

Centrosome Impairments and Consequent Cytokinesis Defects are Possible Mechanisms of Taxane Drugs

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Abstract. Taxol and taxotere are two of the most promising anticancer drugs. To determine the mechanisms responsible for cell death after exposure to low doses of taxane, PC3 cells were treated with taxol and taxotere, and observed with immunofluorescence microscopy. Pericentriolar material dissociation and blockage of normal centrosome separation were found to result in two different abnormal spindle types; multipolar and monopolar spindles, respectively. The majority of abnormal spindles induced by taxol were monopolar spindles, whereas taxotere mostly induced abnormal multipolar spindles. Consequently, monopolar spindle mitosis resulted in cleavage failure, while multipolar spindle mitosis led to the formation of both cleavage failure and multipolar cell division. Multinucleation characterized interphase cells which had undergone cytokinesis defects. These cells subsequently became giant multinucleated cells after several rounds of cell cycle with sustained cleavage failure, and were gradually eliminated through cell death.

Taxanes are effective drugs in the treatment of several tumors, such as ovarian, breast, lung and prostate (1). Taxol and taxotere are important taxanes that have undergone extensive clinical development. The conventional mechanisms of action of taxanes are attributed to their ability to bind microtubules and to prevent their disassembling.

Incubation of cells with a high concentration of taxol results in an increase of microtubule polymer mass and the formation of stable microtubule bundles, that disrupt the

normal polymerization and depolymerization cycle of microtubules (2). Low concentrations of taxol (10 nM) promote the suppression of microtubule dynamics rather than the formation of stable microtubule bundles (3). The mechanism of low concentration is thought to have more clinical significance (4). However, the process by which microtubule deregulation leads to cytotoxicity is not well understood.

Multinucleation is a characteristic of cells in interphase induced by a low concentration of taxane (3, 5) This suggests a catastrophic restitution from abnormal mitosis. Previous studies have confirmed that exposure of cells to low concentrations of taxol is sufficient to delay mitotic progression, but is insufficient to induce a mitotic arrest (5, 6). Also proposed is the hypothesis that dysfunctional spindles cannot properly polymerize and segregate chromosomes (7). Together, these studies imply that impairment of normal mitotic spindle function is critical for taxane-induced cell death.

In this work, we have performed a comparison between the actions of taxol and taxotere. Our results indicate that centrosome impairments directly result in aberrant mitotic spindle formation and mitotic catastrophe. The inferences that can be drawn from these experiments on centrosome impairments and cytokinesis failure are discussed.

Materials and Methods

Cell culture. Androgen-independent prostate cancer cell lines, PC-3, were maintained in culture as monolayers in modified McCoy's 5a medium, supplemented with 5% bovine calf serum. These cells were grown in 5% CO₂ and passed at intervals of 3 days. Equal densities (6.4 x 10³/cm²) of cells were seeded 28 hours before treatments. Cells were treated with or without taxol and taxotere (concentration range: 5 nM to 20 nM) for 2-24 hours, followed by washing and continued incubation in drug-free medium.

Reagents. Mouse monoclonal anti- β tubulin and rabbit polyclonal anti- γ tubulin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); rabbit polyclonal anti-Pericentrin from Berkeley Antibody Company (Richmond, CA, USA); rabbit polyclonal anti-

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Aurora-B from ZYMED Laboratories Inc. (San Francisco, CA, USA). FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG and rhodamine conjugated anti-rabbit IgG were from Biomedica Corp (Foster city, CA, USA). Propidium iodide (PI) was purchased from Sigma-Aldrich. Mounting medium with anti-fading agents was from Biomedica Corp.

Indirect immunofluorescence. For observation of mitotic spindles using fluorescence microscopy, cells were grown in Corning brand tissue culture dishes. For observation of centrosomes with laser scanning confocal microscope (three-laser Nikon TE2000 inverted microscope), cells were plated onto poly (D)-lysine-coated cover slips. The cells were treated with taxane drugs 28 hours after cell seeding. At each time-point during or after drug treatment, cells were fixed with ice-cold methanol for 4°C minutes, blocked for 20 minutes with 10% horse serum in PBS, and incubated overnight with primary antibody (1:250 dilution) at 4°C. After three washes with PBS, the cells were incubated with a secondary antibody (1:250 dilution) overnight at 4°C, stained with 0.5 µg/ml PI for 5 minutes, then washed and mounted using mounting medium containing anti-fading agents. Approximately 100 mitotic cells were counted to calculate the percentage of cells with abnormalities.

