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## Original Paper

# Effects of Paclitaxel With or Without Cremophor EL on Cellular Clonogenic Survival and Apoptosis

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**Paclitaxel is currently formulated in a vehicle of 50% ethanol and 50% polyethoxylated surfactant cremophor EL. Cremophor EL has been reported to reverse P-glycoprotein-mediated multidrug resistance (MDR) at doses which are clinically achievable. It has also been reported to have a cytotoxic effect *per se*. In this study we used two different methods to evaluate the survival of cells exposed to paclitaxel with or without cremophor EL and the vehicle alone. Two laryngeal SCC cell lines (UT-SCC-19A and UT-SCC-29) and two ovarian adenocarcinoma cell lines (UT-OC-3 and UT-OC-5) established in our laboratory were investigated. Northern hybridisation was used to study the *mdr-1* mRNA expression of the cell lines. With sensitive Northern analyses, these four lines yielded *mdr-1* mRNA signals of the expected 4.5 kb size and of variable intensity, generally at higher levels than those in the positive control cell line KB. The 96-well plate clonogenic assay was used to obtain the fraction survival data and apoptosis was recorded by time-lapse video microscopy. Both methods indicate that cremophor EL alone has no effect on cellular survival. Consequently, paclitaxel without cremophor EL is as active as paclitaxel with cremophor EL *in vitro*.** 

**Key words: cremophor EL, paclitaxel, apoptosis, cellular survival, clonogenic assay, multidrug resistance**
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## INTRODUCTION

CREMOPHOR EL IS commonly used as an emulsifying agent in the pharmaceutical and foodstuffs industries [1]. It is also the principal constituent of paclitaxel's clinical formulation vehicle. Recently, this polyoxyethylated castor oil has been reported to be a potent modulator of multidrug resistance (MDR) [2–7] and to block the P-glycoprotein drug efflux pump responsible for the MDR phenotype [8]. In addition, some reports have indicated that cremophor EL might have a cytotoxic effect by itself [5, 9]. Therefore, this vehicle may contribute to some of the clinical activity observed with paclitaxel (Taxol®). We have shown previously that 10 nM paclitaxel solution induces accumulation of laryngeal carcinoma cell lines in the $G_2$/M phase of the cell cycle [10] and the cells died by apoptosis [11]. Several studies using human ovarian carcinoma cell lines have confirmed that paclitaxel principally leads to cell death by inducing apoptosis [12, 13].

The present study was performed to assess the effects of paclitaxel with or without cremophor EL or cremophor EL alone on survival of cells with variable *mdr-1* mRNA expression levels. The effects on the cellular survival were studied using the 96-well plate clonogenic assay and with time-lapse video microscopy recording apoptosis.

## MATERIALS AND METHODS

*Cell lines*

Two laryngeal squamous cell carcinoma (SCC) cell lines (UT-SCC-19A and UT-SCC-29) and two ovarian serous cystadenocarcinoma cell lines (UT-OC-3 and UT-OC-5) established at the University of Turku were used in this study. The SCC lines were derived from glottic larynx and

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the ovarian carcinoma cell lines from primary ovarian tumours. All cell lines were used at relatively low passage numbers (10–44).

*Mdr-1 mRNA analysis by Northern blotting*

RNA (ribonucleic acid) was extracted from the cell lines using the guanidinium isothiocyanate protocol with ultracentrifugation through CsCl cushions [14], 15 μg aliquots were size-fractionated in denaturing 1.2% agarose/formaldehyde gels and blotted on to synthetic nylon membranes (GeneScreenPlus™) by capillary transfer. Subsequent to drying and 2 h baking at + 80°C, the RNAs were further UV-fixed to the membrane. The KB cell line RNA was used as positive control in the *mdr-1* mRNA analyses. The hybridisation and washes were carried out as suggested by the membrane manufacturer, under stringent conditions. The *mdr-1* clone pCHP-1, obtained through the ATCC (depositor J.R. Riordan), detects the ca. 4.5 kb human *mdr-1* mRNA. The XbaI-excised insert of this probe was labelled by the random priming method to high specific activity [15]. Hybridisations and washes were done under stringent conditions, as suggested by the membrane manufacturer. Autoradiography was for various exposure times at − 70°C. The X-ray films were not density-scanned because there is no pre-flashing equipment at our disposal. Therefore, the films would not exhibit a linear response to emission energy and any scanning results expressed as numbers would be bound to be arbitrary to an unknown degree. Instead, only the raw data are represented.

