Cancer Chemother Pharmacol (1994) 33: 331–339

Cancer Chemotherapy and Pharmacology

The influence of Cremophor EL on the cell cycle effects of paclitaxel (Taxol®) in human tumor cell lines

James Liebmann, John A. Cook, Claudia Lipschultz, Diane Teague, Joyce Fisher, James B. Mitchell

Radiation Oncology Branch, National Cancer Institute, Bethesda, MD 20892, USA

Received: 9 June 1993/Accepted: 31 August 1993

Abstract. We have performed DNA flow analysis, mitotic index studies, time-lapse photography, and paclitaxel uptake studies of human tumor cell lines exposed to paclitaxel. DNA flow analysis demonstrated that cells began accumulating in G2/M within 6 hrs of exposure to paclitaxel; by 12 hrs over 50% of cells accumulated in G2/M at all concentrations tested. After 24 hrs of exposure to 10 nM paclitaxel, cells underwent non-uniform mitotic division resulting in multinucleated cells. Of cells treated with 30 nM to 1000 nM paclitaxel, 75% to 85% remained blocked in G2/M for up to 72 hrs. Although a large proportion of cells treated with higher concentrations of paclitaxel (10,000 nM) was blocked in G2/M, a significant proportion (10% to 40%) of these cells was also in G1. Cells exposed to lower concentrations of paclitaxel (10 nM to 1000 nM) in medium containing 0.135% (v/v) Cremophor EL also had a relatively large proportion in G1. Mitotic index studies demonstrated that the paclitaxel-induced G2/M block was initially a mitotic block and that cells remained in mitosis for up to 24 hrs. With additional time of exposure to paclitaxel, mitotic index and time-lapse studies indicated that cells attempted to complete mitosis; however, cytokinesis was inhibited and cells became multinucleated. Time-lapse photography revealed that paclitaxel markedly prolonged the time in mitosis from 0.5 hr to 15 hr. High levels of Cremophor EL (0.135% v/v) markedly reduced the number of cells in mitosis but did not alter the mitotic delay induced by paclitaxel. ^{3}H-paclitaxel uptake studies revealed that high concentrations of Cremophor EL did reduce the rate of uptake of paclitaxel into cells but had little effect on total paclitaxel accumulation. These results confirm that paclitaxel has striking effects on the cell cycle and show that high concentrations of Cremophor EL are capable of inducing a cell cycle block distinct from the mitotic block seen with paclitaxel. These results also demonstrate that cells exposed to paclitaxel for longer than 24 hours attempt to complete mitosis but the process of cytokinesis is inhibited. Together with cytotoxicity data, these results indicate that entry into and exit out of mitosis are prerequisites for paclitaxel cytotoxicity.

Correspondence to: J. Liebmann

Introduction

Paclitaxel is a novel chemotherapeutic agent that is derived from the bark of the Western yew tree [16]. Paclitaxel binds tightly to the β-tubulin subunit of microtubules [12]. Paclitaxel promotes the assembly of microtubules in the absence of microtubule associated proteins and prevents the disaggregation of microtubules that normally occurs at low temperatures or in the presence of low concentrations of calcium [14, 15]. In the presence of paclitaxel, cytoplasmic microtubules are condensed into anomalous bundles and mitotic microtubules form multiple asters [1]. The effects of paclitaxel on microtubules are believed to account for the development of a block in proliferating cells in the G2 or M phases of the cell cycle that is consistently seen after exposure to the drug [14].

Paclitaxel's effects on microtubules and the cell cycle have been known for years and are believed to account in large measure for the cytotoxicity of the drug. However, the exact mechanism of paclitaxel-induced cytotoxicity is not known. We have found that paclitaxel exhibits a number of unique characteristics in in vitro clonogenic assays [4, 8]. First, the dose-response curve of paclitaxel cytotoxicity is initially steep at low concentrations of the drug (<20 nM), but then is flat over a wide range of paclitaxel concentrations (30 nM to 1000 nM). Second, very high concentrations of paclitaxel (10,000 nM), actually improve cell survival compared with lower paclitaxel concentrations. Third, paclitaxel cytotoxicity is time-dependent, with toxicity increasing with increased times of exposure. Fourth, cells in plateau phase of growth are much less sensitive to paclitaxel than are exponentially growing cells. Finally, high levels of Cremophor EL, the diluent in which paclitaxel is

prepared for clinical use, can antagonize paclitaxel cytotoxicity.

In an attempt to determine the mechanism(s) responsible for the cytotoxic properties of paclitaxel, we have conducted a number of studies utilizing DNA flow cytometry, time-lapse photography, mitotic index, and paclitaxel uptake studies. These studies confirm that entry into, and exit out of, mitosis is a likely prerequisite for cell killing by paclitaxel. Further, we have found that Cremophor EL can block cells in G1, prevent the entry of cells into mitosis, and antagonize paclitaxel's cytotoxicity.

Materials and Methods

Chemicals. Paclitaxel powder was supplied by the Cancer Therapy Evaluation Program (CTEP), National Cancer Institute. The powder was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, Mo.) to a stock concentration of 10 mM. Paclitaxel diluted in Cremophor EL was obtained from the Pharmacy Branch of the Clinical Center at the NIH at a stock concentration of 6 mg/ml (7.4 mM). Cremophor EL was obtained from CTEP. Propidium iodide (PI) and RNAse A were purchased from Sigma Chemical Co. ^{3}H-Paclitaxel (specific activity 23 Ci/mmol) was obtained from Research Triangle Institute (Research Triangle Park, NC). The ^{3}H-paclitaxel was found to be 96% pure by thin-layer chromatography and 95% pure by high-pressure liquid chromatography.

Cell culture. The human breast adenocarcinoma line MCF-7 and lung adenocarcinoma line A549 were both obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. For cell experiments, a number of 100-mm petri dishes were plated with 5×10^5 cells. Exponentially growing cells were exposed to various concentrations of paclitaxel or its appropriate diluent 24 h later. For experiments that studied cells in plateau phase of growth, cells were permitted to grow for a minimum of 72 h before they were exposed to paclitaxel. After exposure to paclitaxel for various times, the cells were rinsed, trypsinized, washed in PBS (pH 7.4) and collected for DNA flow analysis and mitotic index survey.