Trypan blue staining assay. 0.1 ml of 0.4% trypan blue stain was added to 0.9 ml of a cell suspension consisting of cells in PBS, mixed, and allowed to stand for 5 minutes at room temperature. A hemocytometer was used for cell counting.

Results

Taxane-induced pericentriolar material dissociation and blockage of normal centrosome separation at mitosis resulted in multipolar spindles and monopolar spindles formation, respectively (Figure 1). In control cells, γ -tubulin and pericentrin displayed a stronger staining of centrosome pairs during mitosis. After taxane treatment for 2 hours, the cells exhibited two kinds of centrosome impairments. In mitotic cells with multi-polar spindles, pericentrin was always located at each pole of the mitotic spindles. However, γ -tubulin was only located at two poles and could rarely be found on other poles. Double staining with pericentrin and γ -tubulin antibodies also confirmed this conclusion (photos not shown here). Because multipolar spindles arose briefly after drug exposure, it could not be explained by centrosome overduplication. Pericentrin and γ -tubulin are important components of pericentriolar material (8, 9). Our results strongly suggest that centrosomes may lose their integrities in these abnormal mitotic cells, and pericentrin may be dissociated from centrosome. In monopolar spindles, γ -tubulin and pericentrin are located at monopoles, which indicated that centrosome pairs were not separated.

Taxol and taxotere induced different morphologies of mitotic spindles (Figure 3). The lowest drug doses that initiated

significant abnormalities of mitotic spindles were 10 nM for taxol and 5 nM for taxotere. A large proportion of abnormal spindles was induced only 2 hours after drug exposure. Furthermore, mono-polar spindle was a common characteristic of spindle malformation in taxol-treated cells; on the other hand, multipolar spindle was a common characteristic in taxotere-treated cells.

Taxane drugs induced cytokinesis defects and multinucleation (Figure 2). Twenty-four hours after exposure to 5 nM taxotere or 10 nM taxol, we found that most of the mitotic cells were blocked at metaphase, while only a very small number of mitotic cells were at later stages of mitosis (anaphase or telophase). From the observation of these later stage of mitotic cells, we found that: i) mitotic cells with monopolar spindles resulted in cleavage failure (Figure 2c); ii) mitotic cells with multipolar spindles resulted in two types of aberrant cell division: multipolar cell division (Figure 2e) and cleavage failure (Figure 2f). We also found that most of interphase cells were multinucleated after 24 hours of drug treatment. These abnormal interphase cells always contained excessive number of centrosomes.

No midbody was detected in cytokinesis failure (Figure 4). Normally, Aurora-B is localized at midzone microtubule during anaphase, and at midbody during telophase. Aurora-B was employed as a marker to label these structures. Our results indicated that midzone microtubules were significantly disrupted and midbody was not built in cytokinesis failure cells (Figure 4c, g).

Mitotic catastrophe during prolonged incubation (Figure 5, Figure 6). To observe the consequences of multinucleation, drugs were withdrawn after treatment for 24 hours, and cells were incubated for longer periods of time. Based on the results obtained by the method of trypan blue staining, these multinucleated cells did not readily die (Data shown in Figure 8). Other rounds of aberrant mitosis were observed. The abnormalities of mitotic spindles seen here were more severe than those seen in cells undergoing primary cell cycle division (Figure 5 a, b, c, d; Figure 6 a, d). Both pericentrin and γ -tubulin were always located at each pole of the mitotic spindles (Figure 5 a, b, c and d). Double staining with pericentrin and γ -tubulin antibodies confirmed this conclusion (photos not shown here). These indicated that mitotic cells were derived from multinucleated cells containing excessive centrosomes rather than normal cells. Additionally, a large number of atypical telophase cells were observed during prolonged incubation. The majority (95%) of these cells was undergoing cleavage failure (Figure 6 b and e). The rest were passing through multipolar cell divisions. Giant cells arose after several rounds of cell cycle (Figure 6 c and f).

