Pergamon

European Journal of Cancer Vol. 33, No. 8, pp. 1299–1308, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00107-X

Original Paper

Enhanced Radioresponse of Paclitaxel-Sensitive and -Resistant Tumours *In Vivo*

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Paclitaxel is a potent chemotherapeutic drug and also has the potential to act as a radioenhancing agent. The latter is based on its ability to arrest cells in the radiosensitive G_2M phases of the cell cycle; the weight of supporting evidence is derived mainly from *in vitro* studies. Our previous *in vivo* experiments identified enhanced tumour radioresponse predominantly attributable to tumour reoxygenation occurring as a result of paclitaxel-induced apoptosis. The current study investigated whether paclitaxel enhanced the radioresponse of tumours which are insensitive to apoptosis induction, but exhibited mitotic arrest, and compared the degree and kinetics of the response to that in tumours which develop apoptosis. The mouse mammary carcinoma MCa-29 (apoptosis sensitive) and the squamous cell carcinoma SCC-VII (apoptosis resistant) were used. In addition, the study investigated whether paclitaxel affected normal skin radioresponse to determine if a therapeutic gain could be achieved. Paclitaxel enhanced the radioresponse of both types of tumours. In the SCC-VII tumour, radiopotentiation occurred within 12 h of paclitaxel administration coincident with mitotic arrest, where enhancement factors (EFs) ranged from 1.15 to 1.37. In MCa-29 tumour, the effect was greater, EFs ranging from 1.59 to 1.91 and occurred between 24 and 72 h after paclitaxel when apoptosis was the predominant microscopic feature of treated tumours and when tumour oxygenation was found to be increased. The acute skin radioresponse and late leg contracture response were essentially unaffected by prior treatment with paclitaxel. Therefore, by two distinct mechanisms, paclitaxel was able to enhance the radioresponse of paclitaxel-sensitive and -resistant tumours, but not the normal tissue radioresponse, thus providing true therapeutic gain. © **1997 Published by Elsevier Science Ltd.**

Key words: paclitaxel, radiation, mitosis, apoptosis, oxygen, cell cycle

Eur J Cancer, Vol. 33, No. 8, pp. 1299–1308, 1997

INTRODUCTION

PACLITAXEL, A natural diterpene compound, has recently been widely investigated for its anticancer activity. The drug displays significant antitumour action against various tumours of expermiental animals [1–3] and is highly effective in the treatment of many common cancers in man including breast and ovary [4, 5]. The cytotoxic action of paclitaxel has been attributed to its ability to bind to cellular tubulin, thereby interfering with tubulin polymer formation and inducing tubular bundling [6, 7]. These changes in cellular tubulin obstruct cell division, resulting in accumulation of cells in the G_2 and M phases of the cell cycle [7, 8]. In addition to inducing mitotic arrest, paclitaxel has been reported to induce apoptotic cell death. This observation has been made in studies with cell cultures [9, 10] and with *in vivo* tumour systems [1–3]. In analysing cellular changes in 16 different murine tumours treated with paclitaxel, we observed that all tumours showed mitotic arrest, although to various degrees, but only some displayed apoptosis as well [3]. However, only paclitaxel-induced apoptosis, and not mitotic arrest, correlated with antitumour efficacy measured

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Received 12 Sep. 1996; revised 7 Jan. 1997; accepted 14 Jan. 1997.

by tumour growth delay [3]. These findings, as well as flow cytometry analysis of tumours treated with paclitaxel [11], clearly indicated that mitotically arrested cells were not inevitably doomed to die but, in some tumours, were capable of continued survival.

Because of its ability to arrest cells in G_2 and M, the most radiosensitive phases of the cell cycle [12–14], paclitaxel has also undergone testing for its radiosensitising potential [15, 16]. Most cell lines have shown an enhanced radioresponse when pretreated with paclitaxel, having enhancement factors in the range of 1.5 to 1.8 [15, 17]. Both actively proliferating and plateau phase cells were radiosensitised, although the former showed higher radiosensitisation [17]. Generally, cell lines that showed significant G_2M block at the time of their exposure to radiation were sensitised by paclitaxel, and this arrest in G_2M was considered the dominant underlying mechanism. There were, however, cell lines that exhibited G_2M arrest but showed no enhanced cell radiosensitivity [16]. The reasons for this are unclear, but possibilities may include the fact that the radioresponse was dominated by other factors such as intrinsic cellular radioresistance and that a fraction of cells considered to be G_2M were in fact radioresistant late S phase cells.

Few studies have addressed the radiomodifying action of paclitaxel *in vivo*. We recently reported that paclitaxel can enhance the radioresponse of a murine mammary carcinoma [18] and an ovarian adenocarcinoma [19] when the drug is administered to mice within 3 days before irradiation. The drug enhanced the rate of tumour cure, delayed the appearance of tumour recurrences and delayed the rate of tumour growth [18, 19]. The degree of radiopotentiation ranged from 1.2 to more than 2.0 and was greater as the time interval between administration of paclitaxel and tumour irradiation was increased. Histological analysis of tumours treated with paclitaxel showed significant mitotic arrest in cells of both tumour types that peaked 9 h after paclitaxel administration and normalised 1–2 days later [1, 2]. Most mitotically arrested cells in both tumours appeared to die by apoptosis or lytic cell death [1]. Because the greatest enhancement of the radiation response was not seen at the time of the highest mitotic arrest but at later times (48–72 h) after paclitaxel treatment, mitotic blockade could not be the only mechanism responsible for radiosensitisation. Using the MCa-4 tumour, we demonstrated that a dominant mechanism of radiopotentiation was reoxygenation of hypoxic cells within the tumour, which resulted from paclitaxel tumour cell killing and subsequent rapid removal of destroyed cells [18].

Our *in vivo* studies were limited to two tumours that responded to paclitaxel by mitotic arrest, apoptosis and resultant growth retardation. This type of response was associated with significant radioenhancement when paclitaxel was combined with tumour irradiation. There is no information about whether and to what extent paclitaxel enhances the radioresponse of tumours that exhibit mitotic arrest but not apoptosis nor growth delay when treated with paclitaxel. Since a percentage of experimental animal tumours [3] and a proportion of human tumours [20–23] do not respond to paclitaxel by regression or growth delay, it is important to establish whether such tumours would show radiopotentiation when pretreated with paclitaxel. We used a murine squamous cell carcinoma, designated SCC-VII, which exhibits only mitotic arrest when treated with paclitaxel [3], to answer this question and to determine whether the magnitude and kinetics of any response differ from those in tumours that exhibit both mitotic arrest and apoptosis. As a comparison we chose the murine mammary tumour MCa-29, since it exhibits both mitotic arrest and apoptotic cell death in response to paclitaxel [3]. We hypothesised that paclitaxel would induce a smaller degree of radiopotentiation in the SCC-VII tumour.

