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Original Paper

Influence of Drug Exposure Parameters on the Activity of Paclitaxel in Multicellular Spheroids

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Paclitaxel is a chemotherapeutic drug which has clinical activity against several solid tumours including ovarian and metastatic breast cancers. Despite extensive preclinical evaluation in several experimental models, no studies have determined the effect of taxol on multicellular spheroids, a model which closely mimics the microregions of solid tumours. MCF-7 human breast carcinoma spheroids were significantly less sensitive than monolayers with IC_{50} values of 14.33 ± 4.51 μM and 0.15 ± 0.09 μM, respectively, following a 1 h drug exposure. Similarly, DLD-1 human colon carcinoma spheroids were also more resistant ($IC_{50} = 33.0 \pm 8.89$ μM) than monolayers ($IC_{50} = 0.36 \pm 0.14$ μM) following a 1 h drug exposure. Paclitaxel was unable to penetrate DLD-1 multicell layers (22 μm in thickness), suggesting that suboptimal drug exposures to paclitaxel occur in cells which reside some distance away from the surface of the spheroid. In the case of DLD-1 spheroids, extending the exposure time to 24 h whilst maintaining the same overall concentration × time ($C \times T$) drug exposure parameters, resulted in greater cell kill ($C \times T$ required to kill 50% of cells = 13.67 ± 3.21 μM/h) compared with 1 h drug exposures ($C \times T$ required to kill 50% of cells = 33.00 ± 8.89 μM/h). Similar results were obtained with MCF-7 spheroids. In monolayers cultures, dose–response curves contained a marked plateau phase (a characteristic feature of cell cycle phase specific drug) and in the case of MCF-7 cells, cell kill was proportional to T as opposed to $C \times T$. These results support the use of prolonged infusions of paclitaxel in the clinic, as extending the duration of drug exposure not only allows more cells to enter sensitive phases of the cell cycle, but would also allow paclitaxel more time to penetrate into avascular regions of solid tumours. It is likely that paclitaxel will only be effective against cells which reside close to tumour blood vessels and combination therapy with bioreductive drugs (such as tirapazamine) may produce synergistic effects *in vivo*. © **1997 Published by Elsevier Science Ltd.**

Key words: paclitaxel, chemosensitivity, spheroids, drug penetration, $C \times T$ exposure

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INTRODUCTION

A CHARACTERISTIC feature of many solid tumours is that their blood supply is inadequate to meet the needs of all cells in the tumour mass [1]. Gradients of oxygen tension, pH, nutrients, catabolites and cell proliferation rates are established, all of which vary as a function of distance from a supporting blood vessel [2, 3]. In addition, many cytotoxic drugs are unable to penetrate through several layers of cells to reach all clonogenic cells within tumours, and drug penetration barriers have been recognised as one of the factors leading to the resistance of solid tumours to many anticancer drugs [4, 5]. Each of these characteristics of solid tumours may influence the final outcome of therapy and experimental models of the tumour microenvironment may contribute to the preclinical evaluation of anticancer drugs. Multicellular spheroids closely mimic many of the characteristic features of solid tumours [6] and provide a valuable tool to study both the efficacy of chemotherapeutic drugs and the kinetics of drug penetration within the controlled environment of an experimental model. Most studies have demonstrated that spheroids are more resistant than monolayers to treatment with anticancer drugs and significant

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drug penetration barriers in multicell spheroids have been identified for several anticancer drugs, particularly doxorubicin [7, 8]. The use of spheroids to identify drug penetration barriers is, however, restricted to those compounds which are either naturally fluorescent (e.g. doxorubicin) or can be radioactively labelled. In preclinical drug development, many compounds do not meet these requirements and there is, therefore, a need to develop alternative assays to assess drug penetration.

Paclitaxel is a clinically active anticancer drug with activity against refractory ovarian cancer, non-small cell lung cancer (NSCLC), metastatic breast cancer and malignant melanoma [9]. Despite extensive preclinical evaluation in both *in vitro* and *in vivo* experimental models, paclitaxel has not, to our knowledge, been evaluated against multicellular spheroids.

The principle aim of this study was to compare the response of MCF-7 human breast carcinoma cells and DLD-1 human colon carcinoma cells exposed to paclitaxel as multicellular spheroids or monolayers. DLD-1 spheroids and monolayers have been extensively characterised and spheroids in particular are well differentiated with tight junctions and glandular structures observed in the viable rim of the spheroid [10]. In addition, we modified and utilised a novel assay (initially described by Cowan and associates [11]) to determine drug penetration through multicell layers which does not require radiolabelled compound or rely on other properties of the compound (i.e. fluorescence). This assay is based upon the addition of paclitaxel to one side of a microporous membrane upon which cells are grown. The appearance of paclitaxel on the other side of the membrane is determined as a function of time using standard analytical techniques (i.e. HPLC). As the diffusion of drugs through multicell layers is time dependent, varying the duration of drug exposure (T) and drug concentration (C) may help to address the problem of poor drug penetration. The influence of drug exposure parameters on the efficacy of paclitaxel against spheroids and monolayers was studied with the aim of determining whether or not a long exposure to low drug concentrations is more active than a short exposure to high drug concentrations whilst the $C \times T$ exposure parameters remained constant.