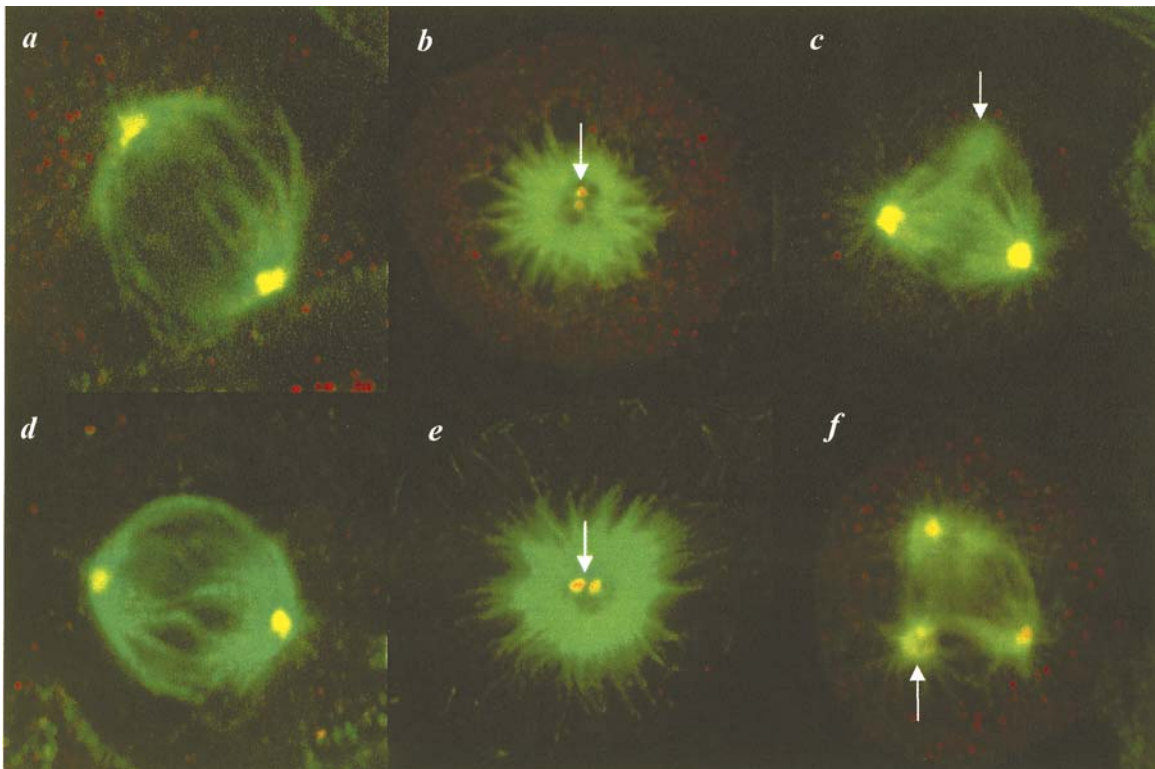


Figure1. Centrosome and mitotic spindle impairments induced by taxol and taxotere. Mitotic spindles were stained by anti- β tubulin in green color. Centrosomes were stained by γ -tubulin (a, b, c) or pericentrin (d, e, f), both in red color. Normal mitotic spindles and centrosomes were shown in control cells (a, d). Monopolar spindles were shown in cells treated with 10 nM taxol for 2 hours (b, e). Arrows in b and e indicate unseparated centrosomes stained by γ -tubulin and pericentrin, respectively. By contrast, multipolar spindles were shown in cells treated with 5 nM taxotere for 2 hours (c, f). Arrows in c and f indicate extra poles. Pericentrin was detected positively on extra pole (f), but γ -tubulin was absent on extra pole (c).

