Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells

David L. Morse,1 Heather Gray,2 Claire M. Payne,3 and Robert J. Gillies2

1Arizona Cancer Center, 2Department of Biochemistry and Molecular Biophysics, University of Arizona, and 3Center for Toxicology: Microbiology and Immunology, University of Arizona, Arizona Health Sciences Center, Tucson, Arizona

Abstract

Apoptosis has long been considered to be the prevailing mechanism of cell death in response to chemotherapy. Currently, a more heterogeneous model of tumor response to therapy is acknowledged wherein multiple modes of death combine to generate the overall tumor response. The resulting mechanisms of cell death are likely determined by the mechanism of action of the drug, the dosing regimen used, and the genetic background of the cells within the tumor. This study describes a nonapoptotic response to docetaxel therapy in human breast cancer cells of increasing cancer progression (MCF-10A, MCF-7, and MDA-mb-231). Docetaxel is a microtubule-stabilizing taxane that is being used in the clinic for the treatment of breast and prostate cancers and small cell carcinoma of the lung. The genetic backgrounds of these cells were characterized for the status of key pathways and gene products involved in drug response and cell death. Cellular responses to docetaxel were assessed by characterizing cell viability, cell cycle checkpoint arrest, and mechanisms of cell death. Mechanisms of cell death were determined by Annexin V binding and scoring of cytology-stained cells by morphology and transmission electron microscopy. The primary mechanism of death was determined to be mitotic catastrophe by scoring of micronucleated cells and cells undergoing aberrant mitosis. Other, nonapoptotic modes of death were also determined. No significant changes in levels of apoptosis were observed in response to docetaxel. [Mol Cancer Ther 2005;4(10):1495–504]
Docetaxel-Induced Mitotic Catastrophe in Breast Cancer

which may be followed by apoptosis. In both non–small cell lung carcinoma (20) and breast cancer cells, including MCF-10 and MCF-7 (21), paclitaxel has a concentration-dependent, biphasic response. At low concentrations, mitotic catastrophe and apoptosis are observed, whereas at high concentrations terminal mitotic arrest and necrosis are observed. In a recent study, Jurkat leukemia cells underwent apoptosis following treatment with paclitaxel, whereas MDA-mb-231 breast cancer cells underwent a slow nonapoptotic mitotic death characterized by multinucleation (22). In mouse fibroblast cells, docetaxel induced death by a mechanism exhibiting characteristics of both apoptotic and necrotic death (23). Docetaxel at the ≥5 nmol/L dose caused human breast cancer cells (BCap3) to initiate mitotic arrest and exhibit DNA laddering and sub-G0 DNA content, which are associated with apoptosis (24); however, these cells were not analyzed for responses associated with mitotic catastrophe, and increases in sub-G0 DNA are also associated with mitotic catastrophe and necrotic death. Human ovarian cancer cells treated with docetaxel exhibited apoptotic death preceded by cells with multifragmented nuclei (25). A recent study suggested that docetaxel-induced cell death is caused by c-Jun NH2-terminal kinase–mediated apoptosis in MCF-7, SK-Br-3, and MDA-mb-231 breast cancer cells (26). Dosages identical to those used in our study were given to cells, and c-Jun NH2-terminal kinase activation was observed; however, the actual mechanism of cell death was not determined.

Unlike apoptosis or senescence, which are “programmed” responses that involve signaling, the likely mechanism for taxane-induced mitotic catastrophe involves the disruption of mitotic cell division by the drug’s inhibition of the dynamic reorganization of microtubules. This disruption results in activation of the mitotic spindle checkpoint in checkpoint-proficient tumor cells. When damage to the mitotic apparatus is excessive, the checkpoint will eventually release (adapt) without the mitotic machinery being repaired; if the checkpoint is completely or partially defective, the cell will arrest transiently or proceed through mitosis without arresting (27, 28). The damage causes the improper segregation of chromosomes resulting in aberrant mitosis, multiple micronuclei, and an eventual necrosis-like death. Although checkpoint signaling may be involved in the drug response, it is not required for mitotic catastrophe to occur. In some cases, cells are observed to undergo apoptosis following mitotic catastrophe (18, 19). However, in mitotic catastrophe, apoptosis is not required for cell death to occur (see below).