MATERIALS AND METHODS

Cell culture

DLD-1 human adenocarcinoma of the colon cells [12] and MCF-7 human breast cancer cells [13] were routinely maintained as monolayer cultures in RPMI 1640 medium (Gibco Life Technologies, Paisley, U.K.) supplemented with 10% heat inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate and penicillin–streptomycin (50 IU/ml/50 μg/ml) (Gibco). Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells were routinely tested for mycoplasma contamination (Mycoplasma Detection Kit, Boehringer Mannheim, Lewes, U.K.) and were found to be free of infection. Multicellular spheroids were prepared by seeding approximately 5×10^5 single cells into T25 flasks (Costar U.K. Ltd, High Wycombe, U.K.) that had been coated with 1% agar (Sigma Chemicals, Poole, Dorset, U.K.) and incubated for 24 h at 37°C. Following incubation, the spheroids were added to a spinner flask (Techne) containing 250 ml of RPMI 1640 growth medium and spheroids were kept in suspension by stirring at 50–60 rpm (Techne biological stirrer). When spheroids reached a diameter of approximately 500 μm with a visible necrotic centre, they were harvested from the spinner flask for chemosensitivity studies.

Test compounds

Paclitaxel (Sigma Chemicals, Poole, Dorset, U.K.) was dissolved in DMSO (Fisher Scientific, Loughborough, Leics, U.K.) and diluted in RPMI to a concentration of 1 mM. The final concentration of DMSO in all chemosensitivity assays did not exceed a concentration of 1% (v/v) which is not toxic to cells under the drug exposure conditions used in this study.

Response of DLD 1 and MCF-7 spheroids and monolayers to paclitaxel

Single cell suspensions prepared from monolayer cultures by trypsinisation were counted using a haemocytometer and 2×10^3 viable cells (in 180 μl of growth medium) were plated into each well of a 96-well plate. Following incubation at 37°C in a humidified atmosphere containing 5% CO_2,

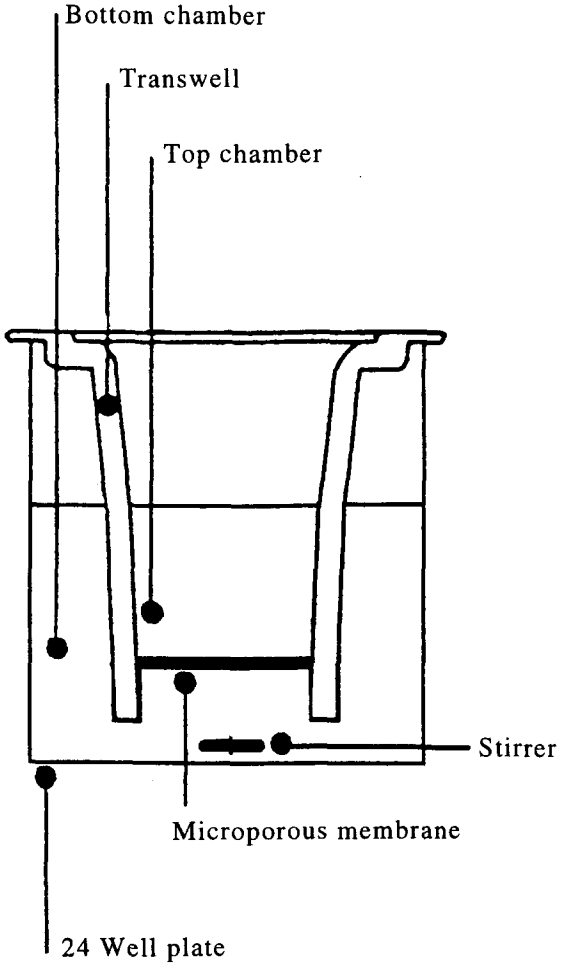

Figure 1. Diagrammatic representation of a transwell insert used to asses the penetration of compounds through multicell layers. Cells are cultured on the microporous membrane (pore size = 3 μm) and paclitaxel added to the upper chamber at a concentration of 100 μM. Samples are taken from the bottom chamber at regular time intervals following drug administration and analysed by HPLC.

