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# Paclitaxel exposure time determines the nature of the interaction with radiation in HeLa cells: the role of apoptosis

F. Yildiz [a,b], R. Perez [a], J.L. Redpath [a,*]

[a] *Department of Radiation Oncology, University of California Irvine, Irvine, CA 92697, USA*
[b] *Department of Radiation Oncology, Hacettepe University, Ankara 06100, Turkey*

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**Abstract**

The purpose of the present study was to investigate further the mechanisms underlying subadditive and superadditive interactions of radiation and paclitaxel treatments *in vitro*. The protocol studied was a fixed radiation treatment (7 Gy) followed either immediately or after a 10 h delay by paclitaxel treatment. Paclitaxel treatment was exposure to either a fixed dose of paclitaxel (10 nM) for varying time intervals up to 25 h, or varying doses of paclitaxel up to 20 nM for a fixed exposure time of 24 h. The cells used were HeLa. Cell survival was assessed by colony forming ability and apoptosis was measured by flow cytometry. The results show that when the paclitaxel exposure time was 24 h superadditive interactions were observed at all paclitaxel doses. A reasonable correlation between surviving fraction as measured by colony forming ability and apoptosis in the attached cells at the end of paclitaxel treatment was observed. The nature of the interaction of radiation and subsequent paclitaxel treatment is critically dependent on the duration of the paclitaxel treatment. 

*Keywords:* Paclitaxel; Radiation; Apoptosis; HeLa cells

## 1. Introduction

Paclitaxel is a potent anticancer drug with activity against a wide variety of human malignancies [1]. It is a potent microtubule stabilising agent which inhibits mitosis by binding to the N-terminal 31 amino acids of the beta-tubulin subunit of microtubules and induces a G2 arrest in cycling cells [1]. The use of concurrent radiation and paclitaxel therapy is an area of increasing interest in the clinical setting. Past studies of radiation–paclitaxel interactions have revealed the nature of the interaction to be variable and dependent on the cell line, as well as on the paclitaxel concentration and exposure time, radiation dose and the scheduling of the two agents [2–17]. These studies have recently been reviewed [4]. Radiation sensitisation, by pretreating cells with paclitaxel, has been hypothesised to be due to blockage of the cells in the radiosensitive G2 region of the cell cycle [16]. In contrast, radioprotection could occur by holding cells in G2 for a prolonged postirradiation period prior to mitosis, thus allowing more time for repair of radiation damage, as has been hypothesised for the effect of basic fibroblast growth factor-4 [18].

From studies performed in our laboratory using a human laryngeal squamous carcinoma cell line, we have evidence that paclitaxel and radiation can interact in a subadditive manner if radiation treatment is followed immediately by paclitaxel exposure and vice versa [9]. Another published study also found an antagonism of paclitaxel toxicity by X-rays in human breast and lung cancer cell lines [12]. These results indicate that prior radiation treatment can alter paclitaxel toxicity as well as prior paclitaxel treatment altering radiation sensitivity. A second study from our laboratory showed that if an interval is placed between the radiation treatment and initiation of paclitaxel exposure, subadditive, additive or supra-additive interactions can be observed depending on the time interval [19]. Analysis of the data revealed a role for the cell cycle distribution at the time of addition of paclitaxel on the toxicity of the drug, with G1 cells being resistant to paclitaxel-induced cell killing. We have subsequently confirmed this conclusion by examining the response of HeLa cell populations enriched in G1 content by growing to very high density

* Corresponding author. Tel.: +1-949-824-7395; fax: +1-949-824-3566.
*E-mail address:* jlredpat@uci.edu (J.L. Redpath).

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followed by serum starvation, as well as in human skin fibroblasts released from contact inhibition (data not shown). Two other recent studies have also described the role of alterations in cell cycle distribution in the nature of the interaction of paclitaxel and radiation [6,7].

Previous studies in our laboratory were carried out using single dose and time exposure paclitaxel protocols. One of these studies did examine the nature of the interaction as a function of radiation dose [19]. The present study was undertaken to include variable paclitaxel treatment protocols, specifically a fixed concentration of paclitaxel (10 nM) for variable exposure times, and a fixed exposure time (24 h) using variable concentrations of paclitaxel following a fixed dose of radiation (7 Gy).