To provide therapeutic benefit, a radioenhancing agent must potentiate the radioresponse of the tumour more than that of normal dose-limiting tissue. In an earlier study, we found that paclitaxel did not significantly sensitise jejunal mucosa when given 1–4 days before irradiation [24], which implied that in cases in which jejunal damage is dose-limiting, paclitaxel would provide significant therapeutic gain. In the present study, we extended investigations of the influence of paclitaxel on radiation injury to other normal tissues, specifically skin and tissues involved in the leg contracture response to radiotherapy.

MATERIALS AND METHODS

Mice

Male C3Hf/Kam mice bred in our specific-pathogen free facility were used at 3–4 months of age. They were housed 5–6 per cage, exposed to 12-h light–dark cycles, and given free access to sterilised pelleted food (Prolab Animal Diet, Agway Inc., Syracuse, New York, U.S.A.) and sterilised water. Animals were maintained in an American Association for Laboratory Animal Care approved facility, and in accordance with current regulations of the United States Department of Agriculture and Department of Health and Human Services. The experimental protocol was approved by, and in accordance with, institutional guidelines established by the Institutional Animal Care and Use Committee.

Paclitaxel

A stock solution (20 mg/ml) was made by dissolving paclitaxel in equal volumes of absolute ethanol (Aaper, Shelbyville, Kentucky, U.S.A.) and Cremaphor EL (Sigma, St Louis, Missouri, U.S.A.) by sonication for 30 min; it was kept in the dark at 4°C for up to 1 week. The final paclitaxel solution was prepared by diluting the stock solution 1:4 with physiological saline and administered within 10 min at a dose of 40 mg/kg body weight by i.v. injection into the tail vein. This dose of paclitaxel was selected on the basis of our earlier study [1] where it showed strong antitumour efficacy but caused no mortality.

Tumour studies

Tumours. Studies were performed using two transplantable syngeneic tumours. MCa-29 and SCC-VII, in their fourth and seventh isotransplant generations, respectively. These tumours originally arose spontaneously and have been stored in liquid nitrogen. Solitary tumours were established in the muscle of the right leg of experimental mice by injection of 10 µl of a single-cell suspension of 5×10^5 tumour cells. The suspensions were prepared by mechanical disruption and enzymatic digestion of parent tumours, as previously described [25]. Once tumours achieved an 8 mm arithmetic mean diameter, confirmed by

measurement of three orthogonal diameters with Vernier calipers, mice were randomly allocated to treatment groups.

Irradiation procedure. Tumours were locally irradiated, using a small animal irradiator (Atomic Energy of Canada, Ottawa, Canada) consisting of parallel opposed ^{137}Cs sources, at dose rates of 6.85–6.59 Gy/min. A single dose of 15 Gy was given in experiments combining paclitaxel and radiation for MCa-29 tumours. Irradiations were applied either under oxic conditions, that is with mice awake, or under hypoxic conditions, in which mice were anaesthetised with pentobarbital (63 mg/kg intraperitoneal injection) and their tumour-bearing limbs clamped proximally for 2 min before and during irradiation. A single dose of 45 Gy was given under oxic conditions to the SCC-VII tumours. The higher dose of radiation was selected for the SCC-VII tumour because of its radioresistance [26], and in this tumour 45 Gy produces a growth delay similar to that produced by 15 Gy in the MCa-29 tumour. In all scenarios, mice were immobilised on a custom jig, and breathed ambient room air; tumours were centred in a 3 cm diameter circular field.

Histological analysis. To determine the effect of paclitaxel on two known cellular hallmarks of exposure to paclitaxel, mitotic arrest and apoptosis, mice were treated with paclitaxel when tumours were 8 mm in diameter and sacrificed 0, 1, 3, 6, 9, 12, 24, 36 or 48 h later. Tumours were removed and placed immediately in neutral buffered formalin. After fixation, 4 μm histological sections were prepared and stained with haematoxylin and eosin (H&E). Tumours were harvested from 4 untreated and 3 treated mice at each time interval. For each tumour type, a total of 28 mice were used.

The micromorphometric method of scoring the percentage of cells in mitosis or apoptosis has been extensively described and illustrated [1, 27]. Briefly, five non-necrotic fields were randomly selected on each tissue section and examined at 400× magnification. 100 nuclei per field were counted and scored as normal interphase, mitotic, or apoptotic. The mitotic and apoptotic indices are presented as a percentage based on counting 1500–2000 nuclei.

Tumour growth delay. In parallel experiments, mice bearing 8 mm diameter tumours were treated with paclitaxel, radiation or paclitaxel followed by radiation at interagent intervals of 1, 3, 6, 9, 12, 24, 36 or 48 h. Tumour growth after treatment was followed by measurement, each day or every other day, until tumours grew beyond 12 mm in diameter. Treatment groups consisted of 5 to 8 mice, and in a number of instances growth delay experiments were repeated: these were for MCa-29, the oxic growth delays for control, paclitaxel and radiation following paclitaxel at 1, 6, 12, 24, 48 and 72 h interval groups; and for SCC-VII the control and paclitaxel only groups: results of replicate experiments were combined.

The effect of treatment on tumour growth was expressed as absolute growth delay (AGD), defined as the difference (in days) taken to grow from 8 to 12 mm in diameter between treated and untreated tumours. For groups treated with the combination of paclitaxel and radiation, an enhancement factor (EF) was calculated as the ratio of the difference in days taken to grow from 8 to 12 mm in diameter between the combination treated tumours and those treated with paclitaxel alone to the AGD of tumours treated with radiation alone.

Measurement of tumour oxygen. Tumour oxygen measurements were performed using the Eppendorf-6650 histograph (Eppendorf, Hamburg, Germany) in groups of 4 mice bearing 8 mm diameter MCa-29 tumours in the right leg, 0, 4, 9, 24, 48 and 72 h after paclitaxel treatment. Mice were awake breathing ambient room air immobilised in a perspex jig. The polarographic needle probe was introduced into the tumour under direct vision after cutting a small (approximately 2 mm diameter) fenestration in the overlying skin. Probe advancement was configured, using a 0.7 mm forward step followed by a 0.3 mm backstroke, to take a measurement every 0.4 mm, along four 6-mm long tracks. Thus, 60 pO_2 measurements were collected per tumour from a total of 24 mice. Details of the experimental setup have been recently published [28]. For each tumour, the median pO_2 and the percentage of pO_2 values ≤ 5.0 mmHg were determined. At each time interval following paclitaxel, a group mean and mean error were calculated. The Mann–Whitney test was used to estimate the significance of changes in pO_2 following paclitaxel.