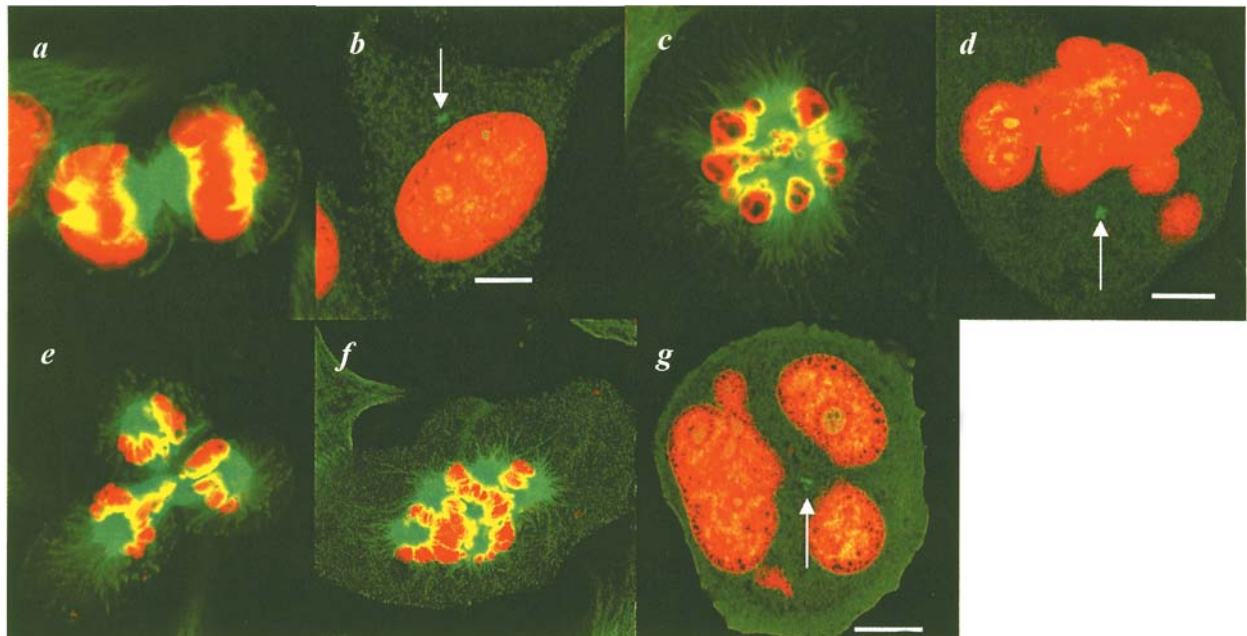


Figure 2. The percentage of mitotic cells with abnormal spindles induced by taxol (TAX) and taxotere (TXT). The cells were treated with taxol and taxotere for 2 hours. Mitotic spindles were stained by anti- β tubulin antibody and observed with fluorescence microscopy. Each bar contains two proportions of abnormal spindle percentage. The remaining percentage of cells were normal mitotic cells. Data are expressed as the mean from three experiments.

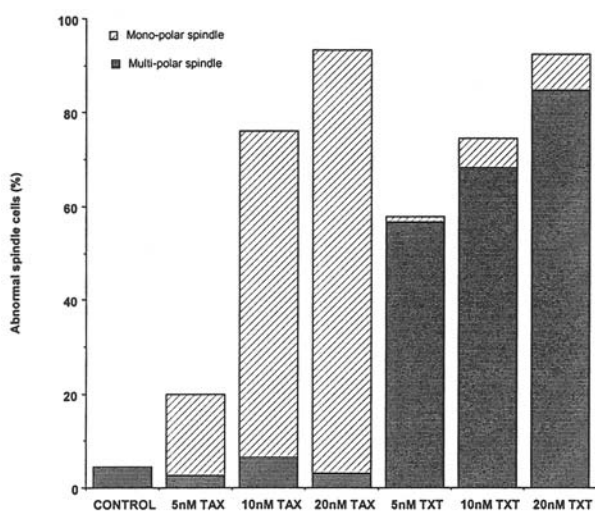


Figure 3. Cytokinesis failure and multinucleation in PC3 cells after treatment with 10 nM and 5 nM taxotere for 24 hours. Mitotic spindles were stained by anti- β tubulin in green color (a, c, e, f). Centrosomes were stained by γ -tubulin in green color (b, d, g). DNA was stained by PI (a, b, c, d, e, f, g) in red color. Panel a and b show a normal anaphase cell and a normal interphase. Cytokinesis defects were induced in taxol (c) and taxotere (e, f)-treated cells. Panel c shows cleavage failure resulting from monopolar spindles. Panel f shows cleavage failure resulting from multipolar spindles. Panel e shows multipolar cell division resulting from multipolar spindles. Multinucleated interphase cells are also shown after treatment with taxol (d) and taxotere (g). Multinucleated cells are characterized by multiple nuclei, cell enlargement and multiple centrosomes. Arrows indicate centrosomes. Bars, 10 μ M.

