

Cell Death Induced by Taxanes in Breast Cancer Cells: Cytochrome c is Released in Resistant but not in Sensitive Cells

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Abstract. *Background:* The aim of the study was to contribute to our understanding of the mechanisms responsible for the resistance of breast cancer cells to taxanes. *Materials and Methods:* Cell cycle characteristics, DNA fragmentation, p53 and p21^{WAF1/CIP1} expression, caspase-3 and caspase-9 activity, cytochrome c release from mitochondria during cell death induction by the taxanes paclitaxel and docetaxel in highly-sensitive MDA-MB-435 and highly-resistant NCI-ADR-RES human breast cancer cells were compared. *Results:* Approximately 300-fold higher concentrations of the taxanes were required to induce death in resistant NCI-ADR-RES cells than in sensitive MDA-MB-435 cells. Cell death induced by the taxanes in both sensitive and resistant cells was preceded by the accumulation of cells in the G₂/M-phase. Neither cell type produced any DNA fragmentation (DNA ladder) typical of regular apoptosis. The p53 and the p21^{WAF1/CIP1} levels did not change in sensitive or in resistant cells during cell death induction by the taxanes. The activity of the executioner caspase-3 increased significantly (2 to 2.5-fold) and, similarly, the activity of caspase-9 increased significantly (2-3.5-fold) in both cell types. However, cytochrome c was found to be released from mitochondria into the cytosol only in the resistant NCI-ADR-RES cells, but not in the sensitive MDA-MB-435 cells. *Conclusion:* The death induced by the taxanes in the studied breast cancer cells can be characterized as an apoptosis-like death, including caspase-3 and caspase-9 activation but not oligonucleosomal DNA fragmentation. However, the mechanisms of death induction by

the taxanes in sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells differ. Cytochrome c is released from the mitochondria in resistant but not in sensitive cells.

The taxanes represent a relatively new class of anticancer drugs. There are two taxanes used in cancer therapy, *i.e.* paclitaxel and docetaxel (1). Paclitaxel (Taxol[®]) was originally isolated from the bark of the Pacific Yew (*Taxus brevifolia*) and is now manufactured in a partly synthetic process (2). Docetaxel (Taxotere[®]) is a semi-synthetic taxane which does not occur naturally. Its precursor is isolated from the needles of the European Yew (*Taxus baccata*) (3). Both taxanes have been used in the therapy of breast, ovarian, lung, esophageal and head and neck cancers (2, 4, 5).

Taxanes are mitotic poisons. They bind to the β subunit of the tubulin heterodimer. This interaction accelerates the polymerization of tubulin, stabilizes the resulting microtubules and inhibits their depolymerization (2, 6). It results in the formation of microtubule bundles in interphase cells and in the formation of deformed microtubule structures called asters, instead of mitotic spindles, during mitosis (7, 8). In this way, the taxanes block progression through the M-phase of the cell cycle (9).

Taxanes induce cell death, supposedly by apoptosis (10, 11). However, the molecular mechanism of cell death induction by the taxanes remains obscure. The relationship between cell death induction and mitotic arrest is unclear (11, 12). Similarly, the role of phosphorylation of the Bcl-2 protein in cell death induction by the taxanes is unclear. Bcl-2 phosphorylation is a hallmark of taxane effects, but it might be related to mitotic arrest rather than to cell death induction (13-15). It has been shown, by several authors (13, 16, 17), that cell death induced by the taxanes is p53-independent. On the other hand, several recent findings concerning cytochrome c release, caspase-9 activation and caspase-3 activation (10, 18-25) support the suggestion that

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the apoptotic mitochondrial pathway is involved in cell death induction by the taxanes, including cell death induction in breast cancer cells.

The aim of the present study was to contribute to our understanding of the mechanisms involved in the cell death induced by taxanes in breast cancer cells and, particularly, to contribute to a better understanding of the mechanisms responsible for the resistance of these cells to the taxanes. The characteristics of cell death induced by the taxanes paclitaxel and docetaxel were compared in human breast cancer cells MDA-MB-435, which were found to be highly sensitive to taxanes, and in human breast cancer cells NCI-ADR-RES, which were found to be highly resistant to taxanes. The cell death induced by paclitaxel or by docetaxel in both types of cells had the characteristics of apoptosis-like death. However, the mechanisms of death induction by the taxanes in sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells differ, since cytochrome c is released from mitochondria only in resistant NCI-ADR-RES but not in sensitive MDA-MB-435 cells.

Materials and Methods

Materials. Paclitaxel was obtained from Bristol-Myers Squibb (Princeton, NJ, USA) and docetaxel from Rhone-Poulenc-Rorer (Vitry sur Seine, France). The mouse monoclonal antibody Pab 240 against human p53 and the mouse monoclonal antibody HZ52 against human p21^{WAF1/CIP1} from Neo Markers (Fremont, CA, USA), and mouse monoclonal antibodies 7H8.2C12 from Zymed Laboratories (San Francisco, CA, USA) and 6H2 B4 from Pharmingen (San Diego, CA, USA) against human cytochrome c were used.

Cells and culture conditions. The human breast carcinoma cell lines MDA-MB-435 and NCI-ADR-RES were obtained from the National Cancer Institute (Frederick, MD, USA). The cells were maintained in a culture medium at 37°C in a humidified atmosphere of 5% CO₂ in air. The culture medium represents a basic medium supplemented with 10% fetal bovine serum (J. Kysilka, Brno, Czech Republic). The basic medium was RPMI 1640 medium containing extra L-glutamine (300 µg/ml), sodium pyruvate (110 µg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml), as described previously (26). For the experiments, cells were harvested employing trypsin (0.2%) + EDTA (0.02%) in PBS.

