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Resistance Mechanisms Determining the *In Vitro* Sensitivity to Paclitaxel of Tumour Cells Cultured from Patients with Ovarian Cancer

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Paclitaxel, a drug which stabilises microtubules, demonstrates marked activity against ovarian cancer. We investigated the sensitivity to paclitaxel of tumour cells from disaggregated solid tumours or tumour-bearing ascites from 7 ovarian cancer patients, and 21 established tumour cell lines (ovarian, melanoma and lung). Response was quantitated by [^{3}H]-thymidine incorporation in 96-well plates or by colony growth. Dose–response curves to paclitaxel were biphasic with a dose-dependent phase providing an IC_{50} value (50% reduction in incorporation) and dose-independent "plateau" phase where the effect was independent of paclitaxel concentration. IC_{50} values ranged from 2.5 to 110 nM with evidence of multidrug resistance in the two most resistant cell lines. The "plateau" killing values varied from 0.1 $\log_{10}$ to >3.4 $\log_{10}$ units reduction, and were found to be significantly correlated ($r = 0.86$; $P < 0.0001$) with logarithmic culture doubling times of the cell lines. Cellular glutathione levels were measured and found not to be significantly associated with response to paclitaxel. The results suggest that the ratio of paclitaxel exposure time to the culture doubling time is a major factor in paclitaxel cytotoxicity. The relationship between tumour cell cytokinetics and paclitaxel pharmacokinetics *in vivo* may therefore be crucial in determining clinical paclitaxel response.

Key words: antitumour, paclitaxel, cell lines, doubling time
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INTRODUCTION

OVARIAN cancer is sensitive to chemotherapy with clinical response rates in the range of 40–60% for platinum-based regimens. Initial response rates, while high, have not translated into permanent remissions, and 5 year survival rates for patients with advanced stage disease are approximately 20% [1]. Recently, ovarian cancer patients who had failed platinum-based chemotherapy have been reported to respond to paclitaxel with a mean overall response rate of 29%, much higher than the rates for other available drugs administered as second-line therapy [2]. Paclitaxel has also been reported to be active in other malignancies such as melanoma [3], breast cancer [4], and non-small cell lung cancer [5]. In spite of these promising results, many patients do not respond to treatment with paclitaxel, suggesting that some tumour cells may be inherently resistant to this agent.

Paclitaxel [6] is an antimitotic agent which stabilises the assembly of microtubules by preventing depolymerisation, thus arresting cells prior to or during mitosis [7–11]. Several types of paclitaxel resistance in cultured cells have been reported. Multidrug resistant cells which overproduce P-glycoprotein are resistant to paclitaxel as well as to drugs such as vincristine, doxorubicin and etoposide [12]. Resistance has been associated with altered α- or β-tubulin subunits, thus altering the kinetics of depolymerisation in the presence of paclitaxel [11]. Paclitaxel resistance may also occur when cells, following treatment, enter another round of DNA replication without dividing (polyploidisation) [13]. Reduction of cellular glutathione by exposure of cultured cells to buthionine sulphoximine also induces resistance to paclitaxel, suggesting a role for glutathione in resistance [14].

The long-term aim of this study was to determine whether paclitaxel responsiveness in the clinic can be predicted by short-term *in vitro* analysis of cultured cells from resected ovarian

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carcinomas. We utilised a [^{3}H]-thymidine (^{3}H-TdR) incorporation method previously applied to melanoma specimens for other drugs [15] to determine the sensitivity to paclitaxel of cells from either enzymatically disaggregated ovarian tumour tissue or ovarian tumour ascites. We have compared these results with corresponding data from a panel of 21 cell lines derived from ovarian cancer, lung cancer and melanoma. The extent to which cell cycle kinetics, multidrug resistance mechanisms and cellular glutathione levels affect sensitivity to paclitaxel *in vitro* has been examined.

MATERIALS AND METHODS

Materials

Paclitaxel samples were kindly provided by Bristol-Myers Squibb (Wallingford, Connecticut, U.S.A.) and by Dr Cynthia Jensen, Department of Anatomy, University of Auckland. α-modified minimal essential medium (α-MEM) and fetal bovine serum (FBS) were obtained from GIBCO/BRL New Zealand (Auckland), insulin–transferrin–selenium supplement from Boehringer-Mannheim (Germany). [^{3}H-methyl]-thymidine (^{3}H-TdR) was obtained from DuPont/NEN Products (Boston, Massachussetts, U.S.A.), mouse epidermal growth factor (EGF) and β-oestradiol from Sigma (St. Louis, Missouri, U.S.A.). Other sources are as previously cited [15].