Flow analysis. Between 5×10^5 and 15×10^5 cells were fixed and stained in 2 ml of 0.1% sodium citrate/0.1% Triton X-100 solution containing 50 µg/ml PI. After at least 24 h in the fixative solution at 4° C, all samples were analyzed using an EPICS V cell sorter (Coulter Electronics, Hialeah, Fla.) with an argon ion laser tuned to 488 nm for excitation. Prior to flow analysis, 50 µg/ml of RNAse A was added to all samples. The PI fluorescence was detected using 514/530 nm filters. Both linear and log DNA histograms were collected and analyzed. For DNA degradation, log histograms are presented. For cell cycle analysis, linear DNA histograms were used and analyzed using previously described computer programs [5].

Mitotic index. Cells were briefly (<10 min) exposed to hypotonic saline (35 mM NaCl) and then fixed with methanol/acetic acid (3:1). Cells were dropped onto microscope slides and stained with crystal violet. Cells were viewed by light microscopy and the number of mitotic, interphase, or multinucleated cells were scored. A minimum of 500 cell nuclei were scored for each time point.

Time-lapse photography. Cells (10×10^3) were plated into 25-mm petri dishes in 3.5 ml of medium and allowed to attach and grow for 24 h. Paclitaxel, Cremophor EL, or DMSO was added to the cells to the appropriate final concentrations after a suitable field of 20–30 cells was identified through the photography system. Culture dishes were viewed using a Nikon inverted microscope (Diaphot-TMD) equipped with an on-stage chamber receiving a flow of humidified 5% CO_2 and air, and located in a 37° C warm room. The phase contrast field of 20–30 cells was recorded at one frame every 4 s through the camera port using a Panasonic WV-CL 300 camera connected to a Panasonic VHS (model AG-6030-P) time-lapse recorder. The tapes could be viewed or used to produce still photographs. To determine the duration that cells were in mitosis, individual cells were identified on the videotapes. Mitosis was considered to have begun when a cell became round and condensed. Mitosis was considered to have ended either with the appearance of cell division or, in the case of many cells exposed to paclitaxel, when the condensed cell again became flat, regardless of whether daughter cells were produced or not.

^{3}H-Paclitaxel binding. Cells were plated into 24-well plates at a concentration of 50,000 cells per well, and 24 h later 0.1 µCi of ^{3}H-paclitaxel was added to each well (8.7 nM final concentration) to assess paclitaxel uptake in exponentially growing cells. To follow the time course of ^{3}H-paclitaxel uptake into cells, medium was aspirated from the plates at various times after the addition of labeled paclitaxel and the cell monolayer was extensively washed with phosphate-buffered saline, pH 7.4. Cells were then lysed with 0.5 ml of a solution containing 1% Triton X-100 and 1% sodium dodecyl sulfate. The lysates were collected and counted in a liquid scintillation counter. To assess specific paclitaxel binding to cells, unlabeled paclitaxel, in final concentrations of 10, 50, 100, 250, or 1000 nM was also added to wells containing ^{3}H-paclitaxel. After 12 h, cells were collected for counting of bound radioactivity as described above. Cells that had been exposed to unlabelled paclitaxel alone were trypsinized and counted. Results are expressed as cpm/10^5 cells.

Results

Flow analysis

Paclitaxel caused a G2/M block in all human cell lines tested, a finding similar to previously reported results [14]. Figure 1 shows the temporal development of the G2/M block in A549 cells exposed to 50 nM paclitaxel. An increase in the G2/M fraction was evident within 6 h of paclitaxel exposure, and over 80% of cells were in G2/M within 24 h. Figure 2 shows the effect of a 24 h exposure to different concentrations of paclitaxel on the cell cycle of A549 cells. At very low concentrations of paclitaxel (5–10 nM), extensive nuclear damage, indicated by the broad peak centered about channel 125, was seen in addition to accumulation of cells in G2/M. Concentrations of paclitaxel up to 1000 nM also resulted in the characteristic G2/M block, although nuclear damage was markedly reduced. However, at very high concentrations of paclitaxel (10,000 nM) a significant proportion of cells remained in G1. All other human cell lines we have studied [8] also showed flow cytometry patterns similar to those exhibited by A549 cells after exposure to paclitaxel, although the extent of the G1 block caused by 10,000 nM paclitaxel did vary between cell lines.

Because of the possibility that the high levels of Cremophor EL that would be present in solutions of 10,000 nM paclitaxel might be responsible for the G1 block, cell cycle analysis was performed on cells incubated in 0.135% (v/v) Cremophor EL and exposed to low levels of paclitaxel (5 nM to 50 nM; Fig. 3). This (0.135%) is the concentration of Cremophor EL that is present in a 10,000 nM solution of paclitaxel prepared from a stock of paclitaxel formulated for clinical use (6 mg/ml paclitaxel in Cremophor EL). The Cremophor EL did prevent accumulation of cells in G2/M