Flow cytometry. Mice bearing 8 mm diameter MCa-29 tumours in the right leg were treated with paclitaxel (40 mg/kg i.v.). At times ranging from 0 to 78 h later, tumours were removed from groups of 3–4 mice and fixed in chilled ethanol. Nuclei were isolated and stained with propidium iodide (PI), according to the method described by Carlton and associated [29]. DNA content was measured using an EPICS 752 flow cytometer (Coulter Corp., Hialeah, Florida, U.S.A.) equipped with narrow-beam (5 μm) excitation optics and a quartz flow cell. Excitation was at 488 nm using a 5 W argon-ion laser operating at 200 mW with a 610 nm long-pass filter. Doublets and clumps were excluded from the analysis by gating on a bivariate distribution of the red peak versus integral signal. 30 000 events were collected in each final histogram. Data were analysed using Multicycle software (Phoenix Flow Systems, San Diego, California, U.S.A.) to determine the percentage of cells in each of the G_1, S and G_2M cell cycle phases. A total of 74 mice were used.

Skin studies

Histological analysis. To determine the effect of paclitaxel on mitotic arrest and apoptosis, groups of 3 mice were either not treated or treated with paclitaxel, and 9, 24 and 72 h later the mice were killed and leg skin was removed and fixed in neutral buffered formalin. Tissue sections of 4 μm were prepared and stained with H&E. Mitoses and apoptoses were scored in the epithelial basal layer at 400× magnification. For each animal, 1000 cells were scored as normal interphase, mitotic or apoptotic. Group means were based on scoring 3000 cells, and 12 mice were used for this assay.

Early and late skin response. Groups of 10 mice were given paclitaxel and then the right hind leg exposed to 10 dose groups of local irradiation ranging from 27 to 55 Gy, 9, 24 and 72 h later under ambient air breathing conditions. Acute skin reactions ranging from dry desquamation to complete moist desquamation of the leg [30] were scored by two observers (NRH and KAM) daily from days 14–28 after irradiation. Data were expressed as the radiation dose required to produce moist desquamation of half the treated leg within this time frame in 50% of the mice (DD_{50}) [30]. On days 29–36, epilation within the treated field was scored

as the percentage hair loss and data expressed as the dose required to cause complete epilation in half the mice (ED_{50}) [31]. Late leg contracture was assayed 160 days after irradiation by measuring the length of the untreated and treated hind legs [32]. Data were expressed as the dose required to produce 4 mm leg contracture in 50% of the mice (LCD_{50}). 400 mice were used for this study.

RESULTS

The analysis presented in Figure 1 shows that paclitaxel caused rapid arrest of cells in mitosis in both MCa-29 and SCC-VII tumours: mitotic figures were already visible in 1 h after treatment. The percentage of arrested cells increased rapidly with time, achieving its peak of $15.6 \pm 1.2\%$ at 9 h after paclitaxel in MCa-29 and a higher peak of $24.7 \pm 1.9\%$ at 6 h after paclitaxel in SCC-VII tumours. Following the peak, the percentage of arrested mitoses in MCa-29 gradually declined, returning to background levels 48 h after treatment. In contrast, the decline in the percentage of mitotically arrested cells in SCC-VII was more rapid, returning to the background level 24 h after administration of paclitaxel. Paclitaxel also induced apoptosis in MCa-29 tumours, but the kinetics differed from that of

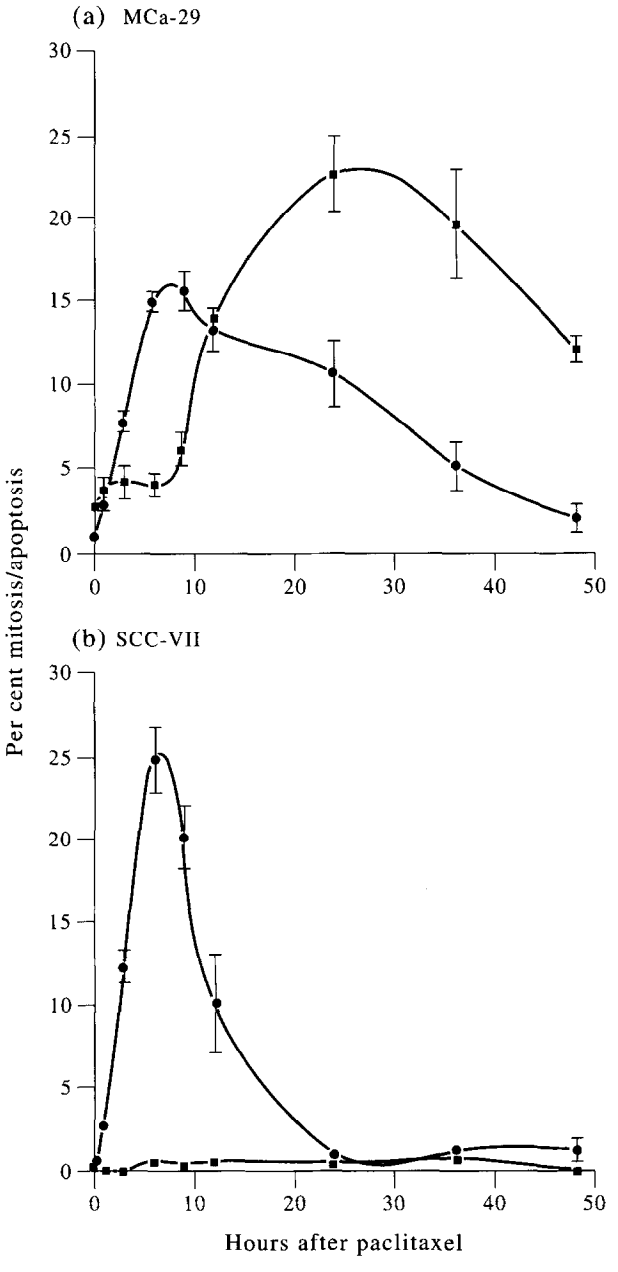

Figure 1. The percentage of cells in mitosis (●), or apoptosis (■), in the MC-29 (a) or SCC-VII tumours (b) following treatment with paclitaxel 40 mg/kg i.v. Shown are the mean and standard errors from 4 untreated and 3 treated tumours at each time point after treatment.

