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Cytotoxicity of Paclitaxel and Docetaxel in Human Neuroblastoma Cell Lines

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Taxanes are an important new class of anticancer agents that inhibit cell division by the unique mechanism of increasing the rate of microtubule assembly and preventing microtubule depolymerisation. Using the colony inhibition assay, we compared the cytotoxicity of paclitaxel and docetaxel in three human neuroblastoma (NB) cell lines, SH–SY5Y, BE(2)M17 and CHP100. Different exposure times (3, 6, 12, 24, 48 and 72 h) and different concentrations ranging from 0.1 nM to 10 μM were tested. Both paclitaxel and docetaxel show antineoplastic activity in human NB cell lines. Taxanes' antitumour activity varied among the different cell lines, CHP100 being the most sensitive and SH–SY5Y the least sensitive. Paclitaxel cytotoxicity appears schedule-dependent, with marked cell kill observed only for exposures of 24 h or longer. Docetaxel cytotoxicity was dependent upon prolonged exposure only in the SH–SY5Y cell line, while an exposure time of 3–6 h resulted in exponential cell kill in the other two cell lines. Docetaxel was more cytotoxic than paclitaxel with a mean ratio of (paclitaxel/docetaxel) IC_{50} values ranging from 2 to 11. For both taxanes, we observed good correlation between cytotoxic effect and percentage of cells blocked in G2/M phase. A cytotoxic effect occurred at concentrations comparable with those achieved in the plasma of patients treated with these agents in initial clinical trials. The full potential of prolonged infusion or repeated daily administrations of taxanes should be explored in clinical studies, and responses to taxanes in neuroblastoma should be assessed in paediatric phase II studies.

Key words: paclitaxel, docetaxel, neuroblastoma, cell lines, cytotoxicity
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INTRODUCTION

NEUROBLASTOMA (NB) is one of the most common childhood tumours, with the third highest incidence after leukaemia and brain tumours. The prognosis for patients with disseminated disease remains poor. The survival rate for patients of age > 1 year with stage IV disease is generally < 10%, and this has not been modified even with aggressive therapeutic protocols. There is a pressing need to develop new and more effective antineoplastic agents for this disease. Over the past few years, an important new class of microtubule-stabilising agents has been shown to exhibit promising antineoplastic activity. Paclitaxel and docetaxel are the first taxanes that have reached early clinical testing. Paclitaxel was extracted from the bark of the Pacific yew *Taxus brevifolia* about 20 years ago, but its poor availability (only 50–150 mg/kg of dried trunk bark can be isolated) has limited an extensive clinical evaluation. Recently, this problem has been, in part, circumvented by the synthesis of docetaxel, a semisynthetic compound derived from the needles of *Taxus baccata*, by esterification of a non-cytotoxic precursor, the 10-

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deacetyl-baccatin III. Taxanes have a unique antineoplastic mechanism, promoting the *in vitro* assembly of stable microtubules and stabilising the formed polymers against depolymerisation [1]. The drug, therefore, results in the formation of a rigid microtubular network that prevents cell division, causing a block in mitosis [2]. In phase II trials in adults, paclitaxel has demonstrated activity in malignant melanoma [3], non-small cell lung carcinoma [4], refractory ovarian carcinoma [5] and in metastatic breast cancer [6]. Only recently, a phase I trial was conducted in children with solid tumours [7] and acute leukaemia [8]. Docetaxel is at an earlier stage of development as compared with paclitaxel, some phase I studies have been published very recently, and phase II trials are currently in progress. Docetaxel appears to have an antitumoral effect similar to paclitaxel. Cytotoxic activities of paclitaxel and docetaxel in NB are unknown. The aim of the present study was to investigate the *in vitro* antineoplastic activity of paclitaxel and docetaxel, and to evaluate the schedule and dose-dependence of cytotoxicity in three human NB cell lines.