*Drug preparation*

All the drugs (paclitaxel, paclitaxel without cremophor EL and cremophor EL) were kindly provided by Bristol-Meyers Squibb. Paclitaxel (Taxol®) was initially dissolved in 0.9% sodium chloride to give a solution of 0.1 mM. Stock solutions were prepared in Ham's F-12 medium containing 10% fetal bovine serum (FBS) to give a solution of 100 nM and stored at − 40°C. Final dilutions of 0.5–3 nM paclitaxel were used for the experiments. Paclitaxel without cremophor EL was initially dissolved in ethanol to give a solution of 1 μM and stored at − 40°C. Stock solutions were prepared in Ham's F-12 medium containing 10% FBS prior to each experiment to give a solution of 100 nM. Cremophor EL was diluted prior to each experiment first in ethanol to give a solution of 1 μM and then immediately in Ham's F-12 medium to give a solution of 100 nM.

*Clonogenic assay*

The 96-well plate clonogenic assay based on limiting dilutions was used. The assay and the cell culture have been described previously [16, 17]. A minimum of three experiments including duplicate plates were performed for each cell line. The cells were harvested with trypsin-EDTA to obtain a single-cell suspension, counted and diluted in Ham's F-12 medium containing 15% FBS. The number of cells plated per well was adjusted according to the plating efficiency (PE) of the cell line. With a stock solution containing 4.167 cells $ml^{-1}$ and diluted in 50 ml of growth medium, a concentration of two cells per well was achieved applying 200 μl of the cell suspension to each well. The desired concentrations of paclitaxel with or without cremophor EL or cremophor EL alone were added to the stock solutions and immediately plated into the 96-well plates. The drugs were allowed to stay in the plates during the whole incubation period. The doses of cremophor EL alone were those used as a solvent in the chosen paclitaxel concentrations. The plates were incubated at 37°C with 5% $CO_2$ for 4 weeks, thereafter the number of wells containing living colonies, consisting of 32 cells or more, was counted using an inverted phase-contrast microscope.

*Time-lapse video microscopy*

One to four days after plating the cells in the culture flasks, medium containing 5 nM paclitaxel (UT-OC-3 and UT-OC-5) or 10 nM paclitaxel (UT-SCC-19A and UT-SCC-29) with or without cremophor EL or cremophor EL only or fresh medium was applied. The medium was equilibrated with 5% $CO_2$ at 37°C in an incubator for 10 min. The culture flask was then capped and transferred to a 37°C heated stage of an inverted microscope (Nikon Diaphot, Nikon Corp., Tokyo, Japan).

Cells were viewed using a phase-contrast optics at 20× objective magnification coupled to a JVC 3CCD KY-F30 video camera (Victor Company, Tokyo, Japan). Time-lapse videorecording was performed so that two successive pictures were taken at 30 s intervals (Panasonic AG-6720A). The videorecorder and the microscope were coupled to a timer (LIBT2, Red Lion, U.S.A.) which lit the microscope lamp for 5 sec in every 30 sec in synchrony with the recorder.

Filming was continued for 96 h. Subsequently the film was viewed frame by frame on a video monitor. The cumulative number of premitoses, mitoses, apoptoses, multinucleated cells and necroses per field were counted at 24-h intervals. Premitosis was considered to have begun when a cell became round and condensed. Mitosis was considered to have ended with the appearance of cell division. A cell was considered to be multinucleated when the condensed cell again became flat without producing a daughter cell. An apoptotic cell death was recorded either when a flat cell condensed rapidly, or an already condensed cell, considered as premitotic, died after violate cytoplasmic pulsation and blebbing. Necrosis was characterised as a non-cycling cell dying after a rapid swelling and rupture of cell membranes. The pH of the medium remained stable during the experiment.