cells were exposed to paclitaxel (at a range of concentrations) for 1 h at 37°C. Following drug exposure, monolayers were washed twice with Hank's Balanced Salt Solution (HBSS) prior to the addition of 200 µl of growth medium. Chemosenstivity was assessed following a 5 day postdrug exposure recovery period using the MTT assay, details of which are described elsewhere [14, 15]. A total of eight wells per drug exposure were used and assays were performed in triplicate. Multicellular spheroids (approximate diameter = 500 µm) were exposed to a similar range of paclitaxel concentrations as monolayers for 1 h. Following drug exposure, spheroids were washed twice in HBSS and disaggregated into a single-cell suspension using trypsin–EDTA. Following disaggregation, cells were washed in HBSS and resuspended in growth medium. Chemosensitivity plates were set up as described above and cell survival was determined 5 days after drug exposure using the MTT assay.

Drug penetration studies

The ability of paclitaxel to penetrate multicell layers was assessed using a modified version of the assay described by Cowan and associates [11]. This assay is based upon the chromatographic analysis of paclitaxel as it crosses cells supported on microporous membranes. Transwell inserts for 24-well culture plates (Costar U.K. Ltd.) have been used to measure drug transport across epithelial cells and the apparatus used is shown in Figure 1. DLD-1 cells were added to the top chamber at an initial density of 5×10^5 cells in 100 µl of growth medium. Following incubation at 37°C for 4 h to allow cells to attach to the membrane, 600 µl of growth medium were added to the bottom chamber and the plate incubated either overnight (to produce a single cell layer) or for a further 3 days (to produce a multicell layer) at 37°C in an atmosphere containing 5% CO_2. The medium in both chambers was changed daily. Paclitaxel (100 µM) was added to the top chamber and 10 µl of medium was removed from the bottom chamber at various time intervals thereafter.

Samples were diluted by the addition of 30 µl of medium prior to the addition of 80 µl of acetonitrile containing an internal standard (*N*-benzylbenzamide, 0.75 µg/ml). Following centrifugation at 4000*g* for 4 min to remove precipitated proteins, paclitaxel concentrations were determined by HPLC according to previously published methods [16, 17]. Calibration curves were constructed using the sample preparation procedures outlined above and the ratios of peak area for paclitaxel to peak area for the internal standard were plotted against paclitaxel concentration. Standard curves were linear within the range 0.058–23.5 µM and the limit of detection was 0.05 µM. The change in paclitaxel concentrations in the bottom chamber of the transwell was determined, as a function of time, in both the prescence and absence of multicell layers. Each assay was performed in triplicate.

Influence of drug concentration (C) and time (T) on chemosensitivity

Cell lines were exposed to a range of paclitaxel concentrations for 1 and 24 h as either monolayer cultures or multicellular spheroids. In the case of 24 h drug exposures, drug concentrations were reduced so that the product of concentration times time (i.e. $C \times T$) remained constant with respect to 1 h drug exposures. Chemosensitivity was determined as described above for both spheroids and monolayers and the results were expressed in terms of percentage cell survival against total drug exposures (i.e. $C \times T$), the units of which are µM/h. Paclitaxel is known to be stable in cell culture medium for several days at 37°C [18].