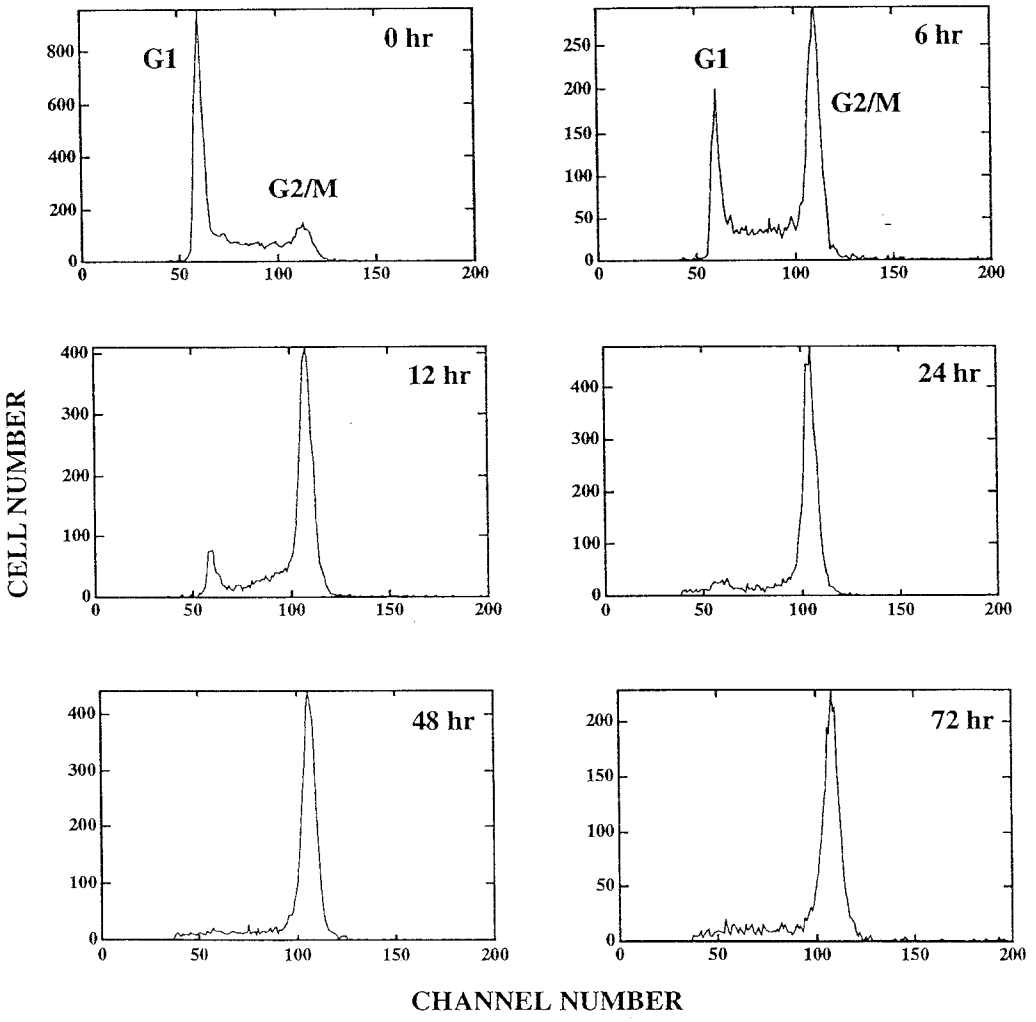


Fig. 1. DNA flow cytometry analysis of A549 cells exposed to 50 nM paclitaxel for increasing periods of time. Cells were exposed to paclitaxel and prepared for flow cytometry as described in "Methods". Of the control cells (*upper left panel*), 45% are in G1, 43% are in S, and 12% are in G2/M. Within 6 h of exposure to paclitaxel (*upper right panel*), a majority of cells are blocked in G2/M, though a significant minority of cells remain in G1. With increasing duration of exposure to paclitaxel, nearly all cells move out of G1 and are blocked in G2/M

after exposure to paclitaxel for 24 h. The nuclear damage that was seen after cells were exposed to 5 nM or 10 nM paclitaxel (Fig. 2B) was also reduced in the presence of 0.135% Cremophor EL. In contrast, high levels of DMSO, an alternative solvent for paclitaxel, did not affect the G2/M cell cycle block normally induced by paclitaxel.

Mitotic index

Because DNA flow analysis cannot distinguish between cells in the G2 or M phases of the cell cycle, cells were fixed and stained to count the proportion of cells in mitosis after treatment with paclitaxel. Table 1 shows the proportion of A549 cells in mitosis or interphase as well as the proportion of cells with multiple micronuclei. During the first 12–24 h of exposure to 50 nM paclitaxel the proportion of mitotic cells increased sharply to between 40% and 60% of total cells. Between 24 and 48 h, however, the proportion of mitotic cells fell and the overwhelming majority of cells (>70%) were multi-nucleated. A far smaller proportion of cells exposed to 10,000 nM paclitaxel, however, was found to be in mitosis compared with cells incubated in 50 nM paclitaxel. Only about 10% of cell nuclei were mitotic at any time after exposure to 10,000 nM paclitaxel.

To determine whether the Cremophor EL diluent was responsible for the lower proportion of mitotic nuclei noted at high concentrations of paclitaxel, cells were incubated in medium containing 50 nM paclitaxel to which Cremophor EL had been added to give a final concentration of 0.135% (v/v). High concentrations of Cremophor EL did result in a marked decrease in the proportion of cells in mitosis compared with cells exposed to identical amounts of paclitaxel in low concentrations of Cremophor EL (Table 1).

Time-lapse photography

Cells were videotaped for up to 72 h during exposure to paclitaxel. Cells not exposed to paclitaxel stayed in mitosis for about 30 min. Concentrations of paclitaxel of between

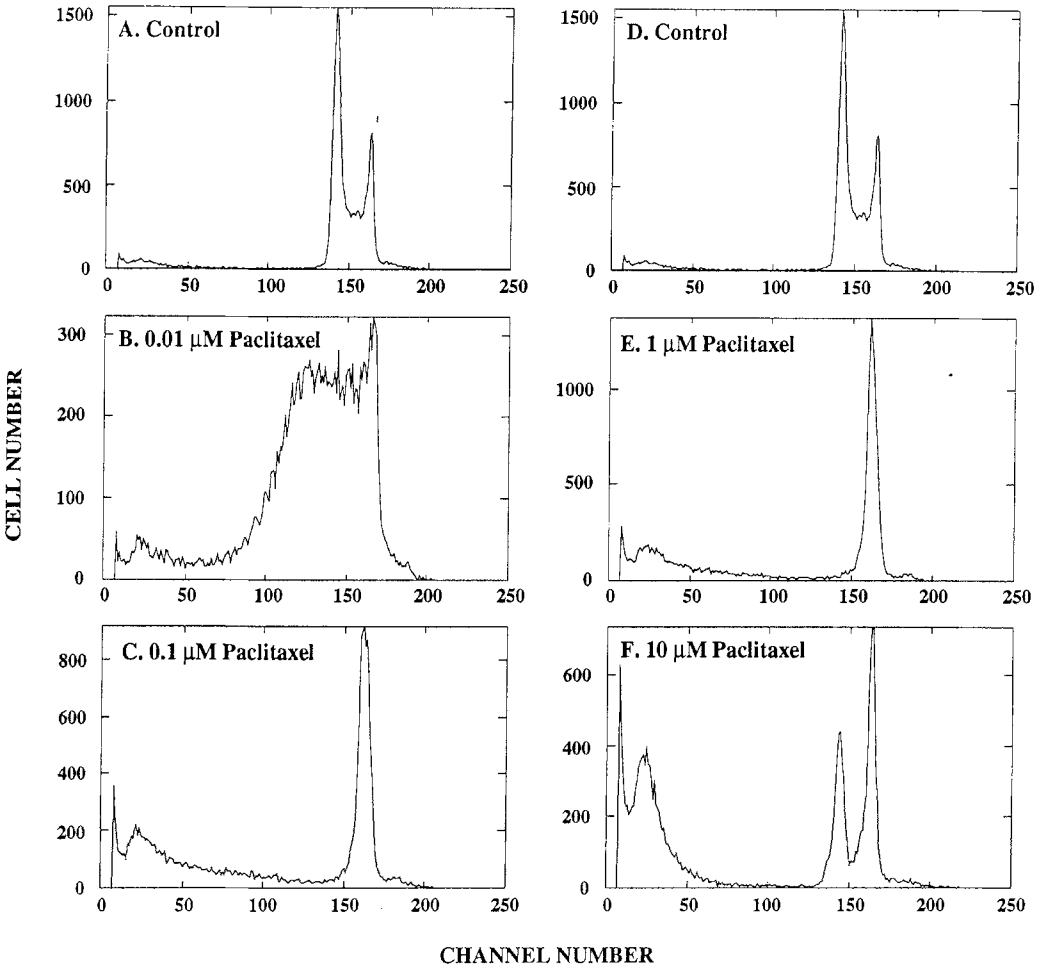


Fig. 2. DNA flow cytometry analysis of A549 cells exposed to increasing amounts of paclitaxel for 24 h. Cells were exposed to paclitaxel and prepared for flow cytometry as described in "Methods". Control cells are shown in *panels A* and *D*. Cells were exposed to 10 nM (*panel B*), 100 nM (*panel C*), 1000 nM (*panel E*), or 10,000 nM paclitaxel (*panel F*)

