ABSTRACT

The kinetics of the time-dependent antitumor effects of paclitaxel are not fully understood; some literature reports indicate a higher activity by prolonging treatment durations, whereas other reports indicate no enhancement under in vitro conditions. The present study was designed to address this controversy and to determine the mechanism of the higher cytotoxicity associated with longer treatment durations. Six human epithelial cancer cell lines (bladder RT4, breast MCF7, pharynx FaDu, ovarian SKOV3, and prostate PC3 and DU145) were used. To determine whether the higher activity observed for the longer treatment durations is due to a delayed exhibition of drug effects and/or a reflection of cumulative effects that required a continuous drug exposure, all treatments produced the same maximum effect at 96 h, although treatments for ≤12 h showed higher IC50s than longer treatments, whereas treatments for ≥24 h showed indistinguishable IC50s; (c) treatment for as brief as 3 h was sufficient to induce apoptosis, which occurred with a lag time of about 24 h, although longer treatments produced a greater extent of apoptosis; (d) the intracellular and extracellular concentrations reached an equilibrium at ~5 h, which rules out slow and/or insufficient uptake as the cause of the lower effects at shorter treatment times (i.e., <24 h); (e) upon removal of drug-containing medium, the amount of drug retained intracellularly was about 10% of the applied dose and was reduced to ~0.5% after three successive washes, separated by 3-h equilibration periods; and (f) the delayed effect of the 3-h treatment was largely due to the drug retained intracellularly, whereas the delayed effect of the 24 h treatment was independent of the drug retained intracellularly. In conclusion, in human epithelial cancer cells, paclitaxel-induced cytotoxicity occurred after termination of drug treatment, which was partly due to the slow manifestation of apoptosis and partly due to the significant amount of drug retained intracellularly. Based on these findings and recognizing that some previous studies measured the immediate effect whereas the other studies measured the delayed effect, we propose that the conflicting data in the literature regarding the effect of treatment duration on paclitaxel activity under in vitro conditions are in part due to the different pharmacodynamics of the immediate and delayed drug effects. Furthermore, differences in the delayed effects for treatments of <24 h and the minimal differences for treatments of ≥24 h indicate that the delayed effect is maximally elicited by 24-h drug exposure.

INTRODUCTION

Paclitaxel is one of the most important anticancer drugs developed in the past two decades. It has shown impressive activity against human solid tumors, i.e., ovarian, head and neck, bladder, breast, and lung cancers (1). Paclitaxel enhances tubulin polymerization, promotes microtubule assembly, and stabilizes microtubule dynamics, resulting in inhibition of cell proliferation and apoptosis (2–4).

One of the challenges regarding the clinical use of paclitaxel is the identification of optimal treatment schedules. Multiple treatment schedules with different infusion durations (1, 3, 24, and 96 h) and different treatment frequency (daily, weekly, and every 3 weeks) are under evaluation in patients. The one completed randomized study in patients to date compares two dose levels, 135 and 175 mg/m², and two infusion durations (3 and 24 h). The results show no differences in the combined response rates at the two doses nor in the response rate for the two treatment durations at the 135 mg/m² dose. However, at 175 mg/m², the 24-h treatment arm shows a higher response rate of 24% compared with the 15% for the 3-h treatment arm, although the relatively small sample size precluded meaningful statistical analysis (5).

The 3-h infusion schedule is approved by the Food and Drug Administration, and there is economic pressure to use this schedule because it can be given in an ambulatory setting. However, preclinical data indicate a greater efficacy for longer treatment durations. For example, increasing the exposure time from 2, 3, or 6 h to 24, 48, 72, or 96 h resulted in a reduced clonogenic survival, a greater G2-M block, and/or lower plating efficiency in human leukemia, ovarian, breast, lung, cervical, astrocytoma, colon, ovarian, and pancreatic tumor cells, and CHO3 cancer cells (6–12). On the other hand, the formation of polyplaid cells or formation of microtubule bundles in human leukemic cells and the cytotoxicity in human ovarian cancer cells were not enhanced by prolonging the exposure time from 4 to 24 h (13, 14). These conflicting results may be caused by biological differences between different cell lines and/or different experimental conditions. Examples of biological differences among cell lines are: (a) lymphoid cells are known to undergo primed apoptosis, which occurs more rapidly than the unprimed apoptosis often found in epithelial cells (14, 15); (b) the paclitaxel-induced microtubule bundling and abnormal aster formation in several leukemia cells show cell type specificity; some cells show an increased response to prolonged

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1 The abbreviations used are: CHO, Chinese hamster ovary; SRB, sulforhodamine B.
treatment times, whereas other cells do not (10). An experimental condition that may alter paclitaxel effect is the use of Cremophor EL as the solvent. Cremophor EL has multiple pharmacological effects that can either potentiate or antagonize paclitaxel activity, i.e., Cremophor EL can block cells in G, phase, reverse the MDR1 P-glycoprotein-mediated resistance to paclitaxel, and produce cytotoxicity (10, 16, 17). Furthermore, the highly lipophilic paclitaxel may favor partition in Cremophor EL, which would alter the intracellular accumulation of paclitaxel. As shown in the present study, the retention of paclitaxel in cells, due to its tight binding to intracellular macromolecules (e.g., microtubules; Ref. 4), may contribute to its delayed effect.