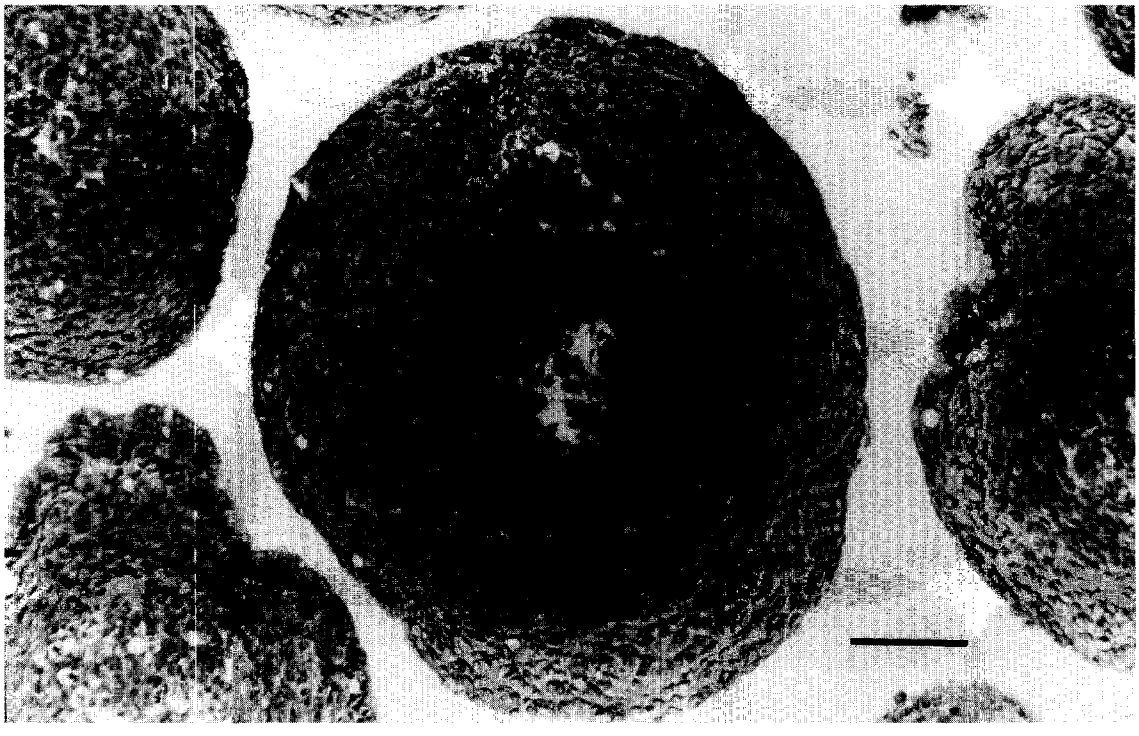

Figure 2. Cross-section through DLD-1 multicellular spheroids. Sections stained with Gomoris Trichrome stain. Bar represents 100 µm.

Histology

DLD-1 spheroids and multicell layers grown in transwells were fixed in Bouins fluid for 1 h, dehydrated in ethanol and embedded in paraffin wax. Sections (5 μm) were cut on a microtome and stained with Gomori's Trichrome (spheroids) and haematoxylin and eosin (transwells). The thickness of the cell layer growing on transwell membranes was determined microscopically using a graduated eyepiece which had been calibrated using a stage micrometer.

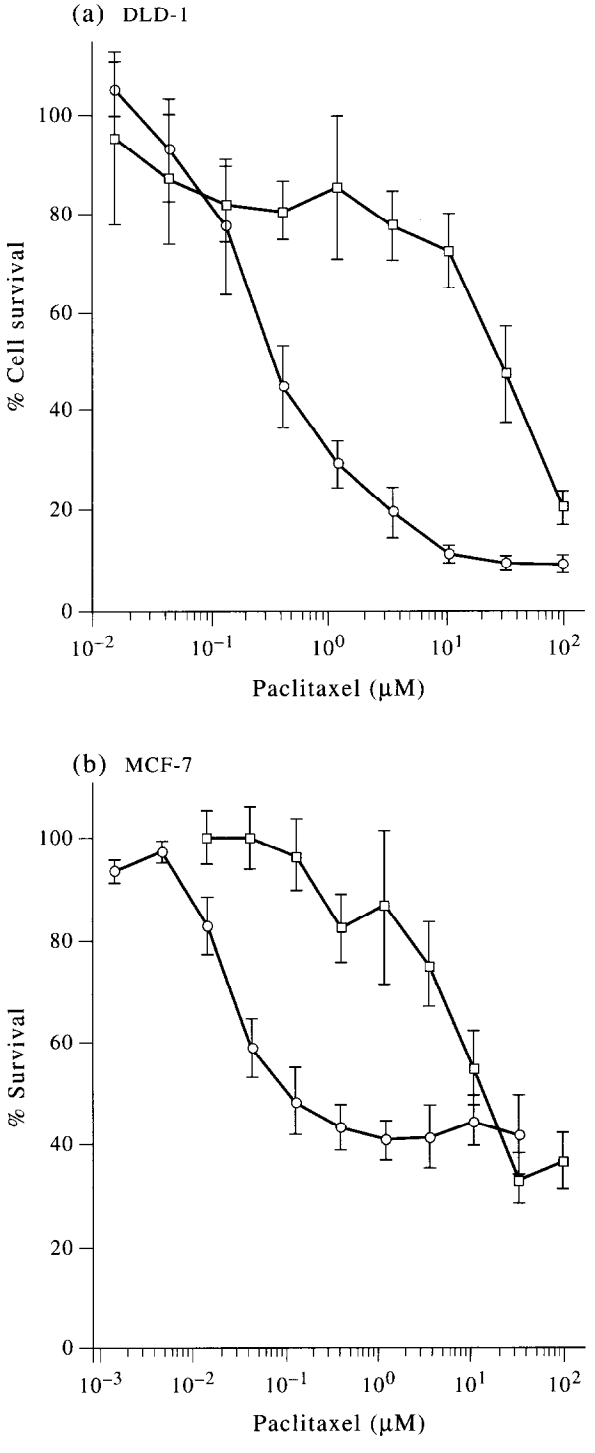

Figure 3. The response of (a) DLD-1 and (b) MCF-7 spheroids (□) and monolayers (○) following a 1 h exposure to paclitaxel. Each point represents the mean of three experiments + standard deviations.