## 2. Materials and methods

### 2.1. Cell line and culture conditions

The HeLa cell line D98/AH-2 was used for the reported experiments. Cells were grown in minimal essential medium supplemented with 5% calf serum, 2 mM L-glutamine and non-essential amino acids, in humidified incubators containing 5% $CO_2$/95% air and kept at 37°C. The plating efficiency of the cells was in the range 0.6–0.7.

### 2.2. Irradiation

Cells were irradiated using a self-shielded Cs-137 gamma irradiator (J. L. Shepherd). The cells were at room temperature during the irradiation and the dose-rate was 1.75 Gy/min.

### 2.3. Flow cytometry

Cells were prepared for flow cytometric analysis of the cell cycle distribution by standard procedures using propidium iodide staining of the DNA [20]. Analysis was performed using a Becton-Dickinson flow cytometer and cell cycle distributions were analysed using MODFIT software.

### 2.4. Treatment protocol

The general treatment experimental plan was to examine the response of HeLa cells to radiation alone, paclitaxel alone and then radiation followed by paclitaxel. Two paclitaxel treatment protocols were used. The first employed a fixed paclitaxel concentration (10 nM) and exposure times over the range 7–25 h. The second employed a fixed exposure time of 24 h and paclitaxel concentrations over the range 5–20 nM. For radiation followed by paclitaxel, the drug treatment was initiated either immediately after irradiation or after a 10 h delay, conditions under which subadditive and superadditive interactions, respectively, were previously observed [19].

The protocol was to treat exponentially growing cultures of HeLa cells ($0.5–1.0\times10^6$ cells/T-25 flask). Duplicate flasks were treated, one for flow cytometric analysis and the other for the assay of cell survival. At the completion of treatment, the cells from one flask were put into suspension, washed three times with medium, and diluted and plated for colony formation. Eight to ten days later the colonies were fixed with alcohol and stained using Crystal Violet; colonies containing $>50$ cells were then counted and surviving fractions calculated in the usual way. Cells from the second flask were harvested for flow cytometric analysis of cell cycle distribution as well as for assessment of the extent of apoptosis (sub-G1 fraction). In addition, the percentage of floating cells in the culture flasks at the end of treatment was recorded since this endpoint has claimed to be a measure of apoptosis [21]. Cremaphor EL, the diluent in which paclitaxel is supplied, has been previously shown to have no effect on cellular radiosensitivity, cell cycle distribution, or cytotoxicity when used at the concentration present in the reported experiments [13]. Paclitaxel stock (Mead Johnson Oncology Products, 6 mg/ml) was obtained from the UCI Medical Center Pharmacy and was stored in a sterile container at 5°C.

## 3. Results

### 3.1. Fixed paclitaxel concentration with variable exposure times

The data in Fig. 1 show survival curves for HeLa cells treated either with paclitaxel alone (10 nM), or with radiation (7 Gy) followed by paclitaxel, as a function of paclitaxel exposure time. Data are shown for paclitaxel treatment initiated either immediately or after a 10 h delay following radiation treatment. In addition, survival curves corrected for the radiation toxicity are shown. The data represent the average of three separate experiments for each treatment condition. It can be seen that when paclitaxel treatment immediately followed irradiation a subadditive interaction was obtained at all exposure times except 25 h. When the addition of paclitaxel to the irradiated cells was delayed by 10 h a superadditive interaction was seen at all exposure times.

The data in Fig. 2 show the per cent apoptosis, as determined by flow cytometric analysis, as a function of paclitaxel (10 nM) exposure time. It can be seen that the induction of apoptosis was compromised by the radiation when paclitaxel was added immediately after irradiation and the paclitaxel exposure time was up to 15 h.