Multinucleated cells were eliminated through cell death (Figure 7, Figure 8). To examine the correlation between multinucleation and cell death, the formation of multinucleated cells was induced by taxol and taxotere treatment for 24 hours, followed by incubation in drug-free media. For treatment of a dose of 5 nM, taxotere induced a significant amount of multinucleation (54%), while this concentration of taxol elicited little (Figure 7). Correspondingly, taxotere induced significant cell death, but taxol did not (Figure 8). For treatment of a dose of 10 nM, large numbers of multinucleated cells (81% for taxol, 94% for taxotere) were induced by two taxanes (Figure 7). As the result, both taxol and taxotere induced considerable cell death (Figure 8).

Discussion

Studies have reported that low concentrations of taxol (~10 nM) strongly retard mitosis at the metaphase/anaphase transition and induce cell death without an increase in microtubule polymer levels or induction of microtubule bundling. Taxol-treated cells in these studies

were seen to have abnormal spindle formation. Aberrant spindle types previously described include monopolar spindles, multi-polar spindles and bipolar spindles with lagging chromosomes (10, 11). Submicromolar concentrations of taxol significantly alter the dynamics of microtubules (3). Other studies indicate that suppression of microtubule dynamics by taxol disrupts the assembly of mitotic spindle formation (10). However, aberrant mitosis was induced only by microtubule-stabilizing drugs and not by destabilizing agents. This suggests that suppression of spindle dynamics cannot fully account for aberrant mitosis (11). Taxol and taxotere share the same mechanisms of microtubule stabilization, even though taxotere is generally found to be more active than taxol (12, 13). Our results showed that taxol and taxotere induced different types of centrosome impairment, strongly suggesting that centrosome impairments could not be secondary consequences due to changes in microtubule dynamics. Centrosome impairments may directly result in abnormal mitotic spindles. These data suggest that centrosome could be a primary target for taxane-induced cell death.

Past studies found that multipolar spindles resulted from pericentriolar material dissociation in taxotere-treated cells (7), and that the centrosome was a target for fluorescent taxoid (14). Our results indicate that blockage of normal centrosome separation and pericentriolar material dissociation result in monopolar spindles and multipolar spindles, respectively. The structure of taxotere differs from that of taxol in two respects. First, taxotere has a 10-hydroxy (-OH) group in place of taxol's 10-acetoxy (-OCOCH₃) group. Second, taxotere has a tert-butoxycarbonyl (-COOC(CH₃)₃) group attached to its 3' nitrogen atom, in place of taxol's benzoyl (-COC₆H₅) group (15). Taxolter metro, another taxol analog which shares the same modifications of taxotere, was also investigated in our laboratory. Our data showed that taxolter metro was more potent than taxol (16). We found that pericentriolar material dissociation was also a common characteristic of taxolter metro-induced centrosome impairments in several cell lines (data not shown here). The results support our proposal that pericentriolar material dissociation would play a key role in cytotoxicity of these taxol analogs. To the best of our knowledge, this is the first report describing the relationship between the specific type of centrosome impairment and taxane cytotoxicity. Further research into this effect is currently being performed in our laboratory.

The results demonstrated that multinucleation was a consequence of centrosome impairments in taxane-treated cells. These multinucleated cells then underwent abnormal mitosis during subsequent cycles and ended in giant multinucleated cells. Recently, the mode of mitotic catastrophe has emerged as an important type of cell death (17-20). It was defined mostly by the morphology of the cells