Cell growth and survival experiments. Cells maintained in the culture medium were harvested by low-speed centrifugation, washed with the culture medium and then seeded at 1x10⁴ cells/100 µl of medium into wells of a 96-well plastic plate. Cell growth and survival were evaluated after 96 h of incubation in the culture medium without taxane (control) and with various concentrations of paclitaxel or docetaxel. The number of living cells was determined by hemacytometer counting after staining with trypan blue (27).

Propidium iodide staining analysis. Cells grown in the culture medium were harvested by low-speed centrifugation, washed with

the culture medium and seeded at 5x10⁶ cells/25 ml of medium into plastic culture flasks. After the required incubation period (24, 48, 72, 96 h) in the culture medium without taxane (control) and with selected concentrations of paclitaxel or docetaxel, the cells were harvested by low-speed centrifugation, stained and analyzed as described previously (27).

DNA fragmentation analysis. Cells previously grown in the culture medium were harvested by low-speed centrifugation, washed with the culture medium and seeded at 5x10⁶ cells/25 ml of medium into plastic culture flasks. After 72 h of incubation in the culture medium without taxane (control) and with effective concentrations of paclitaxel or docetaxel, the cells were harvested by low-speed centrifugation and analyzed employing agarose gel electrophoresis, as described previously (27).

Indirect immunofluorescence analysis. Harvested cells were seeded at 5x10⁶ cells/25 ml, as described above. After 24 h of incubation in the culture medium without taxane (control) and with effective concentrations of paclitaxel or docetaxel, the cells were harvested and stained as described previously (28). Mouse monoclonal antibodies (IgG) Pab 240 against human p53 and HZ52 against human p21^{WAF1/CIP1} were used as primary antibodies. Anti-mouse FITC-conjugated goat antibody (Sigma, St. Louis, MO, USA) was used as the secondary antibody.

Measurement of caspase-3 and caspase-9 activities. Harvested cells were seeded at 5x10⁶ cells/25 ml, as described above. After 24 h of incubation in the culture medium without taxane (control) and with effective concentrations of paclitaxel or docetaxel, the cells were harvested by low-speed centrifugation and analyzed. Commercial colorimetric kits Caspase-3 Assay Kit from Sigma and ApoTarget™ Caspase-9/Mch6/Apaf-3 from Biosource (Camarillo, CA, USA) were used as described previously (29). The total protein content in samples was determined by the bicinchoninic acid assay (30).

Confocal microscopy. Harvested cells were seeded at 1x10⁶ cells/5 ml of medium over glass coverslip coated with poly-L-lysine from Sigma. After 24 h of incubation in the culture medium without taxane (control) and with effective concentrations of paclitaxel or docetaxel, the cells on the coverslip were incubated with 300 nM MitoTracker Red from Molecular Probes (Eugene, OR, USA) at 37°C for 20 min. The cells were washed with the culture medium and fixed in 3.7% formaldehyde for 15 min at 37°C. Fixed cells were washed with PBS and permeabilized with cold (-20°C) acetone for 4 min. After washing with PBS, the cells were incubated with 150 µl of the mouse monoclonal antibody 6H2 B4 against human cytochrome c (6 µg/ml of PBS). After overnight incubation with the primary antibody at 4°C, the cells were washed with PBS and incubated with 150 µl of FITC-conjugated secondary antibody from Sigma-Aldrich (Steinheim, Germany) or FITC-conjugated secondary antibody from Jackson ImmunoResearch Laboratories (Bar Harbor, MA, USA). After 1 h of incubation with the secondary antibody at room temperature, the cells were washed again with PBS. Stained cells were allowed to dry, overlaid with 15 µl of vectashield from Vector Laboratories (Burlingame, CA, USA) and placed on a glass slide. The samples were analyzed with a confocal microscope Leica TCS-SP from Leica (Bannockburn, IL, USA) with Argon (458 nm) and HeNe (543 nm, 633 nm) lasers and with a 100x objective.