The present study was designed to examine the time-dependent cytotoxicity of paclitaxel in several human epithelial cancer cell lines that are originated from several major types of human solid tumors and to determine the mechanisms of the enhanced cytotoxicity associated with longer treatment durations. The possible causes of the conflicting literature data on paclitaxel pharmacodynamics, in light of the new findings, are discussed.

MATERIALS AND METHODS

**Chemicals.** Paclitaxel was a gift from Bristol-Myers Squibb Co. (Wallingford, CT), and [3H]paclitaxel (specific activity, 19.3 Ci/mmol) was from the National Cancer Institute (Bethesda, MD). Cetoxetame sodium was purchased from Hoechst-Roussel (Somerville, NJ), gentamicin from Solo Pak Laboratories (Franklin Park, IL), culture medium (MEM, McCoy’s medium, and RPMI 1640) from Life Technologies, Inc. (Grand Island, NY), and SRB and propidium iodide from Sigma Chemical Co. (St. Louis, MO). All chemicals and reagents were used as received.

**Cell Culture Conditions.** Human cancer cell lines, including the bladder RT4, breast MCF-7 cells, ovarian SKOV3, pharynx FaDu cells, and prostate PC3 and DU145 cells, as well as the CHO cells, were obtained from the American Type Culture Collection (Rockville, MD). RT4 cells were maintained in McCoy’s medium containing 10% FBS, MCF7 cells in MEM and 10% FBS, SKOV3 cells in McCoy’s medium and 9% FBS, FaDu cells in MEM and 10% FBS, and PC3 and DU145 cells in RPMI 1640 and 10% FBS. All culture media were supplemented with 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml cetoxetame sodium. Cells were incubated with complete medium at 37°C in a humidified atmosphere of 5% CO2 in air. For experiments, cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. Cells with >90% viability, as determined by trypsin blue exclusion, were used.

The cell cycle time, or the doubling time in exponentially growing cells, was 33 h for RT4 cells, 30 h for MCF7 cells, 22 h for FaDu cells, 25 h for SKOV3 cells, and 24 h for PC3 and DU145 cells.

**Drug Treatments.** Paclitaxel stock solutions were prepared in ethanol at 10 mg/ml and stored at −70°C. Aliquots of the stock solution was added to culture medium so that the final concentration of ethanol was <0.1%, which does not affect paclitaxel effects (9). Cells were seeded in 96-well microtiter plates (2000–3000 cells/well), and were allowed to attach to the plate surface by growing in drug-free medium for 20 to 24 h. Afterward, cells were incubated with 0.2 ml of culture medium containing 0.01 to 10,000 nM paclitaxel, for seven treatment durations ranging from 3 to 96 h. There were six replicates for each concentration per plate. To avoid potential problems caused by evaporation of medium in wells located at the edge of the plate (which would alter the drug concentration), only the inner wells (rows 2–7 and columns 2–11) in 96-well plates contained cells. The outer wells (rows 1 and 8 and columns 1 and 12) contained only the tissue culture media to serve as blanks.

**Measurement of Overall Drug Effect.** To determine whether the higher activity observed for the longer treatments is due to a delayed exhibition of drug effects and/or a reflection of cumulative effects that require a continuous drug exposure, cells were treated with paclitaxel for 3–96 h and then either: (a) immediately processed for drug effect measurement (immediate effect); or (b) washed, incubated in drug-free medium, and processed for drug effect measurement at 96 h (delayed effect). For the latter, paclitaxel-containing medium was removed at the end of treatment, and the culture plates were rinsed one to three times with 0.2 ml of PBS or serum-free culture medium. The PBS/medium wash mainly removed the residual medium. The washing period (an average of 5 min) was not sufficiently long to allow significant drug efflux from cells (see “Results”). Afterward, cells were incubated with 0.2 ml of drug-free medium until 96 h.