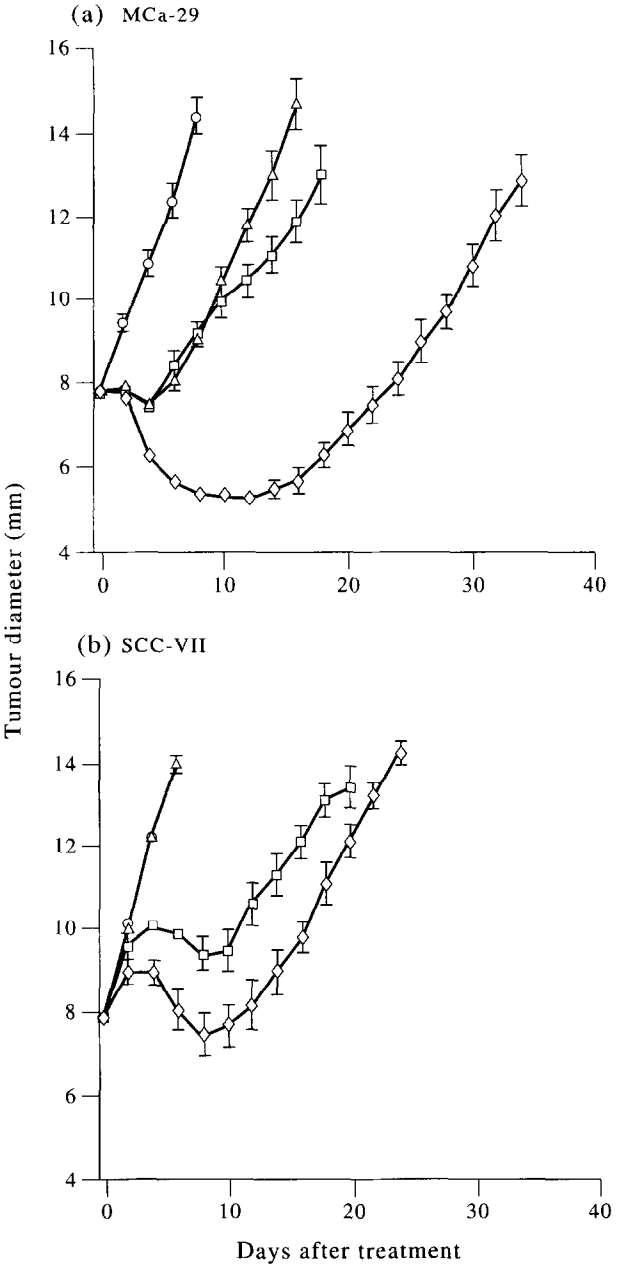

Figure 2. Tumour growth of untreated 8 mm diameter MCa-29 (a) or SCC-VII (b) tumours (○) and of tumours treated with paclitaxel- alone (40 mg/kg i.v.) (△), radiation alone (15 Gy for MCa-29, 45 Gy for SCC-VII) (□), or paclitaxel followed by radiation (at 24 h for MCa-29 or 9 h for SCC-VII) (◇). The 9 h and 24 h time points were selected to illustrate the ability of paclitaxel to potentiate growth delay produced by irradiation in two different tumours.

mitotic arrest. Apoptosis began to increase approximately 9 h after paclitaxel administration and reached its peak of $22.7 \pm 2.3\%$ at 24 h. The percentage then started to slowly decline, although at 48 h it was still above the normal value: $12.1 \pm 0.9\%$ compared to the control value of $2.8 \pm 0.2\%$.

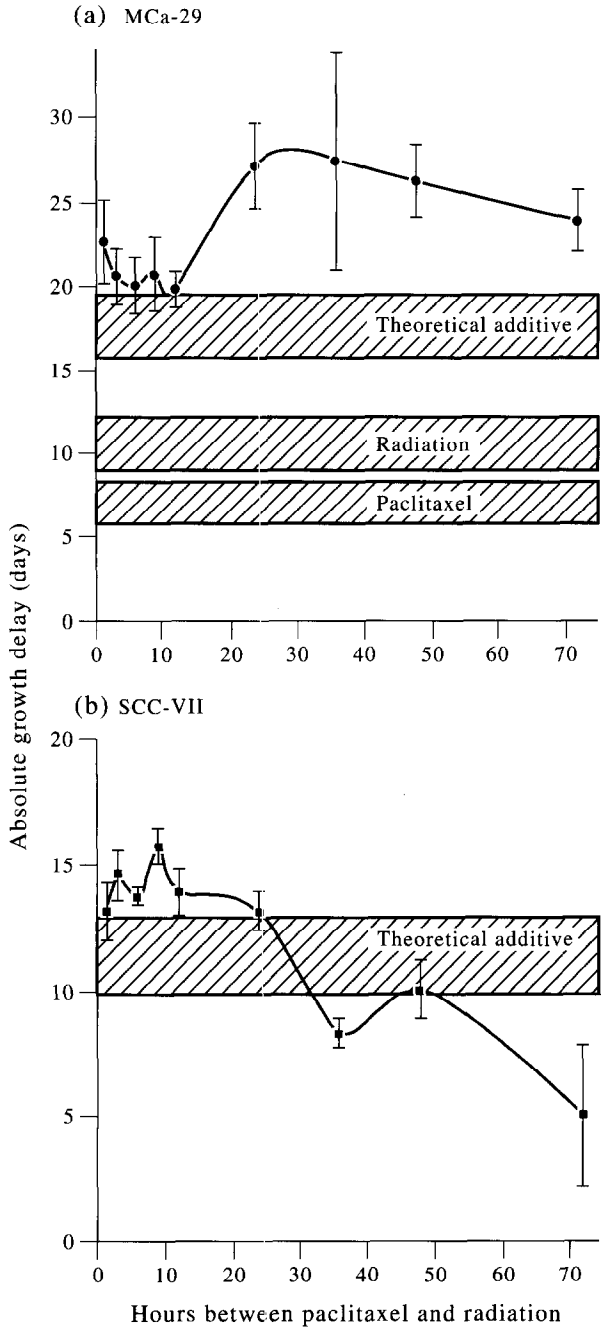

Figure 3. Absolute growth delay (AGD), defined as the time in days for treated MCa-29 (a, ●) or SCC-VII (b, ■) tumours to grow from 8 to 12 mm in diameter minus the time for untreated tumours to grow from 8 to 12 mm, plotted against the interval between paclitaxel administration (40 mg/kg i.v.) and subsequent radiation (15 Gy for MCa-29, 45 Gy for SCC-VII). Hatched bars represent the AGD resulting from treatment with single-agent paclitaxel or radiation, or their sum (theoretical additive effect); bar width corresponds to mean ± standard error. In the case of SCC-VII, the hatched bar indicating theoretical additivity also corresponds to the effect of radiation only.