In general, breast cancers are resistant to the therapeutic induction of apoptosis; this is likely due to the status of gene products, such as Bcl-2, p21, and p53 (29). Therefore, therapies that promote other types of death, such as mitotic catastrophe, may be preferential for use in treating breast cancer. Three human breast cancer cell lines of increasing degree of cancer progression were used for this study: MCF-10A, MCF-7, and MDA-mb-231, representing the nontumorigenic, tumorigenic/nonmetastatic, and metastatic stages of disease, respectively. As suggested by the gene expression phenotypes of these cells (Table 1; see Materials and Methods), we hypothesized that the tumorigenic lines would be at least partially deficient in docetaxel-induced apoptosis but that the normal MCF-10A cells would exhibit apoptosis. However, at the dosages and time points examined in this study, mitotic catastrophe was the predominant mechanism of cell death observed in all three cell lines, with a lesser contribution by other necrotic modes of death and practically no contribution by apoptosis. In MCF-10A and MDA-mb-231 cells, mitotic catastrophe was nearly the only mode of death observed. A mixture of mitotic catastrophe and other nonapoptotic modes of death was observed for MCF-7 cells, with mitotic catastrophe still being the predominant mechanism. Mitotic catastrophe increased with increasing cancer progression, although this may simply be a function of the fraction of proliferating cells at confluence. The absence of apoptotic morphologies was confirmed by transmission electron microscopy (TEM), which is the “gold standard” for detection of apoptosis (30).

Materials and Methods

Materials

Docetaxel was acquired from spent clinical stocks originally purchased from Aventis Pharmaceuticals (Par-sippany, NJ). The commercial sources of reagents used in this study are as follows: epidermal growth factor (EGF; Upstate Biotechnology, Lake Placid, NY), fetal bovine serum (Omega Scientific, Tarzana, CA), trypsin (Invitrogen/Life Technologies, San Diego, CA), Annexin V-FITC apoptosis detection kit (PharMingen, San Diego, CA), and the Diff-Quik system for cytology staining (Dade Behring, Inc., Newark, DE). All other media, chemicals, and reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Statistics

For mode of cell death determination by cytology, at least 500 cells were scored per treatment group and the results were presented as a percentage. In all other cases, the results from at least three individual experiments (n ≥ 3) were averaged to determine mean and SE. Where appropriate, significance was determined by Student’s t test. 95% Confidence intervals are reported for IC50 values.

Cell Lines

MCF-10A cells were obtained from the Michigan Cancer Foundation (now Karmanos Cancer Institute, Detroit, MI), MCF-7 cells were obtained from the Arizona Cancer Center cell culture shared service (Tucson, AZ), and MDA-mb-231 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were passaged weekly in DMEM/F-12 supplemented with 10% fetal bovine serum and 5 mg/mL insulin. MCF-10A medium also contained 20 ng/mL EGF (31). For all assays involving therapeutic response, cells were grown to confluence plus 1 day; the medium was then replaced with medium containing 10 or
Table 1. Status of key pathways and gene products of cell lines used in this study

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>MCF-10A</th>
<th>MCF-7</th>
<th>MDA-mb-231</th>
<th>References</th>
</tr>
</thead>
</table>

100 nmol/L docetaxel or docetaxel carrier as a control and allowed to grow for the indicated duration. Table 1 lists the genetic and functional status of pathways known to be involved in proliferation and therapeutic response for these cell lines.

MCF-10A cells were originally isolated from fibrocystic breast disease, were spontaneously immortalized, and do not form tumors when xenografted into severe combined immunodeficient mice (32). MCF-10A cells have intact cell cycle checkpoint controls (33–38), are aneuploid but have stable genomes comparable with human mammary epithelial cells (36), and have normal proliferation controls, including contact inhibition (31, 39–43) and normal apoptotic machinery (37, 38, 44, 45).