Cell fractionation and Western blot analysis. Harvested cells were seeded at 5×10^6 cells/25 ml, as described above. After 24-h incubation in the culture medium without taxane (control) and with effective concentrations of paclitaxel or docetaxel, the cells (approximately 2×10^7 cell per sample) were harvested by low-speed centrifugation and washed with PBS. The cell pellet was resuspended in STEA medium (250 mM sucrose, 10 mM Tris-HCl, 2 mM EDTA, 0.05% BSA, pH 7.4) supplemented with protease inhibitors (20 μ M leupeptin, 1 μ M pepstatin A, 1 mM PMSF) and incubated on ice for 5 min. The cells were mechanically homogenized using a syringe needle (diameter 0.3 mm). Nuclei and unbroken cells were removed by centrifugation at 800 xg for 10 min. The supernatant was collected into a new Eppendorf tube and centrifuged at 20,000 xg for 15 min at 4°C. The supernatant (cytosolic fraction) was collected into another new Eppendorf tube and the pellet (mitochondrial fraction) was resuspended in 40 μ l of Triton lysis buffer (1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 30 mM NaF, 0.2 μ M leupeptin, 1 mM PMSF, pH 7.6) or SDS lysis buffer. Cytosolic and mitochondrial fractions were homogenized using an ultrasonic homogenizer CP501 (Cole Parmer) and stored at -80°C. The total protein content in both fractions was determined by the bicinchoninic acid protein assay (30). Protein samples (80 μ g) were heated for 5 min at 100°C in the sample loading buffer (SDS lysis buffer containing 5% 2-mercaptoethanol and 0.05% bromophenol blue) and then quickly cooled on ice. SDS-PAGE was performed according to Laemmli (31). Samples were run at a constant current of 30 mA for 90 min, employing mini-Protean 3 (Bio-Rad Laboratories, Hercules, CA, USA), on 12% polyacrylamide gel (4% polyacrylamide stacking gel). Proteins separated by SDS-PAGE were blotted, according to the modified method of Kyhse-Andersen (32) on to 0.2 μ m Protran nitrocellulose membrane from Schleicher & Schuell BioScience (Dassel, Germany) for 2 h at a constant current of 0.2 A using Mini Trans-Blot Cell from Bio-Rad Laboratories. The membrane was blocked with 5% non-fat milk in TBS for 30 min at 4°C and then washed with 0.1% Tween/TBS 3 times. The washed membrane was incubated overnight at 4°C with the primary antibody (0.5 μ g/ml of TBS containing 1% non-fat milk and 0.3% Tween). The mouse monoclonal antibody 7H8.2C12 against human cytochrome c was used as the primary antibody. After the incubation, the membrane was washed with 0.1% Tween/TBS 3 times. The washed membrane was incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibody from Bio-Rad Laboratories (1:10,000 in TBS containing 1% non-fat milk and 0.3% Tween). The membrane was then washed with 0.1% Tween/TBS 3 times. The horseradish peroxidase-conjugated antibody was detected by enhanced chemiluminescence using the ECL chemiluminescence reagent from Pierce Biotechnology (Rockford, IL, USA) and CCD camera LAS1000 (Fuji).

Statistical analysis. Statistical significance of differences was determined using one-way ANOVA and the Student-Newman-Keuls tests.

Results

Effect of taxanes on growth and survival. The effect of the taxanes, paclitaxel and docetaxel, were tested in a wide range of concentrations (0.1-3000 nM) on the growth and survival of several human breast cancer cell lines (MCF-7,

MDA-MB-231, MDA-MB-435, NCI-ADR-RES, SK-BR-3). MDA-MB-435 cells were found to be the most sensitive and NCI-ADR-RES cells the most resistant to cell death induced by the taxanes.

Paclitaxel, at a concentration of 3 nM and higher concentrations, induced death of MDA-MB-435 cells over 96 h of incubation. Docetaxel induced death of MDA-MB-435 cells at a concentration of 1 nM higher concentration during the same incubation period. The D_{50} (dose of taxane producing 50% of living cells in comparison with the control after 96 h of incubation) of paclitaxel was approximately 1 nM and the D_{50} of docetaxel approximately 0.3 nM. On the other hand, paclitaxel induced death of NCI-ADR-RES cells at a concentration of 1000 nM and higher and docetaxel induced death of NCI-ADR-RES cells at concentrations higher than 300 nM. The D_{50} of paclitaxel and docetaxel was approximately 300 nM and 200 nM, respectively (Figure 1). The data showed that approximately 300-fold higher concentrations of paclitaxel or docetaxel were required to induce death in resistant NCI-ADR-RES cells than in sensitive MDA-MB-435 cells. Flow cytometric analysis, after propidium iodide staining, detected a significant accumulation of particles with hypodiploid DNA content in the population of tested cells during 72 h of incubation with death-inducing concentrations of a taxane. In the case of sensitive MDA-MB-435 cells, the accumulation of particles with hypodiploid DNA content was detected for 3 nM and higher concentrations of paclitaxel and for 1 nM and higher concentrations of docetaxel. Relevant data were obtained for the resistant NCI-ADR-RES cells (data not shown).

On the basis of the data presented above, we selected 30 nM paclitaxel and 10 nM docetaxel, for sensitive MDA-MB-435 cells, and 3000 nM paclitaxel and 1000 nM docetaxel, for resistant NCI-ADR-RES cells, as employed effective concentrations, *i.e.* the lowest concentrations with full death-inducing effect. These concentrations of paclitaxel and docetaxel were used in further studies with the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells.

Effect of taxanes on DNA content and fragmentation. Flow cytometric analysis, after propidium iodide staining, showed a significant accumulation of particles with hypodiploid DNA content after 48 h of incubation of the sensitive MDA-MB-435 cells as well as the resistant NCI-ADR-RES cells with effective concentrations (30 nM, 3000 nM) of paclitaxel. This accumulation was connected with induced cell death as demonstrated above. DNA histograms also showed a significant accumulation of cells in the G2/M-phase of the cell cycle detectable after 24 h of incubation with the effective concentrations of the taxanes (Figure 2). This finding demonstrated that cell death induced by the taxanes in both sensitive and resistant cells was preceded by