Patients and tumour samples

Samples of solid tumours or of ascitic fluid were taken from patients undergoing surgery for advanced ovarian cancer. Formal consent was obtained from all patients using guidelines approved by the Auckland Area Health Board Ethics Committee. Immediately after extirpation, fresh tissue was taken to the histopathology laboratory and a portion was placed into ITS medium (α-MEM supplemented with insulin 10 μg/ml, transferrin 10 μg/ml and selenium 10 ng/ml) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml). Solid tumour specimens were disaggregated immediately, or after overnight storage at 4°C in those instances where they were received late in the day. Normal, adipose or grossly necrotic material was removed, and the tumour tissue minced finely using crossed scalpels. Cells were released by digestion of tissue (at 50 mg/ml) with collagenase (1 mg/ml) and DNAase (50 μg/ml) with continuous agitation (stir bar) at 37°C. After 1 h, the suspension was sampled and assessed for the release of refractile cells by phase contrast microscopy. If tissue breakdown was inadequate, digestion was continued further for 1 h. Cells were collected by centrifugation, washed twice in ITS medium and counted using a haemocytometer. Cytospins were prepared for the determination of malignant cells. Cells were also fixed and stained with propidium for examination by flow cytometry as previously described [16]. Characterisation of the proliferating fraction in culture was carried out after 7 days of culture by labelling the cells with ^{3}H-TdR for the last 24 h. Cytospins were prepared for radioautography (emulsion dipping method) and labelled cells identified cytologically.

Cell lines

The melanoma cell lines SK-MEL-28, SK-MEL-2, SK-MEL-5, M19-MEL, MALME-3M, LOX-IMVI, the ovarian cell lines SK-OV-3, OVCAR-8, OVCAR-4, OVCAR-5 and the lung cancer cell lines DMS-114, H23, H522, EKVX and H460 were kindly provided by Mr Richard Camalier, National Cancer Institute, U.S.A. (NCI). The New Zealand melanoma cell lines NZMI, NZM5, NZM6, NZM7 and NZM8 were developed from pathologically confirmed malignant melanoma. None of the patients from whom melanoma tissue had been obtained had received prior radiotherapy or chemotherapy. A full characterisation of lines NZM1 to NZM7 has been reported elsewhere [17]. NZM8, an aneuploid line (2.2 × diploid), was derived from a male with recurrent melanoma (skin nodule) who had received no previous chemotherapy. The New Zealand ovarian line NZOV1 was developed from an ascites fluid from a patient with papillary serous cystadenocarcinoma of the ovary who had received five cycles of carboplatin (1 year prior to sampling), six cycles of chlorambucil (6 months prior to sampling) and megestrol acetate at the time of ascites collection. NZOV1 displayed the cytological characteristics of a papillary carcinoma and cytogenetic analysis indicated a modal chromosome number of 80.

Chemosensitivity assay

Ovarian tumour cell primary cultures were set up in agarose-coated 96-well plates [15] in ITS medium supplemented with FBS (5% v/v), EGF (5 ng/ml), oestradiol (10 nM), penicillin (100 units/ml) and streptomycin (100 μg/ml) in 96-well plates (125 μl/well). Control cell suspensions were set up in quadruplicate at 2-fold dilutions from 940 to 7500 cells/well. Dilution series of paclitaxel were prepared in 3-fold dilution steps at six times the required final concentration in a master 96-well plate using an 8-channel pipette, and 25 μl was transferred to culture wells containing 7500 cells. Cultures were incubated for 7 days in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. ^{3}H-TdR (20 Ci/mmol; 0.04 μCi/well), thymidine and 5-fluorodeoxyuridine (each at a final concentration of 0.1 μM) were added in medium to cultures (20 μl/well) 24 h before harvesting the cultures. Cells were aspirated on to glass fibre filters using a multiple automated sample harvester (LKB Wallac Oy, Finland). The filter discs were washed for 15 s with water, dried, and the amount of tritium retained quantitated as dpm by liquid scintillation. Because the relationship between the number of cells plated and the dpm obtained was not necessarily linear, the relationship between dpm and cells plated in control cultures was used to convert the observed dpm in paclitaxel-treated cultures to equivalent cells/plate. These values were used to construct the dose–response curves. Cell lines were cultured in 96-well plates in α-MEM (except for NZOV1, which was cultured in ITS medium with EGF and oestradiol) containing FBS (5% or 10%, depending on the requirements of the line) penicillin and streptomycin. The seeding density was optimised for each cell line in preliminary experiments, which established a linear relationship between the number of cells inoculated and the amount of ^{3}H-TdR incorporated. Paclitaxel was added as above (and also over lower concentration ranges), and the plates were incubated in 5% CO_2 in air for 5 days unless otherwise indicated. ^{3}H-TdR, thymidine and 5-fluorodeoxyuridine were added in medium to cultures 6 h before harvesting the cultures. Cells were released from the plastic by the addition of pronase (0.5 mg/ml final concentration) dissolved in Ca^{2+}- and Mg^{2+}-free PBS containing 2 mM tetrasodium EDTA. After incubation at 37°C for 1 h, cells were aspirated on to glass fibre filters and the filter discs washed, dried and counted as above. Because radioactivity was related directly to cell number, dose–response curves were derived directly from dpm values. The limit of detection was 20 dpm and values below this figure were set arbitrarily at 20 dpm. IC_{50} values were obtained from duplicate assays [15] and the maximal logarithmic reduction of ^{3}H-TdR incorporation was also determined. Pearson correlation