MCF-7 cells were isolated from a pleural effusion of stage IV invasive ductal carcinoma (46), form tumors when xenografted into severe combined immunodeficient mice but are not metastatic (47), are aneuploid with high chromosomal instability, have a mismatch repair defect (48) and a single-strand break repair defect (49), and are partially defective for the G<sub>1</sub> and mitotic spindle checkpoints (36, 50). MCF-7 cells are proficient for apoptosis but not by a caspase-3-mediated pathway (49, 51) and overexpress Bcl-2. Enhanced proliferation is suggested by EGF-independent growth (31), elevated Her-2/Neu/c-ErbB-2 expression (52), amplified N-ras (53), and rapid phosphorylation of the Rb gene product (43).

MDA-mb-231 cells were isolated from stage IV invasive ductal carcinoma (54), form tumors, and are metastatic when xenografted into severe combined immunodeficient mice (55). These cells are aneuploid and have an intermediate level of chromosomal instability (36), with partial defects in mismatch repair (48) and single-strand break repair (49). Although at least partially proficient for all cell cycle checkpoints (34, 36, 56), MDA-mb-231 cells express mutated p53 (49) and low p21 expression with subduced induction (35). MDA-mb-231 cells are proficient for apoptosis (51, 57) but resistant (58) due to elevated expression of the apoptosis-inhibiting Bcl-x<sub>L</sub> (49). Regulation of proliferation is perturbed by the following...
observations: MDA-mb-231 cells are both estrogen and EGF independent for growth (53, 59), have an activated Ki-ras mutation (53), and deregulated dephosphorylation of Rb (43).

**Cell Proliferation and Cell Cycle**

Population doubling times were determined by plating cells at $1 \times 10^6$, $3 \times 10^5$, and $1 \times 10^5$ cells/mL in 24-well plates and detaching (0.25% trypsin) and counting three wells per day on a hemacytometer for 3 subsequent days and then every other day until the culture reached steady state. The slope of the growth curves during log phase of at least three individual experiments ($n \geq 3$) was used to determine population doubling time. Pretreatment and post-treatment cell cycle status was determined by staining the DNA of $1 \times 10^6$ cells/mL with propidium iodide (PI; modified Krishan buffer) and performing fluorescence-activated cell sorting (FACS) following treatment periods of 0, 8, 16, 24, and 48 hours. FACS analysis was done by the Arizona Cancer Center Flow Cytometry Shared Service Laboratory using a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ). The percentage of DNA at G0-G1, S, and G2-M phases of the cell cycle was determined using the modeling program ModFit LT 2.0 (Verity Software House, Inc., Topsham, ME).

**Nucleoprotein Content Assay**

Post-treatment nucleoprotein content was determined by crystal violet staining (60). A decrease in nucleoprotein is interpreted as resulting from drug-induced cell detachment or decreased proliferation. Cells were grown in 96-well plates, 10,000 per well, and each row of wells was treated with increasing concentration of drug. Following 24 or 48 hours of treatment, the medium was aspirated from each well and washed with medium so that only viable adherent cells remain. Cells were either allowed to grow for an additional 24 hours before fixation or fixed immediately in 0.025% glutaraldehyde for 30 minutes. The nucleoprotein of fixed cells was stained by 0.1% crystal violet for 60 minutes and then destained by multiple washes with double distilled water. After air drying overnight, the remaining crystal violet was solubilized in 0.1 mL of a 100× dilution of glacial acetic acid. Absorbance was measured at 590 nm wavelength in a microplate reader model EL311 (BioTek Instruments, Winooski, VT). The measured absorbance is proportional to the protein content of the living-adhered cells remaining in the well following treatment. Inhibitory concentration values were determined using GraphPad Prism version 3.01 (GraphPad Software, Inc., San Diego, CA) to generate a nonlinear regression line fit.

**Cell Viability Assay**

Cell viability was determined by scoring trypan blue uptake 48 hours after treatment with 10 nmol/L docetaxel or docetaxel carrier as a control. One hundred cells per treatment group were scored and results are reported as a percentage.

