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Research Article

Survival of docetaxel-resistant prostate cancer cells in vitro depends on phenotype alterations and continuity of drug exposure

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Abstract. We evaluated in vitro the effect of paclitaxel and docetaxel on PC-3 and DU-145 prostate cancer cell lines to understand better the downstream events in drug-induced tumor cell death. Taxane treatments of DU-145 cells induced rapid cell death by apoptosis, but in PC-3 cells, treatments achieved growth arrest, followed by extensive karyokinesis resulting in multinucleation, giant-cell formation and delayed cell death. To determine if the giant multinucleated cells were able to produce proliferating and drug-resistant survivors, we first delineated the kinetics of drug activity and cytotoxic dose range. Analysis of both lines by colorimetric and cell viability assays demonstrated improved cytotoxicity of taxanes applied continuously. Selected doses and schedules of docetaxel were used to induce giant multinucleated cells that gave rise to docetaxel-resistant survivors, which remained sensitive to paclitaxel and other chemotherapeutics. Growth and morphology of the recovered clones was similar to parental cells. The resistant phenotype of these clones determined by immunofluorescence and immunoblot was associated with transient expression of the β-tubulin IV isoform and was independent of P-glycoprotein, bcl-2 and bcl-xL. Resistant clones will be useful to model progression of resistance to taxanes and to identify unknown and clinically important molecular mechanisms of cell death and resistance.

Key words. Prostate; cancer; endoreduplication; cytotoxicity; taxane; resistance; phenotype.

Introduction

Paclitaxel and docetaxel, members of the taxane family of anticancer drugs, are utilized as single agents or in combination with other compounds to treat advanced stages of cancer, including hormone-refractory prostate cancer [1]. Both taxanes bind to microtubules and promote their assembly and stabilization. Unfortunately, not all patients respond to taxane chemotherapy and this could result from inefficient induction of tumor cell death following microtubule stabilization by taxanes.

The mechanism of taxane-induced cancer cell death involves multiple intracellular activities of the drugs besides inhibition of microtubular function [2]. Taxanes were proposed to activate apoptosis and induce cytotoxicity via phosphorylation of bcl-2 and bcl-xL, homologous genes that may prevent apoptosis in tumor cells by similar mechanisms [2, 3]. Various cell lines including prostate cancer display complexity and variability in drug-induced apoptotic pathways [4, 5]. Taxanes were also established to frequently induce tumor cell death via

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endoreduplication, a death pathway often occurring following irradiation or exposure to genotoxic agents [6–8]. These and other studies suggest a deficiency of gene products involved in the G1-S checkpoint function of the cell cycle (p53 [9–12]), pRB [9, 12], $p16^{INK4A}$ [12] or $p21^{Waf1/Cip1}$ [11, 13, 14]) that forces reentrance into the G1 phase with a 4N DNA content, a cell cycle defect called endoreduplication.

Survival of tumor cells and disease progression is often associated with gradually increasing intrinsic or acquired resistance to anticancer drugs. Drug resistance can originate through multiple mechanisms, such as changes in cellular drug uptake, metabolic drug deactivation, structural changes in the drug target, or changes in other cellular components that interact with the target, as well as suppression of apoptosis [15]. Resistance to taxanes and other microtubule-binding agents was attributed to overexpression of the MDR1 gene, and its product P-glycoprotein, an ATP-dependent pump-facilitating drug efflux [16] or alterations in cellular microtubule proteins [17–19]. Overexpression of bcl-2 and more so of bcl-xL was found to delay taxane-induced apoptosis [20, 21], and down-regulation of bcl-2 by antisense oligonucleotides chemosensitizes cells to taxanes [21, 22].

In the present report we compared in vitro the effect of taxanes on PC-3 and DU-145 prostate cancer cell lines to understand better the downstream events in drug-induced tumor cell death. The differences in mechanisms and kinetics of cell death were quite remarkable in these cell lines. Taxane treatments of DU-145 cells induced rapid cell death by apoptosis. In contrast, treatments of PC-3 cells achieved growth arrest followed by extensive karyokinesis, multinucleation, giant-cell formation and delayed cell death. Of interest in this regard is that neither of the androgen-independent prostate cancer cell lines DU-145 and PC-3 express functional p53 protein [23] and only PC-3 cells responded to taxanes by endoreduplication. Tumor cells that do not undergo apoptosis are relatively understudied, and PC-3 cells presented a valuable experimental model to determine if the giant multinucleated cells were able to produce proliferating and drug-resistant survivors. Testing this hypothesis by delineating the kinetics and molecular events accompanying drug-induced tumor cell death or recovery of drug-resistant survivors might prove therapeutically important and provide new leads for clinical trials.

Materials and methods

Cell culture

DU-145 and PC-3 human androgen-independent prostate carcinoma cell lines were obtained from the American Tissue Culture Collection (ATCC) and propagated in plastic dishes in RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco BRL), 5% (v/v) fetal bovine serum (FBS; Atlanta Biologicals) and 0.05 mg/ml gentamicin (Gibco BRL). Medium was changed every 48 h unless specified otherwise. Cells were subcultured with 1 × trypsin/EDTA (GibcoBRL) and split at a 1:10 ratio.

Reagents

1) Cytotoxic compounds. The drugs used in this study were obtained as a sterile analytical powder. Taxotere (docetaxel) was provided by Aventis Pharmaceuticals. Taxol (paclitaxel), carboplatin, vincristine, vinblastine, doxorubicin and etoposide were purchased from Sigma. Estramustine was obtained from Pharmacia & Upjohn. Stock solutions of taxotere and taxol were prepared in 50% ethanol at 5 mg/ml. Carboplatin and doxorubicin were reconstituted at 10 mg/ml; vincristine and vinblastine at 1 mg/ml concentration with sterile water; estramustine and etoposide were solubilized in dimethylsulfoxide (DMSO) at 10 mg/ml. All reagents were kept in the dark at 4 °C.

2) Immunological reagents. The following polyclonal (pAbs) and monoclonal (mAbs) antibodies were used for indirect immunfluorescent (IIF) staining of cultured cells and/or for immunoblot analyses: β-tubulin (mAb, IgG1) and its isoforms β-tubulin II (IgG2b), β-tubulin III (IgG2b) and β-tubulin IV (IgG1) were obtained from Biogenex; P-glycoprotein (mAb, clone JSB1, IgG1,) was purchased from Signet. Antibodies against bcl-2 (pAb, PharMingen), bcl-xL (pAb, Zymed) and actin (pAb, Sigma) were used for immunoblot only.

Cytotoxicity assays

1) MTT. DU-145, PC-3 and docetaxel-resistant PC-3 derivatives were plated onto 96-well plates at a density of 5×10^3 cells/well. Cells were allowed to attach for 24 h. Medium was then replaced with an equal volume of fresh medium containing various concentrations of docetaxel, paclitaxel or other drugs. Following a 96-h drug treatment, cultures were incubated with MTT ([3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium bromide) for 3.5 h at 37 °C and fixed in DMSO for 1 h. Optical density values measured at 550 and 630 nm with a microplate reader (Bio-Tek Instruments EL340) were used to determine the size of the surviving fraction relative to untreated cultures. IC_{50} values were defined as the inhibitory concentration for 50% cells. All experiments were performed in triplicate and repeated at least twice.

2) Sulforhodamine B. Cytotoxicity of docetaxel was also evaluated by the sulforhodamine B (SRB) assay developed by Skehan et al. [24]. This colorimetric assay was used to validate the results produced in the MTT-based assay. Triplicate cultures were plated onto 96-well plates at a density of 5×10^3 cells/well. Twenty-four hours later, spent medium was replaced with an equal volume of fresh medium containing docetaxel at concentrations

used in the MTT assay. Following 24, 48, 72 or 96 h drug exposure, cells were fixed with 10% trichloroacetic acid (TCA) and stained with SRB. Absorbances were measured at 490–530 nm wavelength and cell survival was determined relative to untreated control cultures.