cofficients were calculated using SigmaStat (Jandel Scientific, San Rafael, California, U.S.A.).

Clonogenic assays

Cells (5×10^5 per 60 mm culture plate) were seeded and incubated for 24 h in growth medium. The medium was then aspirated and fresh medium containing paclitaxel (0–2 μM) was added to the cells. After incubation for 24 h, plates were rinsed with PBS and non-adherent cells collected. Adherent cells were released from the plastic by trypsinisation, combined with the non-adherent cells, washed once in medium and counted in a haemocytometer. Cells (100 or 1000) were plated in fresh medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 10–14 days. The medium was removed, colonies were stained with methylene blue, and colonies containing more than 50 cells were counted.

Determination of culture doubling time

Cells were subcultured from exponential phase cultures and grown for 1, 2 or 3 days before being harvested and counted in a haemocytometer. A regression line was drawn through the points on a semi-logarithmic scale and used to calculate doubling time, and the standard errors of each regression provided an estimate of the error of the doubling time. This value was, on average, ±18% of the measured doubling time.

Measurement of glutathione content

The method was based on a published method [18] but modified (Dr W.R. Wilson, Department of Pathology, University of Auckland Medical School, personal communication) to allow the assay in 96-well plates. Cells were suspended in 0.4 μM 5-sulphosalicylic acid and frozen until assay. Glutathione standards were prepared in 0.4 μM 5-sulphosalicylic acid. Samples were thawed and loaded in triplicate (50 μl/well) in 96-well plates together with appropriate standards at 4°C. To each well was added 100 μl of a freshly prepared reaction mixture containing 0.22 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), 0.3 mM NADPH, 1.5 units/ml glutathione reductase and 1 mM EDTA in 0.1 M sodium phosphate, pH 7.5. After incubation for 10 min at room temperature, absorbance was determined using a plate reader (reference wavelength 630 nm, test wavelength 410 nm). If the absorbance was above 1.2, the sample was diluted and re-analysed. A regression line was derived from the standards and used to calculate glutathione content per well. Cell volume was estimated on freshly cultured cells using an electronic particle counter (Coulter Electronics).

RESULTS

Chemosensitivity of freshly cultured ovarian cancer cells to paclitaxel

We obtained tumour cells from 13 tumour specimens from 10 patients with ovarian cancer, established the malignant character and histological type of each of the specimens using standard histopathological procedures, and determined the ploidy by flow cytometry. We tested cells for chemosensitivity by exposing them to paclitaxel for 7 days at concentrations up to 2 μM and quantitating the surviving cells by ^{3}H-TdR incorporation (Figure 1). ^{3}H-TdR incorporation by control cultures ranged from 1200 to 14000 dpm per well. For three of the samples, incorporation was independent of the number of cells seeded, and these were discarded from the analysis. Labelled control cultures of OV 13, OV 14 and OV 15 were subjected to radioautography and labelled cells were identified cytologically as malignant. The characteristics of the remaining cultures are shown in Table 1. Over the concentration range tested (25–2000 nM), inhibition was constant within the error of the assay (±0.1 $\log_{10}$ units). These "plateau" inhibition values ranged from 0.1 to 0.9 $\log_{10}$ units and are shown in Table 1.

Chemosensitivity of cell lines to paclitaxel

In an attempt to explain the unusual dose–response curves obtained for the above primary cultures, we exposed five ovarian, 11 melanoma and five lung tumour lines to paclitaxel for 5 days and quantitated the remaining proliferative cells by ^{3}H-TdR incorporation (Figure 1). The dose–response curves were found to be biphasic on a semi-logarithmic plot, with a linear decrease in incorporation up to a particular drug concentration, above which no further decrease was evident. Further assays were carried out at lower paclitaxel concentrations to determine the IC_{50} value, the drug concentration required to reduce ^{3}H-TdR incorporation to 50% of the control. These values, which had a mean coefficient of variation of ±15% in repeat assays, are shown in Table 2. The "plateau" values, corresponding to the maximum effect, are also shown in Table 2 and had a mean coefficient of variation of ±15% in repeat assays.