The cell number was measured using the SRB assay. SRB stains for cellular proteins (18). In brief, after removing the culture medium, plates were rinsed one to three times with 0.2 ml PBS for about 10 min. The cells were then fixed by incubating with 0.05–0.2 ml of 10% trichloroacetic acid at 4°C for 1 h, followed by five washes with distilled water. SRB solution (0.035–0.15 ml, 0.4%) was added at room temperature for 10 min to stain the cells. Afterward, the plate was washed five times using 1% glacial acetic acid and allowed to air-dry overnight. Tris-HCl (0.2 ml, 10 mM) was then added to each well to dissolve the SRB bound to cellular protein, which was measured by absorbance at 490 nm using an EL 340 microplate biospectrometer reader (Bio-Tek Instruments, Inc., Winooski, VT). The absorbance is proportional to the number of cells attached to the culture plate. Therefore, the SRB results represent the overall drug effect, i.e., the combination of cytostatic and apoptotic effects.

In addition to the six human cancer cell lines, the immediate and delayed effects of 24-h paclitaxel treatment were also measured in CHO cells. The goal of this experiment was to directly compare our results with the results of a previous study, which used the CHO cells to demonstrate increasing drug effect with increasing treatment duration (7).

The mechanical agitation during the washing procedures in the SRB assay may, in addition to removing the cells that undergo anoikis or detachment as a part of the apoptosis process, remove the M-phase cells, which is known to increase by paclitaxel treatment. Removal of viable M-phase cells that can recover from drug treatment would introduce artifacts and confound data interpretation. To determine the upper limit of this potential complication, we compared the kinetics of the M-phase block with the kinetics of detachment of cells from the culture flask up to 12 h, or before apoptosis was detectable (see “Results”). The distribution of cells in the cell cycle was determined by standard flow cytometric analysis (19). In brief, cells were stained for DNA with propidium iodide, followed by analysis of DNA content. Single-parameter DNA histograms from gated mode data were analyzed for cell cycle phase distribution using the multicycle AV (Phoenix Flow Systems, San Diego, CA).

The numbers of detached cells and attached cells after paclitaxel treatment were determined using a Coulter counter (Coulter Electronic, Inc., Hialeah, FL). The results showed that after treatment with 20 nm paclitaxel for 3, 6, and 12 h, the fraction of PC3 cells in the M phase was 32, 35, and 46%, and the detached cells represented 3, 7, and 15% of the total cells. The significantly lower fraction of detached cells compared with the M-phase cells indicates that the washing procedures in the SRB assay did not significantly reduce the fraction of viable M-phase cells attached to the culture flask.

**Measurement of Drug-induced Apoptosis.** Apoptosis was measured by two methods. Agarose gel electrophoresis was used to detect DNA fragmentation, and ELISA (Cell Death Detection ELISA kit; Boehringer Mannheim, Germany) was used to quantify the amount of DNA-histone complex released to the cytoplasm. As shown in “Results,” apoptosis was first detected at 24 h after initiation of drug treatment. To determine whether apoptosis occurs with a time delay and whether continuous drug exposure is needed, cells collected immediately after treatment with paclitaxel for various times or cells treated with paclitaxel for 3 h and collected at later time points were analyzed for apoptosis using both methods.

Nucleosomal DNA fragmentation was determined using procedures described previously (19). In brief, DNA was isolated with phenol:chloroform (1:1) and precipitated by adding 0.2 volume of 10 mM NH4Cl and 2 volumes of ethanol. The pellet obtained after centrifugation was resuspended in 100 mM Tris-HCl and 10 mM EDTA (pH 8.0). The amount of DNA was measured on a spectrophotometer at 260 nm, and the purity of DNA was ascertained by a 260:280 absorbance ratio of ≥1.8. Equal amounts of DNA were loaded on a 1.5% agarose gel containing 5 mg/ml ethidium bromide and run at 5 V/cm for 1 h in 1X Tris-acetate/EDTA electrophoresis buffer. The DNA laddering pattern was visualized by UV transillumination and photographed.

For the ELISA, cells harvested by trypsinization and the detached cells dispersed in the culture medium were collected by centrifugation at 250 × g. Fifty thousand cells were lysed in lysis buffer, and the cytoplasmic fractions of the lysates were placed in a flask precoated with mouse antihistone primary
Fig. 1. Immediate and delayed overall effects. Cells were incubated with Taxol for 3-96 h. The immediate effect was determined immediately after drug treatment. The delayed effect was determined at 96 h, irrespective of treatment durations. For example, the delayed effect of the 3-h treatment was measured with a 93-h delay. • 3 h; • 6 h; ▲ 12 h; ▼ 24 h; ♦ 48 h; □ 72 h; ○ 96 h. MCF7 cells did not receive the 6-h treatment. Results are means; bars, SD.

antibody and mouse anti-DNA antibody conjugated to peroxidase. The peroxidase substrate, 2,2'-azido-di-(3-ethylbenzthiazoline sulfonate), was applied, and the absorbance at 405 nm was measured.

