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Original Paper

Antiproliferative Effects of Paclitaxel (Taxol®) on Human Renal Clear Cell Carcinomas *In Vitro*

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The aim of this study was to analyse the direct antiproliferative effects of paclitaxel on 20 different renal clear cell carcinoma (RCCC) cell lines comparing the effects of paclitaxel dissolved in either DMSO or Cremophor® EL/ethanol (Taxol®). The MTT assay was used to determine the growth inhibition of the cell lines by paclitaxel. In addition, micronuclei and microtubule alterations were examined by light and immunofluorescence microscopy. A significant ($P < 0.05$) dose-dependent inhibition of proliferation was evident in 19 out of 20 cell lines after exposure to paclitaxel dissolved in DMSO and in all cell lines after exposure to paclitaxel in Cremophor EL/ethanol. The extent of response markedly varied between the different cell lines ranging from modest effects to reduction of cell viability down to 1–2% of the control. The effects of paclitaxel in Cremaphor EL/ethanol proved to be more pronounced than the effects of paclitaxel dissolved in DMSO. This observation could be explained by additional growth inhibitory effects of Cremophor® EL alone. Light microscopy revealed extensive micronucleus formation after treatment with paclitaxel. However, the failure to demonstrate differences of micronucleus formation in paclitaxel-responsive and non-responsive RCCC cell lines argued against a causal relationship between micronucleus formation and growth inhibition. Immunofluorescence microscopy revealed no differences in the formation of abnormal microtubules in cell lines responsive or non-responsive to the growth inhibitory effects of paclitaxel. Further investigations, therefore, are needed to understand the mechanisms determining the response of RCCCs to paclitaxel treatment. © **1997 Elsevier Science Ltd.**

Key words: renal cell carcinoma, paclitaxel, growth inhibition, microtubule, micronucleus

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INTRODUCTION

HUMAN RENAL clear cell carcinoma (RCCC) is the most frequent malignant tumour of the kidney in adults, with an extremely poor prognosis once the tumour has metastasised and is beyond the reach of curative surgery [1]. Until now, no effective therapeutic regimen has been established against metastatic renal cell carcinoma, with most conventional chemotherapeutic agents, radiotherapy and immunotherapy proving to be largely ineffective [2, 3].

Recently, paclitaxel has been introduced as a novel anticancer agent showing activity against a broad range of human tumours especially drug-resistant ovarian and breast carcinomas as well as non-small cell lung cancer [4–9]. The main molecular target of paclitaxel is the equilibrium between microtubules and tubulin dimers, which form the basic units of microtubules. The ability of paclitaxel to stabilise polymerised tubulin into microtubule bundles results in an efficient inhibition of replication, the cells being blocked in the late G2 or M phase of the cell cycle [10–14]. Microtubules, however, are also indispensable for many interphase functions, including maintenance of cell shape, motility, anchorage, intracellular transport and mediation of signals between cell surface receptors and the nucleus [4, 12]. Therefore, other effects of paclitaxel have also been observed. Thus, paclitaxel has been shown to induce internucleosomal DNA fragmentation and apoptosis [15, 16]; to increase TNF-α (tumour necrosis factor-α) mediated cell cytolysis [17]; and to interfere with tumour cell invasion and metastasis [18, 19]. Furthermore, paclitaxel has been shown to play a role in modulating the inter-

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action of growth factors with their corresponding receptors or the resulting intracellular signalling pathways [20, 21].

The aim of the present investigation was to analyse the antiproliferative effects of paclitaxel on a broad range of 20 different human RCCC cell lines. Thus far, the potential of paclitaxel in the treatment of human RCCC has not been further analysed in clinical studies after disappointing observations in a single phase II trial on 18 RCCC patients with widespread metastatic disease [22, 23].

Since Cremophor® EL, which is used as a diluent in the clinical formulation of paclitaxel, may independently affect tumour cell viability [24–27], we compared the effects of paclitaxel dissolved in either DMSO or Cremophor® EL/ethanol, respectively.

MATERIALS AND METHODS

Cell lines and culture

The 20 cell lines used in our study were established in our laboratory as previously described [28, 29]. The original tumours were typical representatives of RCCC as defined by Thoenes and associates [30] (Table 1). The standard growth medium was Dulbecco's modified medium (DMEM; Gibco Europe, Germany), supplemented with 10% heat-inactivated fetal calf serum, penicillin and streptomycin. The cultures were maintained at 37°C in an atmosphere with 5% CO_2. For subculturing, cells were disaggregated by exposure to 0.05% EDTA (Biochrom, Berlin, Germany). Our studies were performed with cells from passages 30–110.

Reagents

Paclitaxel was purchased from Sigma (Deisenhofen, Germany) and dissolved in DMSO. Paclitaxel dissolved in Cremophor® EL/ethanol (Taxol®) was kindly provided by Bristol-Myers Squibb (Munich, Germany, 1 ml contains 6 mg paclitaxel, 527 mg Cremophor® EL, 49.7% (v/v) dehydrated alcohol, USP). The final concentrations of paclitaxel used were 0.001, 0.01, 0.1, 1 and 10 μM.