Relationship of paclitaxel exposure time to toxicity

We exposed four of the cell lines to paclitaxel for 1, 2, 3 or 4 days (with ^{3}H-TdR added over the last 6 h of incubation) before harvesting and measuring ^{3}H-TdR incorporation. A family of biphasic curves was obtained, showing an increased paclitaxel effect at longer exposure times (Figure 2). Cytologic examination of the cell suspensions at each of these times showed a high percentage of cells with abnormal mitoses. In a separate experiment, we investigated the stability of paclitaxel in culture using a bioassay. After culture for 3 days in the presence of paclitaxel, growth medium was removed and assayed for remaining cytotoxicity by ^{3}H-TdR incorporation assays with NZM7. Within the limits of the assay, paclitaxel was stable under the culture conditions used.

In order to determine whether clonogenic survival curves exhibited the same shape as did the ^{3}H-TdR dose–response curves, four cell lines were exposed for 24 h to paclitaxel, using the same culture conditions as used for the ^{3}H-TdR assay (apart from the exposure time). Survival curves were also approximately biphasic with IC_{50} <25 nM (Figure 3).

Relationships of culture doubling time and glutathione content to paclitaxel sensitivity

We measured the cell culture doubling times for each cell line in order to determine whether they were related to paclitaxel sensitivity. Values ranged from 19 to 100 h (Table 2) with an average standard error of determination of ±18% of the value. Doubling times were not correlated with IC_{50} values, but were significantly correlated ($r = 0.86$; $P < 0.001$) with the maximal or "plateau" degree of inhibition (Figure 4). We also determined glutathione levels corrected for cell volume, for a number of the cell lines (Table 2), but these values were not significantly correlated with either IC_{50} values or "plateau" inhibition.

DISCUSSION

The dose responses of a number of cell lines to paclitaxel, as assayed by ^{3}H-TdR incorporation, can be approximated by the intersection of two straight lines, the first defining the IC_{50} and the second defining the maximum or "plateau" reduction of

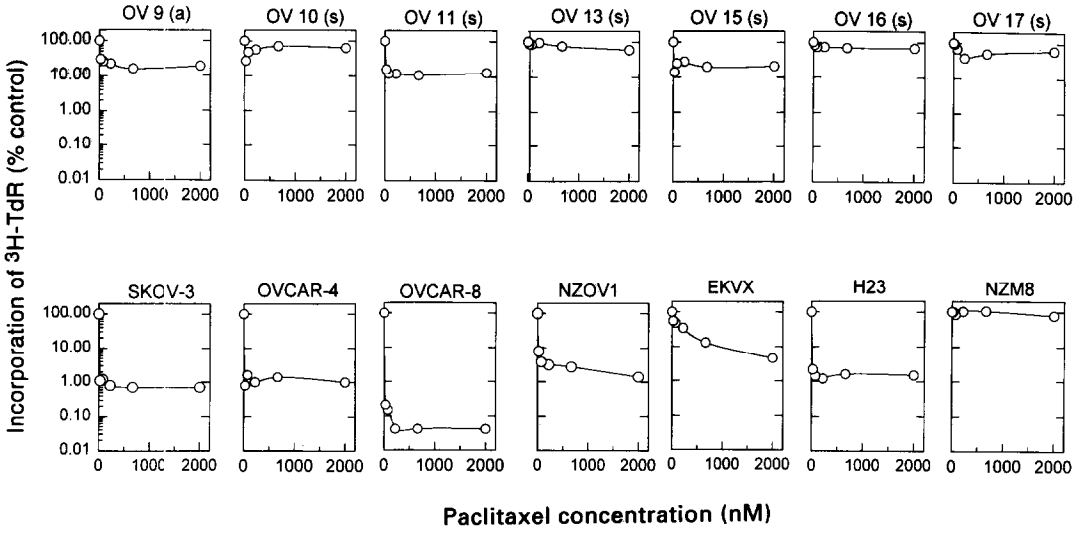

Figure 1. Effect of paclitaxel on ^{3}H-TdR incorporation. Upper panel: primary cultures of ovarian tumour cells from solid tumours (s) or from ascites fluid (a). Lower panel: cell lines.