**FACS Apoptosis Assay**

Annexin V binding assays were done by incubating cells in 10 nmol/L docetaxel or docetaxel carrier for 0, 2, 4, 8, 16, 24, and 48-hours followed by retrieval of detached cells suspended in the buffer and dispersal of adhered cells using cell dispersal buffer (HBSS prepared with K+ or Na+ salts substituted for Mg2+ or Ca2+ salts). Annexin V-FITC staining was done in conjunction with PI to distinguish early from late apoptosis and living from dead cells. Annexin V stains cells with accessible phosphatidylserine. During early apoptosis, phosphatidylserine is translocated to the outer membrane surface. However, cells that are dying and have permeabilized membranes as in early necrosis will have Annexin V–accessible internal phosphatidylserine. PI stains cells with permeable membranes, thus distinguishing cells with permeabilized membranes from those with healthy membranes. Following FACS, fluorescence of PI was plotted over Annexin V-FITC fluorescence. Healthy, nonapoptotic cells have low FITC fluorescence and low PI fluorescence. Early apoptotic cells have high FITC fluorescence (bound phosphatidylserine) but low PI fluorescence (intact membranes). Late apoptotic or early necrotic cells have high FITC fluorescence and high PI fluorescence. Late necrotic or dead cells have low FITC fluorescence but high PI fluorescence.

**Cell Death by Cytology and TEM**

Following 48-hour treatment of cells with 10 or 100 nmol/L docetaxel or docetaxel carrier, cells were detached from the cell culture plate using 0.25% trypsin, resuspended in 20% bovine serum albumin, cytospanned onto slides, fixed, and stained using the Diff-Quik system. Using a ×100 objective lens, at least 500 cells per treatment group were scored as being normal nonmitotic, normal mitotic, or drug-carrier only (Oi). Plots are shown for MCF-10A cells (left), MCF-7 cells (center), and MDA-mb-231 cells (right). Representative FACS histograms, plotted as counts over DNA content (top), are positioned above respective time points.
aberrant mitotic, having multiple micronuclei, having a vacuolated nucleus, being dark and shrunken, or being apoptotic. Images of cytostained cells were generated using a SPOT model 1.50 CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on a Nikon Eclipse E600 microscope (Nikon, Melville, NY). To verify cytologic results, TEM images of cells were acquired following 48-hour treatment with 10 and 100 nmol/L docetaxel and docetaxel carrier. Following treatment, cells were caused to detach using 0.25% trypsin, fixated in 3% glutaraldehyde in phosphate buffer, postfixed in 1% osmium tetroxide, and embedded in Spurr low viscosity resin. Sections (1 μm thick) were prepared and stained with uranyl acetate and lead citrate. TEM was done by the Arizona Research Laboratories Biotechnology Imaging Facility using a Philips CM12 system.

Results
Cell Cycle
Relative proliferation rates of MCF-10A, MCF-7, and MDA-mb-231 cells during exponential growth (doubling time), percent G0 + G1 DNA at confluence (steady-state growth), and G2 + M checkpoint arrest following treatment with 10 nmol/L docetaxel were determined. In these growth conditions, no significant difference between the exponential growth rates of the three cell lines was observed, with doubling times of 1.52 ± 0.23 for MCF-10A cells, 1.60 ± 0.21 for MCF-7 cells, and 1.67 ± 0.23 for MDA-mb-231 cells. However, at confluence, a higher fraction of cells remained proliferating (non-G0/G1) in the tumorigenic and metastatic MDA-mb-231 cells (43.9 ± 2.5%) and tumorigenic MCF-7 cells (27.8 ± 0.5%) compared with the relatively normal MCF-10A cells (18.2 ± 2.5%). As an indicator of checkpoint arrest, the (G2 + M) / G0 + G1 ratios were determined over a period of 48 hours following treatment with 10 nmol/L docetaxel (Fig. 1). MCF-10A cells had a small increase peaking at 8 hours and returned to pretreatment levels by 24 hours. Cell cycle arrest in MCF-7 and MDA-mb-231 cells steadily increased to 24 hours and remained elevated but leveled off with a lesser degree of increase at 48 hours.