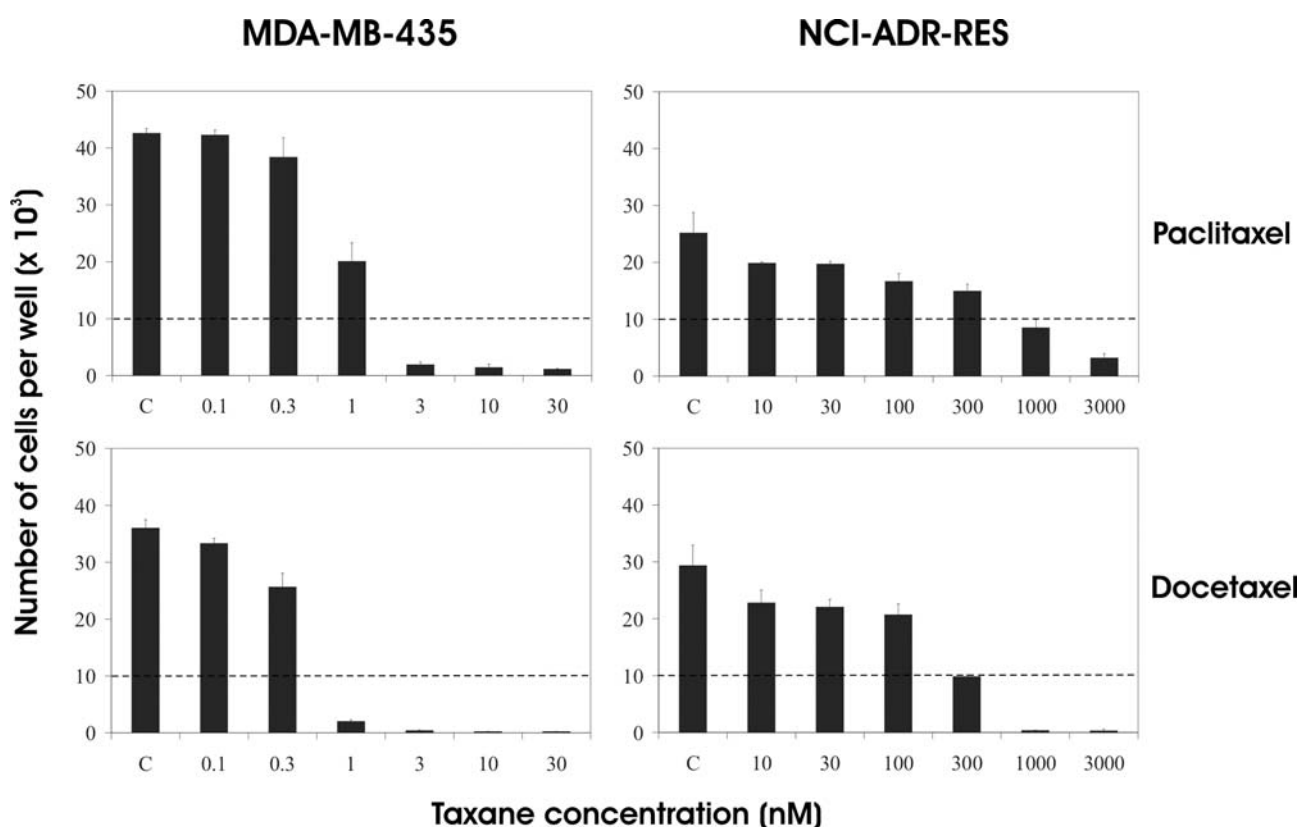


Figure 1. Effect of paclitaxel and docetaxel (0.1-3000 nM) on the growth and survival of MDA-MB-435 and NCI-ADR-RES cells. Control cells (C) were incubated without taxane. The cells were seeded at 10×10^3 cells/100 μ l of medium in the well. The number of cells of the inoculum is shown as a dotted line. The number of living cells was determined (see "Materials and Methods") after 96 h of incubation. Each column represents the mean of 8 separate cultures \pm SEM.

the accumulation or block of cells in the G₂/M-phase. However, in the case of resistant NCI-ADR-RES cells even after 96 h of incubation with the taxanes at the effective concentrations, when nearly all cells were dead (see Figure 1), a significant number of cells was still detected with regular cell cycle DNA content. Similar data were obtained with effective concentrations (10 nM, 1000 nM) of docetaxel (data not shown).

Particles with hypodiploid DNA content, detected by flow cytometric analysis, did not form a typical apoptotic peak of cells with hypodiploid DNA content. Therefore, in order to characterize the cell death induced by the taxanes, we employed DNA fragmentation analysis. DNA fragmentation analysis by agarose gel electrophoresis showed that both sensitive MDA-MB-435 and resistant NCI-ADR-RES cells, after 96 h of incubation with the effective concentrations of paclitaxel or docetaxel, did not produce any DNA fragmentation (ladder) typical of regular apoptosis (Figure 3).

Effect of taxanes on p53 and p21^{WAF1/CIP1} expression. Changes in the expression of p53 in relation to the cell death induced by the taxanes in the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells were assessed. The activation of p53 was also assessed by the induction of p21^{WAF1/CIP1} expression. However, indirect immunofluorescence analysis showed that 24-h incubation with the effective concentrations of paclitaxel or docetaxel did not significantly change either the p53 level or that of p21^{WAF1/CIP1} in the sensitive as well as in the resistant cells (data not shown).

Effect of taxanes on caspase-3 and caspase-9 activities. In order to further characterize the cell death induced by the taxanes in sensitive and resistant cells, the activation of the key executioner caspase-3 and the activation of upstream caspase-9 were tested. The employed colorimetric assay showed that the basal activity of caspase-3 in the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells was similar. After 24 h of incubation with the effective