Table 1. Tumour cells in primary culture

Patient	Age (years)	Tumour			Plateau* (log_{10})
		Reports	Stage	Ploidy	
OV 9	47	Poorly differentiated serous adenocarcinoma of ovary		Aneuploid	0.6 (a)
OV 10	62	Papillary serous tumour of ovary with focal proliferative activity	IV	Aneuploid	0.3 (s)
OV 11	32	Poorly differentiated serous adenocarcinoma of ovary	III	Diploid	0.9 (a) 0.9 (s)
OV 13	82	Poorly differentiated serous adenocarcinoma of ovary	III	Aneuploid	0.1 (s)
OV 15	50	Poorly differentiated serous adenocarcinoma of ovary	IIIc	Not analysed	0.7 (s)
OV 16	65	Moderately differentiated adenocarcinoma consistent with metastatic serous adenocarcinoma of ovary		Aneuploid	0.1 (s)
OV 17	38	Poorly differentiated serous adenocarcinoma of ovary		Not analysed	0.2 (s)

* Maximum decrease in ^{3}H-TdR incorporation (log_{10} units) following exposure to paclitaxel of cells from ascites (a) or solid tumours (s).

incorporation. In the case of the primary cultures, a similar shape of curve is obtained except that the slope of the descending line is not defined. The clonogenic survival curves (Figure 3), within the error of determination, are similarly biphasic, as are those reported by others [14, 19]. Clonogenic survival curves might be expected to be related to ^{3}H-TdR incorporation curves since the first is related to the number of cells outside mitotic arrest, while the second is related to the number of S-phase cells.

A hypothetical model for generation of the biphasic paclitaxel response curves is illustrated in Figure 5. It is assumed in this model that killing in response to paclitaxel occurs only during metaphase arrest, while the rate of progress through other phases in the cell cycle is close to normal. Over the range where reduction of ^{3}H-TdR incorporation is paclitaxel concentration-dependent, it is hypothesised that a proportion of cells undergo paclitaxel-induced irreversible arrest once they enter mitosis, while the remaining cells continue through mitosis to another cell cycle. Paclitaxel is stable under the conditions used in this study, preventing cells from recovering at a late stage of incubation. Over the "plateau" concentration range where reduction of ^{3}H-TdR incorporation is paclitaxel concentration-independent, all cells undergo irreversible paclitaxel-induced arrest or death once they enter mitosis. The number of S-phase cells remaining with time (which is reflected by ^{3}H-TdR incorporation) is the resultant of entry into S-phase of remaining G_1-phase cells and the departure of S-phase cells into G_2/M phase, and is, therefore, independent of paclitaxel concentration. However, the number of remaining S-phase cells will be strongly dependent on exposure time, as supported by results in Figure 2.

Table 2. Tumour cell lines in culture

Line	IC_{50} value* (nM)	Plateau † (log_{10})	Doubling time (h)	Glutathione content (mM)
OVCAR-4	2.5	2.0	34	
OVCAR-5	5.1	1.0	27	
OVCAR-8	4.0	≥3.4	24	
SKOV-3	4.4	2.3	44	11
NZOV1	3.7	1.0	65	
NZM1	2.7	1.4	60	4.9
NZM5	2.5	1.8	30	18
NZM6	3.5	2.3	34	8.5
NZM7	6.5	2.7	24	7.7
NZM8	N.D.	0.1	100	
SK-MEL-2	4.2	2.0	32	
SK-MEL-5	3.8	2.0	26	
SK-MEL-28	4.4	1.7	27	
M19-MEL	3.6	2.1	35	
MALME-3M	3.7	2.0	43	
LOX-IMVI	2.6	2.5	25	41
H460	2.8	≥3.4	19	34
EKVX	110	1.3	72	12
H23	2.6	1.9	25	8.3
H522	3.2	≥3.2	20	39
DMS-114	3.1	2.4	27	9.7

* IC_{50} values. N.D. = not defined because plateau was less than 50% reduction.
† Maximum decrease in ^{3}H-TdR incorporation (log_{10} units).