3) Trypan blue cell viability assay. A direct correlation between the cytotoxicity profiles of docetaxel determined by MTT and SRB colorimetric assays and the actual number of cells surviving the drug treatments was established by the Trypan blue exclusion assay. Cells were plated into six-well plastic dishes at 1×10^4 density. Twenty-four hours later, spent medium was replaced with an equal volume of fresh medium containing docetaxel at concentrations corresponding to those used in MTT and/or SRB assays. Treated cells were harvested with trypsin/EDTA following 48, 72 or 96 h treatment, stained with Trypan blue and counted using a hemocytometer. Cell counts were performed in triplicate for each time point and drug dose. Dependence of the time interval preceding death of the giant multinucleated PC-3 cells on the drug dose was also determined by the Trypan blue exclusion assay. Cells plated in six-well dishes at 2×10^5 cells/well were allowed to attach for 24 h before treatment with docetaxel at 10 ng/ml for 24 h. Three cultures were set for each time point. After incubation, medium containing docetaxel was removed, cells were rinsed twice with phosphate-buffered saline (PBS) and fresh medium was supplied. To remove any residual drug, medium was also changed 3 and 24 h later. For the rest of the observation period, cells were maintained in drug-free medium that was changed every other day. Triplicate cultures were harvested with trypsin/EDTA at each 24-h interval, stained with Trypan blue and counted. The Trypan blue exclusion assay was terminated when the total number of surviving cells was less than 5% and residual cells were monitored by microscopy for long-term survival thereafter. The cell survival curve was presented as the percentage of surviving cells versus time. The effect of higher docetaxel doses (100, 250 and 500 ng/ml) was evaluated similarly. The Trypan blue test for the high-dose treatments was terminated earlier, when the number of residual cells was less than 12% and the cell death course was apparent. The period of long-term survival of the residual cells was monitored microscopically. These experiments were concluded when the number of remaining cells in each culture did not exceed 20–30.

Determination of multinucleation/mitotic index

The rate of multinucleation in PC-3 and DU-145 cells following exposure to taxanes was evaluated microscopically. Multinucleation was used as an objective criterion of cell damage following exposure to the drug. Multinucleation was defined as the occurrence of more than one nucleus of the same or different size in an individual cell. Mitotic/multinucleated cells in untreated control cultures were used as an initial reference point and were included in the pool of multinucleated cells in drug-treated cultures. Cells were plated in six-well dishes at 10^6 cells/well and allowed to attach for 24 h. Cells were then incubated for a total of 96 h in medium containing docetaxel at 0.05, 0.1, 0.25 and 0.5 ng/ml or paclitaxel at 0.05, 0.1, 0.25, 0.5, 1 and 2 ng/ml concentrations. Fresh medium containing drugs was provided after 48 h of treatment. At each 24-h time point, triplicate cultures corresponding to each dose and time point were rinsed with PBS and fixed in 60% ice-cold acetone. Fixed cells were observed and photographed on a Nikon E800 microscope using × 200 phase contrast optics. Between 700 and 1500 cells were scored for each time point and treatment dose using NIH Image 1.6 software and the cell-scoring macro. Multinucleation/mitotic index was estimated as a ratio of multinucleated cells to total number of cells. Resulting values are reported as the percentage of multinucleated cells. A similar study on DU-145 cells was also conducted using a single dose of either taxane corresponding to the IC_{50} concentration for this cell line. Following fixation, cells were stained with DAPI to visualize better nuclei under phase contrast optics combined with epifluorescence.

Induction of giant cells to promote selection of docetaxel-resistant cell lines

PC-3 cells were plated at high density into two 24-well plates and allowed to grow beyond confluency. At high cell densities, the volume of medium was increased and medium changes were performed every 24 h. After 7 days at confluency, when proliferation had significantly diminished, cells were exposed to various doses of docetaxel in two regimens: continuous and intermittent. Cultures treated continuously were exposed to docetaxel for a total of 120 h. In the intermittent regimen, treatments were given for 1 h, every 24 h for the total of 120 h. The docetaxel dose for each culture remained constant during all treatments. During continuous treatments, medium containing taxotere was changed daily to ensure a stable concentration of the drug and a supply of fresh nutrients. Following each treatment in the intermittent regimen, cells were washed twice with PBS to remove residual compound and kept in drug-free medium until the next treatment. Upon completion of the 5-day treatment schedules, cells were passaged at low density and maintained in drug-free medium for 14 days after which they were passaged again to consolidate residual giant cells and cultured similarly for up to 30 days. Emergence of the giant-cell-derived clones was assessed by phase contrast microscopy. Stable and docetaxel-resistant clones were expanded for three passages and then gradually adapted to docetaxel at concentrations of 0.2–0.5 ng/ml. Frozen stocks of drug-resistant PC-3 lines were prepared on passage 3.

IIF staining

IIF staining was performed on preconfluent PC-3 cell lines cultured on plastic Permanox-coated chamber slides (Lab-Tek, Nunc). Slides were rinsed in PBS, fixed in ice-cold acetone for 10 min, air dried and stored frozen at –70 °C until used. Slides were rehydrated and blocked in 5% normal goat serum in PBS for 15 min and then incubated with primary antibodies diluted in PBS for 1 h at ambient temperature. The appropriate dilution of each antibody was at first determined by titration on acetone-fixed frozen tissue sections of human liver for P-glycoprotein, and skin for β-tubulin and its isoforms. Primary antibodies were removed by a 15-min wash in PBS, and secondary antibodies conjugated to a fluorochrome were applied for 45 min in the dark. Slides were rinsed once for 15 min and then washed overnight in PBS. To verify the results, two secondary antibodies were used to detect each primary antibody: FITC-conjugated goat anti-mouse IgG, Fc-specific (Sigma), and Texas-Red-conjugated goat anti-mouse total IgG (Calbiochem). For negative controls, the primary antibody was omitted or a mouse anti-rat mAb or preimmune mouse serum was substituted for primary antibodies. Slides were examined on a Nikon E800 microscope and photographed with a Spot digital camera (Diagnostic Instruments).

Protein extraction and immunoblot analyses

Unsynchronized cells in the logarithmic phase of growth were treated with or without docetaxel (see text for details). For preparation of detergent extracts, cells were washed twice with PBS, scraped off the dishes and lysed in the ice-cold buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 2 mg/ml aprotinin, 5 µg/ml leupeptin and 50 µg/ml phenylmethylsulfonyl fluoride for 30 min. Lysates were centrifuged at 14,000 g for 20 min at 4 °C to pellet insoluble material. For detection of P-glycoprotein, cells were lysed in ice-cold buffer composed of 10 mM Hepes, pH 7.3, 150 mM NaCl, 1 mM $CaCl_2$, 1% (w/v) digitonin, 10 mM iodoacetamide and protease inhibitors as above. Insoluble material was removed by centrifugation at 14,000 g for 30 min at 4 °C. Protein concentrations were determined by the Bradford assay using Bio-Rad Protein Assay reagent. Samples were reduced with 2-mercaptoethanol, boiled for 5 min and 15 µg of each protein lysate was separated by SDS-PAGE on 10 or 12.5% gels and then transferred onto nitrocellulose membrane (Bio-Rad) by electroblotting. To decrease nonspecific reactivity of antibodies, membranes were blocked for 1 h at 37 °C in a buffer composed of 5% fat-free milk and 0.05% Tween in PBS. Blots were then incubated overnight at 4 °C with primary antibodies diluted in PBS containing 5% fat-free milk, 0.05% Tween, 0.5 M glucose and 10% glycerol. Following three washes in PBS/0.05% Tween, blots were developed with HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies using a chemiluminescent HRP substrate (Pierce).