Nucleoprotein Content
As a measure of cell detachment or decreased proliferation following treatment, nucleoprotein content of adhered cells was quantified by crystal violet staining (60) at increasing doses of docetaxel and at different time points following treatment. To assess clonogenic death (senescence), crystal violet staining was also assayed after 48 hours of drug followed by 24 hours in normal medium. From these experiments, IC50 values were determined for all three cell lines and are shown in Table 2. As shown in Table 2, the two cancer lines, MCF-7 and MDA-mb-231, were similarly sensitive to drug at 24 and 48 hours but were less sensitive than the normal MCF-10A cell line. Only MCF-7 cells had a further decrease in crystal violet staining following 24 hours of recovery in normal medium, and drug sensitivity under these conditions was similar to that of MCF-10A cells. From these data, 10 and 100 nmol/L docetaxel were chosen for subsequent experiments, as they bracket the IC50 response in all cell lines under all conditions. The responses at these doses were determined and are presented in Table 3. As shown in Table 3, virtually all of the MCF-10A and MCF-7 cells are destined to die by 48 + 24 hours at the higher dose of 100 nmol/L, whereas nearly one third of the MDA-mb-231 cells survive. Similarly, even the lower dose of 10 nmol/L

Table 2. IC50 docetaxel at 24- and 48-hour chronic treatment, and at 48-hour treatment followed by 24-hour incubation in drug-free medium

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50, 24 hours (95% confidence interval)</th>
<th>IC50, 48 hours (95% confidence interval)</th>
<th>IC50, 48 + 24 hours (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A</td>
<td>3.25e−8 (1.10e−8 to 9.66e−8)</td>
<td>2.24e−8 (1.02e−8 to 4.93e−8)</td>
<td>1.97e−8 (1.49e−8 to 2.63e−8)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>3.87e−7 (8.69e−8 to 1.72e−6)</td>
<td>8.34e−8 (1.03e−8 to 6.73e−7)</td>
<td>1.47e−8 (2.53e−9 to 8.52e−8)</td>
</tr>
<tr>
<td>MDA-mb-231</td>
<td>9.28e−8 (1.63e−9 to 5.28e−6)</td>
<td>5.12e−8 (3.25e−8 to 8.07e−8)</td>
<td>5.00e−8 (3.43e−8 to 7.29e−8)</td>
</tr>
</tbody>
</table>

Table 3. Percent death following treatment with 10 and 100 nmol/L docetaxel at 24- and 48-hour chronic treatment and at 48-hour treatment followed by 24-hour incubation in drug-free medium

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose (nmol/L), docetaxel</th>
<th>% Death, 24 hours (SE)</th>
<th>% Death, 48 hours (SE)</th>
<th>% Death, 48 + 24 hours (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A</td>
<td>10</td>
<td>30.7 ± 6.9</td>
<td>34.4 ± 5.2</td>
<td>30.2 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>68.9 ± 6.9</td>
<td>78.9 ± 5.4</td>
<td>90.0 ± 2.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td>10</td>
<td>4.5 ± 7.7</td>
<td>11.2 ± 13.0</td>
<td>28.0 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25.4 ± 7.7</td>
<td>53.2 ± 15.0</td>
<td>99.6 ± 5.6</td>
</tr>
<tr>
<td>MDA-mb-231</td>
<td>10</td>
<td>13.4 ± 25.6</td>
<td>21.8 ± 2.9</td>
<td>12.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>51.5 ± 31.6</td>
<td>62.5 ± 3.1</td>
<td>68.4 ± 2.8</td>
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</table>
kills significant fractions of the MCF-10A and MCF-7 cells (30% and 28%, respectively) and only a small number (13%) of the MDA-mb-231 cells. Similar to the data of Table 2, the MCF-7 cells were slowest to respond at the low dose, and the MCF-10A and MDA-mb-231 cells did not have a further decrease in cell number following the recovery period.