RESULTS

Response of DLD-1 spheroids and monolayers to paclitaxel

Cross-sections through DLD-1 spheroids with a diameter of approximately 500 μm demonstrated that regions of central necrosis exist at the time of chemotherapy (Figure 2). The activity of paclitaxel against DLD-1 and MCF-7 monolayers and spheroids is presented in Figure 3. DLD-1 multicellular spheroids (Figure 3(a)) were significantly more resistant ($IC_{50} = 33.00 \pm 8.89$ μM) than monolayers ($IC_{50} = 0.36 \pm 0.14$ μM) following a 1 h drug exposure (Table 1). Similarly, MCF-7 spheroids (Figure 3(b)) were more resistant than monolayers with IC_{50} values of 14.33 ± 4.51 μM and 0.15 ± 0.09 μM, respectively (Table 1). MCF-7 cells were more responsive to paclitaxel than DLD-1 cells with monolayer IC_{50} values of 0.15 ± 0.09 μM and 0.36 ± 0.14 μM respectively (Table 1). In the case of monolayer cultures, a distinct plateau in the dose–response curve was present in MCF-7 cells (Figure 3(b)) and to a lesser extent in the case of DLD-1 cells (Figure 3(a)). In both cell lines, increasing the dose of paclitaxel has little effect on cell survival within this plateau phase.

Penetration of paclitaxel through multicell layers

Histological, sections through multicell layers following 3 days incubation at 37°C are shown in Figure 4. In the absence of cells, paclitaxel rapidly crossed the membrane and reached equilibrium within 30 min (Figure 5). In the presence of a single cell layer, penetration into the lower chamber was delayed and equilibrium was approached after approximately 360 min. In the caes of multicell layers (22 μm thick), detectable levels of paclitaxel were observed after 30 min incubation although only a fraction of the administered drug was detectable in the medium following 360 min incubation (Figure 5).

Influence of C × T *on chemosensitivity*

The response of DLD-1 and MCF-7 monolayers and spheroids to different schedules of paclitaxel are presented in Figure 6 and Table 1. In the case of DLD-1 monlayer cultures, no difference in cytotoxicity was observed between a 1 h and 24 h exposure period with $C \times T$ values required to kill 50% of cells of 0.36 ± 0.14 μM/h and 0.29 ± 0.10 μM/h, respectively (Figure 6(a)). Twenty-four hour exposure to paclitaxel of DLD-1 cells grown as spheroids was more effective than if the same dose was given over 1 h with $C \times T$

Table 1. Influence of drug exposure parameters on the response of DLD-1 and MCF-7 cells exposed to paclitaxel as monolayers or multicellular spheroids

Cell line	$C \times T$ value required to kill 50% of cells— 1 h drug exposure (μM/h)	$C \times T$ value required to kill 50% of cells— 24 h drug exposure (μM/h)
DLD-1 (monolayer)	0.36 ± 0.14	0.29 ± 0.10
DLD-1 (spheroid)	33.0 ± 8.89	13.67 ± 3.21
MCF-7 (monolayer)	0.15 ± 0.09	0.03 ± 0.01
MCF-7 (spheroid)	14.33 ± 4.51	6.67 ± 1.15

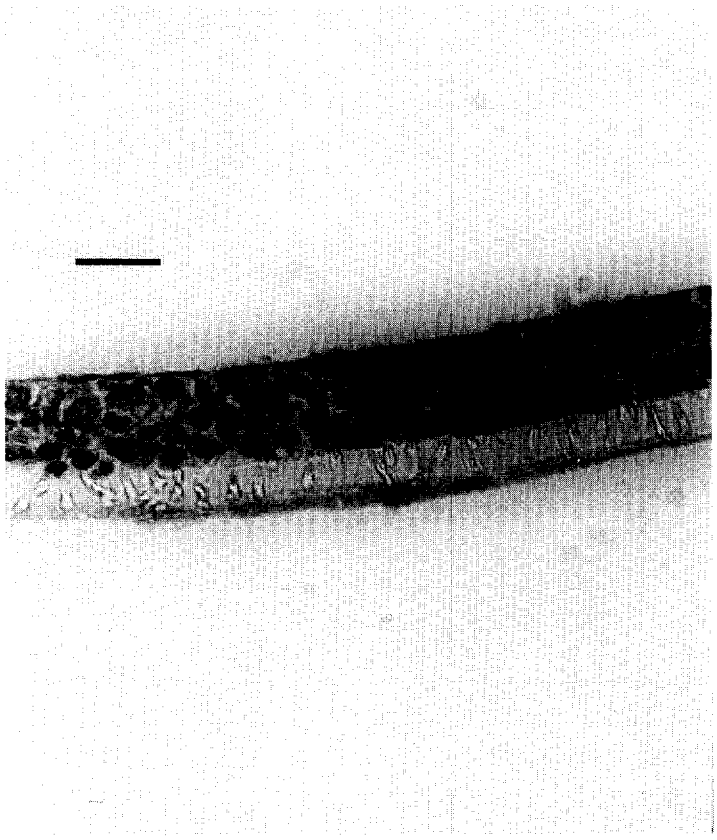

Figure 4. Histological section through a multicell layer 22 μm thick growing on a microporous membrane. Sections are stained with H & E. Bar represents 20 μm.