Clonogenicity assay

Docetaxel-resistant PC-3 cells actively proliferating in the presence of docetaxel for at least ten passages were suspended in 0.45% SeaPlaque Agarose (FMC) containing docetaxel-free 1 × RPMI medium with 5% FBS and plated in 60-mm Petri dishes. Each dish was seeded with 4×10^3 cells and the gels were set up in triplicate for each cell line. Cells were allowed to proliferate for 10 days. Gels were then overlaid with a 3 mg/ml solution of p-iodonitrotetrazolium violet for 24 h to aid visualization of colonies growing in suspension. Colonies composed of more than 30 cells were scored under a dissecting microscope at × 60 magnification.

In a separate set of experiments, five randomly chosen docetaxel-resistant cell lines (8A5, 8B2, 8B6, 9A2, 1-int) growing in medium containing docetaxel were cultured in the absence of docetaxel for seven passages and then assayed for clonogenicity as described.

Results

Cytotoxicity of taxanes in DU-145 and PC-3 cell lines

The mode of taxane-induced cell death was studied in DU-145 and PC-3 cells to identify treatment conditions enabling the least and the greatest cytotoxicity via two distinctly different cell death pathways. We reasoned that a range of doses and treatment regimens with the least cytotoxicity would have the greatest likelihood of facilitating recovery of endoreduplicating PC-3 cells. In turn, knowledge of the conditions leading to the highest levels of cytotoxicity would aid in selecting a more potent compound of choice and could also be clinically relevant.

Three assays were employed to optimize assessment of taxane cytotoxicity: the MTT assay that measures reduction of MTT tetrazolium salts to a blue formazan product by mitochondrial dehydrogenase of viable cells; the SRB assay, which relies on colorimetric determination of total cellular protein, and the Trypan blue dye exclusion assay based on the ability of viable cells to prevent uptake of the dye. Correlation of these three assays was the strongest after a 96-h exposure to docetaxel and of the two colorimetric assays, the MTT test was chosen for all subsequent experiments. The IC_{50} values for docetaxel determined by analysis of cell survival curves were 0.3–0.4 ng/ml for PC-3 cells and 0.6 ng/ml for DU-145 cells. The IC_{50} values for paclitaxel were 1.5–1.6 ng/ml for PC-3 cells and 3.1 ng/ml for DU-145. Comparison of the IC_{50} values indicated that the toxicity of docetaxel was 5.2 times higher than paclitaxel in DU-145 cells and 3.75–5.3 times higher in PC-3 cells; docetaxel, therefore, was chosen for the subsequent studies.

Since the estimated IC_{50} values were representative of the cumulative cytotoxicity induced by continuous treatments at a constant dose over a predetermined period of time, we further investigated whether cytotoxicity varied with the length of exposure. DU-145 and PC-3 cells were seeded onto microplates as for the standardized 96-h MTT test but incubated with docetaxel for periods of 24, 48, 72 or 96 h. Following drug exposure, drug-containing medium was replaced with drug-free medium and all cells were fixed for MTT measurement at 96 h. Comparison of the IC_{50} values revealed that prolongation of drug exposure improves cytotoxicity as shown by the gradual decrease in the IC_{50} values from 0.9 to 0.6 ng/ml in DU-145 cells and 0.9 to 0.3 ng/ml in PC-3 cells.

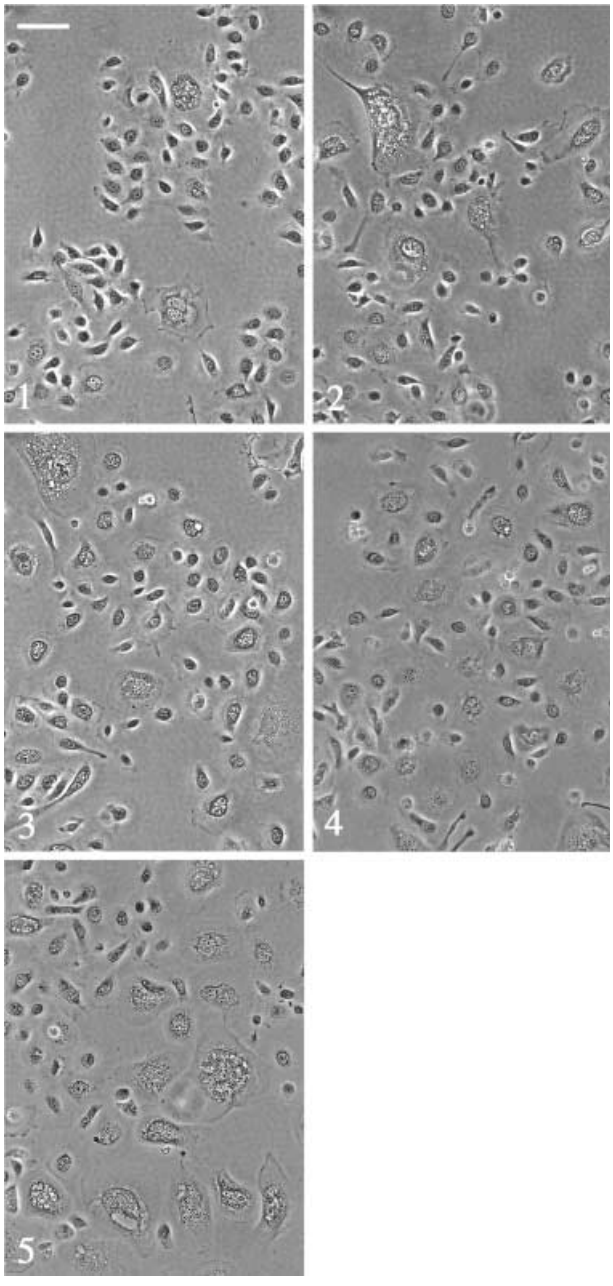
To understand better the time-dependent drug activity at various concentrations, we investigated the effect of low and higher doses separately. Using the trypan blue exclusion test, after a 96-h treatment with docetaxel, concentrations from 0.5 to 1.5 ng/ml did not induce any cell loss but were cytostatic to DU-145 cells. The lowest concentration range effectively inducing death of more than 90% cells over the same period was determined to be 2–4 ng/ml. Concentrations above 4 and up to 250 ng/ml used in this study displayed increasingly high levels of cytotoxicity. Microscopic assessment of multinucleation/mitotic index indicated that the initial number of DU-145 cells displaying an enlarged nucleus, multiple nuclei or nuclear fragmentation did not exceed 2.6%. This ratio did not change appreciably following 96 h continuous treatment with docetaxel or paclitaxel at the IC_{50} concentration for each drug, suggesting that the taxanes did not induce delayed cell death via endoreduplication in DU-145 cells, but initiated apoptosis instead.