Cellular Uptake and Efflux of Paclitaxel. The rates of paclitaxel uptake into and efflux from cells were studied. PC3 cells were plated in six-well plates (30,000 cells/ml in each well). One day after seeding, 1 ml of medium containing 10 nM [3H]paclitaxel (specific activity, 19.3 µCi/nmol) was added. An aliquot (0.1 ml) of medium was removed at predetermined times to analyze for the remaining drug concentration. After aspirating the remaining medium, cells were washed with 0.25 ml of ice-cold versene twice and then harvested.
Cells were treated with paclitaxel for 3–96 h and then either (a) immediately processed for drug effect measurement (immediate effect); or (b) washed, incubated in drug-free medium, and processed for drug effect measurement at 96 h (delayed effect). Note that the immediate and delayed effects are identical for the 96 h treatment. Mean ± SD of three to five experiments, six replicates per data point for each experiment, are shown.

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### Table 1: Immediate and delayed overall effects of taxol

![Table 1](https://example.com/table1.png)

**Table Legend:**

- **Maximum effect:** $E_{max}$ (%)
- **RT4:**
  - Immediate: 12 ± 2
  - Delayed: 84 ± 5
- **MCF7:**
  - Immediate: 10 ± 3.8
  - Delayed: 81 ± 7.0
- **SKOV3:**
  - Immediate: 3.9 ± 5.3
  - Delayed: 87 ± 4.7
- **PaDu:**
  - Immediate: 0
  - Delayed: 89 ± 5.2
- **PC3:**
  - Immediate: 6.4 ± 2.2
  - Delayed: 98 ± 0.3
- **DU145:**
  - Immediate: 7.0 ± 5.0
  - Delayed: 91 ± 5.3

**Effective concentration:** $IC_{50 - 90}$

### Results

**Effect of Removal of Intracellularly Retained Drug on the Delayed Drug Effect.** To examine the contribution of the intracellularly retained drug on the delayed effect, we measured the effect of successive washes, or successive reduction of residual drug in cells, on the delayed effect of the 3-h treatment. This treatment duration was selected because it showed the most dramatic differences between the immediate effect and the delayed effect measured at 96 h. A second experiment used the 24-h treatment duration, because this treatment produced the same delayed effect at 96 h as the 48-, 72-, and 96-h treatments (see "Results").

**Statistical Analysis.** Statistical analysis was performed using SAS. Differences in mean values between immediate and delayed effects were analyzed using paired and unpaired Student’s t-tests.

**RESULTS**

In all six cell lines, paclitaxel treatment resulted in two types of concentration-effect relationships, i.e., immediate and delayed effects. For the remainder of this report, immediate effect refers to the drug effect that was measured immediately after termination of treatment. Delayed effect refers to the drug effect that was measured after a delay. The delayed effects were measured in two ways. The first was to measure the drug effect at 96 h, irrespective of the treatment duration. This measurement indicates whether the different treatment durations yield different effects at 96 h. The second was to treat cells for 3 h, remove the drug-containing medium, and measure the drug effect at 6, 12, 24, 48, 72, and 96 h. This measurement indicates the kinetics of the delayed drug effect. Note that the immediate effect and

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**Note:** NA, not available.

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**Equation:**

$$E = (E_0 - R_c) \cdot \left(1 - \frac{C}{K_e + C}\right) + R_c$$

This equation is a modification of the more commonly used equation that describes a sigmoidal concentration-effect relationship that encompasses a spectrum of effect from 0 to 100% (20). Because nearly all paclitaxel treatments produced <100% reduction of cell number (see "Results"), it was necessary to include a term $R_c$, which represents the residual unaffected fraction. $E$ is the cell number remaining after drug treatment and equals the absorbance reading of the SRB assay for drug-treated samples. $E_0$ is the cell number in untreated controls and equals the absorbance reading for untreated controls. $(E_0 - R_c)$ equals the maximum effect ($E_{max}$). C is the drug concentration. $K_e$ is the drug concentration at one-half of $(E_0 - R_c)$. n is a curve shape parameter.

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**Analysis of Pharmacodynamic Data.** The relationship of paclitaxel-induced effect and drug concentration was analyzed by computer fitting the following equation to the experimental data using nonlinear least square regression (NLIN; SAS, Cary, NC):

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Cells were treated with paclitaxel for 3–96 h and then either: (a) immediately processed for drug effect measurement (immediate effect); or (b) washed, incubated in drug-free medium, and processed for drug effect measurement at 96 h (delayed effect).