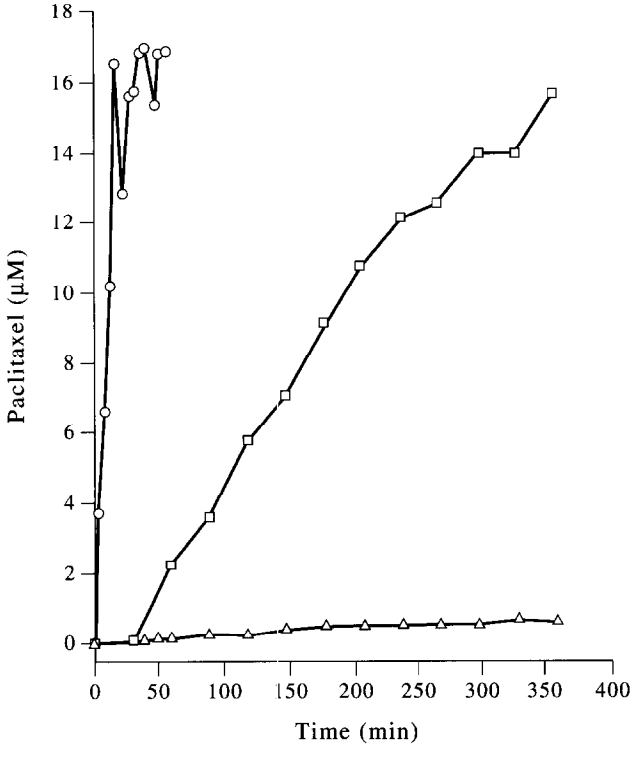

Figure 5. Penetration of paclitaxel across microporous membranes in the absence of cells (○) and in the presence of a single cell layer of DLD-1 cells (□) and a multicell layers of cells (22 μm thick, △).

values required to kill 50% of cells being 13.67 ± 3.21 μM/h and 33.00 ± 8.89 μM/h, respectively (Figure 6(b)). In the case of MCF-7 cells, increasing the duration of drug exposure resulted in increased cell kill in both monolayer (Figure 6(c)) and spheroid cultures (Figure 6(d)). In MCF-7 monolayer cultures, increasing the duration of drug exposure effectively lowered the plateau phase of the dose–response curve (Figure 6(c)).

DISCUSSION

The results of this study clearly demonstrate that both DLD-1 and MCF-7 cells grown as multicellular spheroids are significantly more resistant to paclitaxel than the same cells grown as monolayers (Figure 3). Similar results have been obtained with the mechanistically related vinca alkaloid compounds, vincristine and vinblastine and the relative resistance of spheroids has been attributed to the fact that these compounds do not readily penetrate multicell spheroids [19–21]. Similar conclusions can be drawn in the case of paclitaxel as it does not readily penetrate multicell layers in the assay described in this study (Figure 5). However, it should be stressed that other factors are likely to contribute to the poor response of spheroids including alterations in cell shape (the contact effect [22]) and particularly cell kinetic factors. Paclitaxel is known to cause the polymerisation and stabilisation of microtubules [23], and it has been demonstrated that paclitaxel is a cell cycle phase specific drug with cells in late G2 phase and mitosis being more sensitive than cells in interphase [24]. The presence of a plateau phase in dose–response curves is a characteristic fea-

