drugs. This effect is evident from the black bars (paclitaxel) in Fig. 1D being taller than the colored bars (nocodazole and K5I). The effect was almost a 2-fold increase in apoptotic index for paclitaxel compared with the next most toxic drug for H460 and HCT116. This consistently higher E_{max} for paclitaxel is not evident in the mitotic arrest response, indicating that it is not an artifact of using subsaturating concentrations of the other drugs. We also noticed a stronger apoptotic response to paclitaxel than other drugs in time lapse and Western blot assays (see below). Thus, the ability of the drugs to promote apoptosis, when normalized to approximately equal mitotic index, is not equal; paclitaxel is significantly more toxic. This observation may have important implications for how apoptosis is triggered.

**Sensitivity to an alternative apoptotic trigger.** To test if sensitivity to apoptosis in our cell panel was unique to antimitotics, we measured the apoptotic response to staurosporine, a broad spectrum kinase inhibitor that is often used to trigger apoptosis, although its precise mechanism is unknown. We used 1 μmol/L staurosporine and a 12-hour time point, as preliminary investigation of concentrations and times (not shown) showed that our qualitative conclusion is not sensitive to changing these variables. Figure 2A shows the apoptotic index for staurosporine, with the cell lines ordered by sensitivity to paclitaxel as in Fig. 1. A general correlation in sensitivity is evident by the bars mostly decreasing from left to right. This is quantified with correlation coefficients in Table 3. Sensitivity to staurosporine, measured by apoptotic index at 12 hours, correlated fairly well with 72-hour cell survival ratio for the three antimitotic drugs (coefficients, ~0.62 to ~0.7). This correlation was less good than it was for the antimitotics with each other, as evident from both the coefficients in Table 2, and the outlier status of U2OS in Fig. 2A. We also measured apoptosis triggered by Brefeldin-A, which activates the ER stress pathway, and found that sensitivity across our panel roughly correlated with staurosporine and the antimitotics (data not shown). We tried to correlate with sensitivity to a DNA-damaging reagent, necarzinostatin, but were unable to obtain fast enough killing in most of our cell lines for work in 384-well plates. We conclude that

<table>
<thead>
<tr>
<th>Table 3. Pearson correlation coefficients between 72-h cell survival ratio and the phenotypic index variables in Fig. 3</th>
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<tr>
<td>72-h cell survival ratio</td>
</tr>
<tr>
<td>Paclitaxel</td>
</tr>
<tr>
<td>Nocodazole</td>
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<tr>
<td>K5I</td>
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</table>
Antimitotics are not unique and sensitivity to different apoptotic triggers broadly correlates in our panel of cell lines.

Antimitotics induce death that depends on both caspases and mitosis. Most of the cell death in our panel occurred directly from mitosis (see below), and it has been controversial whether this type of death is caspase-dependent (9). To test this, we used the broad-spectrum caspase inhibitor zVAD-FMK. We focused initially on one drug-sensitive line, HeLa. As expected, zVAD-FMK almost completely blocked PARP cleavage (Fig. 2B, left). It also largely rescued the 72-hour cell survival (Fig. 2B, right). The observed survival ratios of 0.6 to 0.9 indicate that the majority of cells are not dying in antimitotic + zVAD-FMK, whereas almost all HeLa cells die in antimitotic alone (survival ratio, <0.05). We then repeated the zVAD-FMK test on other cell lines that showed appreciable levels of apoptosis. In each case zVAD-FMK strongly boosted the cell survival ratios to near 1 (data not shown). We concluded that most of the cell death in our assays was due to classic apoptosis. We note three important caveats to this conclusion: zVAD-FMK is not completely specific for caspases and may inhibit other proteases potentially involved in cell death; in a nonadherent cell line not included in this panel, we found that death in mitosis caused by antimitotics was largely insensitive to zVAD-FMK and thus presumably not caspase mediated;3 and finally, the cells in our panel rescued from death using zVAD-FMK were far from normal. Their nuclei often displayed highly abnormal morphologies, and the apparent size of their cytoplasm was variable. We suspect these rescued cells would, in many cases, die or senesce if observed for longer. Thus, it is likely that other death and senescence pathways also play a role in the response to antimitotics in our panel, as noted by others (9, 17, 18).