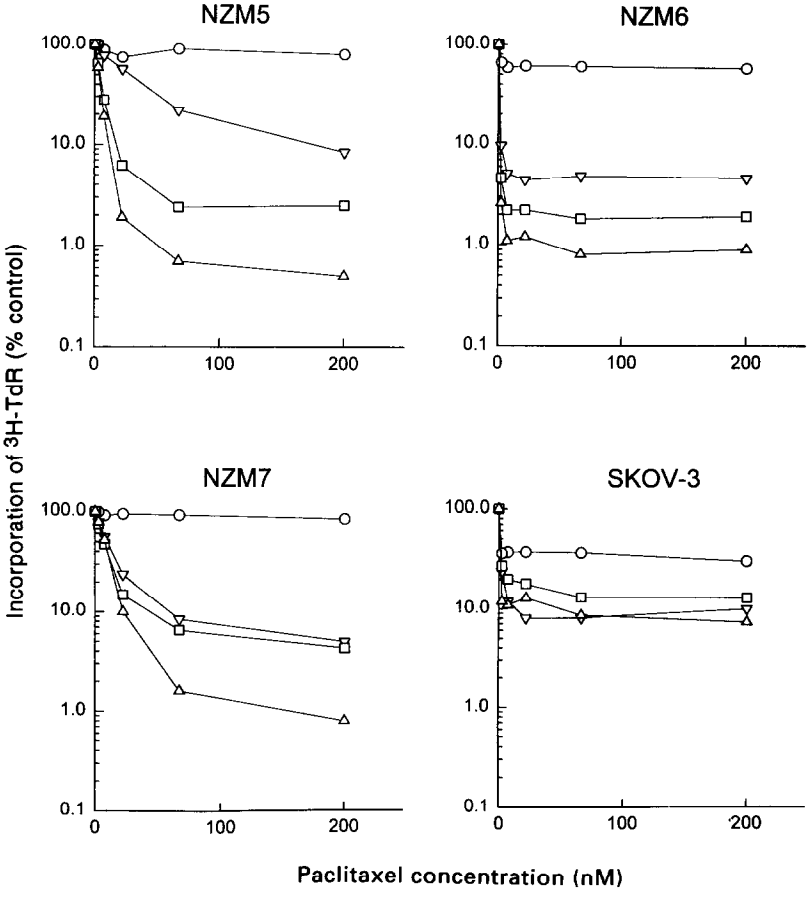

Figure 2. ^{3}H-TdR dose–response curves for four cell lines after exposure to paclitaxel for 24 (○), 48 (▽), 72 (□) or 96 (△) h. ^{3}H-TdR was added during the last 6 h of each incubation.

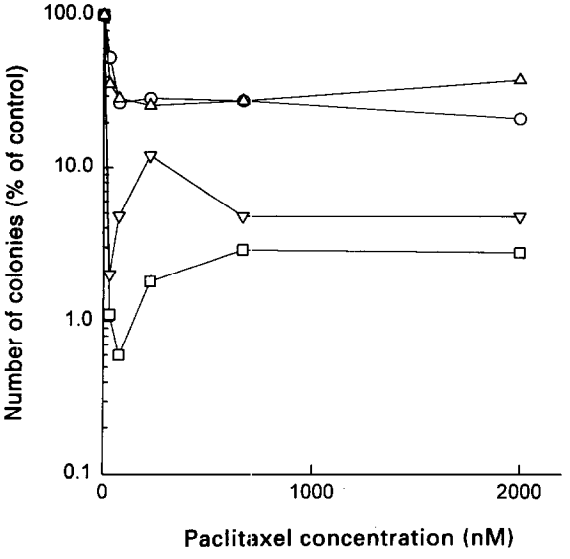

Figure 3. Clonogenic survival curves of SKOV-3 (○), NZM7 (△), H522 (▽) and LOX-IMVI (□) after exposure to paclitaxel for 24 h.

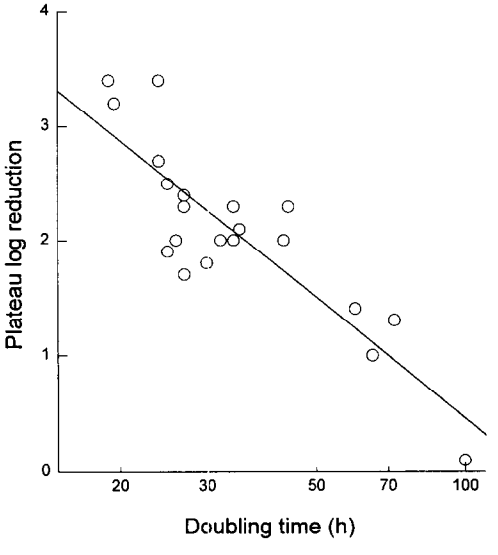

Figure 4. Relationship between "plateau" values of the paclitaxel dose–response curves and the measured doubling times of the cultured cell lines. Values for OVCAR-4, H460 and H522 are equated to the lower limit of detection.