Unlike DU-145 cells, PC-3 cells rapidly underwent hypertrophy and endoreduplication following exposure to taxanes, the end result being formation of giant multinucleated cells surviving for extended periods of time. When attached to substratum, the giant cells stayed viable, as shown by negative Trypan blue staining. Following trypsinization, the majority of giant cells reattach when passaged to a new dish. Preliminary analyses by phase contrast microscopy suggested the lowest concentration inducing multinucleation in nearly all PC-3 cells within a 24-h treatment period was 10 ng/ml, and the lowest concentration reaching the same effect over a 96-h period was 0.5 ng/ml. We further delineated kinetics of the docetaxel-induced onset of multinucleation at the low range of the drug concentrations by calculating the mitotic/multinucleation index microscopically. Endoreduplication was induced by treatments with docetaxel at 0.05–0.5 ng/ml doses over a 96-h period and triplicate cultures were fixed and analyzed at each 24-h time point (fig. 1A). As shown in figure 1B, higher concentrations within this dose range were able to induce multinucleation quite rapidly, but a longer incubation time was required to achieve an almost equivalent effect using lower doses. Similar results were obtained using paclitaxel assayed in the dose range 0.05–2 ng/ml. This assay also indicated that sensitivity to docetaxel versus paclitaxel was 2.5–4 times greater, in agreement with the result obtained using the MTT assay.

The time- and dose-dependent onset of endoreduplication induced in PC-3 cells with low-dose docetaxel was further investigated to determine the dose dependence of the delay period preceding death of the giant multinucleated PC-3 cells. Figure 2 illustrates the kinetics of delayed cell death following 24 h treatment with docetaxel of exponentially growing PC-3 cells determined by the Trypan blue exclusion test. Treatments with 10 ng/ml docetaxel produced an initially small decline in cell numbers during the first 48 h posttreatment, a steep decline over the next 5 days followed by a very gradual decline thereafter. After 11–12 days posttreatment, the 5–10% of cells that were still viable remained in culture for up to 5 weeks. Treatments of PC-3 cells with higher docetaxel doses of 100, 250 and 500 ng/ml for 24 h facilitated rapid cell loss of up to 80% cells within 2 days. The remaining 20% of cells underwent multinucleation and delayed cell death similar to the giant cells induced by low concentrations of docetaxel, but the period preceding death was almost twofold shorter and occurred 2.5–3 weeks following the initial treatment. Apparent abbreviation of the delay period preceding PC-3 cell death and close similarity of cytotoxic profiles produced by the high-dose treatments indicated activation of alternative cell death mechanisms in PC-3 cells that complement cytotoxicity associated with endoreduplication.

Induction of the giant cells and selection of docetaxel-resistant cell lines

Our initial attempts to select giant-cell-derived docetaxel-resistant lines by exposing actively proliferating PC-3 cells to the drug at concentrations as low as 0.01 ng/ml for various periods of time resulted either in complete cell kill or inability to induce endoreduplication in the majority of cells. We then undertook an alternative approach to create resistant lines based on the finding that taxanes have a less potent cytotoxic effect on nonproliferating than on actively proliferating cells [25]. Growth inhibited at confluency, PC-3 tumor cells had formed multilayered cultures and reversibly ceased rapid proliferation. We reasoned that lack of active proliferation in growth-arrested cultures might diminish cytotoxic activity of docetaxel at the low-dose range 0.05–0.5 ng/ml tested earlier and as a result fail to induce profound multinucleation. We therefore incubated cells with docetaxel at the range of concentrations 10–250 ng/ml, which in preliminary studies was more cytotoxic. Cells were also treated in two time regimens: intermittent, to shorten the exposure time while maintaining overall longevity of the treatment, and



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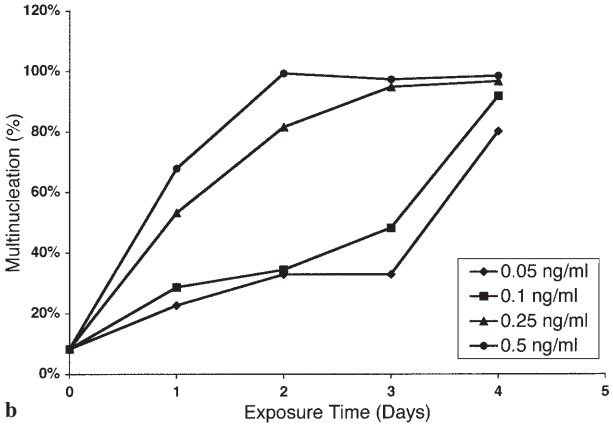

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Figure 1. (*a*) Progressive hypertrophy and endoreduplication in naïve PC-3 cells treated with 0.25 ng/ml docetaxel for 96 h determined by phase contrast microscopy. The high extent of multinucleation reflects ongoing mitotic activity. 1, 0 time; 2, 24 h; 3, 48 h; 4, 72 h; 5, 96 h. Magnification ×200; bar, 100 µm. (*b*) Time- and dose-dependent kinetics of multinucleation detected in PC-3 cells by phase contrast microscopy following treatment with various low doses of docetaxel. Higher concentrations within this dose range induced multinucleation quite rapidly in the majority of cells, but a longer incubation time was required to achieve an almost equivalent effect using lower doses.

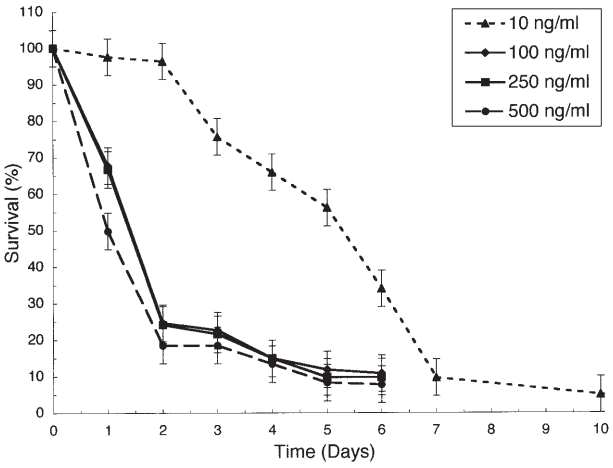

Figure 2. Kinetics of giant multinucleated PC-3 cell survival following 24 h incubation with low (10 ng/ml) and high (100, 250, 500 ng/ml) doses of docetaxel determined by the Trypan blue cell viability assay. Long-term survival of the residual giant cells is not shown.

continuous, as in preliminary studies of cytotoxicity. Treatment regimens used in this experiment are described in Materials and methods and the selection criteria and the end result stages are outlined in table 1.

Following the 5-day treatment schedules, cells were passaged into T-25 culture dishes at low density and examined daily for death/survival by phase contrast microscopy. Treatment of all cultures by either continuous or intermittent administration of docetaxel at every dose used in this experiment induced profound multinucleation of PC-3 cells and formation of giant cells. Giant-cell cultures induced by continuous incubation with docetaxel at 100 and 250 ng/ml rapidly senesced over the first 10–14 days. The remaining 40 treated cultures that were able to survive beyond 14 days were passaged into 12-well plates to consolidate residual giant cells and monitored microscopically for death/survival. The first resistant clone composed of proliferating and single nucleated cells emerged 15 days following the last treatment. Phase microscopy evaluation conducted every other day by two investigators (A. M. and E. S.) indicated that not more than 50 giant cells and no single nucleated cells remained in any of the treated cultures by this time.

Table 1. Summary of the developmental steps and selection criteria for the new drug-resistant cultures emerging from giant endoreduplicating PC-3 cells.

Docetaxel concentration (ng/ml)	10	20	50	100	250
Continuous regimen					
1. Treatment of cultures (24/24/120)	10	10		4	4
2. Passage at low density	10	10		4	4
3. 14-day survival of giant cells/pass	10	10		0	0
4. 30-day survival of giant cells	10	8		0	0
5. Yield of proliferative survivors	7	0		0	0
6. Cultures with sustained growth	5				
7. Cultures resistant to docetaxel	3				
New cell line designation	9A2, 9A3, 9A4				
Intermittent regimen					
1. Treatment of cultures (1/24/120)	8		4	4	4
2. Passage at low density	8		4	4	4
3. 14-day survival of giant cells/pass	8		4	3	3
4. 30-day survival of giant cells	7		4	2	3
5. Yield of proliferative survivors	7		1	2	2
6. Cultures with sustained growth	6		1	1	1
7. Cultures resistant to docetaxel	4		1	1	1
New cell line designation	8C3, 8B2, 8D2, 1-int		5-int	8A5	8B6

Aggregate numbers indicate number of cultures induced under various conditions, those surviving endoreduplication and those yielding the new cultures with a substantial level of drug resistance. See details in the text.