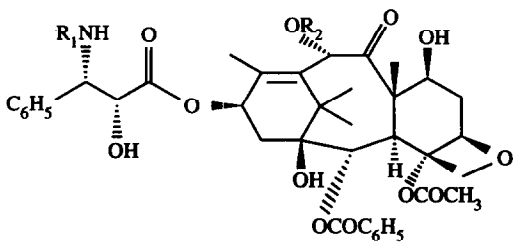
MATERIALS AND METHODS

Drugs

Paclitaxel was a gift from Bristol-Myers Squibb (Wallingford, U.S.A.), and docetaxel was a gift from Rhône-Poulenc Rorer (Vitry sur Seine, France). Stock solutions of paclitaxel and docetaxel were prepared in ethanol (20 mM) and aliquots were stored frozen at −20°C. Immediately before their use, stock solutions were diluted at least 1:1000 (v/v) in growth medium and rediluted thereafter as required. The final concentration of ethanol was < 0.1% and was non-cytotoxic to the cell lines. The structures of paclitaxel and docetaxel are shown in Figure 1.

Cell lines and culture conditions

The three human NB cell lines, SH-SY5Y, BE(2)M17 and CHP100, were a gift from Dr J.L. Biedler (Memorial Sloan-Kettering Cancer Center, New York, U.S.A.). The SH-SY5Y cell line was grown in a mixture of Eagle's minimum essential medium and Ham's F12 (1:1) (Bio Whittaker, Verviers, Belgium) supplemented with non-essential amino acids, L-glutamine (2 mM) and 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Israel) at 37°C. The CHP100 and the BE(2)M17 cell lines were maintained as monolayer culture in RPMI 1640 medium (Bio Whittaker) with 10% heat-inactivated FCS and 2 mM L-glutamine at 37°C in humidified 5% CO_2. Cells were maintained in logarithmic growth by harvesting with trypsin-EDTA solution and seeding before cells reached confluency. Cells were seeded 48 h before an experiment to ensure exponential growth during drug exposure. At least three different experiments in duplicate were performed for each cell line tested for both paclitaxel and docetaxel.



Docetaxel: $R_1 = -COOC(CH_3)_3$ $R_2 = -H$
Paclitaxel: $R_1 = -COC_6H_5$ $R_2 = -COCH_3$

Figure 1. Structural formulae of paclitaxel and docetaxel.

Flow cytometry

Progression of cells through cell cycle and block in the mitotic phase were tested by flow cytometry. Exponentially growing cells from the three NB cell lines, exposed to drug concentrations varying from 1 nM up to 10 μM, were removed at various intervals during incubation, resuspended in 1 ml of PBS–EDTA, diluted with 3 ml of 95% ethanol to the final concentration of 3×10^6/ml and stored at 4°C. Before analysis, cells were washed twice in PBS, stained with 2 ml propidium iodide (50 μg/ml) in 0.1% sodium citrate together with 25 μl RNA-ase and 25 μl 0.1% Nonidet. Cells were then incubated overnight at 4°C in the dark. All samples were run through a FACScan flow cytometer (Becton-Dickinson, Palo Alto, California, U.S.A.) equipped with an argon laser emitting at 488 nm. The percentage of cells in the G1, S and G2/M cell cycle phases were calculated using a Cellfit Software (B–D). A minimum of 10 000 events was acquired in list mode for each determination.

Colony forming assay

Cells in exponential growth (approximately 5×10^5/25 cm^2) were incubated with medium containing paclitaxel or docetaxel at concentrations ranging from 0.1 nM to 10 μM (usually 6–7 graded concentrations per assay) for 12, 24, 48 and 72 h for paclitaxel, 3, 6, 12, 24 and 48 h for docetaxel, exposure times being established in a preliminary test. For exposure over 24 h, fresh medium containing the drug was supplied daily. Cells were then washed with PBS, harvested with trypsin–EDTA and counted using the Trypan Blue dye exclusion test. In order to obtain 150–200 colonies in the control tubes, an appropriate number of cells was seeded in RPMI medium (DMEM + Ham's 1:1 for SH-SY5Y cell line) containing 20% FCS, 1% glutamine and 0.3% agarose. The cells were then incubated at 37°C for approximately 3–4 weeks before visual colony count. Treated cultures are expressed as a percentage of the control cultures, which were given the value of 100%. The mean ± S.D. of colony counts from replicate cultures were calculated, and survival curves were plotted on a semilogarithmic scale with the percentage surviving fraction against drug concentration. All results represent mean values for 3–4 independent experiments. The IC_{50} values (drug concentration required to inhibit colony formation by 50%) were determined for both drugs at the different exposure times.