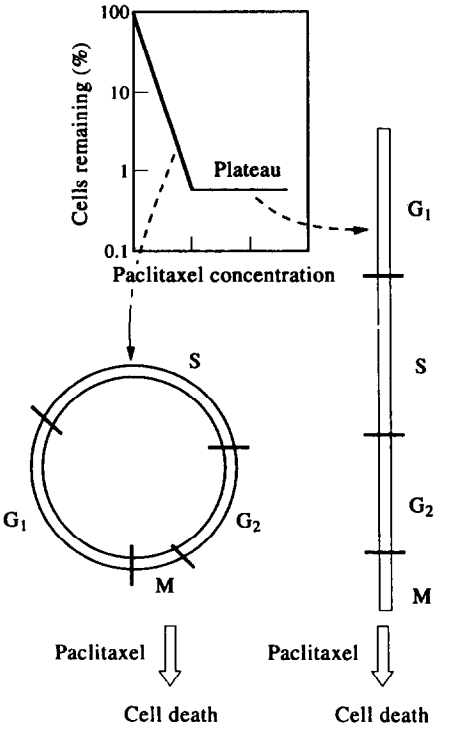

Figure 5. Model for the action of paclitaxel, as explained in the text.

The paclitaxel concentration-dependent phase of the dose–response curves (Figure 5), as quantitated by the IC_{50} value (Tables 1 and 2), provides a measure of intrinsic paclitaxel resistance. IC_{50} values of three of the primary tumours (OV 13, OV 16 and OV 17) and one of the cell lines (NZM8) could not be assessed because the "plateau" values were less than 0.3 $\log_{10}$ units. Of those primary cultures which could be assessed, IC_{50} values were less than 25 nM (Figure 1), while of the cell lines, 18 had IC_{50} values of <6 nM. Only two lines (NZM7 and EKVX) had higher IC_{50} values (Table 2). One of these (EKVX) was an NCI line which has been demonstrated to contain measurable amounts of both P-glycoprotein and MDR1 mRNA [20]. The other (NZM7) has not been tested for P-glycoprotein but has been shown to exhibit transport multidrug resistance by a cell culture method [17]. Seven of the NCI lines have detectable levels of MDR1 mRNA, but none of these have detectable amounts of P-glycoprotein [20], arguing against the presence of transport multidrug resistance. Taken together, these results suggest that P-glycoprotein may be responsible for the observed paclitaxel resistance in EKVX and NZM7. Other types of resistance, as discussed in the Introduction, may contribute to the remaining variability of IC_{50} values.

The major finding of this study has been the significant relationship between "plateau" killing by paclitaxel and measured culture doubling times (Figure 4). This relationship, together with the data in Table 1, suggest that the doubling times of tumour cells in the primary cultures are long. It might be thought that if paclitaxel, at the concentration for "plateau" killing (Figure 5), prevents all cells from re-entering the cell cycle, then a paclitaxel exposure of one cell cycle time would completely eliminate S-phase cells and thus abolish ^{3}H-TdR incorporation. However, cell cycle times in cultured mammalian cell populations are heterogeneous with most of the variability occurring in G_1-phase [21]. Moreover, cell cycle times are not normally distributed and behave as though the passage of cells past a defined transition point located in G_1-phase is a stochastic event [22]. Various mechanisms have been suggested to explain this behaviour [23]. As a result of this property, if cell division is halted selectively at mitosis, the proportion of G_1-phase cells (prior to the transition point) would be expected to decrease with time in a non-linear manner, as approximated by an exponential. The exact relationship between time elapsed and proportion of G_1-phase cells remaining may vary according to the cell line

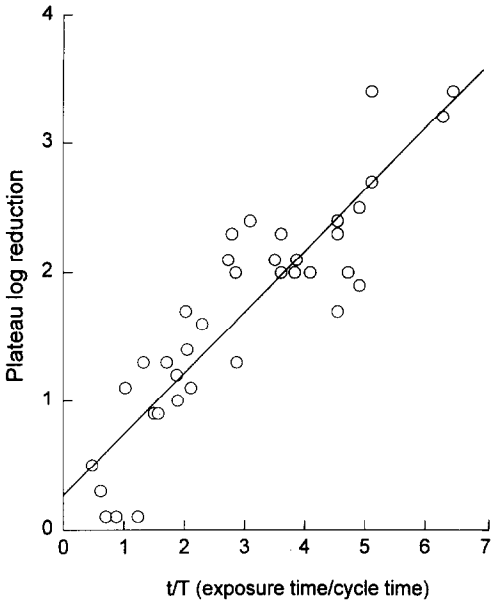

Figure 6. Relationship between t/T, the total exposure time divided by the culture doubling time and the $\log_{10}$ "plateau" values derived from the paclitaxel dose–response curves. Results are derived from Table 2 and Figure 2.

used, accounting for differences in the time-dependent effects of paclitaxel for the different cell lines in Figure 2.