Cell Viability
To determine whether cell killing occurred in response to docetaxel, trypan blue uptake was scored 48 hours after treatment with the lowest dosage of docetaxel used (10 nmol/L) or with docetaxel carrier as a control. MCF-10A cells had 18% lower viability, MCF-7 cells had 17% lower viability, and MDA-mb-231 cells had 10% lower viability in treated versus untreated cells.

Apoptosis Assay by FACS
To determine the proportion of cell death due to apoptosis, Annexin V binding was determined at 0, 2, 4, 8, 16, 24, and 48 hours following treatment with 10 nmol/L docetaxel in tandem with untreated controls (61). For all three cell lines and all time points, post-treatment increases in Annexin V binding in the absence of PI staining compared with controls were minimal and not significant, with a maximal increase of 4.68% ($P = 0.21$) at 8 hours for MCF-10A cells, 1.05% ($P = 0.35$) at 16 hours for MCF-7 cells, and 0.97% ($P = 0.39$) at 8 hours for MDA-mb-231 cells (data not shown). A significant increase in Annexin V + PI costaining was observed for MCF-10A cells at 16 hours after treatment. Costaining increased by 17.8% ($P = 0.003$). Because this increase in costaining was not preceded with increased staining by Annexin V alone at earlier time points, it is attributed to loss of membrane integrity due to a necrotic type of cell death.

Cell Death Assays by Cytology and TEM
Figure 2 shows both cytology and TEM images from untreated MCF-7 cells and those treated with 10 nmol/L docetaxel. Normal nonmitotic nuclei (Fig. 2A and E) are juxtaposed with multiple micronucleated cells (Fig. 2B and F), treated cells undergoing aberrant mitosis (Fig. 2D and H).

Figure 2. Mitotic catastrophe in MCF-7 cells following 48-h treatment with 10 nmol/L docetaxel. Shown are Diff-Quik-stained cells (A–D), TEM images (E–H), normal untreated nuclei (A and E), treated cells with multiple micronuclei (B and F), untreated cells undergoing normal mitosis (C and G), and treated cells undergoing aberrant mitosis (D and H).

Figure 3. Other forms of death in cells treated with docetaxel. Shown are Diff-Quik-stained cells (A–C), TEM images (D–F), MCF-7 cells with large vacuolated nuclei (A and D), small nonapoptotic MCF-7 cells with dark-staining nuclei and cytoplasm (B and E), an MDA-mb-231 apoptotic cell (C), and a MCF-10A apoptotic cell (F). In addition, the MCF-7 cells in (D and E) are being engulfed via phagocytosis by viable cells in the culture.
and F). Normal mitotic cells (Fig. 2C and G) are juxtaposed with cells undergoing aberrant mitosis (Fig. 2D and H). Figure 3 illustrates other nonmitotic modes of death observed in these samples. Cells with large vacuolated nuclei were observed (Fig. 3A and D), with the cell in Fig. 3D being phagocytosed by another cell. Nonapoptotic cells with condensed nuclei and cytoplasm were also observed (Fig. 3B and E) along with apoptotic cells (Fig. 3C and F). By TEM only, cells with condensed mitochondria were observed (Fig. 4). The mitochondria of these cells have a “state III” conformation (ref. 62; e.g., energized mitochondria), which is hypothesized to represent a compensating effect in cells struggling to maintain adequate energy metabolism. Recent studies also indicate that condensed mitochondria can be observed following a drop in mitochondrial membrane potential (63).

From these preparations, a minimum of 500 cells per condition were scored for morphologies and the results are presented in Table 4. For all three cell lines at all doses, apoptosis accounted for <0.5% of all cells scored. Mitotic catastrophe (aberrant mitoses + multinucleated cells) was the major cause of death for all cell lines and treatments. Other nonapoptotic forms of death increased with dose for MCF-10A and MCF-7 cells but did not increase for MDA-mb-231 cells. MDA-mb-231 cells had the highest number of dead cells at all doses, with mitotic catastrophe being the only relevant cell death response.