In contrast to MCa-29 tumours, SCC-VII showed no apoptotic response to paclitaxel.

We next investigated whether paclitaxel would enhance the radioresponse of MCa-29 and SCC-VII tumours and whether such enhancement was associated with the cellular effects of paclitaxel observed above. Both paclitaxel alone and 15 Gy irradiation alone were effective in delaying the growth of MCa-29, but their combination, where paclitaxel was given 24 h before radiation, delayed it more than the additive effect of the individual treatments (Figures 2 and 3). The enhancement factor (EF), calculated at the tumour regrowth level of 12 mm, was 1.91 (Figure 4). Paclitaxel had no effect on the growth of SCC-VII, whereas 45 Gy delayed it by 11.5 days. Although paclitaxel was ineffective by itself against SCC-VII, it potentiated the effect of radiation when given 9 h before (Figure 2), producing an EF of 1.37 calculated at the tumour regrowth level of 12 mm (Figure 4).

The magnitude of the potentiation of radioresponse of both tumours varied depending on the length of time between paclitaxel administration and tumour irradiation (Figure 4). There was a clear time-dependent distinction in the response between the two tumours. In the case of MCa-29, paclitaxel-induced potentiation of the radiation-induced growth delay was small when paclitaxel was administered within 12 h before tumour irradiation. When the interval between paclitaxel administration and tumour irradiation was 24–72 h, potentiation in radiation-induced tumour growth delay was achieved (Figure 3(a)). The EFs, presented in Figure 4, ranged from 1.59 to 1.91, being highest when paclitaxel was administered 36 h before irradiation. In general, more profound enhancement was found when the

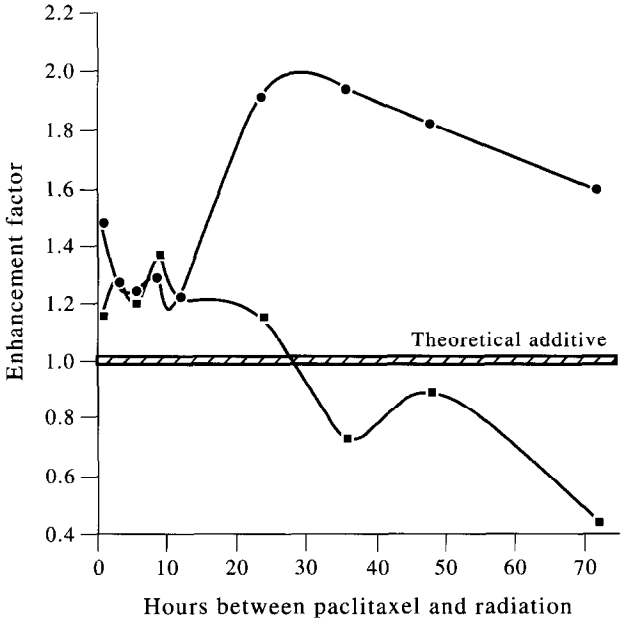

Figure 4. The enhancement factor (EF) for MCa-29 (●) or SCC-VII (■) treated with paclitaxel (40 mg/kg i.v.) followed by radiation (15 Gy for MCa-29, 45 Gy for SCC-VII). EF was calculated as the difference in the time (in days) taken for tumours treated with paclitaxel and radiation to grow from 8 to 12 mm in diameter to that of tumours treated with paclitaxel only, divided by the AGD of tumours treated with radiation only.

cellular response to paclitaxel was dominated by apoptosis rather than mitotic arrest (Figure 1(a)). It should be noted that because only one (15 Gy) radiation dose was used here, the EFs could not be defined as the ratio of isoeffective radiation doses, as was performed in our earlier studies [18, 19]. However, the present analysis provided a similar EF (1.91, see above) to the EF obtained in another study, where paclitaxel increased the MCa-29 tumour cure rate by 1.93 at the isoeffective TCD_{5-} dose (radiation dose yielding 50% tumour cure) when paclitaxel preceded tumour irradiation by 24 h (unpublished).

In contrast to the response of MCa-29, the radioresponse of SCC-VII was enhanced by paclitaxel only when given within 12 h (Figure 3(b)), at which time paclitaxel-induced mitotic arrest was demonstrated (Figure 1(b)). The highest EF (1.37) was observed when paclitaxel preceded tumour irradiation by 9 h. Thus, in the absence of cell killing by paclitaxel only, the observed enhancement was attributable to paclitaxel-induced mitotic arrest. When paclitaxel was given 36–72 h before tumour irradiation, the absolute growth delay was smaller than that obtained by irradiation only. However, it should be noted that at these times after paclitaxel administration, the tumours had grown larger before radiation exposure (8 mm versus 9–10 mm) (Figure 2(b)).

Radioenhancement was higher in MCa-29 than in SCC-VII tumours and was similar to that previously reported for MCa-4 and OCa-1 tumours [18, 19]. Since MCa-29, MCa-4 and OCa-1, unlike SCC-VII, exhibit apoptosis, as well as mitotic arrest, it was logical to assume that greater radioenhancement was mediated by mechanisms other than or in addition to mitotic arrest. We previously postulated that reoxygenation of radioresistant hypoxic tumour cells due to loss of cells by paclitaxel-induced apoptosis was likely to account for increased radioenhancement and provided experimental evidence that this indeed occurred in the MCa-4 tumour [18]. The supporting evidence was that MCa-4 tumours treated with paclitaxel showed an increased pO_2 and that they did not exhibit potentiation of radioresponse when irradiated under hypoxic conditions. To determine whether this mechanism of reoxygenation underlay any induced potentiation of radioresponse in MCa-29 tumour, we treated 8 mm tumours with paclitaxel and then at various times thereafter subjected them to polarographic pO_2 measurements or rendered them hypoxic and then exposed them to 15 Gy local tumour irradiation. pO_2 measurements (Figure 5) showed a decrease in the percentage of values less than or equal to 5.0 mmHg, which was significant 24, 48 and 72 h after paclitaxel at 59.9 ± 7.5, 51.0 ± 9.7 and 38.4 ± 5.3, compared to $92.3 \pm 0.9\%$ in untreated tumours (all $P = 0.021$). There was a corresponding increase in the median pO_2 value, although this achieved statistical significance only at 72 h ($P = 0.021$) when the median pO_2 was 9.8 ± 0.8 mmHg compared to 1.4 ± 0.2 in untreated tumours.