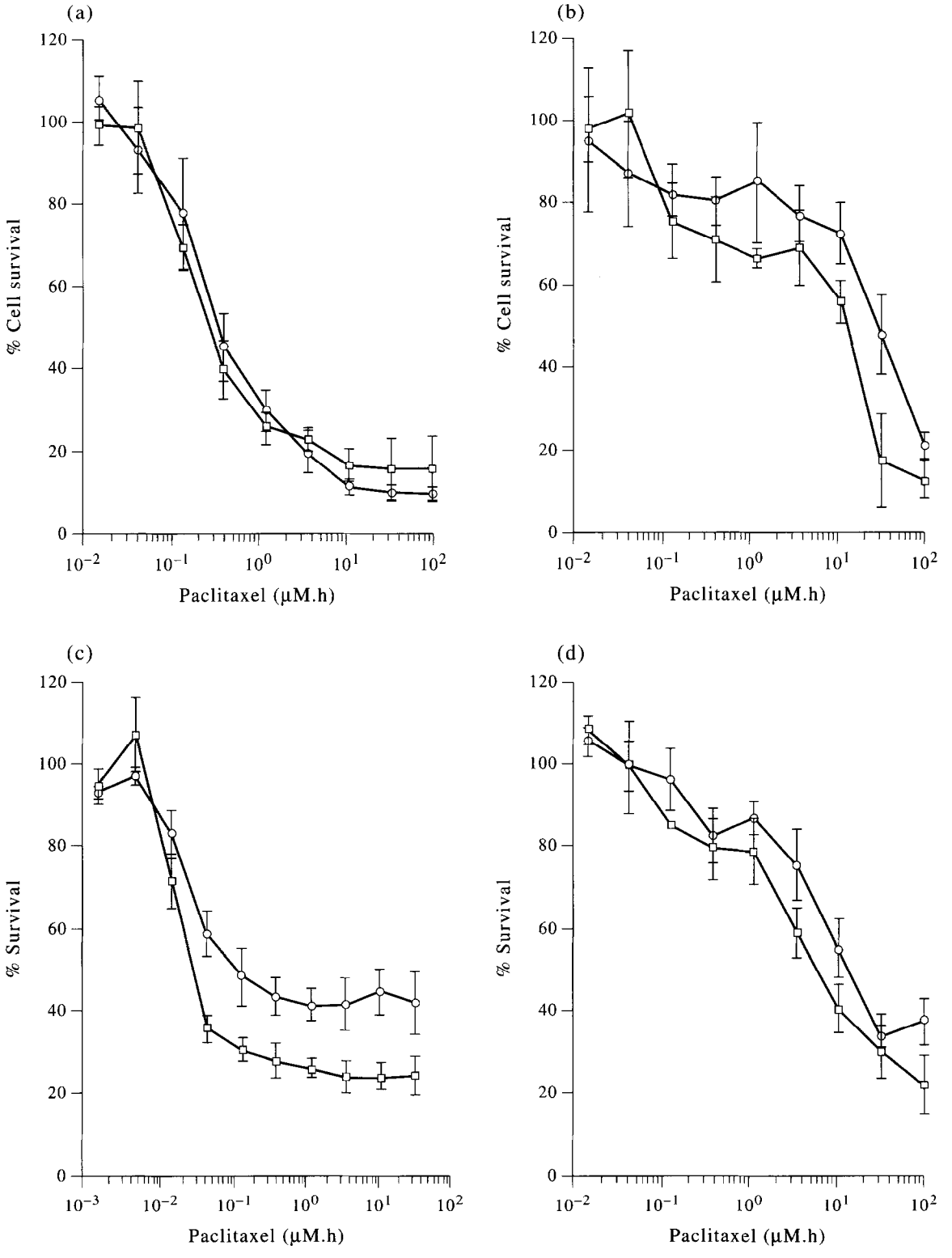

Figure 6. Influence of drug $C \times T$ exposure parameters on the response of DLD-1 (a) monolayers or (b) spheroids and (c) MCF-7 monolayers or (d) spheroids to paclitaxel. In each experiment, cells were exposed to paclitaxel for 1 h (○) or 24 h (□) at doses which ensure that the product of $C \times T$ remained constant for each data point. Values presented are the means of three independent experiments ± standard deviations.

ture of a cell cycle phase specific cytotoxin [25] and paclitaxel fits this pattern well in several cell lines [24, 26, 27], including MCF-7 and DLD-1 cells (Figure 3). In addition, non-proliferating cells are significantly less responsive to paclitaxel than exponentially growing cells [26]. As multicell spheroids are known to contain gradients of cell proliferation [6], the relative resistance of DLD-1 spheroids to paclitaxel compared with monolayers is therefore likely to

be caused by a combination of both poor drug penetration and cell kinetic factors. Whilst the poor penetration of paclitaxel through cellular masses may adversely influence the activity of paclitaxel in solid tumours, this property may conversely be useful if paclitaxel were administered into pleural or ascitic fluid. The slow penetration of paclitaxel from the third space into the tumour and vascular spaces would ensure prolonged therapeutic levels in the fluid and continuous exposure of cells to paclitaxel. The regional delivery of paclitaxel in this way would have the same effect as giving a prolonged infusion, and clinical studies could prove interesting from both a therapeutic and pharmacokinetic standpoint.