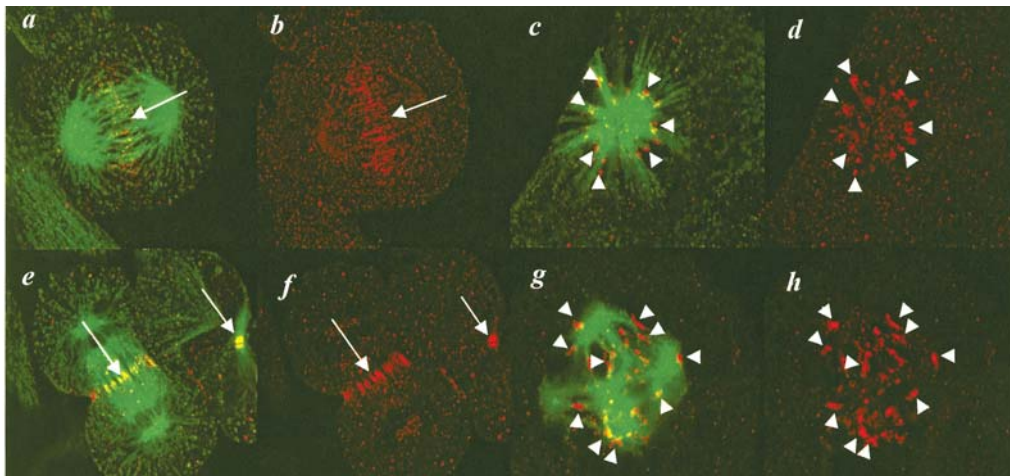


Figure 4. No midbody was detected in cytokinesis failure cell. Cells were stained with anti- β tubulin in green color and anti-aurora B in red color. Panels a, c, e and g show the superimposed images of double staining. Panels b, d, f and h show single staining by anti-Aurora B antibody (red). Arrows indicate Aurora B located at both midzone microtubules and midbodies in control cells during metaphase, anaphase and telophase (a, b, e, f). Arrowheads indicate that midbody was not built in cytokinesis failure cells (c, d, g, h), respectively.

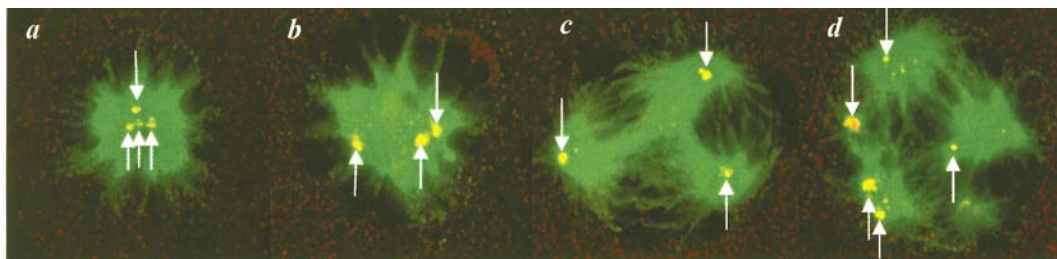


Figure 5. Multiple centrosomes in abnormal spindle cells during prolonged incubation. PC3 cells were treated with 10 nM taxol (a, b) and 5 nM taxotere (c, d) for 24 hours, then were incubated in drug-free media for 48 hours. Mitotic spindles were stained by anti- β tubulin in green color (a, b, c, d). Centrosomes were stained by γ -tubulin (a, c) and pericentrin (b, d), both in red color. Arrows indicate multiple centrosomes stained positively with γ -tubulin or pericentrin antibodies.