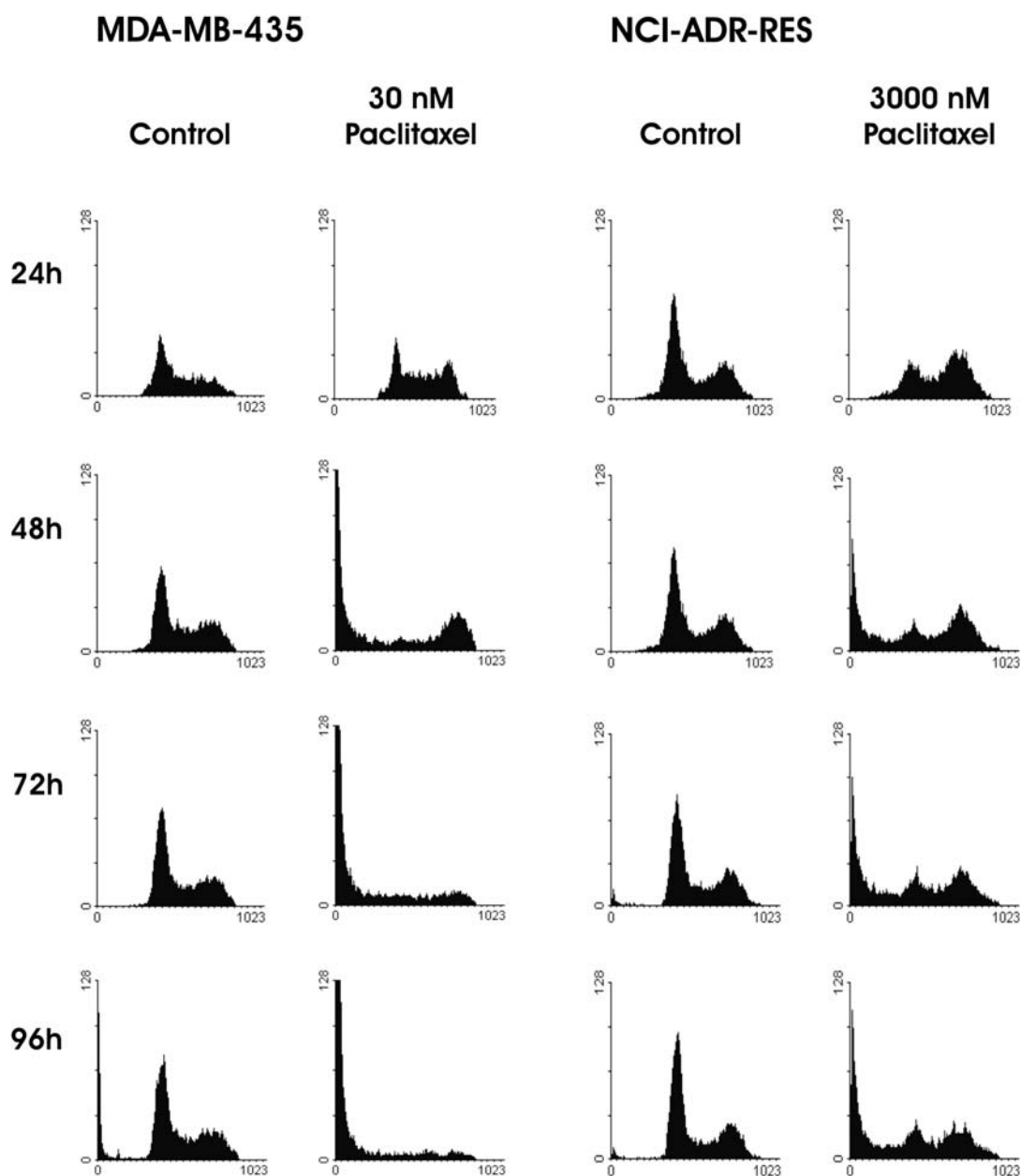


Figure 2. Effect of effective concentrations of paclitaxel (30 nM and 3000 nM) on the DNA histogram of sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. After the incubation period (24, 48, 72, 96 h), the cells were stained with propidium iodide (see "Materials and Methods") and analyzed by flow cytometry.

concentrations of paclitaxel or docetaxel, the activity of caspase-3 in both sensitive and resistant cells increased significantly (approximately 2 to 2.5-fold). The effective concentrations of paclitaxel (30 nM) and docetaxel (10 nM) for the sensitive MDA-MB-435 cells did not produce any increase of caspase-3 activity in the resistant NCI-ADR-RES cells (Figure 4).

The employed colorimetric assay showed that the basal activity of caspase-9 in sensitive and resistant cells was also similar. The activity of caspase-9 in the sensitive MDA-MB-435 cells increased significantly (approximately 3 to 3.5-fold) after 24-h incubation with the effective concentrations of paclitaxel or docetaxel. In the resistant NCI-ADR-RES cells, a significant increase in caspase-9 activity

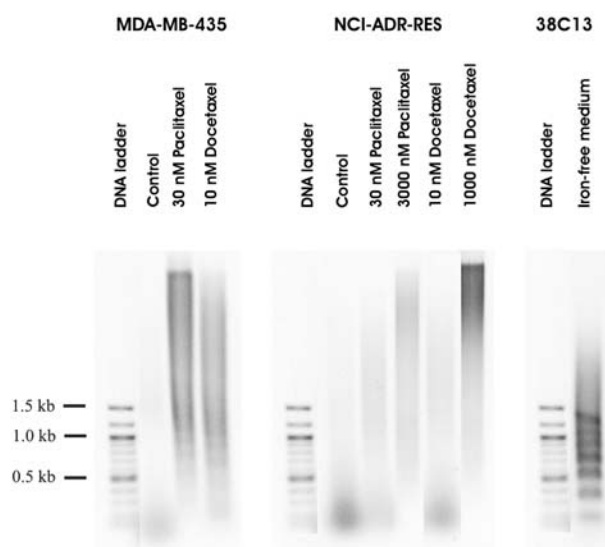


Figure 3. Effect of paclitaxel and docetaxel at effective concentrations on DNA fragmentation in sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. DNA fragmentation was determined (see "Materials and Methods") after 96 h of incubation. Control DNA ladder and typical apoptotic DNA fragmentation in 38C13 cells (induced by iron deprivation) are shown.