**Kinetics of Intracellular Drug Retention.** Fig. 4 shows the kinetics of [3H]paclitaxel uptake into and efflux from PC3 cells. The half-lives for the intracellular and extracellular drug concentrations to reach an equilibrium during uptake and efflux were 1.8 and 1.5 h, respectively. Accordingly, the fraction of the intracellularly retained drug that was released during the 5-min wash would be <4%. Under the experimental conditions used to determine the delayed drug effect, i.e., replacing the drug-containing medium with drug-free medium and one wash (for <5 min) without allowing the extracellular concentration to equilibrate with the intracellular concentration, ~10% of the applied dose (at an initial concentration of 10 nM) remained in the cells (Table 4). Three successive washes, with a 3-h waiting period each, allow for the equilibration of intracellular and extracellular concentrations, reduced the residual radioactivity to less than ~0.5% of the applied dose. Note that the data in Table 4 are presented as percentages of the applied dose, whereas the data in Fig. 4 are presented as actual intracellular drug concentration on a per cell basis.

**Contribution of Intracellular Drug Retention to Delayed Overall Effect.** Fig. 5 shows the effect of reducing the intracellularly retained drug on the delayed effect of the 3- and 24-h treatments in PC3 cells. For both treatments, the delayed overall effects in samples with or without washes were greater than the immediate effect, which is consistent with the findings in Fig. 1. However, removal of residual drug had different effects for the two treatments. For the 3-h treatment, the rank order of the delayed overall effect was the same as the rank order of the amount of residual drug, i.e., zero wash > one wash > two washes > three washes, and the delayed effect in the sample washed three times was nearly identical to the immediate effect. In contrast, the delayed effects in the 24-h treatment were indistinguishable, irrespective of the number of washes.

**Results on CHO Cells.** The immediate and delayed overall effects of paclitaxel in the CHO cells were comparable with the findings in human cancer cells. The delayed overall effects of the 24-h treatment, measured at 48 and 72 h (i.e., with a 24- and 48-h delayed), were identical to the immediate effects of the 48- and 72-h treatments (data not shown).

**DISCUSSION**

The present study was designed to examine the relationship between treatment duration, concentration, and effect of paclitaxel, the

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*NS, not significantly different; NA, not applicable because the data were not obtained.

the delayed effect at 96 h are the same for the 96-h treatment, because its effect was measured only once at 96 h.

**Overall Effect.** The left panels of Fig. 1 show the immediate overall effects of paclitaxel treatments of 3–96 h. For all six cell lines, paclitaxel produced sigmoidal concentration-effect relationships; drug effect increased with increasing drug concentration and increasing treatment time. The sensitivity of individual cell lines to paclitaxel varied as indicated by the different E\text{max} and IC\text{50} values (Table 1). For each treatment time, the E\text{max} was achieved at a relatively low concentration (between 5 and 30 nM) with no further increases in overall effects of paclitaxel treatments of 3-96 h. For all six cell lines, the E\text{max} and decreased the IC\text{50} (Table 1).

The delayed overall effects for different treatment times (3-72 h) were compared with the standard overall effect. In contrast, the delayed overall effects in samples with or without washes were greater than the immediate effect, which is consistent with the findings in Fig. 1. However, removal of residual drug had different effects for the two treatments. For the 3-h treatment, the rank order of the delayed overall effect was the same as the rank order of the amount of residual drug, i.e., zero wash > one wash > two washes > three washes, and the delayed effect in the sample washed three times was nearly identical to the immediate effect. In contrast, the delayed effects in the 24-h treatment were indistinguishable, irrespective of the number of washes.

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PACLITAXEL PHARMACODYNAMICS

**Table 2 Differences between immediate and delayed overall effect**

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<td>&lt; 0.001</td>
</tr>
<tr>
<td>DU145</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*NS, not significantly different; NA, not applicable because the data were not obtained.

**Kinetics of Intracellular Drug Retention.** Fig. 4 shows the kinetics of [3H]paclitaxel uptake into and efflux from PC3 cells. The half-lives for the intracellular and extracellular drug concentrations to reach an equilibrium during uptake and efflux were 1.8 and 1.5 h, respectively. Accordingly, the fraction of the intracellularly retained drug that was released during the 5-min wash would be <4%. Under the experimental conditions used to determine the delayed drug effect, i.e., replacing the drug-containing medium with drug-free medium and one wash (for <5 min) without allowing the extracellular concentration to equilibrate with the intracellular concentration, ~10% of the applied dose (at an initial concentration of 10 nM) remained in the cells (Table 4). Three successive washes, with a 3-h waiting period each, allow for the equilibration of intracellular and extracellular concentrations, reduced the residual radioactivity to less than ~0.5% of the applied dose. Note that the data in Table 4 are presented as percentages of the applied dose, whereas the data in Fig. 4 are presented as actual intracellular drug concentration on a per cell basis.