Figure 6 shows the effect of hypoxia on tumour radioresponse. Tumours exposed to radiation only under hypoxic conditions had a lower radioresponse than that in tumours irradiated under air-breathing conditions (see Figure 3(A)). Paclitaxel enhanced the effect of radiation in hypoxic tumours, but the effect was variable as indicated by large standard errors. Radioenhancement was apparent only when paclitaxel administration preceded tumour irradiation by 6, 24 or 48 h, at which times the calculated EFs were 2.69, 2.94 and 2.18, respectively. However, it should be noted that these EFs were calculated from growth delay data with broad error bars (Figure 6), implying considerable heterogeneity in the effect. These findings also show that in MCa-29, paclitaxel can radiosensitise hypoxic tumour cells, at

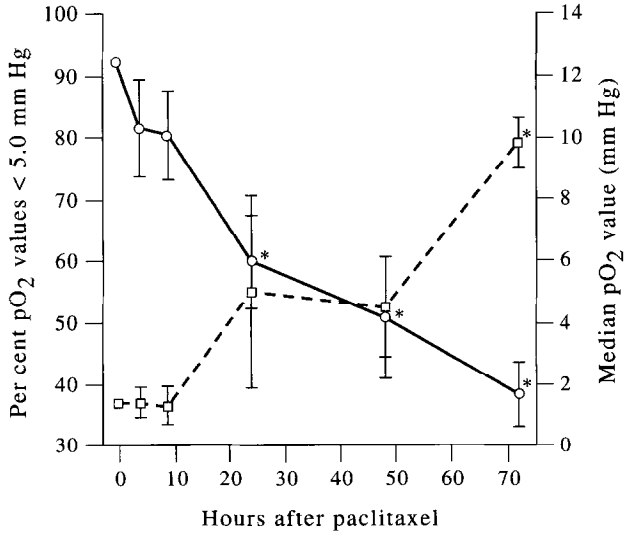

Figure 5. The effect of paclitaxel (40 mg/kg i.v.) treatment of 8 mm MCa-29 tumours on tumour oxygenation, illustrated by the percentage of measured pO_2 values ≤5.0 mmHg (○), or the median pO_2 value (□). Points represent the mean and standard error of four tumours at each time point. Adjacent asterisk indicates value significantly different from untreated control.

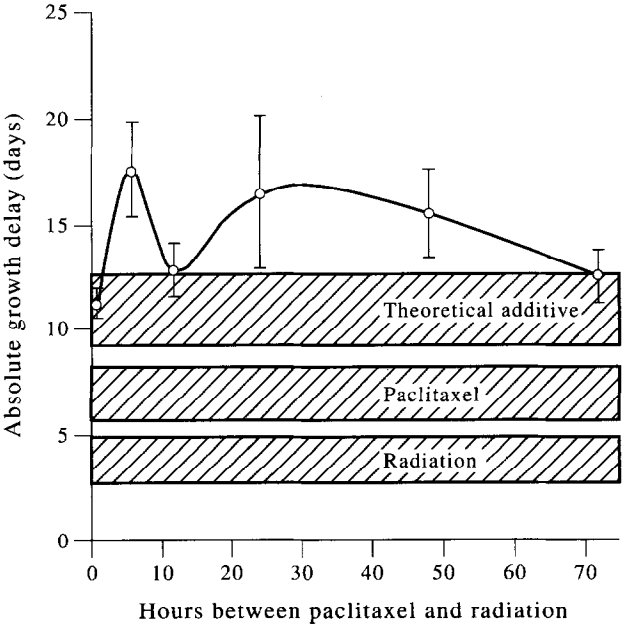

Figure 6. Absolute growth delay (AGD) (see legend of Figure 3 for definition) of MCa-29 tumours treated with paclitaxel (40 mg/kg i.v.) and then irradiated under clamped hypoxic conditions (○). Hatched bars represent the AGD resulting from treatment with single-agent paclitaxel or radiation, or their sum (theoretical additive effect); bar width corresponds to mean ± standard error.

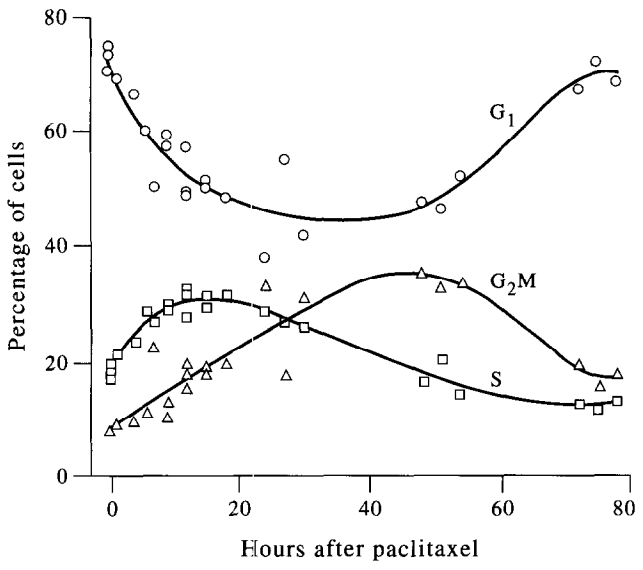

Figure 7. Percentage of cells in the G1 (○), S (△) and G_2M (□) cell cycle phases following treatment of 8 mm diameter MCa-29 tumours with paclitaxel (40 mg/kg i.v.). Points represent the mean from three tumours at each time point, data were fitted with a polynomial function.

least in a percentage of tumours. A possible explanation for the radioenhancement of hypoxic cells is that these cells were arrested in G_2 or M when exposed to radiation. This is partly confirmed in Figure 1 where the percentage of cells in mitosis following paclitaxel peaked between 6 and 12 h and further supported by Figure 7 which shows that the percentage of cells in the G_2M phase was elevated to 33.2 ± 1.4 and 35.7 ± 2.8 at 24 and 48 h, respectively, from a baseline of $8.1 \pm 0.3\%$.