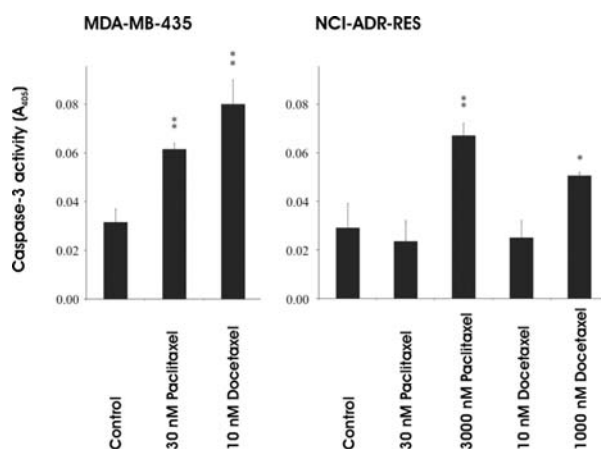


Figure 4. Effect of paclitaxel and docetaxel at effective concentrations on caspase-3 activity in sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. After 24 h of incubation, the activity of caspase-3 was measured as the absorbance of the cleaved product of a caspase-3 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see "Materials and Methods"). Each column represents the mean of 2 experimental values \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with control values.

(approximately 2 to 2.5-fold) was also detected. It seemed that the increase in caspase-9 activity was less pronounced in the resistant than in the sensitive cells (Figure 5), as in the case of caspase-3 activity (Figure 4).

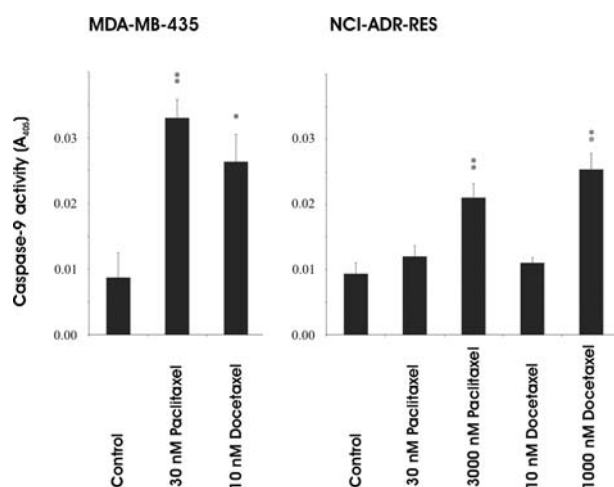


Figure 5. Effect of paclitaxel and docetaxel at effective concentrations on caspase-9 activity in sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. After 24 h of incubation, the activity of caspase-9 was measured as the absorbance of the cleaved product of a caspase-9 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see "Materials and Methods"). Each column represents the mean of 3 experimental values \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with control values.

Effect of taxanes on cytochrome c release. In order to complete the information on caspase-3 and caspase-9 activation during the cell death induced by taxanes in the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells, the cellular distribution of cytochrome c was assessed. Confocal microscopy, after indirect immunofluorescence staining, showed (Figure 6) that cytochrome c was, under the control conditions, *i.e.* incubation without taxane, localized in the mitochondria in both sensitive and resistant cells. After 24 h of incubation with the effective concentrations of paclitaxel, cytochrome c was found to be released from the mitochondria into the cytosol in the resistant NCI-ADR-RES cells but, surprisingly, was not found to be released in the sensitive MDA-MB-435 cells. The effective concentration of paclitaxel (30 nM) for the sensitive MDA-MB-435 cells did not lead to cytochrome c release in the resistant NCI-ADR-RES cells. Similar data were obtained with effective concentrations of docetaxel (data not shown). These data were confirmed by Western blot analysis after cell fractionation (Figure 7). Cytochrome c was detected solely in the mitochondrial fraction in sensitive as well as in resistant cells under the control conditions. In the resistant NCI-ADR-RES cells incubated 24 h with the effective concentrations of paclitaxel or docetaxel, cytochrome c was also detected in the cytosolic fraction, while in the sensitive MDA-MB-435 cells incubated with the effective concentrations of paclitaxel or docetaxel, cytochrome c stayed in the mitochondria.