A total of 20 colonies composed of single nucleated, relatively small and actively proliferating cells emerged from 19 separate cultures by the end of the observation period. The last clone emerged 23 days following the final day of treatments and the experiment was terminated on day 30. All new PC-3 clones were derived from a single giant cell recovering in different PC-3 cultures with one exception: culture 8B2 arose from two giant cells within one parental culture. Both 8B2 clones were pooled at passaging and then maintained as a single culture.

Of the 19 recovered cultures, 14 showed sustained proliferation and were kept for further investigation. The remaining 5 clones failed to expand beyond 200 cells. The highest yield of resistant clones was associated with the minimal docetaxel dose of 10 ng/ml applied in either intermittent- or continuous-treatment regimens (see table 1). Intermittent exposure of eight cultures at 10 ng/ml resulted in the isolation of six clones (8A2, 8B2, 8C3, 8D2, 8D3 and 1-int) and continuous exposure yielded five clones from the ten cultures treated (9A2, 9A3, 9A4, 9D3 and 1c). Thus both continuous and intermittent regimens produced comparable cytotoxicity and yield of survivors at this low dose. Significant differences in recovery yield of resistant clones were observed at docetaxel doses increased beyond minimal. The intermittent treatments of four cultures/dose at 50, 100 and 250 ng/ml docetaxel resulted in a fairly high survival rate of giant cells and each dose yielded one resistant clone, designated 5-int, 8A5 and 8B6, respectively. This is in contrast to poor survival of giant-cell cultures following continuous treatments with 20, 100 and 250 ng/ml docetaxel, when similar doses facilitated accelerated cell loss and did not yield any proliferative survivors.

Recovered clones at passage one were composed of cells with diverse morphologies. A significant percentage of cells within each clone did not survive longer than two passages or 2 weeks. To further select for the most drug resistant cultures, the level of resistance to docetaxel in recovering clones was first tested by determining IC_{50} values (see below). Because the MTT assay measures the relative rate of growth inhibition induced by various drug concentrations compared to untreated cultures, it does not clearly indicate whether these concentrations are cytostatic, cytolytic or both. We therefore tested the new cultures for their ability to maintain sustained growth in the presence of docetaxel in growth medium by incubating the cells with increasing concentrations of the drug.

Of the 14 lines evaluated, 10 were able to continue active proliferation when grown in medium containing docetaxel at concentrations up to the IC_{25} for each line without induction of multinucleation and/or giant cells, unlike parental cells. After obtaining confirmation of the substantial levels of drug resistance in the new PC-3 lines, docetaxel at the IC_{25} concentration for each cell line was added to growth medium during regular maintenance unless indicated otherwise. The remaining four clones (9D3, 8A2, 8D3 and 1c) were less resistant and demonstrated gradual growth arrest and formation of giant cells even at concentrations equivalent to the IC_{10}. These four lines were excluded from further investigation.

Resistance to docetaxel by cytotoxicity assays

Table 2 demonstrates relative levels of resistance to docetaxel in the ten new lines compared to parental PC-3 cells as determined by MTT cytotoxicity assays. Cells were assayed prior to adaptation to docetaxel in growth

Table 2. Relative resistance of docetaxel-resistant PC-3 cell clones compared to parental cells as determined by MTT cytotoxicity assays.

Cell line designation	IC_{50} (passage 3, before adaptation to docetaxel)	Relative resistance (RR1)	IC_{50} (passage 30, after adaptation to docetaxel)	Relative resistance (RR2)	Degree of change (RR1/RR2)
PC-3	0.4	1.00	0.4	1.0	1.00
9A2	1.5	3.75	2.5	6.25	1.67
1-int	0.9	2.25	4.5	11.00	4.89
8C3	3.0	7.50	1.7	4.25	0.57
8D2	1.0	2.50	1.4	3.50	1.40
8B2	4.4	11.00	4.4	11.00	1.00
8B6	2.9	7.25	5.6	14.00	1.93
9A4	1.3	3.25	2.1	5.25	1.62
8A5	1.2	3.00	2.0	5.00	1.67
5-int	1.2	3.00	1.8	4.50	1.50
9A3	3.5	8.75	2.5	6.25	0.71

Cells were incubated with increasing concentrations of docetaxel for 96 h as described in Materials and methods. PC-3 clones were propagated in docetaxel-free growth media for 72 h to allow complete drug efflux prior to the cytotoxicity assay. IC_{50} values are presented in ng/ml.

medium and at every fifth passage up to 30 passages following growth in the presence of docetaxel. Contrary to our expectations, long-term maintenance of the resistant cell lines in the presence of docetaxel in growth medium did not strongly alter the degree of resistance in most lines with an exception of 1-int and 8B6 (fig. 3). This result indicated relative stability of the new cell lines under constant cytotoxic pressure, but also suggested some clonal differences, a variation that may have clinical implications.

Growth characteristics and clonogenic properties of the docetaxel-resistant lines

When grown in the absence of docetaxel, the ten selected docetaxel-resistant lines displayed a morphology resembling parental PC-3 cells. Resistant cells remained strongly adherent, and morphologically heterogeneous, appearing as round, oval or spindle-shaped cells that were equal to or smaller than PC-3 cells (fig. 4). Of all the lines only one (8A5) was composed of cells approximately twice the size of parental PC-3 cells, and was morphologically the most homogeneous of the ten cell lines. Addition of docetaxel to growth medium during routine maintenance reversibly induced loss of apparent morphological heterogeneity resulting in cultures composed of oval or round shapes. The growth rate of the new cultures was close but not identical to the parental line. Although we did not quantify the growth rate for each line, judging by the passage dates of cultures maintained in parallel, the growth rates of the ten resistant lines were approximately equal to or slightly greater than the parental PC-3 line, even when propagated in the presence of docetaxel.

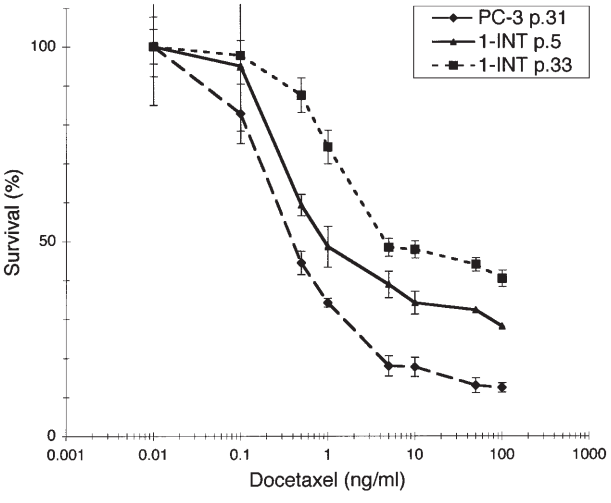

Figure 3. Example of spontaneous progression of resistance to docetaxel in the 1-int clone determined by a frequently repeated MTT assay.