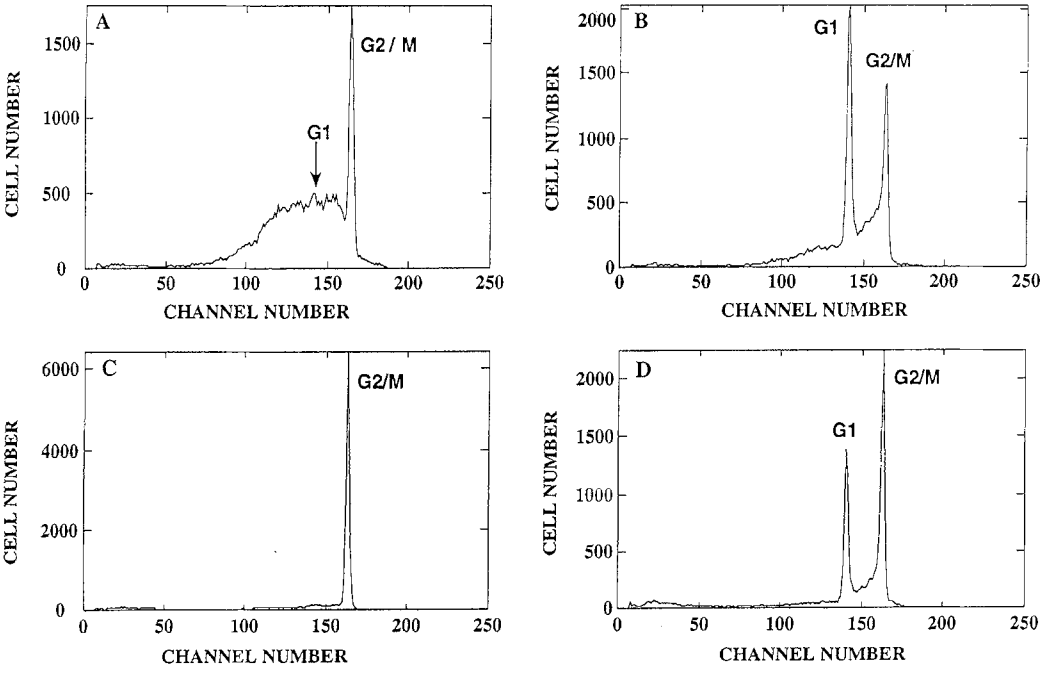


Fig. 3. DNA flow cytometry analysis of A549 cells exposed for 24 h to 10 nM or 50 nM paclitaxel in the presence or absence of 0.135% (v/v) Cremophor EL. Cells were exposed to paclitaxel and/or Cremophor EL and prepared for flow cytometry as described in "Methods". *Panel A* shows cells exposed to 10 nM paclitaxel only. *Panel B* represents cells exposed to 10 nM paclitaxel in the presence of Cremophor EL. *Panel C* shows cells exposed to 50 nM paclitaxel only. *Panel D* represents cells exposed to 50 nM paclitaxel in the presence of Cremophor EL

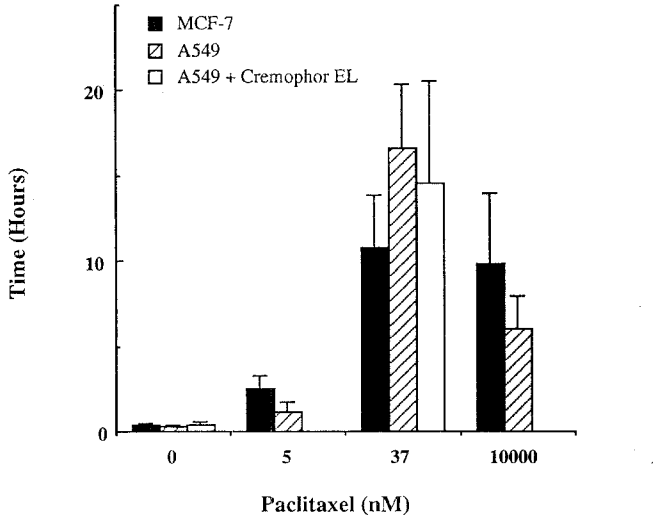


Fig. 4. Time that cells remained in mitosis during exposure to paclitaxel. Cells were videotaped with time-lapse photography as described in "Methods". Individual cells were followed on tape and the time that each cell stayed in mitosis was determined. Values shown are means ± S. D.

Table 1. Distribution of A549 cells in the cell cycle and as multi-nucleated cells after exposure to paclitaxel. After exposure to paclitaxel for the times indicated, cells were fixed, stained, and counted as described in "Methods". Where the addition of "Cremophor" is indicated, cells were incubated in 0.135% (v/v) Cremophor EL during exposure to paclitaxel. Where the addition of "DMSO" is indicated, cells were incubated in 0.135% (v/v) DMSO during exposure to paclitaxel. Values expressed are percentages of total cells ± SD

Paclitaxel	Time (h)	Interphase	Mitosis	Multinucleated
0	–	97.6±0.3	2.4±0.2	–
50 nM	6	79.2±1.7	20.0±1.6	0.8±0.4
50 nM	12	54.2±1.6	43.5±1.6	2.2±0.5
50 nM	24	16.7±1.3	66.5±1.7	16.7±1.3
50 nM	48	3.1±0.7	22.4±1.6	74.6±1.7
50 nM + Cremophor	24	69.8±2.0	19.0±1.7	11.2±1.4
50 nM + DMSO	24	15.6±1.6	41.8±2.2	42.6±2.2
10,000 nM + Cremophor	24	65.6±1.8	12.1±1.2	22.3±1.5
10,000 nM + DMSO	24	8.8±1.2	56.7±2.1	34.5±2.0