*Data analysis*

The fraction survival data as a function of paclitaxel dose with or without cremophor EL or cremophor EL dose were fitted by the linear quadratic equation $S = \exp[-(\alpha D + \beta D^2)]$. The $IC_{50}$ values, the drug concentration causing 50% inhibition of clonogenic survival, were obtained from the fitted dose–response curves and the AUC (the area under the survival curve) values were calculated by numerical integration. The ratio of apoptoses to mitoses was calculated to compare the growth potential of the non-treated and treated cells.

## RESULTS

We measured previously the sensitivity of the UT-OC-3 and UT-OC-5 cell lines to paclitaxel using the 96-well plate clonogenic assay [18]. The $IC_{50}$ values for paclitaxel in the UT-OC-3 and UT-OC-5 cell lines were 1.3 nM and 1.4 nM, respectively. Paclitaxel sensitivities of the two head and neck cell lines used in this study were tested with the same assay; the $IC_{50}$ value for UT-SCC-19A cells was 2.5 nM and that for UT-SCC-29 cells 1.9 nM. In the current study, we tested the *mdr-1* mRNA expression of the cell lines. Upon optimisation of the Northern assay conditions for *mdr-1* mRNA

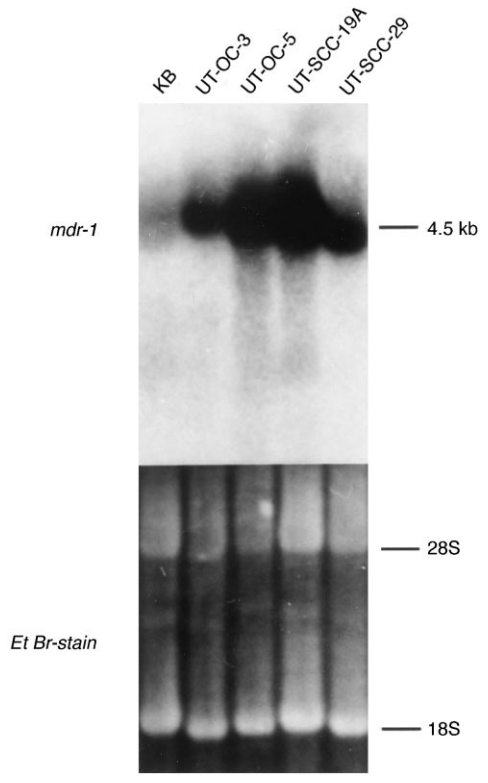


**Figure 1. Analysis of *mdr-1* mRNA by Northern analysis. 5 d exposure of a filter hybridised to a human *mdr-1* cDNA probe, as described in the Materials and Methods, displays clear single-band signals corresponding to the known 4.5 kb size of *mdr-1* mRNA. An ethidium bromide (EtBr) stained image of the filter after transfer (not gel prior to transfer) confirmed both the even loading and quality of the RNAs, as well as the efficacy of the capillary transfer process. The filter was photographed with a measure bar and the location of the band was confirmed with the known location and size of the 28S ribosomal RNA.**

detection, the four cell lines were found to contain *mdr-1* mRNA at levels comparable to (UT-OV-3) or clearly in excess of the positive control cell line, KB (Figure 1).

The clonogenic survival of cells exposed to paclitaxel with or without cremophor EL was tested using identical doses of paclitaxel. The doses of cremophor EL alone were those used as a solvent in the chosen paclitaxel concentrations. The solvent had no effect on cell survival. Consequently, the survival curves for paclitaxel without cremophor EL were similar to the survival curves of paclitaxel with cremophor EL. The AUC values for paclitaxel with cremophor EL were 1.5–3.2 nM and 1.5–3.0 nM for paclitaxel without cremophor EL, respectively. The $IC_{50}$ values for the cell lines calculated from fitted dose–response curves varied from 1.4 to 2.9 nM for both paclitaxel with and without cremophor EL (Table 1). The AUC and $IC_{50}$ values of cremophor EL could not be calculated because the solvent had no measurable effects in these four cell lines.

The results obtained with time-lapse video microscopy are shown in Table 2. The values are given as the ratio of apoptoses to mitoses corresponding to the growth potential of the cell line and moreover, the number of apoptoses per the sum of initial cell number is given. As expected from our previous experiments [11], the control cultures of all cell lines showed frequent mitoses. Apoptoses were also seen in the control cultures, representing 4–35% of the initial cell number. The ratio of apoptoses to mitoses varied from 0.072 to 0.21. Cremophor EL did not affect these values. Paclitaxel with or without cremophor EL acted similarly: the used doses blocked mitosis considerably and the cells died morphologically by apoptosis. No necrosis was detected.