Discussion
The type of cell death resulting from a given therapy is determined by the mechanism of action of the drug, dosing regimen of the therapy, and genetic background of the cells being treated. Nonapoptotic cell death (i.e., mitotic catastrophe) is the major response to docetaxel in three human breast lines at the dosages and timing used in this study. This action of taxanes may be partly responsible for their efficacy in treating breast cancers, which may be apoptosis deficient. Absolutely no apoptotic response was observed for MCF-7 cells, which overexpress the apoptosis-inhibiting Bcl-2 and do not express caspase-3, a major component of the effector or executionary phase of the majority of apoptotic signaling pathways (ref. 49; Table 1). In contrast, MCF-10A and MDA-mb-231 cells had slight (<0.5%) but measurable numbers of apoptotic figures. FACS assays for apoptosis support the cytologic results, with minimal and insignificant increases in Annexin V binding following treatment of all three cell lines. In addition, the cytology and Annexin V results were supported by TEM images of treated cells (64). TEM is considered to be the gold standard for detection of apoptosis (30). For TEM, multiple preparations were made for each cell line and treatment condition. Each preparation was scanned thoroughly and only two examples of apoptosis were observed in all of the preparations. In contrast, other mechanisms of death were ubiquitously observed in TEM images.

As scored by cytology, mitotic catastrophe is the predominant mode of death for all docetaxel dosing regimens and all human breast cancer cell lines used in this study. In general, the percentage of cell death by this mechanism was higher in cells of increasing cancer progression. At the highest dose, the normal MCF-10A cell line had the lowest measure of cell death (15%), with mitotic catastrophe being the major component (12%); the tumorigenic MCF-7 cells had a considerably higher percentage of death (56%), with mitotic catastrophe being the major component (46%); and the tumorigenic and metastatic MDA-mb-231 cells had the highest percentage of death (80%), with nearly all cell death attributed to mitotic catastrophe (78%). The TEM images support the cytologic results, because cells undergoing aberrant mitoses and having multiple micronuclei were common observations following docetaxel treatment of all three cell lines.
Table 4. Results of scoring cell death morphologies of Diff-Quik-stained cytospins (n ≥ 500 cells)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose (nmol/L, docetaxel*)</th>
<th>Normal mitotic</th>
<th>Aberrant mitoses</th>
<th>Multinucleate</th>
<th>Other nonapoptotic cell death</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A</td>
<td>0</td>
<td>98.8</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>10</td>
<td>76.0</td>
<td>0.0</td>
<td>2.2</td>
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<td></td>
<td>100</td>
<td>85.0</td>
<td>0.0</td>
<td>3.4</td>
<td>23.8</td>
<td>14.6</td>
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<tr>
<td>MCF-7</td>
<td>0</td>
<td>97.7</td>
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<td>1.1</td>
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<td>0.2</td>
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<td></td>
<td>10</td>
<td>81.6</td>
<td>0.5</td>
<td>6.1</td>
<td>6.1</td>
<td>17.8</td>
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<td></td>
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<td>0.7</td>
<td>10.7</td>
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<td>55.8</td>
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<tr>
<td>MDA-mb-231</td>
<td>0</td>
<td>94.0</td>
<td>2.3</td>
<td>0.9</td>
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<td>2.0</td>
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<td>19.3</td>
<td>0.4</td>
<td>11.3</td>
<td>66.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* 0 nmol/L dose contained diluted docetaxel carrier.
† Other nonapoptotic cell death includes vacuolated nuclei and nonapoptotic darkly stained nuclei.

Long-term assays show that the percentage of cells undergoing mitotic catastrophe is proportional to the decrease in clonogenic survival and that these morphologies ultimately result in cell death (11, 13, 22). Without performing clonogenic assays, viability (trypan blue incorporation) measurements showed a loss of membrane integrity and ultimate cell death 48 hours after treatment with the lowest dosage used. Percentages of cell death measured by this assay were comparable with percentages scored by both biomass (nucleoprotein) assay and cytology.