RESULTS

Clonogenic assay

Figure 2 shows the dose–survival curves of the SH-SY5Y (Figure 2a), BE(2)M17 (Figure 2b) and CHP100 (Figure 2c) cell lines obtained following 12, 24, 48 and 72 h exposures to paclitaxel at concentrations ranging from 0.1 nM to 10 μM. In SH-SY5Y and BE(2)M17 cell lines, minimal inhibition of colony formation (IC) occurred following 12 h exposure, even with drug concentrations up to 10 μM. With the same exposure time, CHP100 appeared more sensitive and a plateau at approximately 50% inhibition was achieved even at the higher concentrations. Following 24 h exposure and after an initial exponential phase, BE(2)M17 and SH-SY5Y cell lines reached a plateau before 1 log cell kill was achieved. Only the 48 and 72 h exposures resulted in a marked cytotoxic effect for BE(2)M17 and SH-SY5Y cell lines with a 2 log cell kill achieved at 1000 nM (SH-SY5Y) and 100 nM (BE(2)M17) for 48 h exposure, at

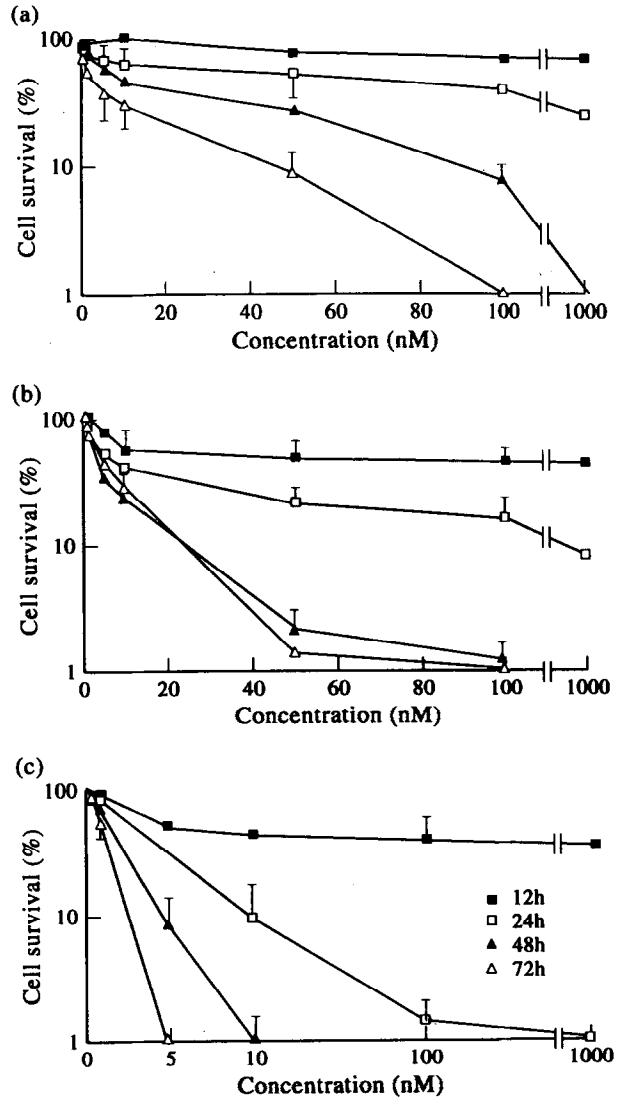


Figure 2. Dose–survival curves of (a) SH–SY5Y; (b) BE(2)M17; and (c) CHP100 cells exposed to different paclitaxel concentrations for various times. Survival was determined by a colony formation assay. Symbols, mean of triplicate values; bars, S.D. S.D. < 5% are not reported.