**Contribution of Intracellular Drug Retention to Delayed Overall Effect.** Fig. 5 shows the effect of reducing the intracellularly retained drug on the delayed effect of the 3- and 24-h treatments in PC3 cells. For both treatments, the delayed overall effects in samples with or without washes were greater than the immediate effect, which is consistent with the findings in Fig. 1. However, removal of residual drug had different effects for the two treatments. For the 3-h treatment, the rank order of the delayed overall effect was the same as the rank order of the amount of residual drug, i.e., zero wash > one wash > two washes > three washes, and the delayed effect in the sample washed three times was nearly identical to the immediate effect. In contrast, the delayed effects in the 24-h treatment were indistinguishable, irrespective of the number of washes.

**Results on CHO Cells.** The immediate and delayed overall effects of paclitaxel in the CHO cells were comparable with the findings in human cancer cells. The delayed overall effects of the 24-h treatment, measured at 48 and 72 h (i.e., with a 24- and 48-h delayed), were identical to the immediate effects of the 48- and 72-h treatments (data not shown).

**DISCUSSION**

The present study was designed to examine the relationship between treatment duration, concentration, and effect of paclitaxel, the

**Table 3 Effect of treatment duration on delayed overall effects**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>E\text{max}</td>
<td></td>
</tr>
<tr>
<td>RT4</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>NS</td>
</tr>
<tr>
<td>SKOV3</td>
<td>NS</td>
</tr>
<tr>
<td>FaDu</td>
<td>NS</td>
</tr>
<tr>
<td>PC3</td>
<td>NS</td>
</tr>
<tr>
<td>DU145</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS, not significantly different; NA, not applicable because the data were not obtained.

**Time Course of Apoptosis.** Fig. 2 shows the results of agarase gel electrophoresis of DNA extracted from PC3 cells, and Fig. 3 shows the ELISA detection of cytoplasmic DNA-histone complex in these samples. The results of the two methods are qualitatively similar. We compared the kinetics of apoptosis in cells that were treated either for 3 h only or continuously with paclitaxel. In both cases, DNA fragmentation and cytoplasmic DNA-histone complex were first detected at 24 h and subsequently increased with time. The ELISA results also showed that the extent of DNA fragmentation in continuously treated cells was higher than in cells treated for only 3 h. Similar results were obtained in RT4, FaDu, and DU145 cells (data not shown).
electrophoresis. A. the immediate effect was measured directly after cells were treated for 3- to 93-h delay at 6, 12, 24, 48, 72, and 96 h. M. DNA marker VII (Boehringer Mannheim). Lane 1, control; Lanes 2–8, effects measured at 3, 6, 12, 24, 48, 72, and 96 h.

For the delayed effect, cells were treated for 3 h and then incubated with drug-free medium, and the drug effect was measured with a 3- to 93-h delay at 6, 12, 24, 48, 72, and 96 h. M. DNA marker VII (Boehringer Mannheim). Lane 1, control; Lanes 2–8, effects measured at 3, 6, 12, 24, 48, 72, and 96 h.

The immediate effect was measured immediately after continuous treatment for 3-96 h: •¿, immediate effect measured immediately after continuous treatment for 3-96 h; •¿, delayed effect of the 3-h treatment. Means are shown; bars, SD (n = 3). Some SDs are smaller than the symbols.

DNA-histone complexes in the cytoplasm of paclitaxel-treated PC3 cells (200 nM drug concentration) was measured using ELISA. Treatment conditions are as described in Fig. 1. The immediate effect measured immediately after continuous treatment for 3-96 h: •¿, delayed effect of the 3-h treatment. Means are shown; bars, SD (n = 3). Some SDs are smaller than the symbols.

Time course of drug-induced apoptosis, and the contribution of drug retention in cells to drug effect. The results showed the following: (a) paclitaxel produced cytotoxicity that was exhibited immediately after treatment (immediate effect) and with a delay after treatment was terminated (delayed effect; Table 1 and Fig. 1); (b) the immediate and delayed effects showed different pharmacodynamics. The immediate effect increased with treatment duration and drug concentration. For the delayed effect, all treatments produced the same maximum effect at 96 h, although treatments for ≤12 h showed higher IC_{50} than longer treatments, whereas treatments for ≥24 h showed indistinguishable IC_{50} (Tables 1–3); (c) treatment for as brief as 3 h was sufficient to induce apoptosis, which occurred with a lag time of about 24 h, although longer treatments produced a greater extent of apoptosis (Figs. 2 and 3); (d) the intracellular and extracellular concentrations reached an equilibrium at ~5 h, which rules out slow and/or insufficient uptake as the cause of the lower effects at shorter treatment times (Fig. 4); (e) upon removal of drug-containing medium, the amount of drug retained intracellularly was about 10% of the applied dose and was reduced to ~0.5% after three successive washes, separated by 3-h equilibration periods (Table 4); (f) the delayed effect of the 3-h treatment was largely due to the drug retained intracellularly, whereas the delayed effect of the 24-h treatment was independent of the drug retained intracellularly (Table 4 and Fig. 5).