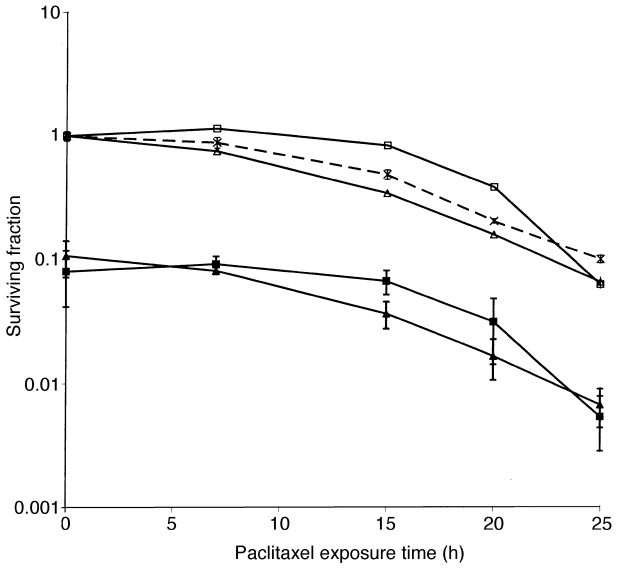


Fig. 1. Survival of HeLa cells as a function of time of exposure to 10 nM paclitaxel. Data are shown for the treatment of asynchronously growing populations with paclitaxel alone (×), 7 Gy immediately followed by paclitaxel (■) and 7 Gy followed by 10 h postirradiation holding prior to initiation of paclitaxel treatment (▲). Survival curves corrected for radiation-induced killing are shown for immediate (□) and delayed (△) paclitaxel treatments.

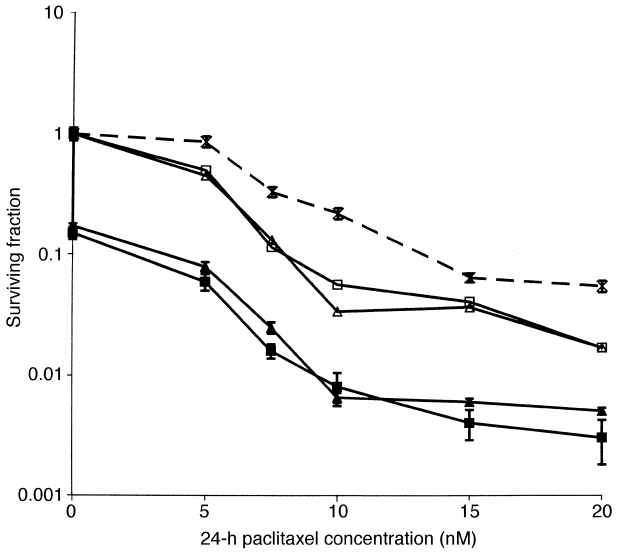


Fig. 3. Survival of HeLa cells as a function of 24 h exposure to a range of paclitaxel concentrations. Data are shown for the treatment of asynchronously growing populations with paclitaxel alone (×), 7 Gy immediately followed by paclitaxel (■) and 7 Gy followed by 10 h postirradiation holding prior to initiation of paclitaxel treatment (▲). Survival curves corrected for radiation-induced killing are shown for immediate (□) and delayed (▲) paclitaxel treatments.

### 3.2. Fixed exposure time (24 h) with variable paclitaxel concentrations

The data in Fig. 3 show survival curves for HeLa cells treated either with paclitaxel alone for 24 h, or with radiation (7 Gy) followed by 24 h of paclitaxel, as a function of paclitaxel concentration. Data are shown for paclitaxel treatment initiated either immediately or after a 10 h delay following radiation treatment. In addition, survival curves corrected for the radiation toxicity are shown. The data represent the average of two separate experiments for each treatment condition. It can be seen that for this treatment protocol, which involves a long paclitaxel exposure (24 h), that a super-additive interaction was seen at all paclitaxel concentrations whether added immediately or 10 h after irradiation. This finding is also in agreement with the 25 h exposure time point in Fig. 1.