5 nM and 10,000 nM increased the duration of mitosis up to 15 h (Fig. 4). Although mitotic index and time-lapse studies showed that high levels of paclitaxel or Cremophor EL (0.135%) resulted in a reduced rate of entry of cells into mitosis, Fig. 4 demonstrates that mitosis was prolonged in those cells that entered mitosis, regardless of the concentration of Cremophor EL in the medium. Cells treated with paclitaxel that entered a prolonged mitotic state eventually re-formed the nuclear membrane and attached to the plate surface; however, the process of cytokinesis was inhibited and cell division rarely occurred. Instead, cells developed multiple nuclei and frequently continued to attempt to go through mitosis but were unable to divide into daughter cells (Fig. 5). High concentrations of paclitaxel (10,000 nM) or of Cremophor EL resulted in a reduced rate of mitosis formation as compared with cells exposed to lower amounts of paclitaxel. These high concentrations of paclitaxel also caused the formation of multiple cytoplasmic vacuoles (Fig. 5). Cytoplasmic vacuoles were also noted in cells exposed to 0.135% Cremophor EL alone (results not shown).

^{3}H-paclitaxel uptake

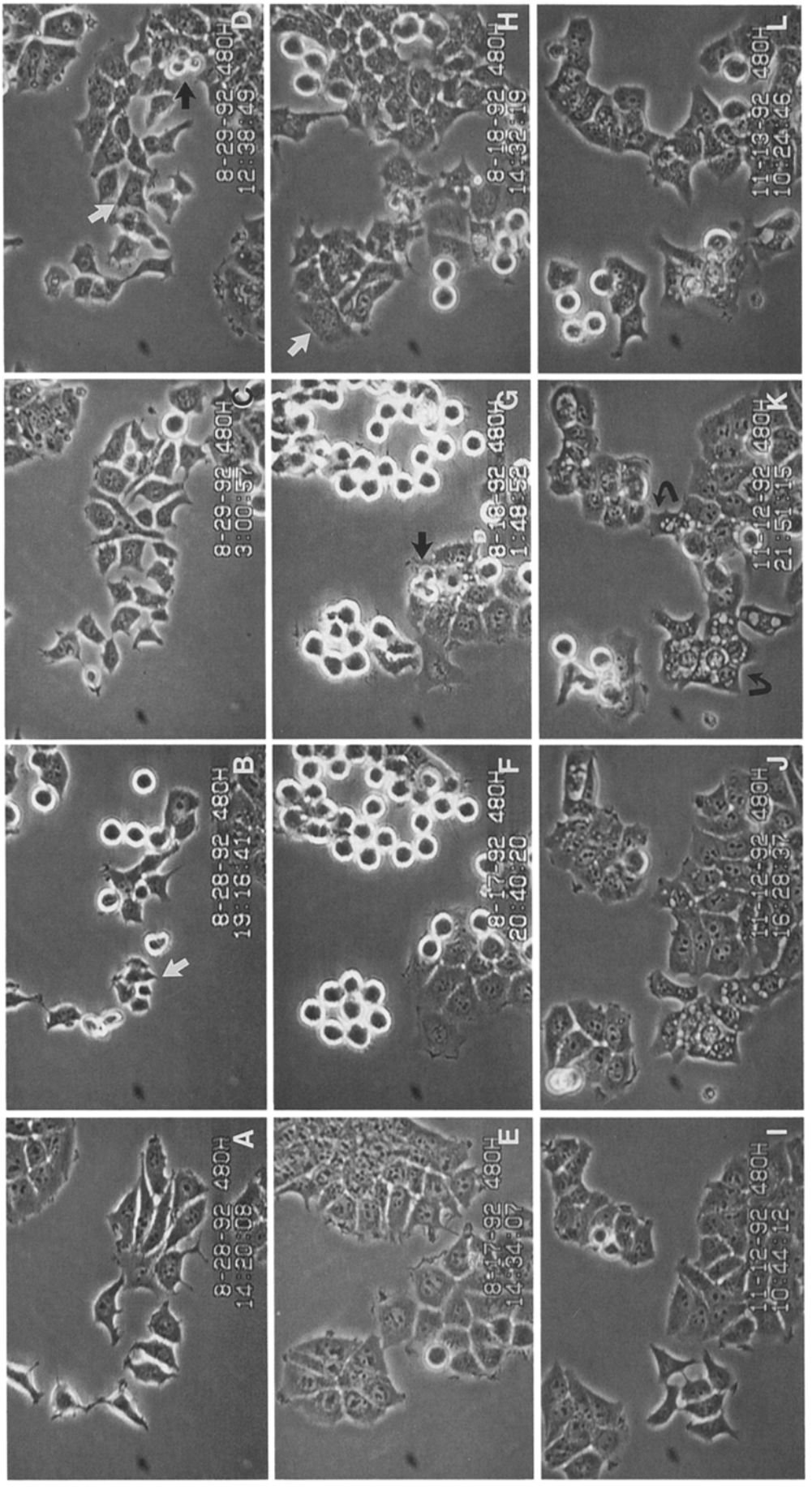
Incubation of cells in high concentrations of Cremophor EL reduced the rate at which cells accumulated ^{3}H-paclitaxel (Fig. 6). In contrast, high levels of DMSO had no effect on paclitaxel uptake. In A549 cells, the total accumulation of ^{3}H-paclitaxel was slightly reduced by 0.135% (v/v) Cremophor EL; however, the total uptake of ^{3}H-paclitaxel into MCF-7 cells was not affected by Cremophor EL (Fig. 6).