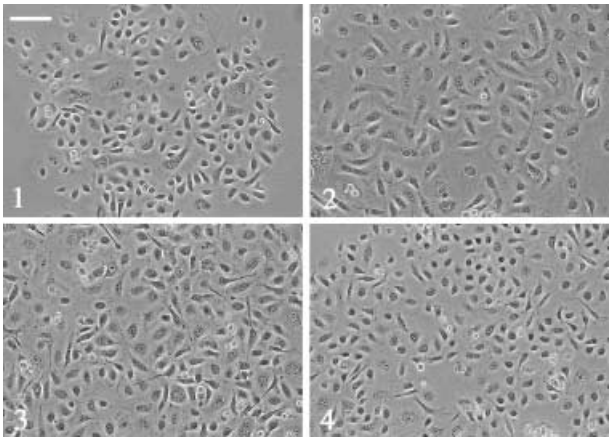

Figure 4. Morphology of the docetaxel-resistant PC-3 clones propagated in the absence of docetaxel in medium by phase contrast microscopy indicates close similarity with parental cells. 1, 1-int; 2, 8A5; 3, 8B6, 4, 8D2. Magnification × 200; bar, 100 µm.

Of note, one of the new cultures (9A4) demonstrated sustained rapid growth but persisted in forming giant multinucleated cells reaching up to 10–15% of the total cell population with or without docetaxel in the medium.

The ability of the docetaxel-resistant PC-3 cells to form colonies in soft agar suspension was compared to the parental cell line. Resistant and naive cells were suspended at low density in soft agar and allowed to proliferate for 10 days. Colonies composed of more than 30 cells were scored. This assay aimed to quantify single clonogenic or stem-like cells with high proliferative potential, capable of attachment-independent proliferation and contributing a minority to the total cell population in culture [26]. We reasoned that the colony-forming properties of the new PC-3 cell lines that were clonally derived from some of the few surviving giant cells could differ greatly from the original parental cultures and this might explain the substantial drug resistance. As evident from figure 5A, most of the resistant lines showed levels of clonogenicity similar to the parental PC-3 cells. The two exceptions were 8D2 and 8A5 that generated almost twice as many colonies. The clonogenicity of several randomly selected lines maintained in docetaxel-free medium was also tested. The purpose of this experiment was to determine if the presence of docetaxel in the medium exerted constant selective pressure and thus presented a growth advantage to the most aggressive, drug-resistant and presumably clonogenic cells. Figure 5B demonstrates that the clonogenic potential of the resistant cells did not change considerably.

In short, these results suggested that the majority of the resistant cell lines emerging from the giant multinucleated ancestors retain many of the growth properties manifested by parental cells but acquire a substantial degree of drug resistance. Some of the known potential mechanisms conferring resistance to taxanes were investigated in this work and the results are provided below.

β-Tubulin isotype analysis by IIF and Western blot

At passage 3, IIF staining of the docetaxel-resistant PC-3 derivatives with β-tubulin-specific antibodies as well as antibodies specific to the β-tubulin II and III isoform showed essentially unchanged levels of expression compared to the parental line. This is in contrast to the β-tubulin IV isoform that was detected in less than 1% of the parental cells but was strongly expressed in all of the drug-resistant cultures. De novo expression of this β-tubulin isoform was confirmed by immunoblot analysis (fig. 6) and stability of expression was monitored by IIF every fifth passage. Expression of β-tubulin IV diminished to undetectable levels in all of the lines by passage 15, or 12 passages following adaptation of the lines to low-dose docetaxel in the medium. This loss occurred rapidly in some lines (by passage 10) and more gradually in others.

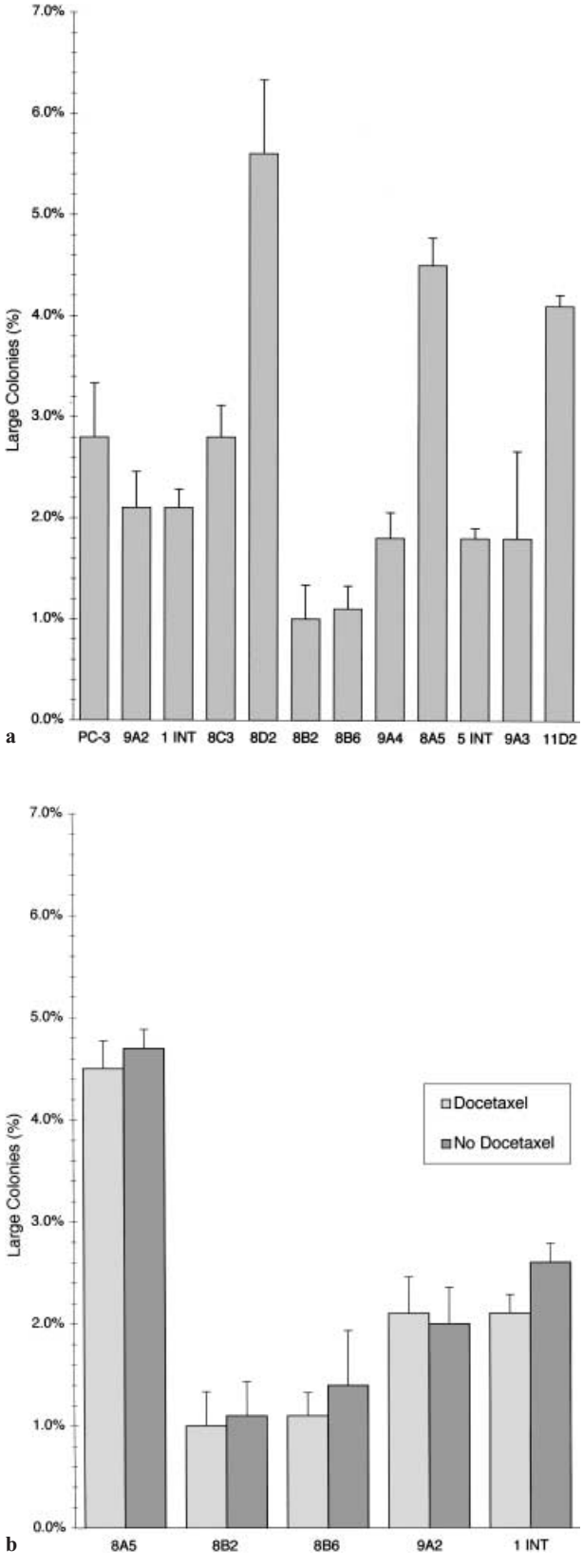

Figure 5. (*a*) Clonogenic potential of docetaxel-resistant PC-3 clones propagated in the presence of docetaxel. (*b*) Comparison of clonogenicity of selected docetaxel-resistant PC-3 clones propagated in the presence or absence of docetaxel.

Expression of P-glycoprotein

IIF analysis of the resistant clones indicated that only two (1-int and 8D2) of the ten lines analyzed expressed low but detectable levels of P-glycoprotein. The pattern of staining was consistent with plasma membrane localization of the reactive protein and was also confirmed by the Western blot assay (fig. 6). Expression of this molecule did not persist and it became undetectable by passage 10, or seven passages following adaptation of resistant lines to docetaxel in the medium. Because most of the lines were P-glycoprotein negative or the expression of this molecule was rapidly lost from the positive lines, we concluded that mediation of docetaxel resistance in PC-3 clones was unlikely to be due to P-glycoprotein function.

Resistance to docetaxel is not bcl-2 and/or bcl-xL mediated

Western blot analyses of docetaxel-resistant PC-3 cells cultured in the presence of docetaxel in the growth medium showed levels of bcl-2 and bcl-xL protein expression identical or close to the base levels detected in the parental PC-3 (fig. 6). This result was true for both early (3–5) and late (25–30) passage cultures. Since the levels of these two antiapoptotic proteins remained unchanged even in the presence of docetaxel in growth medium, we concluded that neither bcl-2 nor bcl-xL contribute significantly to docetaxel resistance in PC-3 cells.