Most death triggered by antimitotics depends on passage into or through mitosis (9), but some cell lines may be sensitive at other cell cycle stages, especially to agents that perturb interphase microtubules. To test directly if entry into mitosis is required for death, we pretreated cells for 12 hours with aphidicolin, an inhibitor of DNA polymerase, which arrests cells in S phase. As shown in Fig. 2C, death triggered by paclitaxel (chosen as the most toxic of the antimitotics) was mostly inhibited when cells were prearrested in S phase. Seventy-two–hour survival ratios of 0.7 to 0.8 were observed after pretreatment with aphidicolin compared with 0.1 to 0.2 without pretreatment. Similar protection from death was observed when cells were pretreated with purvalanol, a potent inhibitor of cdk1 and cdk2, which arrests cells at both G1-S and G2-M transitions, and thus blocks entry into mitosis by alternative mechanisms (data not shown). Therefore, in our cell panel, paclitaxel-induced death largely requires entry into mitosis.

Cell cycle timing of death induced by antimitotics. To test if cell cycle timing of death correlated with drug sensitivity, we used time-lapse phase-contrast microscopy in unsynchronized populations. Three relevant events were easily scored: entry to mitosis, exit from mitosis, and cell death (Supplementary Fig. S2). Essentially, all the cell deaths we observed occurred either directly from mitosis or after exit without cleavage. At the drug concentrations used, essentially no cells completed normal cytokinesis. All the cell lines exhibited considerable cell-to-cell variation in duration of mitotic arrest and time from onset of mitosis to death (or exit to death), whose precise kinetics we will report elsewhere. Here, we

focus on the question of whether death occurred directly from mitosis or after exit (Supplementary Table S1). We observed excellent correlation between sensitivity to apoptosis in the time-lapse and fixed cell experiments, confirming that the different observation conditions did not greatly perturb cell responses, and we again noted higher toxicity of paclitaxel compared with the other drugs.

The fraction of cells dying directly from mitosis versus after exit was different for each cell line and drug, although it tended to correlate between drugs. In the more death-sensitive cell lines, the majority of cells died in mitosis by a ratio that varied from ~30:1 (HeLa) to ~1.6:1 (H460). In U2OS cells, the majority of cells died after exit. For the cell lines that were highly resistant to death, such as MCF7 and PC3, most cells exited after the prolonged mitotic arrest and then remained in an abnormal G1-like state for extended period of time. Thus, the time-lapse data define three broad and overlapping phenotypes: death-sensitive with execution mainly in mitosis, death sensitive with execution mainly after exit, and death insensitive.

Profiling molecular events in apoptotic signaling. To characterize drug responses at a molecular level, we performed immunoblotting for a panel of proteins, and/or their posttranslational modification states, which were previously implicated in apoptotic signaling in antimotic response, or more generally in apoptosis regulation, including proteins involved in the DNA damage and ER stress responses (see the complete list in Materials and Methods). For this extensive analysis, we chose the two clinically relevant drugs (paclitaxel and K5I) and six cell lines representative of different degree of death sensitivity and cell cycle timing of death (HeLa, HCT116, U2OS, A549, RPE, and PC3, listed in descending order of sensitivity). We performed Western blots at 12 time points after drug addition to unsynchronized cells and ran gels with the different time points for one cell line side by side (e.g., Fig. 3A), as well as different cell lines for one time point (e.g., Fig. 3B) to allow quantitative comparisons. Exact timing and extent of changes varied in replicate experiments, but qualitative conclusions were highly reproducible.