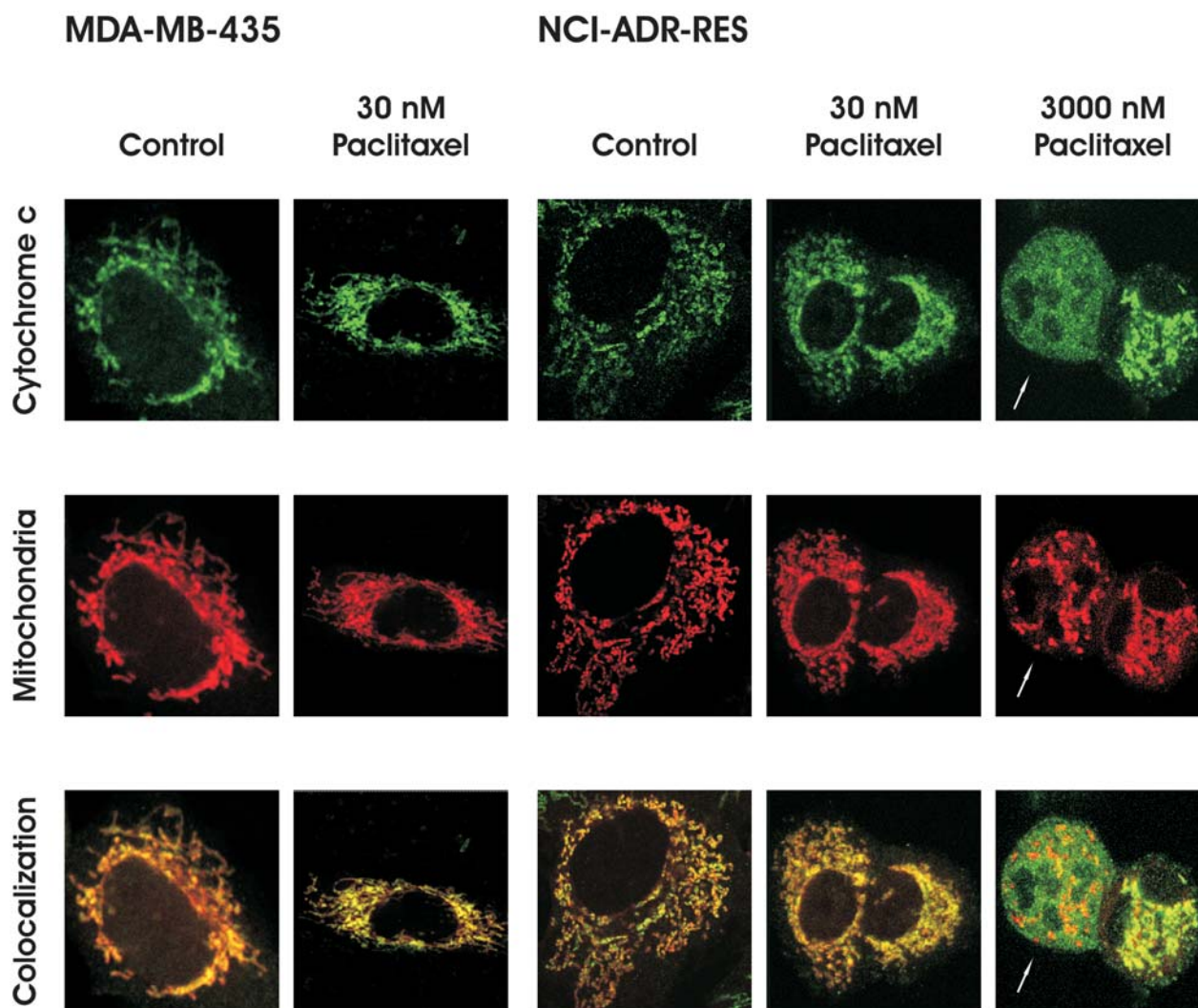


Figure 6. Effect of paclitaxel at effective concentrations on cellular distribution of cytochrome *c* in sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. After 24 h of incubation, the localization of cytochrome *c* and the position of mitochondria were detected by confocal microscopy, following staining with MitoTracker Red (mitochondria) and indirect immunofluorescence staining (cytochrome *c*) with monoclonal antibody 6H2 B4 against human cytochrome *c* (see "Materials and Methods"). The localization of cytochrome *c* (green), of mitochondria (red) and the co-localization of cytochrome *c* and mitochondria within the cell are shown. The presented cell with released cytochrome *c* is indicated by an arrow. The data shown were obtained in 1 representative experiment of 2 independent experiments.

Discussion

In order to contribute to our better understanding of the mechanisms involved in the cell death induced by taxanes in breast cancer cells, and particularly to contribute to our understanding of the mechanisms responsible for the resistance of breast cancer cells to taxanes, we studied cell death induced by the taxanes paclitaxel and docetaxel in the human breast cancer cell lines MDA-MB-435 and NCI-ADR-RES. MDA-MB-435 cells are highly sensitive, while NCI-ADR-RES cells are highly resistant to death induction by the taxanes.

The dose-response effect of paclitaxel and docetaxel showed that approximately 300 times higher concentrations of the taxanes are required to induce death in the resistant NCI-ADR-RES cells than in the sensitive MDA-MB-435 cells (see Figure 1). Thus, highly sensitive MDA-MB-435 cells and highly resistant NCI-ADR-RES cells represent a good model for studies concerning the mechanisms of cell death induction by the taxanes, as well as the mechanisms of resistance to death induction by the taxanes. As employed effective concentrations, *i.e.* the lowest concentrations with full death-inducing effect, we here selected 30 nM paclitaxel

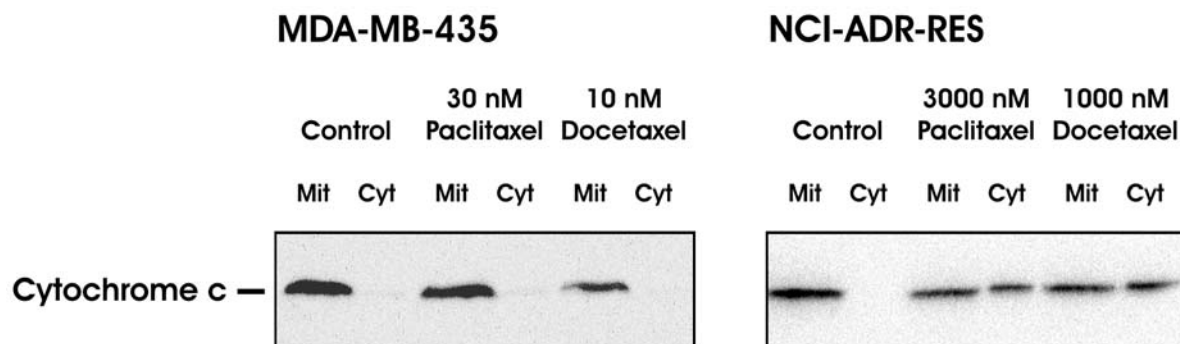


Figure 7. Effect of paclitaxel and docetaxel at effective concentrations on cytochrome c release from mitochondria in sensitive MDA-MB-435 and resistant NCI-ADR-RES cells. Control cells were incubated without taxane. After 24 h of incubation, the level of cytochrome c was determined by Western blot analysis, employing the specific monoclonal antibody 7H8.2C12 against human cytochrome c, (see "Material and Methods") in mitochondrial (Mit) and cytosolic (Cyt) fractions. The data shown were obtained in 1 representative experiment of 2 independent experiments.

and 10 nM docetaxel for the sensitive MDA-MB-435 cells and 3000 nM paclitaxel and 1000 nM docetaxel for the resistant NCI-ADR-RES cells (see Figure 1).