The slow apoptosis induced by paclitaxel is consistent with the {\textsuperscript{51}}Cr release data in human ovarian cells (12), DNA strand breakage in human breast cancer cells (21), and appearance of apoptotic cells in histocultures of human head and neck tumors (22), all showing a 16–24 h delay between drug treatment and cell death detection.

The finding that the intracellularly retained drug contributes to the delayed effect of only the 3-h treatment and not the 24-h treatment is likely related to the cell cycle specificity of paclitaxel effects. The latter has been demonstrated by the reduced drug sensitivity of confluent cells compared with exponentially growing cells, the inverse correlation between sensitivity and cell doubling time, the antagonism induced by other agents that block cells in the G, or S phase, and by the reduced drug effect in cells arrested in S phase by lowering of intracellular pH (10, 23–27). Paclitaxel-induced cell cycle arrest and apoptosis occur mainly in M phase (4, 28). For treatments with duration of less than one cell cycle time, the fraction of cells that pass though the M phase and are subjected to drug toxicity is determined by the duration when the drug is present. In this case, the drug released from intracellular depots will in effect extend the drug treatment time and thereby increase the fraction of cells that are subjected to drug toxicity, and therefore determine the delayed effect.

Fig. 2. Kinetics of paclitaxel-induced DNA fragmentation. DNA was extracted from paclitaxel-treated PC3 cells (200 nM drug concentration) and analyzed by agarose gel electrophoresis. A, the immediate effect was measured directly after cells were treated continuously for 3, 6, 12, 24, 48, 72, and 96 h. B, for the delayed effect, cells were treated for 3 h and then incubated with drug-free medium, and the drug effect was measured with a 3- to 93-h delay at 6, 12, 24, 48, 72, and 96 h. M. DNA marker VII (Boehringer Mannheim). Lane 1, control; Lanes 2–8, effects measured at 3, 6, 12, 24, 48, 72, and 96 h.

Fig. 3. Kinetics of paclitaxel-induced apoptosis measured by ELISA. The amount of DNA-histone complexes in the cytoplasm of paclitaxel-treated PC3 cells (200 nM drug concentration) was measured using ELISA. Treatment conditions are as described in Fig. 2. •¿, immediate effect measured immediately after continuous treatment for 3-96 h; •¿, delayed effect of the 3-h treatment. Means are shown; bars, SD (n = 3). Some SDs are smaller than the symbols.

Fig. 4. Paclitaxel uptake into and efflux from cells. PC3 cells were treated with 10 nM [\textsuperscript{3}H]taxol. At predetermined times, the radioactivity in the medium and trypsinized cells was determined.

Table 4. Taxol retention in PC3 cells

<table>
<thead>
<tr>
<th></th>
<th>Zero wash</th>
<th>One wash</th>
<th>Two washes</th>
<th>Three washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>88.8 ± 2.1</td>
<td>5.8 ± 1.03</td>
<td>3.7 ± 0.79</td>
<td>1.57 ± 0.45</td>
</tr>
<tr>
<td>Cells</td>
<td>11.2 ± 2.1</td>
<td>5.7 ± 0.81</td>
<td>2.1 ± 0.51</td>
<td>0.58 ± 0.31</td>
</tr>
</tbody>
</table>
For treatments that are equal to or longer than one cell cycle time, all cells would have passed through the M phase at least once in the presence of the drug. In this case, the drug released from intracellular depots will not increase the affected cell fraction and therefore does not contribute to the delayed effect. This hypothesis is supported by the decreases in the IC_{50} for the delayed effects when treatment duration was progressively increased, reaching a constant value at and after 24 h, or approximately one cell cycle time (Tables 1 and 3). This is also consistent with our previous finding that the apoptotic effect of paclitaxel in human prostate tumor histocultures was not enhanced by increasing the treatment duration from 24 to 96 h (29).

The contradicting data in the literature on the importance of treatment duration (6–10, 12, 13) can be reconciled based on our present findings. In these earlier studies, the intracellularly retained drug was not removed and would therefore contribute to the delayed drug effect. This would explain the finding that prolonging drug treatment from 4 to 24 h did not enhance the formation of polytubule complexes and microtubule bundles nor the maximum reduction in cell number, when the drug effect was measured at 24, 48, or 72 h (12, 13). This is confirmed by our finding that the maximum delayed overall effect was independent of the treatment duration (i.e., unaltered E_{max}; Table 3). On the other hand, the higher IC_{50} observed for treatments shorter than one cell cycle time compared with treatments longer than one cell cycle time, as reported in several studies (6–8, 10), is comparable with our finding (Table 3) and is likely due to the M phase specificity of paclitaxel effect as discussed above. Finally, the apparent enhancement of drug effect associated with the longer treatment times found in one study, which measured drug effect immediately after treatment (9), is likely due to the delayed manifestation of drug effect. This is confirmed by our finding that the delayed drug effect accounted for the higher cytotoxicity associated with the longer treatment durations and does not require continuous drug exposure (Tables 1 and 2 and the results in CHO cells).