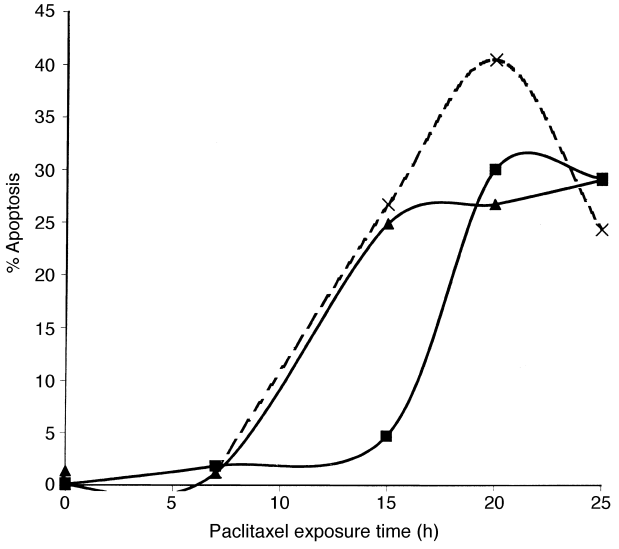


Fig. 2. The percentage of apoptotic cells in the attached cells at the end of the paclitaxel and combined radiation and paclitaxel treatments described in Fig. 1. Symbols are as in Fig. 1.

The data in Fig. 4 show the % apoptosis, as determined by flow cytometric analysis, as a function of paclitaxel concentration for a fixed 24 h exposure time. It can be seen that the induction of apoptosis was seen under both treatment conditions, i.e. when paclitaxel was added either immediately or 10 h after irradiation, for a paclitaxel exposure time of 24 h.

### 3.3. Correlation of apoptosis and cell survival

Figs. 5 and 6 show the dependence of survival on apoptosis for the various treatment groups mentioned above. As can be seen there was a reasonable correlation for most experimental conditions. However, other factors must be involved in the combination treated populations since whilst the apoptotic yield was less than paclitaxel only, the surviving fraction was also less. This is explicable if the major cause of death in paclitaxel only-treated populations is apoptosis and in irradiated cells is reproductive (mitotic) death.

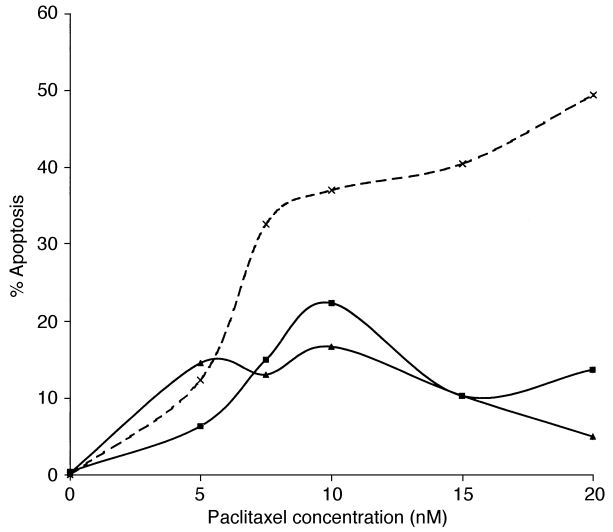


Fig. 4. The percentage of apoptotic cells in the attached cells at the end of the paclitaxel and combined radiation and paclitaxel treatments described in Fig. 3. Symbols are as in Fig. 3.

Previous studies have used floating cells as a measure of apoptosis in the attached population [21]. We therefore measured the yield of floating cells at the end of all treatments as well as examining their DNA content by flow cytometric analysis. We found no correlation between the yield of floating cells and apoptosis in the attached population, particularly at greater paclitaxel exposures where the yield of floating cells was always significantly larger than the apoptotic yield as measured flow cytometrically by the sub-G1 fraction. Furthermore, analysis of the DNA content of the floating cells in combined treatment flasks revealed them to consist principally of G2/M cells (e.g. Fig. 7), which is not surprising since these represent the cell subpopulation which is typically less adherent in tissue culture, with little evidence of apoptosis. In contrast, analysis of the attached cells (as discussed above) did show significant apoptosis, particularly at the higher paclitaxel concentrations (Fig. 7). Thus, at least with HeLa cells under these treatment conditions, measurement of the yield of floating cells does not provide a reliable indicator of apoptotic activity.