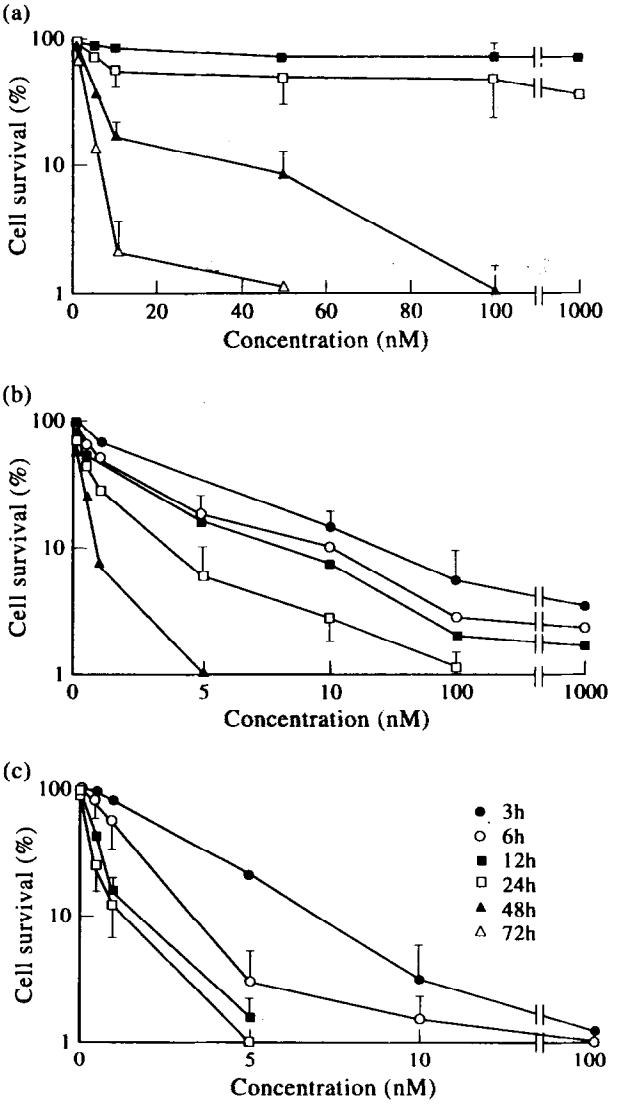


Figure 3. Dose–survival curves of (a) SH–SY5Y; (b) BE(2)M17; and (c) CHP100 cells exposed to different docetaxel concentrations for various times. Survival was determined by a colony formation assay. Symbols, mean of triplicate values; bars, S.D. S.D. < 5% are not reported.

100 nM (SH–SY5Y) and at 50 nM (BE(2)M17) for 72 h exposure. CHP100 cell line showed 1 log cell kill at 10 nM following 24 h exposure. The 48 h and 72 h exposures resulted in complete cell kill at 10 nM and 5 nM, respectively. These results suggest a schedule dependency of *in vitro* cytotoxicity of paclitaxel in the three cell lines tested.

Figure 3 shows the dose–survival curves for SH–SY5Y (Figure 3a), BE(2)M17 (Figure 3b) and CHP100 (Figure 3c) cell lines exposed to docetaxel for 3, 6, 12, 24, 48 and 72 h at concentrations ranging from 0.1 nM to 10 μM. Although docetaxel appears slightly more active compared with paclitaxel, the SH–SY5Y cell line showed a pattern similar to that observed with paclitaxel. BE(2)M17 appeared more sensitive to docetaxel and 1 log cell kill was achieved following a 6 h exposure of 10 nM. However, a plateau phase was observed with up to 12 h exposure. CHP100 not only appeared more sensitive to docetaxel, but also showed a dose-dependent cell kill. With this cell line, 1 log cell kill was achieved at approximately 7 nM following 3 h exposure. Prolonged docetaxel exposure on BE(2)M17 (72 h) and on CHP100 (48 h and 72 h, data not shown) produced a complete cell kill even at the lowest doses. The IC_{50} values obtained after the different exposure times are listed in Table 1. The comparative cytotoxicity expressed as the ratios of mean IC_{50} paclitaxel/docetaxel showed that docetaxel is approximately 2-, 10- and 11-fold more active in the SH–SY5Y, CHP100 and BE(2)M17 cell lines, respectively.