This increase in death by mitotic catastrophe may be caused by ineffective checkpoint arrest due to a partial defect in the mitotic spindle checkpoint or the higher rates of proliferation in confluent cells of increasing metastatic potential (Table 1). At least 18% of MCF-10A cells are proliferative (i.e., in non-G0/G1) at steady-state growth (confluence), whereas 28% of the MCF-7 cells and 44% of MDA-mb-231 cells are proliferating at steady state. Cells actively proceeding through the cell cycle would be more susceptible to mitotic block and subsequent mitotic death. This could also explain the apparently discrepant results between the dose-responses that show the MDA-mb-231 cells to be least sensitive (Tables 2 and 3) and the cytologic scoring (Table 4) that shows the MDA-mb-231 cells to be most sensitive. Because the MDA-mb-231 culture is replenishing itself more rapidly, fewer cells would be lost (as determined by crystal violet staining), although there is a higher proportion of cell death (as determined by cytology). Furthermore, the number of cells scored as being “normal” following treatment may have been ultimately destined for death, causing a disconnect between cytology and survival.

Modes of cell death other than mitotic catastrophe and apoptosis were detected by cytology and TEM. Other forms of death scored by cytology were small, dark nonapoptotic cells and cells with highly vacuolated nuclei at varying degrees of disintegration. These types of death are possibly due to disruption of the cellular infrastructure in terms of uptake, secretion, transport, and energy metabolism due to the mechanism of action of the drug. An inhibition of energy metabolism is implicated by the observation of state III mitochondria (Fig. 4), which may represent the energized state of mitochondria (62). It is also possible that the increase in condensed mitochondria may represent a loss of mitochondrial membrane potential (63), although this loss in membrane potential does not translate into apoptosis. In contrast to mitotic catastrophe, which requires cells to progress through mitosis, all cells regardless of their cell cycle status would be susceptible to these nonapoptotic forms of death. Hence, the nonmetastatic MCF-10A and MCF-7 cells are more competent for contact inhibition than the metastatic MDA-mb-231 cells, thus are more likely to be quiescent (G0), and have a higher proportion of cell death by these other means (Table 4).

In conclusion, mitotic catastrophe is the primary mode of death observed in response to the treatment regimen with docetaxel in the human breast cancer cell lines included in this study, with additional death attributed to other nonapoptotic mechanisms. The proportional mixture of cell death responses was cell line specific and could be due to differential regulation of proliferative controls, contact inhibition, or cell cycle checkpoints (Table 1). Both MCF-7 and MDA-mb-231 cells have compromised contact inhibition and altered proliferative signaling (i.e., amplified ras (MCF-7) and activated Ki-ras (MDA-mb-231)). Both have partial defects in the mitotic spindle checkpoint, the checkpoint activated by microtubule-hyperpolymerizing drugs, such as docetaxel. MDA-mb-231 cells have mutated p53, which is implicated in checkpoint maintenance. In addition, both MCF-7 and MDA-mb-231 cell lines are partially resistant to apoptosis due to elevations in the apoptosis-inhibiting products Bcl-2 (MCF-7) and Bcl-XL (MDA-mb-231) and caspase-3 defects in MCF-7 cells. Therefore, it is not surprising that treatment of these two cell lines with docetaxel resulted in death primarily by mitotic catastrophe with no significant increase in apoptosis. In addition, the mechanism of action of docetaxel, the binding to microtubules,
would favor a type of cell death associated with aberrant assembly and separation of chromosomes during mitosis. However, the normal MCF-10A cells, which are proficient for proliferative controls, contact inhibition, checkpoint controls, and apoptosis, were also negative for a significant increase in apoptosis. In contrast, MCF-10A cells had lower levels of mitotic catastrophe possibly due to a proficient G1 checkpoint and fewer numbers of proliferative cells due to normal regulation of proliferation and contact inhibition. This study underscores the importance of evaluating the specific mechanism of cell death in breast cancer cells (i.e., apoptosis, necrosis, and mitotic catastrophe) because chemotherapeutic agents that target the mitotic spindle may be most effective at killing these apoptosis-resistant cells.

References
Docetaxel-Induced Mitotic Catastrophe in Breast Cancer

Molecular Cancer Therapeutics

Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells

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