Resistance to other cytotoxic compounds

We chose four of the ten resistant lines showing various degrees of resistance to docetaxel to evaluate sensitivity to other chemotherapeutic compounds compared to parental cells. Despite apparent phenotypic alterations, the four resistant lines did not display a significant change in sensitivity to estramustine, doxorubicin, *Vinca* alkaloids and etoposide, but demonstrated slight cross-resistance (1.7- to 2.5-fold) with carboplatin (table 3). Taxotere resistant cells did not show equivalent levels of cross-resistance to taxol, which increased only 1.5- to 3-fold compare to 3.5- to 14-fold for taxotere. These findings suggest that the molecular pathways of resistance to docetaxel in these cell lines are different from those counteracting taxol. The data are consistent with previous clinical observations suggesting differences in activity and pattern of cross-resistance between the two taxanes [2, 27].

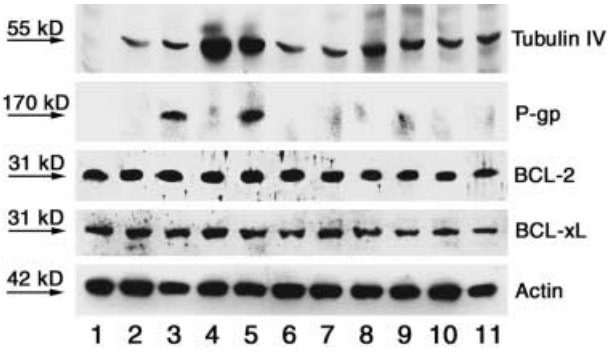

Figure 6. Immunoblot analyses of parental PC-3 and docetaxel-resistant PC-3 clones on passage 3–4. Actin expression indicates even protein load of lanes. 1, PC-3 parental; 2, 9A2; 3, 1-int; 4, 8C3; 5, 8D2; 6, 8B2; 7, 8B6; 8, 9A4; 9, 8A5; 10, 5-int; 11, 9A3.

Stability of the resistant phenotype

To examine stability of the resistant phenotype, the new PC-3 cultures maintained in the presence of docetaxel for at least 10 passages were placed in drug-free medium in an attempt to obtain drug-sensitive revertants. However, after 3 months or 9–12 passages of growth in drug-free medium, reversal of the resistant phenotype did not occur, as assessed by the MTT cytotoxicity assay.

Progression of resistance

Spontaneous progression of drug resistance is a common clinical outcome leading at first to partial and eventually complete failure of cytotoxic therapy. We surmised that adaptation of the new PC-3 clones to docetaxel in medium might enable modeling of such a condition and thus become invaluable for identification of specific molecular events accompanying the progression of resistance. We diligently tested new cell lines by MTT assay every fifth passage to identify changes in resistance to docetaxel, if any, by comparing IC_{50} values. Table 2 indicates that seven out of ten lines progressed to various degrees, while the other three lines retained or decreased initial levels of docetaxel tolerance. Even though progression of resistance was not immediately associated with loss of β-tubulin IV expression, all of the established lines were, nonetheless, subjected to IIF analysis of β-tubulin IV expression. This effort resulted in finding that β-tubulin IV was detected again in the 8B6 line on passage 27, or 24 passages following growth in drug-containing medium, although the level of expression was considerably lower than initial. Reappearance of β-tubulin IV in this cell line coincided with an increase in the level of resistance to docetaxel determined by the MTT assay. This second round of β-tubulin IV expression did not persist and it was not detected in 8B6 cells following another three passages. Another cell line, designated 1-int, also demonstrated a substantial increase in resistance following long-term maintenance in docetaxel, but we were unable to detect reoccurrence of the β-tubulin IV isoform in these cells. Bearing in mind that the sporadic and unstable expression pattern of P-glycoprotein could be similar to the β-tubulin IV isoform, we assayed the 1-int and 8D2 clones by IIF every fifth passage but were unable to detect P-glycoprotein again.

Of great interest was to evaluate whether the already resistant PC-3 clones would increase their level of resis-

Table 3. Cytotoxicity profile of docetaxel-resistant PC-3 cell lines compared to parental cells by the MTT cytotoxicity assay.

Cytotoxic compounds	Cell line designation								
	PC-3 IC5(	8B2		8B6		8D2		11D2	
		IC_{50}	RR	IC_{50}	RR	IC_{50}	RR	IC_{50}	RR
Docetaxel	0.40	4.4	11	5.6	14	1.4	3.5	4.8	12
Paclitaxel	1.5	3.0	2	4.5	3	2.2	1.5	3.0	2
Vinblastine	0.25	0.25	1	0.30	1.2	0.50	2.0	0.25	1
Vincristine	0.80	0.40	0.5	0.80	1	0.60	0.8	0.70	0.9
Doxorubicin	21	29	1.4	27	1.2	22	1	25	1.2
Etoposide	1.0×10^2	1.1×10^2	1.1	1.3×10^2	1.3	0.9×10^2	0.9	1.2×10^2	1.2
Carboplatin	6.0×10^3	1.2×10^4	2	1.5×10^4	2.5	1.0×10^4	1.7	1.4×10^4	2.3
Estramustine	3.0×10^3	4.0×10^3	1	4.1×10^3	1	4.0×10^3	1	3.8×10^3	1

Selected cell lines at passage (p) 25 (p 5 for 11D2) were incubated with increasing concentrations of drugs for 96 h as described in Materials and methods. Medium was depleted of docetaxel for 72 h prior to the cytotoxicity assays to allow complete drug efflux. IC_{50} values are presented in ng/ml. RR, relative resistance.

tance to docetaxel when forced to undergo another cycle of endoreduplication. Highly clonogenic and moderately docetaxel resistant cell clone 8D2 was challenged for 24 h with 10 ng/ml docetaxel, a dose lethal to the parental line. Following drug exposure, actively proliferating 8D2 cells were allowed to recover in drug-free medium. This dose of docetaxel effectively induced multinucleation and formation of giant cells in all of the 8D2 cells but the emergence of single nucleated proliferating survivors from several giant precursors was noted on days 7–8, or twofold faster than in the parental 8D2 clone. Multiple cell clones that arose were pooled together to form another cell line designated 11D2. The cytotoxicity profile of this cell line is illustrated in table 3. The growth and morphological properties of 11D2 cells were similar to the parental 8D2 line. However, 11D2 cells were 3.4 times more resistant to docetaxel than the parental 8D2, but showed no dramatic changes in sensitivity to other drugs. Evaluation of clonogenic potential in soft agar indicated 4.1% of 11D2 cells formed large colonies, representing a minor deviation from the parental 8D2 clone (fig. 5A). Similar to other docetaxel-resistant PC-3 clones, this newest cell line was immediately adaptable to growth in medium with docetaxel at the IC_{25} concentration. Most important, when actively proliferating 11D2 cells were challenged with 10 ng/ml docetaxel for 24 h, not more than 20–30% of these cells responded by multinucleation and became giant, while others resumed active proliferation upon removal of the drug. This cellular response demonstrated levels of resistance to docetaxel substantially higher than in both 8D2 clone and PC-3 cells, and in agreement with the MTT test.

Initial IIF analyses of the 11D2 line was performed on the third passage following recovery and identified strong expression of the β-tubulin IV isoform and negative reactivity with anti-P-glycoprotein antibody. Western blot analyses confirmed these data and also showed that levels of bcl-2 and bcl-xL expression did not change appreciably. As previously detected in other docetaxel-resistant PC-3 clones, expression of β-tubulin IV declined to undetectable levels following 3 weeks or three to four passages in medium containing docetaxel. The absence of antibody-reactive microtubules detectable at first throughout the cytoplasm but not in spindle poles was rapidly replaced in a fraction of mitotic cells by reactive tubules forming metaphase asters, a pattern not observed in other resistant PC-3 cell lines (data not shown).