## 4. Discussion

We have previously observed that both additive and subadditive interactions can be obtained when paclitaxel treatment follows radiation treatment, and the nature of the interaction is dependent on the time post-irradiation of the addition of paclitaxel [9,19]. We have

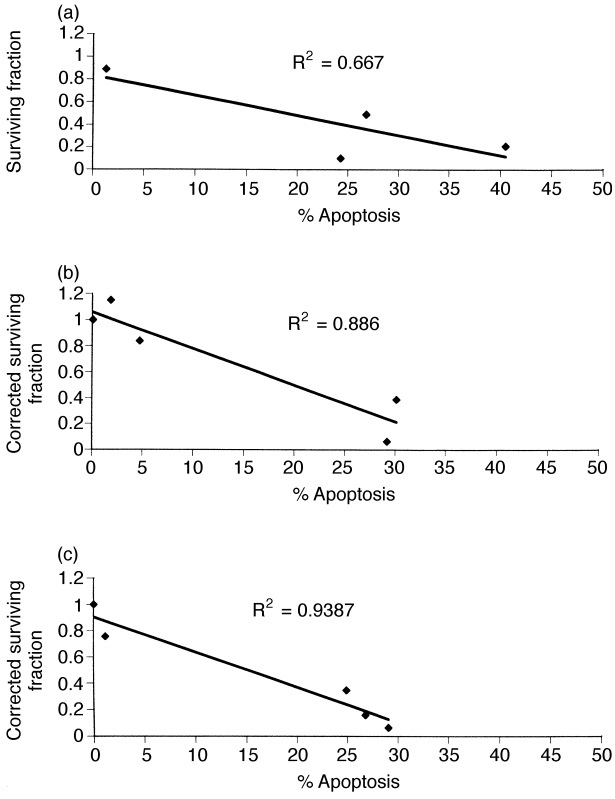


Fig. 5. Correlation of surviving fraction and apoptosis for the data shown in Figs. 1 and 2. (a) Paclitaxel only; (b) 7 Gy immediately followed by paclitaxel; (c) 7 Gy with a 10 h delay prior to initiating paclitaxel.

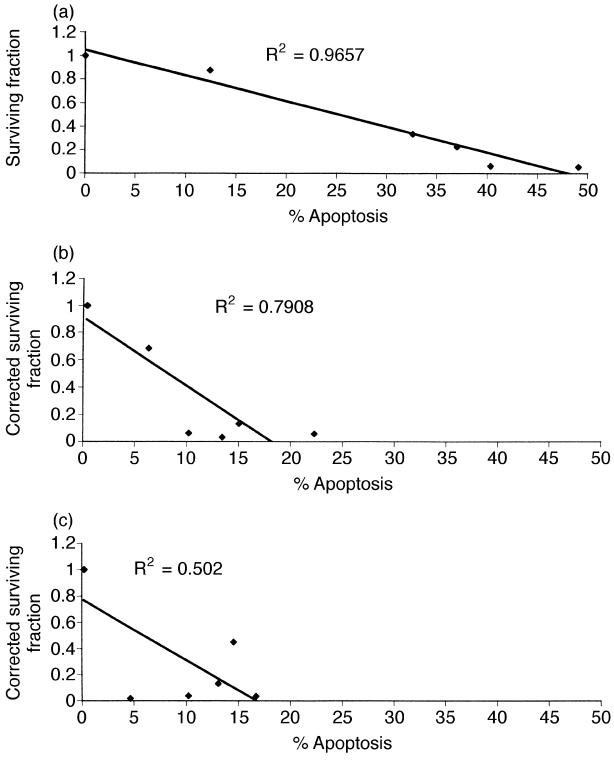


Fig. 6. Correlation of surviving fraction and apoptosis for the data shown in Fig. 3 and 4. (a) Paclitaxel only; (b) 7 Gy immediately followed by paclitaxel; (c) 7 Gy with a 10 h delay prior to initiating paclitaxel.

confirmed these earlier conclusions from single paclitaxel dose/exposure time studies now using a range of exposure times for a fixed paclitaxel concentration of 10 nM (Fig. 1). Our earlier studies all involved relatively short paclitaxel exposure times (< 18 h). In the present study we have used up to 25 h exposure times and note at this longer exposure time subadditivity was lost and only superadditivity was seen (Figs. 1 and 3). Furthermore, superadditivity was obtained over a range of paclitaxel concentrations (5–20 nM) when the exposure