Flow cytometry

The percentages of cells blocked in G2/M phase were examined. Figure 4 shows the curve fitted for 24, 48 and 72 h paclitaxel and docetaxel exposures in the SH–SY5Y cell line. Up to the 100 nM dose, paclitaxel (Figure 4a) did not cause any notable mitotic block, while at the same concentration, docetaxel (Figure 4b) induced between 40% (24 h) and 70% (72 h) cell block in the G2/M phase. There is a good relationship between the doses required for both drugs to increase the percentage of

Table 1. Cytotoxicity of paclitaxel and docetaxel in human neuroblastoma cell lines at different exposure times

Cell line	Exposure (h)	IC_{50} (nM) Paclitaxel	IC_{50} (nM) Docetaxel	Ratio IC_{50} (Paclitaxel/Docetaxel)
BE(2)M17	3	n.r.	3.23	
	6	n.d.	1.60	
	12	n.r.	1.57	
	24	5.27	0.58	9.09
	48	3.12	0.24	13.00
	72	3.92	n.d.	
CHP100	3	n.r.	2.20	
	6	n.d.	1.04	
	12	5.01	0.50	10.20
	24	2.86	0.28	10.21
	48	1.43	n.d.	
	72	0.91	n.d.	
SH-SY5Y	12	n.r.	n.r.	
	24	n.r.	10.62	
	48	7.09	3.70	2.13
	72	3.76	1.77	2.12

n.d., not done; n.r., not reached.

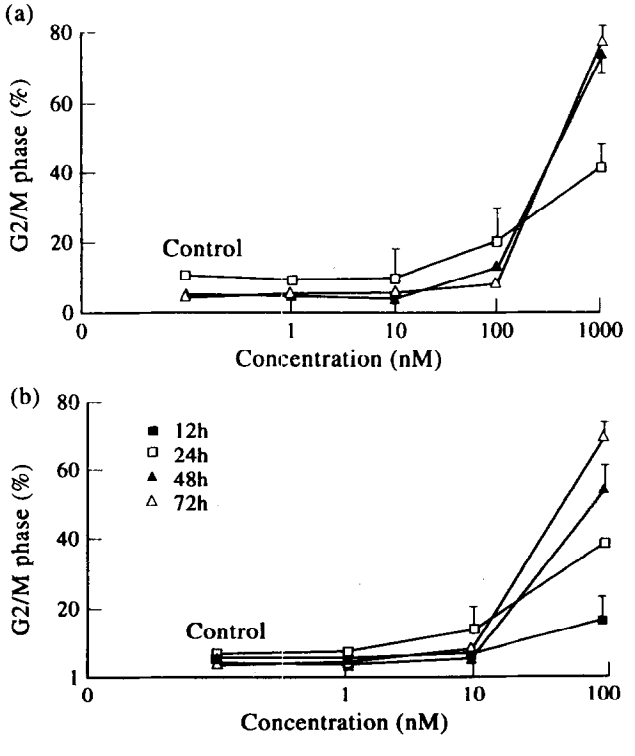


Figure 4. Dose–response for (a) paclitaxel; and (b) docetaxel inducing mitotic block in SH-SY5Y cell line exposed to different concentrations for various times. Symbols, mean of triplicate values; bars, S.D. S.D. < 5% are not reported.

cells blocked in mitosis and the cell kill evaluated by the clonogenic assay. Figure 5 shows the DNA histograms of the BE(2)M17 cell line continuously exposed to 100 nM of either paclitaxel or docetaxel. Starting from the untreated cell, the progression of mitotic cells from diploid to octaploid DNA content through the tetraploid DNA content is shown in sequence. It appears that cells escape the mitotic block and begin new rounds of DNA synthesis without prior cytokinesis.