Discussion

Taxanes are widely used for the treatment of prostate cancer and other neoplasms; however, patients often succumb to the disease. In the present report we evaluated in vitro the effect of paclitaxel and docetaxel on PC-3 and DU-145 prostate cancer cell lines to understand better the mode of taxane cytotoxicity and identify molecular and cellular events accompanying drug-induced cell death and emergence and progression of drug resistance. The taxanes induce different cell death pathways in PC-3 and DU-145 cells and we at first focused on determining the cytotoxic dose range and kinetics of drug activity. Even though PC-3 cells appeared more resistant than DU-145 cells by the MTT assay, the effective range of concentrations was overall similar in both cell lines as defined microscopically. These studies established the lowest range of drug concentrations effectively inducing delayed PC-3 cell death by multinucleation, and cytostasis or death of DU-145 cells via apoptosis. The standardized MTT cytotoxicity assays, cell viability tests and measurement of the mitotic/multinucleation index indicated a concentration- and time-dependent mode of cytotoxic activity displayed by both taxanes. Most important, similar results obtained in both cell lines suggested that the increased

duration of drug exposure achieved improved cytotoxicity independently of the tumor cell death pathway.

To promote emergence of proliferative survivors from endoreduplicating PC-3 cells, we induced giant cells using various schedules and doses of docetaxel applied to both actively proliferating or slow-cycling cell cultures. Incubations of slow-cycling cells generated an array of PC-3 cell lines that arose from multinucleated giant cells and also showed significant levels of resistance to docetaxel. The majority of the resistant PC-3 clones emerged after the minimal dose treatments in both intermittent and continuous regimens. Prevalent outgrowth of the resistant clones followed treatments with a relatively low drug concentration of 10 ng/ml and independently from the treatment schedule, suggesting a dose-limiting effect. As in preliminary studies of drug activity, we also observed accumulation of a cytotoxic effect during continuous exposure that lead to decreased survival rate of the giant-cell cultures in most instances. In contrast, reduction of the exposure duration using a similar range of concentrations allowed the recovery of some drug-resistant survivors. Most of the derived lines demonstrated substantial levels of resistance to docetaxel by MTT assay and were able to sustain growth without induction of endoreduplication in culture medium supplemented with docetaxel at concentrations effectively inducing multinucleation and death of the parental cells. The pattern of drug resistance in the new PC-3 cell lines also appeared to be uniquely specific to docetaxel since cross-resistance with other chemotherapeutic compounds including paclitaxel was not observed.

Following determination of the degree and specificity of resistance to docetaxel as well as morphologic and growth characteristics of the new lines, we compared their clonogenic potential to parental cells to gain insights into the cellular pathways underlying initiation and progression of drug resistance. The majority of human solid tumors are genetically unstable [28]. Drug treatments further amplified this inherent instability during giant-cell formation and during recovery of proliferating PC-3 survivors. This was shown by the tremendous morphologic heterogeneity during slow reestablishment of the resistant PC-3 clones and by instability of several clones that quickly senesced. Instability affected the entire course of giant-cell death and recovery and has lead to notable cellular selection resulting in preferential survival of the few giant cells and resistant clones. Subsequent adaptation of the new clones to growth in the presence of docetaxel to reveal the most resistant cells applied additional cytotoxic pressure that could have enhanced instability and selection. Nevertheless, the majority of the selected docetaxel-resistant PC-3 clones displayed morphologic and clonogenic properties closely resembling the parental line. This outcome suggests highly conserved and fairly efficient molecular mechanisms facilitating recovery of prolific survivors from the multinucleated precursor cells. Observed stable levels of clonogenicity also suggest that the molecular mechanisms maintaining tumor stem cell lineage by regulating the ratios of clonogenic and nonclonogenic tumor cells are also conserved and remained essentially intact in this model system. The presence or absence of docetaxel in the growth medium did not promote selection for clonogenic cells with stem-like characteristics, neither did an additional round of death/recovery from multinucleation in 11D2 cells. In addition, the degree of resistance to docetaxel determined by the MTT assay did not correlate with the levels of clonogenicity. Therefore, we concluded that even though the process of cellular selection for the most resistant cells is actively taking place during giant-cell death and recovery, there have to be simultaneous additional phenotypic modifications to account fully for the substantial levels of resistance in the new clones.

Phenotype characterization of the new PC-3 cell lines using Western blot and IIF techniques indicated emergence of some of the known and suggested the existence of unknown molecular mechanisms conferring resistance to docetaxel in this experimental model. The expression of P-glycoprotein in recovered PC-3 cells was of short duration and rare, making it unlikely that P-glycoprotein mediates resistance to docetaxel in PC-3 cells. No changes in levels of the antiapoptotic proteins bcl-2 and bcl-xL were observed, suggesting that suppression of apoptosis via these molecules was not a major pathway of resistance to docetaxel in these cells. De novo expression of the β-tubulin IV isoform in the resistant lines was an early and common outcome that implied a direct relevance to primary docetaxel resistance. During long-term maintenance of new lines in growth medium containing docetaxel, β-tubulin IV expression decreased. This loss was not associated with a decreased level of resistance to docetaxel as correlated with frequently repeated MTT assays. Based on these data, we concluded that the transient expression of the β-tubulin IV isoform indicated ongoing additional modifications to docetaxel target proteins. These changes could have resulted in reduced affinity binding to tubulin due to multiple translational or post-translational tubulin modifications, a possibility consistent with work of other investigators [29].

To refine our understanding of the role of β-tubulin IV in docetaxel resistance, we are currently evaluating the pattern of its expression immediately after recovery from the giant-cell stage and during long-term maintenance in docetaxel-free growth medium. The developed in vitro model and new cell lines with a unique pattern of cross-resistance will also be helpful to identify unknown molecular mechanisms of resistance specific to docetaxel. By comparing gene expression patterns in parental and resistant cells using microarray technology, identifying molecules involved in the initiation and progression of resis-

tance to docetaxel seems feasible. This inducible in vitro model should also be useful to discern molecular pathways involved in the formation of giant-cells, recovery from the giant-cell phase and survival of the drug-resistant descendants.

The appearance of multinucleation observed in taxane-treated PC-3 cells is an indicator of chromosome breakage [30] resulting in polyploid cells that exhibit multiple different forms of apoptosis. Very recently, polyploid giant cells induced by DNA-damaging radiation were demonstrated to provide a survival mechanism for p53 mutant lymphoma cells [31]. Further exploration of the process of giant-cell formation and breakdown suggested delayed apoptosis in giant cells as a mechanism allowing reorganization and repair of damaged chromatin following single low-dose radiation exposure [32]. Tremendous similarity in the process of PC-3 giant-cell formation and survival observed in our investigation and reported for irradiated lymphoma cells suggests a common cellular response to different cytotoxic insults. In both cases, the molecular mechanisms supporting long-term survival of the giant cells are likely aimed at improving recovery through gradual activation of repair mechanisms. Our studies suggest a possibility to facilitate profound damage to the tumor cells and prevent emergence of drug resistant survivors by continuous and prolonged taxane treatments at reduced dose. We are hopeful that continuous drug administration at an optimal dose range will improve cytotoxicity without compromising tolerability in the clinical setting. Recently developed oral formulations for both docetaxel and paclitaxel [33, 34] could provide the means for continuous drug delivery, as an alternative to frequent infusional therapy, and allow for clinical testing of this hypothesis.

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