## DISCUSSION

We previously determined the $IC_{50}$ values for paclitaxel in the UT-OC-3 and UT-OC-5 cell lines [18], and here we tested two laryngeal SCC cell lines with the same 96-well plate clonogenic assay to obtain $IC_{50}$ values for paclitaxel. The ovarian carcinoma cell lines studied previously are more

*Table* 1. *The AUC (area under the survival curve values) and $IC_{50}$ (drug concentration causing* 50% *inhibition of clonogenic survival) of paclitaxel with or without cremophor EL*

| Cell line | Paclitaxel with cremophor EL | | Paclitaxel without cremophor EL | |
|---|---|---|---|---|
| | AUC ± S.D. (nM) | $IC_{50}$ ± S.D. (nM) | AUC ± S.D. (nM) | $IC_{50}$ ± S.D. (nM) |
| UT-OC-3 | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.5 ± 0.1 | 1.4 ± 0.1 |
| UT-OC-5 | 1.8 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.6 ± 0.2 |
| UT-SCC-19A | 3.2 ± 0.1 | 2.9 ± 0.1 | 3.0 ± 0.1 | 2.9 ± 0.1 |
| UT-SCC-29 | 1.5 ± 0.1 | 1.4 ± 0.2 | 1.5 ± 0.1 | 1.4 ± 0.1 |

S.D., standard deviation; UT-OC, ovarian adenocarcinoma cell line; UT-SCC, laryngeal squamous cell carcinoma cell line.

*Table* 2. *The ratio of apoptoses to mitoses and the number of apoptoses per the sum of initial cell number (in parenthesis) at* 96 *h of time-lapse video microscopy. The results are given as the average* ± 1 *S.D.*

| | UT-OC-3 | UT-OC-5 | UT-SCC-19A | UT-SCC-29 |
|---|---|---|---|---|
| Control | 0.15 ± 0.07 (13/76, 17%) | 0.072 ± 0.05 (4/100, 4%) | 0.15 ± 0.1 (13/106, 12%) | 0.21 ± 0.07 (56/162, 35%) |
| Cremophor EL | 0.054 (2/33, 6%) | 0 (0/42, 0%) | 0.23 (10/37, 27%) | 0.41 ± 0.03 (33/82, 40%) |
| Paclitaxel* | 5 (20/43, 47%) | 1.43 (20/41, 49%) | ∞ (40/83, 48%) | ∞ (76/98, 78%) |
| Paclitaxel without cremophor EL* | 9 (27/47, 57%) | ∞ (19/39, 49%) | ∞ (27/40, 68%) | 13.2 ± 0.33 (50/86, 58%) |

*UT-OC-3 and UT-OC-5 cell lines 5 nM and UT-SCC-19A and UT-SCC-29 cell lines 10 nM of paclitaxel with or without cremophor EL. S.D., standard deviation. ∞ = no mitoses.

sensitive to paclitaxel than the laryngeal SCC cells. However, in both carcinoma types the $IC_{50}$ values are in the nanomolar range and the clinically achievable concentrations have usually been reported to be clearly higher [19]. Taxol®, the clinical formulation of paclitaxel contains 6 mg/ml paclitaxel in 50% cremophor EL and 50% ethanol. Cremophor EL has been suggested to have biological effects *per se* [5, 9] and to reverse P-glycoprotein mediated MDR [2–7]. In the current study the cytotoxic effects of paclitaxel and cremophor EL were measured in cells exhibiting varying *mdr-1* mRNA expression levels. Paclitaxel with or without cremophor EL had very similar effects on the cell survival and apoptosis. In addition, cremophor EL at similar doses as used as solvent in the chosen paclitaxel concentrations did not have any growth inhibitory effect in these cells.