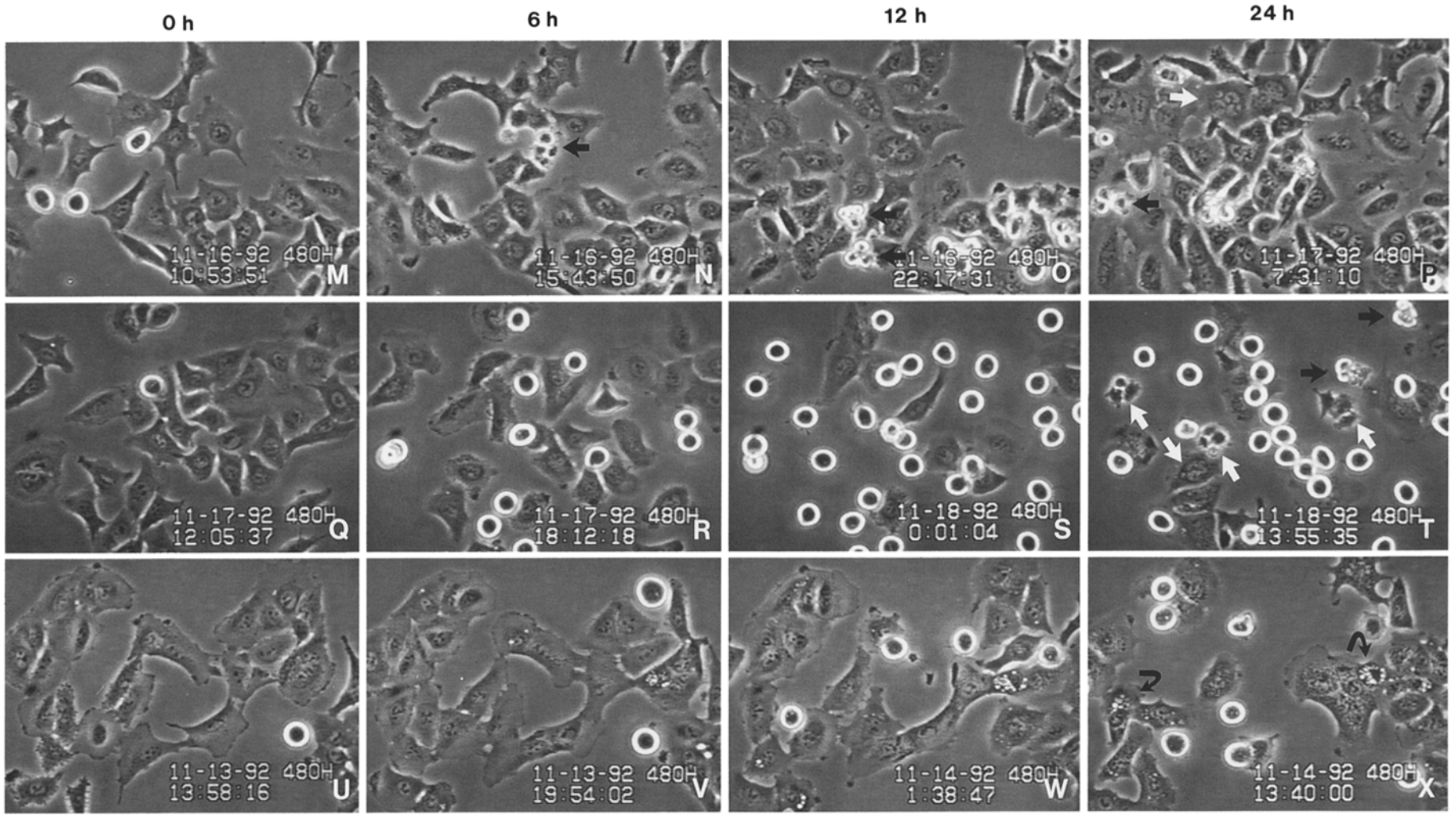
Discussion

We have shown, in rodent and a variety of different human tumor cell lines, that paclitaxel has a unique dose-response cytotoxic effect [4, 8]. All lines exposed to paclitaxel for 24 h that we have studied exhibit a sharp decline in cell survival at low (≤20 nM) concentrations of the drug. However, each line also demonstrates a plateau in survival at concentrations of paclitaxel above about 30 nM. At very high concentrations of paclitaxel (10,000 nM) several cell lines show an increase in cell survival. We have also found that the cytotoxicity of paclitaxel is highly dependent on the time that cells are exposed to the drug. Little or no cytotoxicity is seen in cells that are treated with paclitaxel for less than 12 h. Cytotoxicity increases in all cell lines as time of exposure to paclitaxel increases, so that cell killing after 72 h is as much as 200 times greater than that seen after 24 h of paclitaxel treatment. Further, we have noted that cells in the plateau phase of growth are markedly more resistant to paclitaxel than are cells growing exponentially.

Although paclitaxel avidly binds to tubulin to promote the formation and prevent the dissolution of microtubules [10], the exact mechanism of paclitaxel-induced cytotoxicity is unknown. The DNA flow cytometry studies reported here and by others [9, 14] show that exposure of

Fig. 5. Phase microscope images of cells monitored by time-lapse video. *Panels A* through *L* are MCF-7 cells; *panels M* through *X* are A549 cells. Paclitaxel concentrations are designated for each *row*. *Column headings* indicate time points after addition of paclitaxel to the medium. The mitosis phase (rounded cells) persists for hours in cells exposed to paclitaxel. Multidirectional nuclear "divisions" (*straight black arrows* in *D, G, N, O, P,* and *T*) result in micronucleated cells (*white arrows* in *B, D, H, P,* and *T*). Far fewer cells entered mitosis when cultures were treated with 10,000 nM paclitaxel than with exposure to lower paclitaxel concentrations; 10,000 nM paclitaxel also resulted in the appearance of cytoplasmic vacuoles (examples at *bent arrows* in *K* and *X*).


0 h
6 h
12 h
24 h
8-28-92 480H
14:20:08
A
8-28-92 480H
19:16:41
B
8-29-92 480H
3:00:57
C
8-29-92 480H
12:38:49
D
8-17-92 480H
14:34:07
E
8-17-92 480H
20:40:20
F
8-18-92 480H
1:48:52
G
8-18-92 480H
14:32:19
H
11-12-92 480H
10:44:12
I
11-12-92 480H
16:28:37
J
11-12-92 480H
21:51:15
K
11-13-92 480H
10:24:46
L


0 h
6 h
12 h
24 h
11-16-92 480H
10:53:51
M
11-16-92 480H
15:43:50
N
11-16-92 480H
22:17:31
O
11-17-92 480H
7:31:10
P
11-17-92 480H
12:05:37
Q
11-17-92 480H
18:12:18
R
11-18-92 480H
0:01:04
S
11-18-92 480H
13:55:35
T
11-13-92 480H
13:58:16
U
11-13-92 480H
19:54:02
V
11-14-92 480H
1:38:47
W
11-14-92 480H
13:40:00
X