DISCUSSION

Both paclitaxel and docetaxel exerted antineoplastic activity against the three human NB cell lines tested, although they showed a substantial difference as far as activity and schedule dependence was concerned. In general, cytotoxicity occurred at concentrations comparable with those achieved in the plasma of patients, treated with these agents in initial clinical trials [9, 10]. An up to 13-fold difference of sensitivity to both drugs was noted among the three cell lines. Differential drug sensitivity is a common finding in NB cell lines and reflects the natural phenotypic heterogeneity of the cell population present in the human tumour [11]. We studied three cell lines with different morphological, biochemical, karyotype and growth characteristics. Growth characteristics and, in particular, mean cell doubling time of the lines tested were related to cytotoxicity, with a higher proportion of cell death observed with a shorter cell cycle. Paclitaxel cytotoxicity was more dependent on exposure duration than on dose, and only above a certain exposure time did cytotoxicity become dose-dependent. Critical exposure time varied between the cell lines: 1 log cell kill was achieved following 24 h exposure for BE(2)M17 and CHP100, while a similar effect was achieved for SH-SY5Y above 48 h exposure. Plateau survival curves have been reported with most cell lines tested, although 50% or more inhibition of colony formation has been observed with shorter exposures of 2 or 4 h [12–15]. In animal tumour models, paclitaxel's schedule dependency is less clear [16], and it appears that both dose and schedule may affect antitumour activity and toxicity. In our *in vitro* system, the antitumour activity of docetaxel was less dependent on exposure time compared with paclitaxel. Docetaxel cytotoxicity appeared clearly schedule-dependent only in one cell line (SH-SY5Y) with a 1 log cell kill achieved above 48 h exposure. In the other two lines tested, an exponential cell kill was noted, and in CHP100, a 3 h exposure was sufficient to achieve a 1 log cell kill.

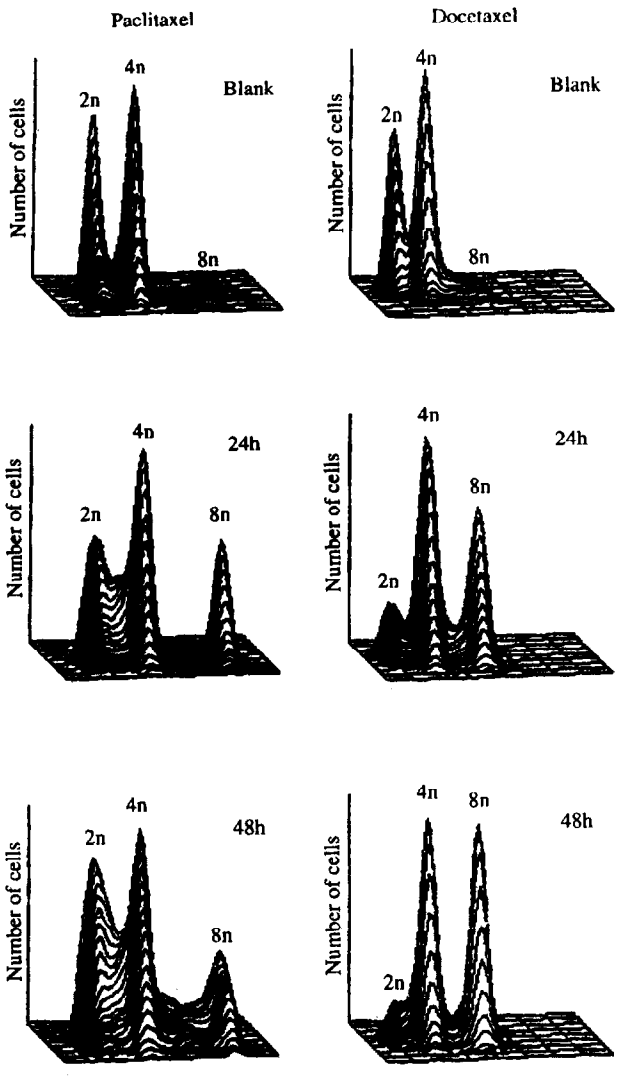


Figure 5. DNA histograms showing the progression of mitotic BE(2)M17 cells exposed to paclitaxel (left) and docetaxel (right) at 100 nM concentration.

Previous *in vitro* studies with docetaxel are not unequivocal, and it appears that in some cell lines a short exposition time, such as 1 h, is sufficient to produce a 1 log cell kill [13, 14]. One study in an *in vivo* animal model suggests that the antitumoral effect of docetaxel is dose-dependent [17].