Docetaxel was found to be more effective than paclitaxel in both sensitive and resistant cells. Approximately 3 times higher concentrations of paclitaxel were required to induce death (see Figure 1). This finding is in agreement with some clinical experiences (33). However, the difference found under *in vitro* conditions does not necessarily reflect the situation under *in vivo* conditions, because other factors are involved under *in vivo* conditions. On the other hand, on the basis of our data, it seems highly probable that the mechanisms of death induction in breast cancer cells by paclitaxel and docetaxel are the same or very similar.

The key executioner caspase-3 and upstream caspase-9 are activated in both sensitive MDA-MB-435 and resistant NCI-ADR-RES cells when death is induced by the taxanes (see Figures 4 and 5). This fact strongly supports the suggestion that apoptotic death is involved. However, flow cytometric analysis after propidium iodide staining, as well as DNA fragmentation analysis by agarose gel electrophoresis, did not show typical apoptotic oligonucleosomal fragmentation of the DNA (see Figure 2 and 3). Therefore, the cell death induced by the taxanes in sensitive as well as in resistant breast cancer cells has rather the characteristics of an apoptosis-like death. Our finding, that the accumulation of cells in the G₂/M-phase of the cell cycle precedes the appearance of dead cells, supports the suggestion that the mitotic arrest (9) resulting from taxane application could be involved in the initial stages of death induction in breast cancer cells by these drugs.

Our data, concerning the expression of p53 and p53-regulated p21^{WAF1/CIP1}, strongly support the suggestion of other reports (13, 16, 17) that p53 is not involved in taxane-induced cell death. Concerning the mechanisms of the cell

death induced by the taxanes in the breast cancer cells, one can consider the involvement of the apoptotic mitochondrial pathway when taking into account the activation of caspase-9 and caspase-3 in sensitive MDA-MB-435 cells, as well as in resistant NCI-ADR-RES cells (see Figure 4, Figure 5). The activation of caspase-9 by paclitaxel was detected by Kottke *et al.* (20) and Razandi *et al.* (22) in several breast cancer cell lines. Similarly, the activation of caspase-3 by paclitaxel and paclitaxel analogs was detected by several groups (20, 21, 23) in several breast cancer cell lines, including the studied NCI-ADR-RES cells (25). On the other hand, Ofir *et al.* (34) demonstrated that cell death induced by paclitaxel in MCF-7 breast cancer cells is caspase-9- and caspase-3-independent.

Taking into account the data concerning the activation of caspase-9 and caspase-3, one could suppose that the mechanisms of death induction by the taxanes in the sensitive MDA-MB-435 and resistant NCI-ADR-RES cells are similar. In this case, the resistance of NCI-ADR-RES cells is not related to the cellular mechanism of death induction, and it therefore seems probable that the resistance is mainly based on the expression of the multidrug resistance (MDR) transporter P-glycoprotein representing an efflux pump (35-38).

The application of effective concentrations of both taxanes led to cytochrome c release from the mitochondria into the cytosol in resistant NCI-ADR-RES cells. It demonstrated that the apoptotic mitochondrial pathway of cell death induction, concerning cytochrome c release and subsequent caspase-9 and caspase-3 activation, is probably involved here. However, cytochrome c was not released when cell death was induced by effective concentrations of both the taxanes in the sensitive MDA-MB-435 cells. This really surprising finding was confirmed by two independent methods (confocal microscopy and Western blot analysis). Cytochrome c release

after paclitaxel treatment was demonstrated in several breast cancer cell lines (10, 20, 24). Audjit and Vuori (10) also mentioned cytochrome c release after paclitaxel treatment in MDA-MB-435 cells, however, relevant data were not shown in their paper. Our data concerning cytochrome c release strongly support the suggestion that the mechanisms involved in cell death induced by taxanes in the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells differ. There is a possibility that the activation of caspase-9 is not decisive for the death induction by taxanes in the sensitive MDA-MB-435 cells and that the activation of caspase-9 just represents an accompanying effect.

Taken together, we conclude that the death induced by the taxanes paclitaxel and docetaxel in both studied breast cancer cell lines can be characterized as an apoptosis-like death including the activation of caspase-9 and caspase-3, but not oligonucleosomal DNA fragmentation. However, the mechanisms of death induction by the taxanes in the sensitive MDA-MB-435 cells and resistant NCI-ADR-RES cells differ. Cytochrome c is released from the mitochondria in the resistant NCI-ADR-RES cells but not in the sensitive MDA-MB-435 cells. The apoptotic mitochondrial pathway, involving cytochrome c release from the mitochondria and subsequent activation of caspase-9 and caspase-3, is considered in the case of the death induction in the resistant NCI-ADR-RES cells. The mechanism of the death induction in the sensitive MDA-MB-435 cells remains obscure. In order to answer the question, whether differing mechanisms of death induction by taxanes in breast cancer cells produce differing sensitivity/resistance of the cells to taxanes, further studies are required.

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