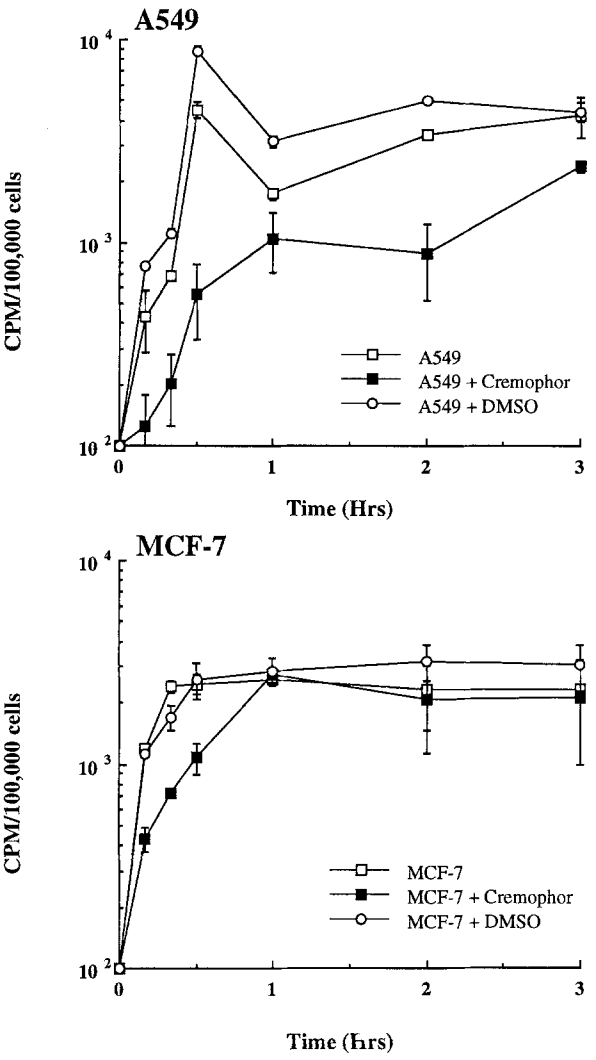


Fig. 6. Uptake of paclitaxel into A549 (above) and MCF-7 (below) cells. Cells were incubated in 3H-paclitaxel and collected at various times as described in "Methods"

exponentially dividing cells to paclitaxel rapidly results in a block in the G2/M phases of the cell cycle. The extent of the G2/M block was somewhat dependent on paclitaxel concentration. Cells incubated in 5 nM to 10 nM paclitaxel were able to complete mitosis after about 12 h; however, cell division occurred in a non-uniform manner, leading to the production of multinucleated cells. Above paclitaxel concentrations of 30 nM, cell accumulation at the G2/M border became noticeable within 6 h of exposure to the drug and persisted for at least 72 h with continued paclitaxel exposure. However, mitotic index measurements demonstrated that, though cells exposed to any level of paclitaxel less than 1000 nM were initially blocked in mitosis, with time most cells did attempt to complete mitosis. All lines had a significant proportion of cells in mitosis after 12–24 h of exposure to paclitaxel. With continued exposure to paclitaxel, the proportion of mitotic cells decreased and the overwhelming majority of cells developed multiple micronuclei. Time-lapse photography of cells exposed to paclitaxel confirmed the development of multiple micronuclei with increasing time of exposure to the drug. Thus, although cells exposed to paclitaxel maintain a G2/M DNA content as determined by flow studies, these cells were not statically blocked in mitosis. Rather, they progressed through mitosis but were unable to divide and instead developed multiple micronuclei.

It may be more accurate to refer to paclitaxel-treated cells that have a tetraploid DNA content but have progressed through mitosis as tetraploid G1 cells. Our DNA flow cytometry studies separate cells on the basis of DNA content and so cannot distinguish between G2, M, or tetraploid G1 cells. However, using bromo-deoxyuridine labeling, we have found that paclitaxel-treated tetraploid or multi-nucleated human cells do not reinitiate DNA synthesis (data not shown). This is in contrast to some paclitaxel-treated rodent cells, which appear to be able to reinitiate DNA synthesis [3, 9] and acquire a DNA content of 8n or greater (n.b., diploid content = 2n).

Our results are consistent with those recently reported by Lopes et al. [9], who found that the human ovarian cancer cell line A2780 progressed through mitosis in the presence of paclitaxel but was unable to successfully divide. Lopes et al. also noted the emergence of a fraction of A2780 cells with DNA content of 8n after exposure to paclitaxel. We also have found that a large fraction of the rodent cell line V79 acquire a DNA content of 8n or greater with prolonged exposure to paclitaxel [3]. However, we have seen very few human cells progress to a DNA content of 8n after exposure to paclitaxel [8]. The fraction of A2780 cells in the study by Lopes et al. which developed a DNA content of 8n appeared to be small in contrast to the large fraction (>50%) of rodent cells that eventually acquire an 8n DNA content during paclitaxel exposure [3, 9]. The reason for these differences in the emergence of cells with DNA content of 8n or greater in different cell lines is not known. However, there are significant differences in the doubling (cell cycle) times between the A2780 cells (16 h) and the cell lines we used in the present study (24 h). It is possible that intrinsic differences in cell cycle progression between the different cell lines could account for the differences in the number of 8n cells.

We have found that concentrations of Cremophor EL, equivalent to that which would be present in a 10,000 nM solution of paclitaxel, inhibit the cytotoxicity of paclitaxel [8]. The current studies demonstrate that Cremophor EL blocks the cell cycle effects of paclitaxel. High concentrations of Cremophor EL reduced the percentage of cells in G2/M and increased the percentage of cells in G1 after exposure to paclitaxel as assessed by flow cytometry. Mitotic index studies confirmed that 0.135% (v/v) Cremophor EL reduced the proportion of mitotic nuclei observed in a population of cells exposed to paclitaxel. Our time-lapse photography studies of cells also showed that high concentrations of Cremophor EL reduced the number of cells that entered mitosis in the presence of paclitaxel. However, time-lapse photography revealed that once cells progressed into mitosis paclitaxel markedly prolonged the duration of mitosis regardless of the level of Cremophor EL in the medium. These results again confirm the ability of paclitaxel to block cells in mitosis. Furthermore, these results suggest that high levels of Cremophor EL antagonize paclitaxel by preventing cells from entering mitosis. We

have noted antagonism of paclitaxel cytotoxicity under other conditions that reduce the proportion of cells that enter mitosis, including glutathione depletion by L-BSO [7], simultaneous or concurrent use of cell cycle active chemotherapeutic agents [6], and maintenance of cells in plateau phase of growth [8].