To be therapeutically beneficial when combined with radiation, paclitaxel should not enhance the response of normal tissues or enhance it to a smaller degree than the tumour response. To test this, we investigated whether paclitaxel modified the acute skin response to radiation as assessed by desquamation and epilation, and the late radiation response of the leg using the leg contracture assay.

We first determined the ability of paclitaxel to cause mitotic arrest and(or) apoptosis in skin epithelial cells. The mitotic index of normal mouse leg skin was $0.4 \pm 0.1\%$ and the baseline apoptotic index was $0.2 \pm 0.1\%$. Nine hours after paclitaxel treatment the mitotic index was elevated to $5.9 \pm 0.4\%$ and declined to baseline levels ($0.3 \pm 0.1\%$) 24 and 48 h after treatment. At no time after paclitaxel treatment was there an increase in the apoptotic index which fell to 0.1–0.3% for all groups. Thus, normal skin showed a pattern of response similar to that of the SCC-VII tumour, i.e. early mitotic arrest but no subsequent apoptosis.

Results of the three assays of skin response to the combined paclitaxel plus radiation treatment are shown in Table 1. The desquamation dose (DD_{50}) was 46.7 Gy in mice not treated with paclitaxel and was reduced to 43.9 Gy in mice irradiated 9 h after paclitaxel, when the mitotic index was elevated. The epilation dose (ED_{50}) was also slightly reduced 9 h after paclitaxel at 45.7 Gy compared to 47.1 Gy in radiation-only control mice, but the 95% confidence limits overlapped. As was seen for desquamation, epilation at 24 and 72 h was not notably different from the radiation-only controls. Late radiation-induced leg contracture was unaffected by prior treatment with paclitaxel.

DISCUSSION

Two important issues relevant to the therapeutic application of paclitaxel when combined with radiation were addressed by the experiments described in this study: whether paclitaxel enhances the radioresponse of tumours regardless of whether they are sensitive or resistant to the drug as a single agent and whether the combination of paclitaxel and radiation increases the therapeutic ratio of radiotherapy. The findings of our experiments provided positive responses to both questions.

MCa-29 was used as an example of paclitaxel-sensitive tumours and SCC-VII as an example of paclitaxel-resistant tumours. When 40 mg/kg paclitaxel was given, MCa-29 growth was substantially delayed (Figure 2(a)), while the growth of SCC-VII was unaffected (Figure 2(b)). At the cellular level, however, paclitaxel caused mitotic arrest in both tumours. The kinetics of arrest were somewhat different between the two tumours in that the arrest peaked sooner (at 6 h after paclitaxel) and disappeared earlier (by 24 h after paclitaxel) in SCC-VII than in MCa-29, where it peaked at 9 h and was still present 48 h after paclitaxel administration. The difference in the time of peak mitotic arrest may be related to differences in cell proliferation kinetics between the two tumours. While both tumours exhibited mitotic arrest, it was only MCa-29 that also exhibited apoptosis. Apoptosis appeared after mitotic arrest; it peaked

Table 1. The effect of combination paclitaxel and radiation on skin reaction

Treatment	Desquamation		Epilation		Leg contracture	
	DD_{50}*	DMF	ED_{50}†	DMF	LCD_{50}‡	DMF§
Radiation	46.7 (45.0–48.3)		47.1 (45.4–48.9)		44.6 (41.7–47.4)	
Paclitaxel + radiation (9 h)	43.9 (42.7–45.2)	1.06	45.7 (44.3–47.5)	1.03	44.7 (41.9–49.5)	1.00
Paclitaxel + radiation (24 h)	46.2 (44.9–47.4)	1.01	46.5 (44.7–48.2)	1.02	44.0 (41.4–46.4)	1.01
Paclitaxel + radiation (72 h)	46.8 (45.4–48.1)	0.99	49.4 (47.7–51.3)	0.95	45.3 (42.8–47.4)	0.97

*DD_{50} is the dose of radiation required to produce moist desquamation of half of the leg in 50% of the mice. The 95% confidence intervals are shown in parentheses.

†ED_{50} is the dose of radiation required to produce complete epilation of the irradiated field in 50% of the mice at 29–36 days. The 95% confidence intervals are shown in parentheses.

‡LCD_{50} is the dose of radiation required to produce 4 mm of leg contracture in 50% of the mice at 160 days. The 95% confidence intervals are shown in parentheses.

§DMF is the ratio of the dose in mice treated with radiation alone to that in the combined paclitaxel and radiation treated mice.

at 24 h and was still significantly above the background level 48 h after administration of paclitaxel. Importantly, it was apoptosis and not mitotic arrest that correlated with the antitumour efficacy of paclitaxel. This correlation was previously established using 16 different murine tumours [3].

When paclitaxel was combined with local tumour irradiation, it enhanced the tumour radioresponse of both paclitaxel-sensitive MCa-29 and paclitaxel-resistant SCC-VII tumours. However, the degree of radioenhancement and the time between paclitaxel administration and radiation exposure required to achieve maximum enhancement differed for the two tumours. MCa-29 responded best when the time interval between treatments was long (24–72 h), at which time the EFs ranged from 1.59 to 1.91. In contrast, SCC-VII showed maximum radioenhancement within 3–12 h after paclitaxel where EFs were 1.15–1.37. The differences in radioenhancement between MCa-29 and SCC-VII tumours can be explained on the basis of their different cellular responses to paclitaxel as a single agent. The major difference between the two tumours is that when treated with paclitaxel MCa-29 exhibits apoptosis and SCC-VII does not (Figure 1). We have shown previously that, following paclitaxel treatment of tumours which exhibit both mitotic arrest and mitosis, it is the cells arrested in mitosis that die predominantly by apoptosis [1]. Thus, since in such tumours many of these mitotically arrested cells, which are highly radiosensitive, are destined to die even without being exposed to radiation, the radioenhancement observed in these tumours could not be explained solely on the basis of accumulation of these cells at the time of irradiation. The findings shown in Figure 3(a), that the highest enhancement of MCa-29 radioresponse was not coincident with the peak of mitotic arrest, supports this reasoning. Other mechanisms must thus be considered, primarily reoxygenation of radioresistant hypoxic cells as a consequence of the loss of large numbers of tumour cells to paclitaxel-induced apoptosis. By reducing the total number of tumour cells, more oxygen may remain available for cells that survived the treatment and more oxygen may be brought to the tumour as a result of improved circulation [18, 33].