Figure 3A (HeLa/paclitaxel) is representative of the antimitotic response in a death-sensitive cell line. Results were very similar in HCT116 and with K5I. Mitotic arrest, measured using phosphorylated H3, peaks at ~24 hours. Apoptosis, measured by cleavage of PARP1 (and also cleavage of caspase-8 and caspase-9; see Supplementary Figs. S3–7), starts at ~24 hours and peaks later,
past 48 hours in this experiment. In general, phosphorylated H3 levels and cleaved PARP1 levels measured by Western blot correlated well with the automated microscopy measurements in Fig. 1 for all cell lines and drugs. Cleavage of caspase-8 and caspase-9 correlated with cleavage of PARP1 for all cell lines and drugs, consistent with a common downstream apoptotic program in all sensitive cell lines.

The DNA damage marker phosphorylated H2AX (34) was strongly induced late in the response in all lines except the highly resistant PC3, and even there, it was induced to some extent. Paclitaxel was markedly more effective than K5I in triggering this response in partially death-insensitive lines (compare 32–72 hour lanes for A549 and RPE between Fig. 3B and C). This may be another indication of the greater toxicity of paclitaxel than K5I. We do not know what triggers the phosphorylated H2AX signal and have not proved that it reports on DNA damage in this context. We observed strong phosphorylated H2AX signals in some cases where we detected little or no PARP1 cleavage (e.g., RPE/paclitaxel and A549/K5I). Another possible DNA damage signal, up-regulation of p53, was observed in p53-positive cell lines,3 correlating with increase in phosphorylated H2AX. However, phosphorylation of Chk1 and Chk 2 was not detected, even in cells with a very strong phosphorylated H2AX response. We also probed for proteins important for ER stress response, including Bip, phosphorylated H2AX response. We also probed for proteins important for ER stress response, including Bip, phosphorylated H2AX, and CHOP, but no up-regulation was observed.

Induction of apoptosis in all cell lines coincided with degradation of XIAP, an inhibitor of apoptosis (e.g., Fig. 3A). XIAP levels remained constant in cells and drugs where PARP1 was not cleaved (Supplementary Figs. S3–7). Similar correlation of XIAP degradation with initiation of apoptosis has been noted in other studies of response to chemotherapeutics (16). This suggests XIAP acts as a buffer that would be titrated out before death occurs. However, the level of XIAP in untreated cells did not correlate well with sensitivity to death.

BH3 family proteins are key regulators of apoptosis, and their expression levels have often been suggested to correlate with sensitivity (35). However, we were unable to find any consistent correlations between expression levels of the family members we tested and sensitivity in our system. Bcl2 shifted to a slower migrating band when cells were arrested in mitosis, presumably due to mitotic phosphorylation, but this was equally true for all the lines. Levels of the other BH3 family proteins we measured did not change until very late in the response, and we saw no evidence of Bid cleavage.

We measured activity of the stress-sensitive kinases p38 and JNK using phosphorylated antibodies. p38 showed some phosphorylation in untreated cells, and the levels tended to increase in the apoptotic phase of the response, especially in more sensitive cells (Fig. 3). Phosphorylated JNK levels also increased somewhat during the apoptotic phase of the response, especially in more sensitive cells. These data are consistent with a role for both p38 and JNK pathways in the response, but correlations with sensitivity were not universal, suggesting they are only one factor governing sensitivity.

Discussion

By profiling phenotypic responses to three antimitotic drugs across a panel of cell lines, we found similar responses to the different drugs, large variation in the tendency to undergo apoptosis, and much smaller variation in the duration of mitotic arrest. Thus, response of the mitotic spindle and checkpoint was relatively constant between cell types and between drugs, whereas response of the apoptotic machinery was much more variable between cell types. Most death in our study occurred by caspase-dependent apoptosis and required entry into mitosis, although its execution could occur directly from mitosis or after exit. Before discussing possible molecular bases of these observations, we will touch on the question whether they are relevant to understanding drug responses in tumors.