The results of the present study demonstrate the difficulties of using in vitro findings to design the optimal treatment schedules for paclitaxel in patients, in part because of the confounding variables under in vivo conditions that cannot be readily predicted or simulated under in vitro conditions: (a) in vitro studies using cell lines, with the exception of the clonogenic assay, determine the drug effect immediately after treatments and do not take into account the delayed drug effect. On the other hand, clinical efficacy of a drug is determined by its immediate and delayed effects. For a drug such as paclitaxel, studies should be expanded to include the measurement of the delayed effect to define its pharmacodynamics. The same consideration applies to other drugs that are known to induce the slow, unprimed apoptosis pathway typical for epithelial cells, rather than the more rapid primed apoptosis pathway often associated with leukemia cells (14, 15); (b) the demonstration of the importance of intracellularly retained drug on the delayed drug effects indicates that in vitro–in vivo extrapolation on paclitaxel pharmacodynamics needs to account for the differences of intracellular pharmacokinetics under in vitro and in vivo conditions.

One factor is the binding of paclitaxel to macromolecules and proteins in plasma and in cells. The release of paclitaxel from its binding sites in deep tissues will also serve to increase the residence time of the drug in the body. We have also shown that the paclitaxel binding to proteins in culture medium, which contains only 10% serum and therefore relatively low protein concentrations compared with undiluted serum, is lower than its binding to human plasma proteins (30). This would result in a higher unbound fraction that partitions into cells under in vitro conditions, as compared with in vivo conditions. Another factor is the presence of Cremophor EL, which is the vehicle for the clinical formulation of paclitaxel and has been used in some in vitro studies as the solvent for paclitaxel (10, 12, 16). A preliminary study in our laboratory showed that replacing 0.1% ethanol with 0.03% Cremophor EL as the solvent for paclitaxel reduced the intracellular drug accumulation, possibly because the highly lipophilic paclitaxel favors partition in Cremophor. Under in vitro conditions, the apparent volume of distribution of Cremophor EL is severalfold lower than that of paclitaxel (31), indicating that Cremophor EL is not taken up in tissues to the same extent as paclitaxel. Hence, the intracellular retention of paclitaxel at the tissue level under in vivo conditions may differ from in vitro conditions, where Cremophor EL is present together with paclitaxel throughout the treatment duration; (c) the importance of treatment duration relative to the cell cycle time indicates the need of accounting for the differences in cytokinetics among the slowly growing human solid tumors and the exponentially growing cells in monolayers. Another potentially complicating factor is the drug delivery to three-dimensional solid tumors, which is likely to differ from the drug delivery to the monolayer cultures.

In summary, results of the present study indicate that in human epithelial cancer cells, paclitaxel-induced cytotoxicity continued be-

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yond termination of drug treatment, which was partly due to the slow manifestation of apoptosis and partly due to the significant amount of drug retained intracellularly. The differences in the delayed effects for treatments of ≤ 12 h and the lack of differences for treatments of ≥ 24 h indicate that the delayed effect is maximally elicited by 24-h drug exposure. Although the results are consistent with an M phase-specific injury as reported previously (4, 27), additional studies to determine the effects of treatment time on treatment outcome, in relation to the cell cycle time, are warranted. Our results highlight the complex relationships between the cell cycle specificity of paclitaxel toxicity, the intracellular pharmacokinetics, and the kinetics of the immediate and delayed drug effects. One approach to evaluate the contribution of the various intertwining dynamic processes to the time- and concentration-dependent drug effects (and therefore the design of optimal treatment schedules) is to use computational approaches, which are emerging tools for solving complex and dynamic biological processes. Computational models that can account for the time-dependent changes in drug concentration in plasma, the kinetics of drug uptake into and efflux from tumor cells, the cell cycle kinetics of tumor cells, and the time-dependent effects of paclitaxel are potentially useful for identifying the optimal treatment schedules for this important anticancer drug.

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REFERENCES

Pharmacodynamics of Immediate and Delayed Effects of Paclitaxel: Role of Slow Apoptosis and Intracellular Drug Retention

Jessie L-S. Au, Dong Li, Yuebo Gan, et al.


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