Three findings in the present study suggest that reoxygenation was a significant underlying mechanism for the observed potentiation of MCa-29 radioresponse. First, maximum radiopotentiation occurred when paclitaxel preceded radiation by 1–3 days, times when large numbers of tumour cells were lost by apoptosis. Second, direct measurements of tumour oxygenation showed that pO_2 increased as the time after paclitaxel administration increased. Increased pO_2 values imply better tumour oxygenation that coincided with enhanced tumour radiopotentiation. Finally, when tumour irradiation was given under hypoxic conditions, either no enhancement of the radiation was observed, or if observed, it was associated with broad heterogeneity. The failure to eliminate fully the potentiation of tumour radioresponse by making tumours artificially hypoxic implies that in this tumour an additional mechanism is responsible for radiopotentiation, perhaps induced synchrony of cells that escaped apoptotic death. This was suggested by flow cytometry findings (Figure 7) which showed that, at the time of maximum radioenhancement, a large percentage of tumour cells were in the G_2M phase of the cell cycle. Thus, both reoxygenation and G_2M arrest are likely to be responsible for the paclitaxel-induced enhancement of radioresponse of the MCa-29 tumour.

Potentiation of radioresponse of MCa-29 was similar to that previously reported for another mammary carcinoma, MCa-4 [18] and ovarian carcinoma OCa-1 [19] both in magnitude and kinetics of development. A common property of these tumours is that they responded to paclitaxel by both mitotic arrest and apoptosis, as well as by significant tumour growth delay [3]. In a study designed to determine mechanisms of paclitaxel-induced radiopotentiation in the MCa-4 tumour, we observed that a dominant mechanism was tumour cell reoxygenation [18]. Taken together, the observations of the present study and those reported previously [18, 19] demonstrate that paclitaxel is capable of inducing potentiation of the radioresponse of tumours that are sensitive to paclitaxel as a single agent. The mechanisms responsible for the enhancement in this type of tumour are tumour cell reoxygenation and mitotic arrest.

Compared to the radioenhancement observed in MCa-29 tumours, the radioenhancement of SCC-VII was smaller (EF = 1.37) and was only observed soon after paclitaxel treatment, when mitotic arrest was present. It is logical to attribute this enhancement to mitotic arrest, rather than to tumour reoxygenation, for several reasons. First, single-agent paclitaxel caused no measurable tumour growth delay, suggesting minimal cytotoxicity. Second, although considerable mitotic arrest occurred after paclitaxel administration, no paclitaxel-induced apoptosis occurred. Finally, SCC-VII tumours treated with paclitaxel increased in size 24–48 h after administration of the drug, and, as we previously observed [34], SCC-VII tumours became progressively more hypoxic as they enlarged as determined by tumour oxygen measurements.

It is unclear why in response to paclitaxel SCC-VII exhibited mitotic arrest but not apoptosis, whereas MCA-29 tumour exhibited both, but the reasons are likely to be multiple including the functional state of genes involved in apoptosis regulation. Recently, we investigated the involvement of *P53* in paclitaxel-induced apoptosis of MCA-4 carcinoma, which responds to paclitaxel similarly to the MCA-29 tumour, and in the inability of paclitaxel to induce apoptosis in SCC VII [35]. Although paclitaxel upregulated *P53* expression in both tumours, the association between this upregulation and induction of apoptosis was unclear. Studies by other investigators have shown that apoptosis induced by paclitaxel may be *P53*-dependent [36] or *P53*-independent [37] or be regulated by other genes such as *BCL-2* [38].

The potential clinical usefulness of any radioenhancing agent depends on its ability to achieve therapeutic gain, that is to maximise antitumour effects while minimising normal tissue toxicity. In these experiments, the response of normal skin was assessed following treatment with paclitaxel and radiation. Maxmimum radiosensitisation, as determined by DD_{50} (DMF 1.06), occurred 9 h following paclitaxel treatment. Importantly, the enhancement was substantially less than that observed at this time for the antitumour effect of paclitaxel and radiation on both SCC-VII (EF = 1.37 at 9 h) and MCa-29 (EF = 1.59–1.91 at 24–72 h). Thus, therapeutic gain was achieved. Interestingly, the increased radiosensitivity of skin 9 h after paclitaxel was coincident with the elevated mitotic index, implying a similar dominant mechanism of radioenhancement as occurred in SCC-VII,

namely G_2M arrest. The enhancement of skin radiosensitivity was, however, smaller than that of the SCC-VII tumour which is in agreement with the lower mitotic arrest of proliferative cells in the skin observed after administration of paclitaxel. In the case of radiation-induced leg contractures, paclitaxel had no influence on radiation injury.

Overall, our data show that paclitaxel can enhance the response to radiation of murine tumours that are either sensitive or resistant to this drug. The enhancement, however, was more pronounced in paclitaxel-sensitive tumours, because in such tumours at least two mechanisms of radiopotentiation were involved—G_2M arrest and tumour reoxygenation—whereas mitotic arrest appeared solely responsible for the radioenhancement in paclitaxel-resistant tumours. This study also established that paclitaxel provides therapeutic gain for both paclitaxel-sensitive and -resistant tumours when combined with radiation, since it did not markedly affect acute skin response or late radiation-induced leg contracture. Therefore, our data demonstrate that paclitaxel could well be a successful potentiator of radiotherapy in the clinic. It should be noted, however, that these studies were performed using single doses of radiation, and that in order to assess more accurately the radiotherapeutic potential of paclitaxel, additional research is warranted where paclitaxel is combined with fractionated irradiation. Based on the present findings, one could anticipate that in order to achieve radiopotentiation of tumours that respond to paclitaxel by significant apoptosis, paclitaxel would be required to be given for several days before initiation of radiotherapy. In contrast, radiopotentiation of tumours that respond to paclitaxel only by mitotic arrest, would be achieved by intermittent paclitaxel irradiation administrations where radiation should be given at the peak of mitotic arrest. In addition to this type of preclinical investigation, studies addressing the duration of paclitaxel administration are also warranted.

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Acknowledgements—This work was supported in part by NIH Program Project Grant CA-06294. The paclitaxel used in these experiments was a gift from Dr John Whisnant (Baker-Norton Pharmaceuticals, Miami, Florida). We are grateful to Lane Watkins and his staff for care of the mice used in this study. We wish to thank Mr Walter Pagel for editorial advice and Mrs Patricia Norfleet for assistance with the preparation of this manuscript.