If an exponential rate of departure of cells from G_1-phase is assumed, the proportion (P_t) of G_1-phase cells at any time (t) after exposure to paclitaxel at concentrations high enough to block cell division will be described by the relationship

$$P_t = P_0 e^{-kt/T}$$

where P_0 is the proportion of G_1-phase cells at zero time, k is a constant and T is the cycle time. After an interval (corresponding approximately to the S-phase transit time) the proportion of S-phase cells will have a similar dependence on t. If k is approximately constant, the model predicts that the logarithm of the remaining S-phase cells at time t after paclitaxel exposure should be a linear function of t/T. To test the model, the data for all cell lines from Table 2 and Figure 2 were combined and plateau response values plotted against t/T (Figure 6). A highly significant positive correlation was obtained ($r = 0.90$; $n = 37$; $P < 0.0001$).

The implications of the regression in Figure 6 for the clinical use of paclitaxel emphasize the critical roles of both pharmacokinetics and cytokinetics. If the maximum extent of killing of tumour cells by paclitaxel is a function of the number of cycle times of drug exposure, then the tumour cell cycle time and the retention time of paclitaxel in tumour tissue will be critical in determining therapeutic response. The mean residence time of paclitaxel in pharmacokinetic studies is 6–10 h [24], but in tumour ascites following intraperitoneal administration paclitaxel is retained for extended periods [25]. The retention of paclitaxel within solid tumour tissue would be expected, on the basis of the behaviour of other lipophilic, highly protein bound molecules such as amsacrine [26], to be considerably longer than that in plasma because of the poor degree of vascularisation. Detailed studies on paclitaxel retention by tumour tissue will be of considerable value. The correlation shown in Figure 4 raises the question of whether *in vivo* tumour doubling times will be related to clinical response rates to paclitaxel. For tumours with long cell cycle times, it might be necessary to administer paclitaxel either continuously or intermittently over several days. However, unacceptable host toxicity could result from such schedules, and as an alternative, paclitaxel might usefully be combined with an agent with a different cell cycle specificity such as cisplatinum [27, 28].

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Growth Regulation by All-trans-retinoic Acid and Retinoic Acid Receptor Messenger Ribonucleic Acids Expression in Gastric Cancer Cells

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Retinoic acid has been recognised as a pivotal compound in cell differentiation, proliferation and malignant transformation. We investigated the effects of all-trans-retinoic acid on cell growth and the expression of retinoid nuclear receptor mRNAs in gastric cancer cells *in vitro*. Cell growth was quantified by measuring total cellular DNA. The growth of two of the five gastric cancer cell lines tested (SC-M1 and TSGH9201) was inhibited by all-trans-retinoic acid at concentrations ranging from 1×10^{-8} M to 1×10^{-6} M. Growth inhibition was associated with G0/G1 phase arrest as determined by flow cytometric analysis. Northern blot analysis showed that all five cell lines expressed mRNA for retinoic acid receptors α and retinoic x receptor α and β. Retinoic acid receptor β mRNA was only expressed in TSGH9201 and TMK-1 gastric cancer cell lines. Two RARγ mRNA transcripts (3.2 and 3.0 kb) were detected in SC-M1 and TSGH9201 cells. RA-resistant cells had markedly decreased levels of the 3.2 kb RARγ transcript. All-trans-retinoic acid had a cytostatic effect on the growth of some gastric cancer cells, which may be associated with the expression of retinoic acid receptors.

Key words: retinoic acid, retinoic acid receptor, gastric cancer, growth regulation
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INTRODUCTION

THE RETINOIDS are a pharmacological class consisting of vitamin A (retinol) and related derivatives, such as retinoic acid (RA), that display a wide range of biological functions in cellular differentiation, proliferation, vision, immune function and malignant transformation [1]. Recent reports of dramatic antitumour effects by RA on acute promyelocytic leukaemia as well as squamous cell cancer of the uterine cervix and the skin have encouraged the exploration of the use of retinoids in the therapy of various types of cancer.

The effects of RA are mediated through specific receptors. Two structurally related retinoid nuclear receptors exist: retinoic acid receptors (RARs) and retinoic x receptors (RXRs). Three types (α, β and γ) of receptors have been identified for each of them [2–7]. All-trans-retinoic acid (tRA) is a ligand for RARs. However, 9-cis RA is a bifunctional ligand that binds both RAR and RXR [8]. All the receptors belong to the steroid/thyroid superfamily. Their expression is tissue specific and developmentally regulated. RARs and RXRs consist of six distinct functional domains, designated A to F [9]. The DNA binding C domain and the RA binding E domain are highly conserved between receptors. RARs are thought to activate gene expression by binding to direct repeats of pentameric sequences called retinoic acid response elements. Genes such as RARβ and alcohol dehydrogenase are known to be regulated by RA [1]. Control of growth and differentiation pathways may be mediated in part by