Immunohistochemical P-glycoprotein staining or *mdr-1* mRNA expression have been detected in 48–63% of ovarian carcinoma biopsies [20, 21]. Cremophor EL potentially enhances antitumour activity, since it has been reported to reverse P-glycoprotein-mediated MDR *in vitro* [2–7]. High concentrations of cremophor EL have markedly reduced the number of human breast and lung carcinoma cells in mitosis without altering paclitaxel induced mitotic delay. The data by Liebmann and colleagues suggest that high levels of cremophor EL can reduce the rate of accumulation of paclitaxel into cells [6]. The results obtained from *in vitro* and *in vivo* experiments have given somewhat varying results. Cremophor EL has been reported to reverse MDR *in vitro* but not in tumour bearing mouse models [22]. Nygren and co-workers have evaluated the efficiency of cremophor EL with a non-clonogenic assay measuring metabolic activity in 492 tumour samples from various malignancies and in one P-glycoprotein deficient myeloma cell line. They reported that cremophor EL and paclitaxel in ethanol were almost identical in cytotoxicity in tumour samples, whereas in the P-glycoprotein negative cell line the vehicle was less cytotoxic [9]. In a recent study the effects of cremophor EL were evaluated using the same assay in parental cell lines and their drug-resistant sublines showing different mechanisms of resistance. The solvent had little effect on the parental cell lines but showed some antitumour activity in the resistant cell lines [23].

Cremophor EL was also without significant cytotoxic effect when measured using a clonogenic assay in human lung carcinoma and glioblastoma cell lines. The *mdr-1* mRNA status of the cell lines was not reported (L. Plasswilm, Kantonhospital, Basel, Switzerland). No increased toxicity after the addition of cremophor EL to chemotherapeutic regimens has been noticed in normal tissues with low endogenous expression of *mdr-1* mRNA [3].

In the current study performed with four human cell lines showing varying *mdr-1* mRNA expression levels all above that found in the KB cell line used as a positive control, cremophor EL had no effect on cell survival measured either with a clonogenic assay or time-lapse video microscopy. These results indicate that cremophor EL concentrations corresponding to those used in clinical formulation of paclitaxel do not have an independent effect on cell survival. This is in agreement with the aforementioned studies, since in cell lines, paclitaxel with and without cremophor EL was highly active and the solvent alone was active only at high concentrations [9, 23], whereas the non-clonogenic model using tumour cells from patients showed that cremophor EL contributed to the efficacy of paclitaxel *in vitro* [23].

Varying results concerning the effects of cremophor EL reported in different studies can be due to different methods and exposure times used. In addition, the information of *mdr-1* status of the studied cells has often been missing. A clonogenic assay is a reliable method to measure the reproductive survival of tumour stem cells capable of clonal expansion. Our study included evaluation of the effects of cremophor EL both after a long, 4-week, drug exposure used in the clonogenic survival assay and after a shorter exposure time of 96 h in time-lapse video microscopy.

In our previous study, performed with seven SCC lines, 10 nM paclitaxel rapidly induced a premitotic block which usually leads to apoptotic cell death [11]. This is in line with other DNA flow cytometry studies showing that exposure of exponentially proliferating cells to paclitaxel rapidly results in a block in the G2/M phase of the cell cycle. The extent of the G2/M block is somewhat dependent on the paclitaxel concentration [6, 10, 24]. Cells in mitotic arrest after exposure to paclitaxel are copious after 24 h when observed with time-lapse video microscopy [10].

Time-lapse photography studies of human breast and lung adenocarcinoma cell lines have shown that high concentrations of cremophor EL reduce the number of cells entering mitosis in the presence of paclitaxel [6]. Moreover, it has been found that once cells progress into mitosis, paclitaxel markedly prolongs the duration of mitosis regardless of the level of cremophor EL in the medium [6]. In the present study time-lapse video microscopy showed that cremophor EL could not induce a premitotic block like paclitaxel with or without the solvent (data not shown).

In summary, cremophor EL, the solvent of the cytotoxic drug paclitaxel, had neither a suppressing nor enhancing effect on cell survival in two adenocarcinoma and two SCC cell lines expressing varying levels of *mdr-1* mRNA. In this study, the lack of antitumour activity of the agents tested was detected both with a clonogenic survival assay after a 4-week drug exposure and with time-lapse video microscopy during a shorter exposure time of 96 h.


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