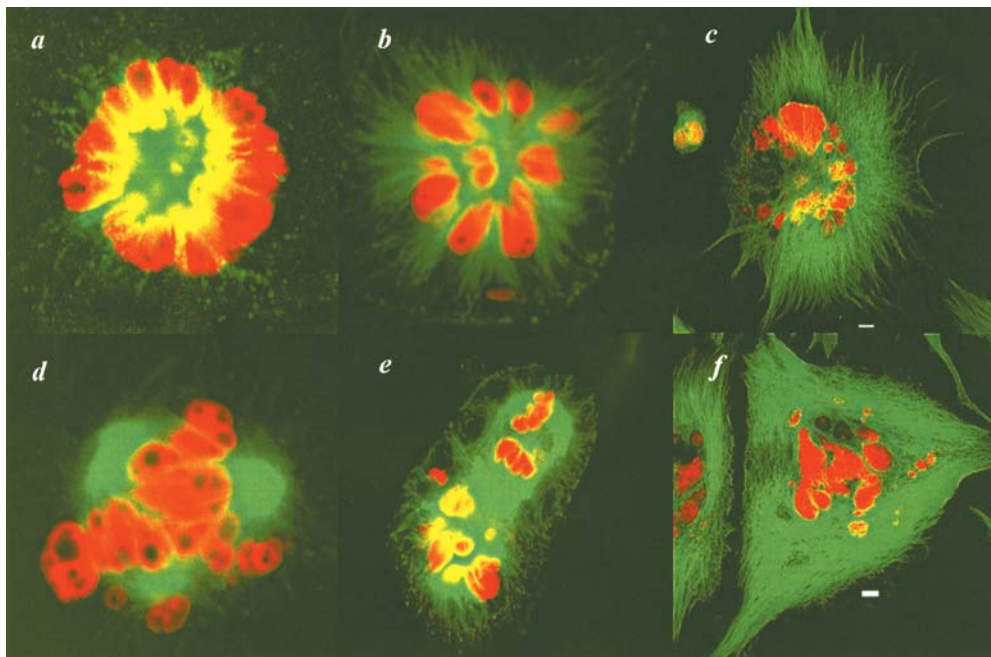


Figure 6. Abnormal mitosis and giant multinucleated cells during prolonged incubation after taxane drug treatment. PC3 cells were treated with 10 nM taxol (a, b, c) and 5 nM taxotere (d, e, f) for 24 hours, then incubated in drug-free media for 48 hours. Microtubules were stained by β tubulin in green color. DNA was stained by PI in red color. Panel a shows monopolar spindles. Panel d shows multipolar spindles. Panel b shows cleavage failure resulting from monopolar spindles. Panel e shows cleavage failure resulting from multipolar spindles. Panels c and f show giant multinucleated cells. Bar, 10 μ M.

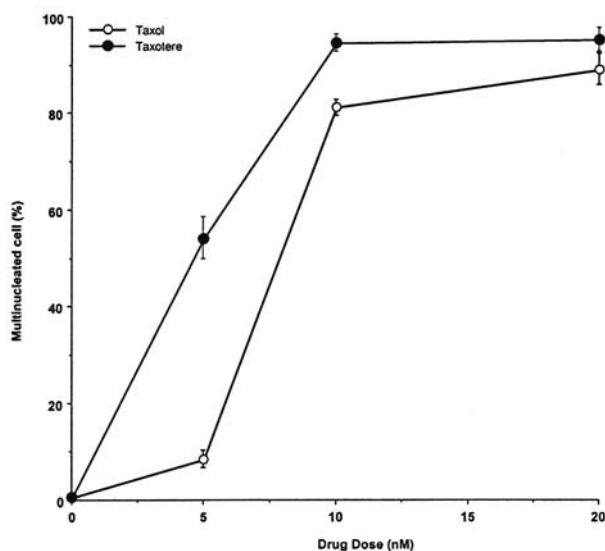


Figure 7. The percentages of multinucleated interphase cells induced by taxanes. PC3 cells were grown in petri dishes 28 hours before drug treatment. The cells were treated with taxol or taxotere for 24 hours. Then, the cells were observed with phase contrast microscopy. The percentage of multinucleated cells was calculated by using the formula: the numbers of multinucleated cell / the total numbers of cells. Data are expressed as mean \pm SD from two experiments.

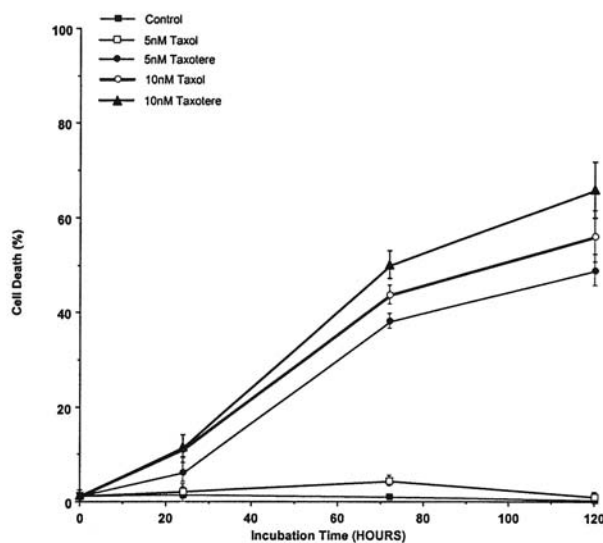


Figure 8. The percentages of dead cells after treatment with two taxanes. PC3 cells were grown in petri dishes 28 hours before drug treatment. These cells were treated with taxol or taxotere for 24 hours, and then incubated in drug-free media during prolonged incubation. Dead cells were stained by trypan blue staining. The percentage of dead cells was calculated by using the formula: the numbers of trypan blue-positive cells / the total numbers of cells. Data are expressed as the mean \pm SD from two experiments.

(giant, multinucleated); and believed to be a response of cancer cells to DNA damage (21). Our data showed that taxane induced cell death through mitotic catastrophe. They strongly suggest that mitotic catastrophe would also be a consequence of impairments of centrosomes and mitotic spindles. Our previous studies revealed that apoptosis in several cancer cell lines was significantly induced by taxanes (22). Our new results show that part of multinucleated cells were eliminated through apoptosis, others through non-apoptotic cell death (data not shown here). Our separate and forthcoming manuscript will present these non-apoptotic data.

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