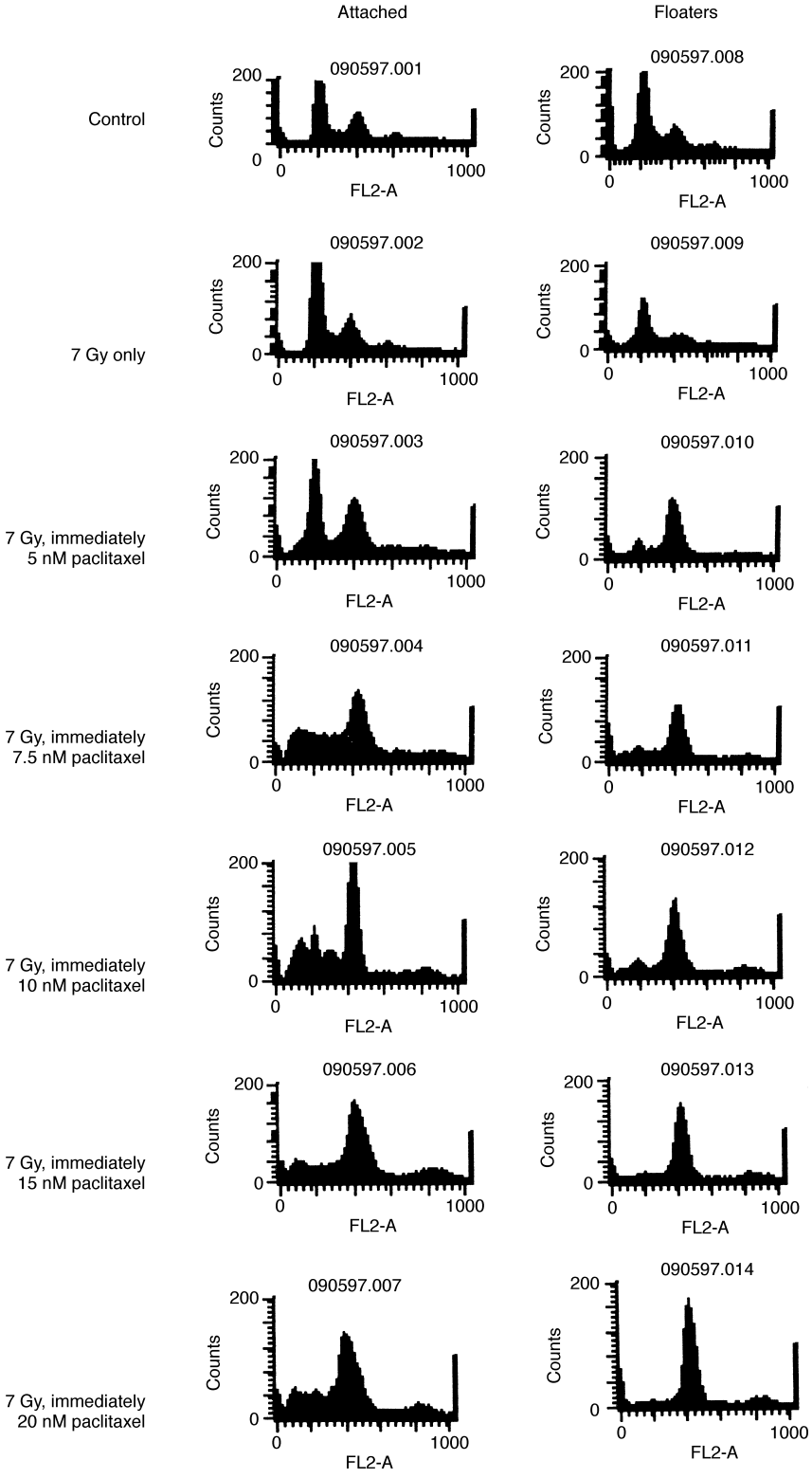


Fig. 7. Comparison of DNA profiles for attached and floating cells at the end of the treatments described for Fig. 3.