In a study of 11 breast cancer patients treated with paclitaxel before surgery, Symmans et al. (36) measured mitotic arrest and apoptosis by serial biopsies every 24 hours after the first round of treatment. Both responses were quite variable between patients and the two responses correlated poorly. The overall clinical response, measured as the fraction of the original cancer remaining after several rounds of drug treatment, correlated very well with apoptosis and poorly with mitotic arrest. These observations agree with our cell culture data in finding that mitotic arrest is largely uncorrelated with apoptosis and tumor cell death and point to apoptosis as the important variable for clinical response. Considering animal model data, a pair of studies from the Milas group (37, 38) is particularly relevant for comparison with our data. A panel of 15 mouse tumor lines, grown by serial passage in mice, were treated with a single dose of paclitaxel or docetaxel, and animals were sacrificed at time intervals for quantitative histology. Most of the cancer lines showed a peak of mitotic arrest, but only some showed a subsequent peak of cell death. Again, the extent of cell death and mitotic arrest correlated poorly, and tumor shrinkage correlated with death much more than mitotic arrest. These studies are entirely consistent with our cell culture data with one important exception. Milas et al. reported that, after paclitaxel administration, death mostly occurred after mitotic exit and was scored as classic apoptosis by histology, whereas with docetaxel, it mostly occurred directly from mitosis, with extensive release of cell contents and leukocyte recruitment, and was scored as non-apoptotic death. We scored both death in mitosis and death after exit as apoptotic, since they both involved PARP1 cleavage and were blocked by caspase inhibitor. This discrepancy probably reflects a difference between histologic and molecular definitions of apoptosis. Overall, our results are concordant with the human and mouse studies, suggesting that variation in sensitivity to apoptosis is more important in determining tumor response to antimitotics than variation in sensitivity to mitotic arrest.

What are likely molecular sources of variation in sensitivity to apoptosis? Sensitivity in our panel correlated between antimitotics and other stress-inducing reagents, suggesting variation in the general stress sensing and apoptotic machinery dominates over variation in mitotic specific pathways. The question becomes, what makes cancer cells more or less sensitive to death triggered by multiple stressors? There is a large literature on this topic, but no clear and general answers have emerged. We were also unable to obtain a clear-cut answer thus far, and our data failed to support some previous suggestions (19–21). We did confirm that two stress-activated protein kinase pathways, JNK and p38, both previously implicated in paclitaxel-induced cell death (22–24), were activated by antimitotics, and to some extent, the degree of activation correlated with drug sensitivity. The strongest correlation we found was loss of XIAP (a negative regulator of apoptosis) during

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1p53 status of human cell lines can be found in a web database: http://www.p53.iarc.fr/index.html.
treatment, which occurred only in death sensitive lines. Unfortunately, from the perspective of predicting responses in patients, levels of this protein before treatment did not seem to correlate with its disappearance during treatment.

Ours may be the first study to carefully compare phenotypic responses of a nonmicrotubule antimitotic, KSI, to antimicrotubule drugs, across a panel of cell lines. The three drugs in our study seemed indistinguishable with respect to duration of mitotic arrest at saturating drug concentration. Their ability to promote apoptosis was also very similar, as were the molecular correlates of death that we measured by Western blotting. The highly similar cell responses to KSI and microtubule drugs indicate that apoptosis is triggered mainly by mitotic arrest and checkpoint activation and not by other consequences of changes in microtubule dynamics. The only significant difference we noted between the drugs was a greater extent of apoptosis and its molecular correlates caused by paclitaxel compared with KSI and nocodazole. This difference was subtle (<2-fold) and only evident in lines with intermediate sensitivity to apoptosis, but in those lines, it was reproducible with both microscopy and molecular assays. The slightly greater toxicity of paclitaxel, normalized to equal mitotic arrest, suggests it may activate additional pathways that lead to apoptosis. Molecular identification of these pathways is an important future direction.

Although human cancer cell lines cannot represent the complexity of real tumors, they provide a model for studying molecular mechanisms involved in drug responses, and to the limited extent that data are available, we argue our results are concordant with tumor data. We observed large variation in apoptosis responses and less in spindle assembly and checkpoint responses and suggest the former may be the dominant source of variation in clinical responses to antimitotics in drug-naïve tumors. Improvements in chemotherapy may come from combining antimitotics with drugs that appropriately modulate apoptotic pathways.

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References


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