Others have shown that Cremophor EL, the diluent in which paclitaxel is prepared for clinical use, is a biologically active solvent [2, 18]. Our studies confirm that this diluent has biologic effects and suggest that high levels of Cremophor EL can reduce the rate of accumulation of paclitaxel into cells. Cremophor EL can antagonize the cytotoxicity of paclitaxel in human tumor cell lines. Though disruption of the cell cycle appears to be one mechanism through which Cremophor EL antagonizes paclitaxel, other possible modes of action cannot be excluded. In particular, we have found that Cremophor EL also slightly decreased the total accumulation of paclitaxel by A549, but not MCF-7 cells. It is possible that the reduced rate of paclitaxel accumulation by cells in the presence of high levels of Cremophor EL could contribute to antagonism of paclitaxel cytotoxicity. However, the total accumulation of paclitaxel by MCF-7 cells did not appear to be affected by Cremophor EL, and total accumulation in both cell lines was maximal within 3–6 h of exposure of cells to the drug. We have found that very high levels of Cremophor EL can antagonize paclitaxel cytotoxicity during exposures to the drug of as long as 72 h. It seems unlikely, therefore, that a reduction in rate of accumulation of paclitaxel that would only influence the first few hours of a cell's exposure to the drug, can account for prolonged antagonism of paclitaxel cytotoxicity.

Serum paclitaxel concentrations of 10,000 nM have been achieved in patients given short infusions of the drug [17]. Though the pharmacokinetic properties of paclitaxel have been well described [13], there is no information on the pharmacokinetics of Cremophor EL in humans. It is possible that this lipid solvent is rapidly cleared by the liver when given i.v. to patients. If this is the case, then the Cremophor EL levels used in these studies may not be maintained in humans, even if paclitaxel serum levels of 10,000 nM or greater are achieved. Recently, however, i.p. administration of paclitaxel has been reported in patients with ovarian cancer [11]. Patients given paclitaxel by the i.p. route achieve peritoneal paclitaxel concentrations in excess of 100,000 nM. Nothing is known about the clearance of Cremophor EL from the peritoneum. On the basis of the results reported here and elsewhere [8], if high levels of Cremophor EL introduced into the peritoneum were to persist for a prolonged period, one might expect that the solvent would interfere with the clinical response of intraperitoneal tumors to paclitaxel.

In summary, we have shown that paclitaxel causes cells to accumulate in G2/M within a few hours of exposure. Although high concentrations of paclitaxel (10,000 nM) caused a large proportion of cells to remain in mitosis, a significant proportion of cells also remained in G1 in the presence of 10,000 nM paclitaxel. High concentrations of Cremophor EL reduced the paclitaxel-induced accumulation of cells in mitosis. However, Cremophor EL did not affect paclitaxel-induced prolongation of mitosis. Cremophor EL lowered the rate of accumulation of paclitaxel into cells; though total accumulation of paclitaxel by A549 cells was reduced by Cremophor EL, the diluent had no effect on total accumulation of paclitaxel by MCF-7 cells. These results are consistent with the hypothesis that paclitaxel is cytotoxic to cells only while they are in mitosis [9]. Whether cells exposed to paclitaxel are destined to die once in mitosis or whether they must attempt to complete mitosis before death is certain is unknown. However, our results clearly indicate that maneuvers that prevent the entry of cells into mitosis should antagonize paclitaxel cytotoxicity.

References

1. Brenner SL, Brinkley BR (1982) Tubulin assembly sites and the organization of microtubule arrays in mammalian cells. Cold Spring Harbor Symp Quant Biol 46: 241
2. Chaung LF, Israel M, Chuang RY (1991) Cremophor EL inhibits 12-*O*-tetradecanoylphorbol-13 acetate (TPA)-induced protein phosphorylation in human myeloblastic leukemia ML-1 cells. Anticancer Res 11: 1517
3. Cook JA, DeGraff W, Teague D, Liebmann JE (1993) Radiation sensitization of Chinese hamster V79 cells by paclitaxel. Radiat Oncol Invest (in press)
4. Cook JA, Liebmann J, Sullivan F, Hahn S, Teague D, DeGraff W, Mitchell JB (1993) Paclitaxel-mediated cytotoxicity in Chinese hamster V79 cells. Cancer Chemother Pharmacol (in press)
5. Fox MH (1980) A model for the computer analysis of synchronous DNA distributions obtained by flow cytometry. Cytometry 1: 71
6. Hahn SM, Liebmann JE, Fisher J, Cook JA, Mitchell JB, Kaufman DK (1993) Paclitaxel in combination with doxorubicin and etoposide: possible antagonism in vitro. Cancer (in press)
7. Liebmann JE, Hahn SM, Cook JA, Lipschultz CA, Mitchell JB, Kaufman DC (1993) Glutathione depletion by L-buthionine sulfoximine antagonizes taxol cytotoxicity. Cancer Res 53: 2066
8. Liebmann JE, Cook JA, Lipschultz C, Teague D, Fisher J, Mitchell JB (1993) Cytotoxic studies of paclitaxel (Taxol®) in human tumor cell lines. Br J Cancer (in press)
9. Lopes NM, Adams EG, Pitts TW, Bhuyan BK (1993) Cell kill kinetics and cell cycle effects of taxol on human and hamster ovarian cell lines. Cancer Chemother Pharmacol 32: 235
10. Manfredi JJ, Horwitz SB (1984) Taxol: an antimitotic agent with a unique mechanism of action. Pharmacol Ther 25: 83
11. Markman M, Rowinsky E, Hakes T, Reichman B, Jones W, Lewis JL Jr, Rubin S, Curtin J, Barakat R, Phillips M, Hurowitz L, Almadrones L, Hoskins W (1992) Phase I trial of intraperitoneal taxol: a Gynecologic Oncology Group study. J Clin Oncol 10:1485
12. Rao S, Horwitz SB, Ringel I (1992) Direct photoaffinity labeling of tubulin with taxol. J Natl Cancer Inst 84: 785
13. Rowinsky EK, Caenave LA, Donehower RC (1990) Taxol: a novel investigational antimicrotubule agent. J Natl Cancer Inst 82: 1247
14. Schiff PB, Fant J, Auster LA, Horwitz SB (1978) Effects of taxol on cell growth and in vitro microtubule assembly. J Supramol Struct [Suppl] 2: 328
15. Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly in vitro by taxol. Nature 277: 665
16. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 93: 2325
17. Wiernik PH, Schwartz EL, Strauman JJ, Dutcher JP, Lipton RB, Paietta E (1987) Phase I clinical and pharmacokinetic study of taxol. Cancer Res 47: 2486
18. Woodcock DM, Jefferson S, Linsenmeyer ME, Crowther PJ, Chojnowski GM, Williams B, Bertoncello I (1990) Reversal of the multidrug resistance phenotype with Cremophor EL, a common vehicle for water-insoluble vitamins and drugs. Cancer Res 50: 4199