time was 24 h (Fig. 3). Previous *in vitro* studies with long-term (>24 h) paclitaxel exposure have involved giving the drug prior to irradiation [5,17]. In the case of melanoma and lung cancer cell lines this resulted in radiosensitisation [17]. However, only additivity was seen in cervical cancer cell lines [5]. What is it about the longer paclitaxel exposure time that can lead to superadditive interactions when given post-irradiation and which appear to be independent of the cell cycle distribution at the time of initiation of the paclitaxel treatment? A clue was provided by the flow cytometric analysis of the attached cell populations at the end of combined treatment. Radiation (7 Gy) alone in these cells revealed little apoptosis (<5%) for up to 24 h post-treatment, whereas paclitaxel alone induced significant apoptosis (50–60%) over a similar time period (Figs. 2 and 4). When paclitaxel and radiation were combined, the yield of apoptotic cells was intermediate. For the combined treatment protocol involving variable treatment times with a fixed paclitaxel concentration of 10 nM, the yield of apoptotic cells was dependent on the paclitaxel exposure time as well as the interval between the end of radiation and initiation of the paclitaxel treatment (Fig. 2). Only at the longer paclitaxel treatment times of 20 and 25 h did the intertreatment interval lose any impact on the yield of apoptotic cells. At the shorter treatment times, little apoptosis was seen for those cells exposed to paclitaxel immediately after irradiation, i.e. conditions where a subadditive interaction was seen (Fig. 1). In the case of combined treatments where the protocol involved variable paclitaxel concentrations for a fixed 24 h exposure time the yield of apoptotic cells was largely independent of paclitaxel concentration and intertreatment interval (Fig. 4). Thus, it would appear that the yield of apoptotic cells was having an impact on the survival of the treated populations. This was confirmed in the analysis shown in Figs. 5 and 6 where a dependence of survival on apoptosis was observed for the various treatment groups.

These *in vitro* studies indicate that when radiation is combined with postirradiation paclitaxel in the treatment of HeLa cells a prolonged paclitaxel exposure of at least 24 h was necessary to obtain a superadditive effect. This effect occurred at all paclitaxel concentrations tested (5, 10 , 15 and 20 nM). This superadditive response appeared to occur even with the schedule of radiation immediately followed by paclitaxel, which we have shown previously [9,19] as well as here (Fig. 1) to result in a subadditive response for paclitaxel exposure times of less than 25 h. The study of postirradiation exposures is relevant to the clinical combination of these agents since even in so-called concurrent treatment even with short-term paclitaxel infusion it is not clear that paclitaxel is always given prior to radiation. In addition, in situations where long-term infusions may be considered in combination with radiation therapy, it is inevitable that post-irradiation paclitaxel exposure will be involved if a daily radiation fractionation scheme is used.

Extrapolation of this observation with HeLa cells to clinical applications of the combination of paclitaxel and radiation in the treatment of cervical cancer would imply that prolonged (>24 h) continuous infusion immediately post-irradiation is more likely to result in a superadditive (radiosensitising) effect than a short-term (3-h) infusion. Many clinical trials in a variety of cancers (breast, lung ovary) which include the combination of paclitaxel and radiation have used a weekly 3 h infusion along with concurrent radiation with some success. Studies of combined paclitaxel, again given as a 3 h infusion, and radiation in cervical cancer are much more limited ([2,22,23] and GOG 9803 and 9804) and the final results of these studies are not yet available. Longer-term infusions of paclitaxel are under study, either alone or in combination with other chemotherapeutic agents [23] or radiation [15]. To the best of our knowledge no combined radiation with long-term paclitaxel infusion trials are currently underway in cervical cancer patients.

The data presented here provide a mechanism associated with the apparent radiosensitisation seen with prolonged paclitaxel exposures following irradiation in HeLa cells, namely that the superadditive effect may be a consequence of an increased contribution of apoptotic cell death at longer paclitaxel exposure times. They also suggest that protocols involving long-term paclitaxel infusion in combination treatment of cervical cancer with radiation should be considered.

## Acknowledgements

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