Docetaxel appears to be more cytotoxic than paclitaxel, and this finding is in agreement with a number of previous reports [13, 14, 18]. We found differences among the cell lines, and the mean ratio of IC_{50} (paclitaxel/docetaxel) ranged from 2 to 11. Although it is believed that the two agents share the same mechanism of action, it has been shown that docetaxel is more active than paclitaxel in promoting tubulin polymerisation [19], and that tubulin polymers generated by the two drugs differ structurally from each other [20]. These differences may explain, in part, the higher activity of docetaxel compared with paclitaxel that we observed. However, in clinical studies, the recommended dose of paclitaxel for phase II trials is approximately 2.5-fold higher than that of docetaxel [9, 10, 21, 22]. These data indicate that increased antitumoral activity of docetaxel is paralleled by increased toxic effect on normal tissues. In clinical trials, paclitaxel has been administered for periods of time varying from 3 to 96 h; however, the most frequently used schedule has been 24 h infusion every 3 weeks. Increased toxicity has been noted with prolonged infusions, but is is unclear if the antitumour effect is also similarly enhanced. Saturable pharmacokinetics has been reported for paclitaxel above a certain dose [10]. This may be an additional variable when comparing dose and schedule dependency, and suggests that pharmacokinetic studies should be part of clinical trials, evaluating the effects of dose and schedule on toxicity and tumour response. At present, data comparing different infusion duration are available only from one clinical trial of relapsed ovarian cancer treated with paclitaxel [23]: two doses, 135 and 175 mg/m^2 and two infusion durations, 3 and 24 h, were compared. Although the 24 h arm appeared more toxic, no major differences were noted between the 24 h and 3 h arm in terms of response and survival rates. Most phase I studies with docetaxel utilise the 1 h infusion since no acute reaction has been noted with the short-term infusion. Administration over an extended time period of up to 5 days has also been used in phase I studies, although comparative data on tumour response and toxicity are not available yet [22]. As compared to paclitaxel, docetaxel is at an earlier stage of clinical testing and other schedules, such as weekly administration, are being tested.

The block in G2/M phase is a well known consequence of cell exposure to taxanes, and it has been proposed as a possible mechanism leading to cell death [1]. With both paclitaxel and docetaxel, we found good correlation between block in G2/M phase and cytotoxicity. Similar observations have been made in other cell lines [15, 24]. When exposed to paclitaxel and docetaxel at doses and exposures that are cytotoxic, cells progressed into one or two new rounds of DNA synthesis without undergoing cytokinesis, and therefore become tetraploid and octaploid. Interestingly, docetaxel appears more potent in inducing polyploid cells compared with paclitaxel. Clinical studies should take into account that the cell kinetics of a tumour population appear to represent a major determinant of paclitaxel antitumoral activity. In addition, the full potential of prolonged infusion or repeated daily administration of taxanes should be explored in clinical studies, and responses to taxanes in neuroblastoma should be assessed in paediatric phase II studies.

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Gene Expression and Protein Localisation of Calcyclin, a Calcium-Binding Protein of the S-100 Family in Fresh Neuroblastomas

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Calcyclin gene, a Ca^{2+}-binding protein with homology to S-100, has been found to be expressed at different levels in leukaemic cells and in other tumour cells. We recently reported the expression of the gene in human neuroblastoma (NB) cell lines, and suggested a possible role of calcyclin in cell differentiation. To extend our findings, we investigated the expression of the gene in NB cells induced to differentiate by retinoic acid (RA), using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Time-course experiments employing LA-N-5 cells showed that calcyclin mRNA appeared 2 h after RA treatment, long before the cells were blocked in the G1 cell-cycle phase and before the neurite-like structures outgrew from the cell bodies. This suggests the involvement of the gene in the early phase of cell differentiation. Furthermore, we investigated mRNA expression in a series of fresh neuroblastomas. NB tumours showed a heterogeneous pattern of calcyclin expression, although calcyclin seemed to be expressed more frequently in cases with a favourable Shimada histology. We also studied the expression of the protein in formalin fixed and paraffin embedded tissues, by using a specific anticalcyclin antibody. The protein was detected in stromal cells which characterise a more mature histological type, and in nerve sheaths, whereas neuroblasts were negative. The tissue that expressed calcyclin protein showed a Schwann-like differentiation and, unlike S-100 protein, calcyclin was expressed in the perineurium.

Key words: neuroblastoma, calcyclin, S-100, gene expression, retinoic acid, cell differentiation, polymerase chain reaction

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