Several studies have examined the effect of drug concentration and duration of drug exposure on paclitaxel induced cell kill [24, 28–30], although the majority of studies have varied just one of these parameters, resulting in changes in overall drug $C \times T$ exposure parameters. Valid information on schedule dependency can only be obtained via studies where C or T are varied in such a way that the overall $C \times T$ drug exposure remains constant. Few studies have examined the effects of $C \times T$ within the experimental constraints outlined above, although in CHO cells [24] cell kill was proportional to T, with longer exposure times being significantly more toxic than short exposures (within the same $C \times T$ value). Cell kinetic factors could account for the increased potency of paclitaxel over prolonged drug exposures as more cells would enter the sensitive phase of the cell cycle leading to dose–response curves which are proportional to T as opposed to the product of $C \times T$. In the case of MCF-7 cells, cell kill was proportional to T as opposed to $C \times T$ (Figure 6(c)) and in this cell line, paclitaxel behaves as a classical cell cycle phase specific drug. In the case of DLD-1 cells, however, cell kill was proportional to drug $C \times T$ exposure parameters, with no benefit (in terms of cell kill) seen if paclitaxel was given for a long time at low drug concentrations compared with a short exposure to high concentrations of drug (Figure 6(a)). The fact that taxol-induced cell kill in DLD-1 monolayer cultures was proportional to $C \times T$ (Figure 6(a)) suggests that paclitaxel is not behaving as a classical cell cycle phase specific drug in this cell line. It is of interest to note that paclitaxel can induce exponential dose–response curves in HL-60 cells, suggesting that paclitaxel does not behave as a cell cycle phase specific drug in all cell lines [27]. These effects have largely been attributed to the fact that microtubules are critical for many cellular functions besides mitosis [31], and it is conceivable that paclitaxel induced toxicity occurs by disturbance of other cellular functions, particularly in well-differentiated cell lines. Minor differences in the relative sensitivity of MCF-7 and DLD-1 cells were observed, with DLD-1 cells being more resistant ($IC_{50} = 0.36 \pm 0.14$ μM) than MCF-7 cells ($IC_{50} = 0.15 \pm 0.09$ μM). The P-glycoprotein (Pgp) status of cells has been implicated in the mechanism of resistance of cells to paclitaxel [9], and as DLD-1 cells have elevated levels of Pgp [32] relative to MCF-7 cells (unpublished data), the multi drug resistance phenotype may play a role in determining inherent sensitivity to paclitaxel.

Whilst $C \times T$ experiments have been performed in monolayer or suspension cultures, no studies (to our knowledge) have investigated the effects of drug $C \times T$ exposure parameters on the response of multicellular spheroids. In view of the fact that penetration into a cellular mass is time dependent, $C \times T$ relationships in spheroids may be quite different from those obtained in monolayer cultures, particularly for drugs which have difficulty in penetrating multicell layers. In this study, DLD-1 multicellular spheroids were significantly more responsive to paclitaxel when exposed for 24 h as opposed to 1 h (Figure 6(b)). The $C \times T$ parameters required to kill 50% of cells were 13.67 ± 3.21 and 33.00 ± 8.89 μM/h for 24 h and 1 h timed exposures, respectively, suggesting that cell kill in the spheroid model is proportional to T as opposed to $C \times T$ which is the case in monolayer cultures. Similar results were obtained with MCF-7 multicellular spheroids (Figure 6(d), Table 1). Increased drug penetration may account for the increased sensitivity of spheroids following a 24 h exposure, particularly as the penetration of paclitaxel through multicell layers is time dependent (Figure 5). These results suggest that cytotoxicity for poorly perfused solid tumours may be increased by giving paclitaxel as a prolonged infusion, thereby allowing more time for the drug to penetrate several layers of cells. In normal tissues, which are well vascularised (where penetration barriers to paclitaxel are likely to be less), cytotoxicity may not increase significantly provided that drug exposure parameters (i.e. $C \times T$) remain constant.

In conclusion, the results of this study demonstrate that MCF-7 and DLD-1 multicellular spheroids are significantly less responsive to paclitaxel than the same cells grown as monolayers. Drug penetration barriers are likely to play a major role in determining the relative resistance of spheroids and extending the duration of drug exposure, whilst maintaining the same overall drug $C \times T$ exposure parameters may circumvent some of these problems. These results support the use of prolonged infusions (e.g. 24 h) of paclitaxel in the clinic, although it should be stressed that manipulating $C \times T$ parameters does not result in the complete reversion of spheroid sensitivity to monolayer levels (Figure 6). The cell kinetic evidence, together with the fact that paclitaxel is unable to penetrate multicell layers, points to the fact that cells which reside some distance away from a supporting blood vessel are likely to be relatively resistant to treatment with paclitaxel. Combination therapy with drugs which are activated within hypoxic regions of solid tumours (i.e. bioreductive drugs such as tirapazamine [33]) may lead to synergistic effects, and further studies to evaluate this proposal are currently in progress.

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