Control experiments were performed to exclude the possibility that the effects observed were due to the diluents DMSO, ethanol or Cremophor® EL/ethanol.

Table 1. Data on the staging and grading of the original tumours and concentrations of paclitaxel required to reduce cell viability to 50% of the control after exposure for 120 h (= IC_{50} in μM)

Cell line	Staging and grading of the original tumours	IC_{50} (μM) Paclitaxel in DMSO	IC_{50} (μM) Paclitaxel in Cremophor EL/ethanol
clearCa-1	pT 3a, G2	8.22	0.79
clearCa-2	pT 3a, G3	4.26	1.53
clearCa-3	pT 3a, G2	1.31	0.30
clearCa-4	pT 3a, G1	1.73	0.29
clearCa-5	pT 3b, G3	0.10	0.02
clearCa-6	pT 3b, G3	0.05	0.02
clearCa-7	pT 2, G3	35.18	1.47
clearCa-8	pT 3b, G2	23.63	1.24
clearCa-9	pT 2, G1	11.34	1.93
clearCa-10	pT 3b, G2	5.87	1.42
clearCa-11	pT 2, G2	0.03	0.01
clearCa-12	pT 3a, G2	134.71	4.71
clearCa-13	pT 2, G2	9.76	1.76
clearCa-14	pT 3b, G3	5.46	0.37
clearCa-15	pT 3a, G3	130.12	2.64
clearCa-16	pT 3a, G2	23.91	0.86
clearCa-17	pT 3a, G3	11.18	0.65
clearCa-18	pT 2, G2	1.26	0.29
clearCa-19	pT 2, G2	0.56	0.11

MTT assay

Tumour cells in the exponential growth phase were harvested from culture flasks using 0.05% EDTA. The cells were washed in standard growth medium and counted using a haemocytometer. The cells were then transferred to 96-microwell plates (Gibco, Europe, Germany) at 10 000 cells per well in standard growth medium. The cells were allowed to adhere for 24 h in 5% CO_2 at 37°C. The tumour cells were then exposed to paclitaxel dissolved in either DMSO or in Cremophor® EL/ethanol (for concentrations see above). The first column of each microwell plate served as a blank and the second was used as a control containing tumour cells in standard growth medium without drug supplement. The plates were incubated for 120 h at 37°C and 5% CO_2 without further renewal of growth medium. Each microwell plate was inspected by phase contrast microscopy as an additional control of plausibility and then the number of living tumour cells was determined by the colorimetric MTT assay, which measures the number of viable, mitochondrial-dehydrogenase active cells [31]. The per cent viability of each well was calculated from the following:

$$\text{per cent viability} = \frac{\text{absorbance of test} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100$$

The data presented are the mean ± standard deviation from eight replicate wells per microwell plate and three replicate microwell plates per cell line. Data from the MTT assays were analysed by means of Student's *t*-test. A *P*-value of less than 0.05 was considered to be significant.

The 50% inhibitory drug concentration (IC_{50} value) was statistically determined by SSPS (probit-analysis).

Light microscopy and immunofluorescence microscopy

Exponentially growing cells were transferred to sterile glass slides and then allowed to adhere for 24 h in 5% CO_2 at 37°C. After 24 h, the tumour cells were exposed to paclitaxel dissolved in either DMSO or Cremophor® EL/ethanol (for concentrations see above).

The slides were washed, air dried and stained with haematoxylineosin after exposure to paclitaxel for 120 h. 500 consecutive cells on each slide were scored to determine the frequency of micronuclei.

For immunofluorescence the cells were washed, fixed with methanol for 5 min, permeablised with cold acetone for 30 s and air dried. The slides were incubated with the mouse anti-alpha-tubulin antiserum (clone B-5-1-2; dilution 1: 2500; Sigma, Germany) for 30 min at room temperature, washed and stained with FITC-conjugated antibodies (dilution 1: 60; DAKO, Germany). Coverslips were mounted in a solution of *p*-phenylenediamine and glycerol. On each slide 500 consecutive cells were scored to determine the frequency of paclitaxel-induced microtubule alterations.

RESULTS

The effects of paclitaxel dissolved in DMSO or in Cremophor® EL/ethanol on the proliferation of 20 different human RCCC cell lines are summarised in Figure 1. A significant ($P < 0.05$) dose-dependent inhibition of proliferation became evident in 19 out of 20 cell lines after exposure to paclitaxel in DMSO and in all cell lines after exposure to paclitaxel in Cremaphor® EL/ethanol.

Effects of paclitaxel dissolved in DMSO

The extent of response to paclitaxel dissolved in DMSO markedly varied between the different cell lines, ranging

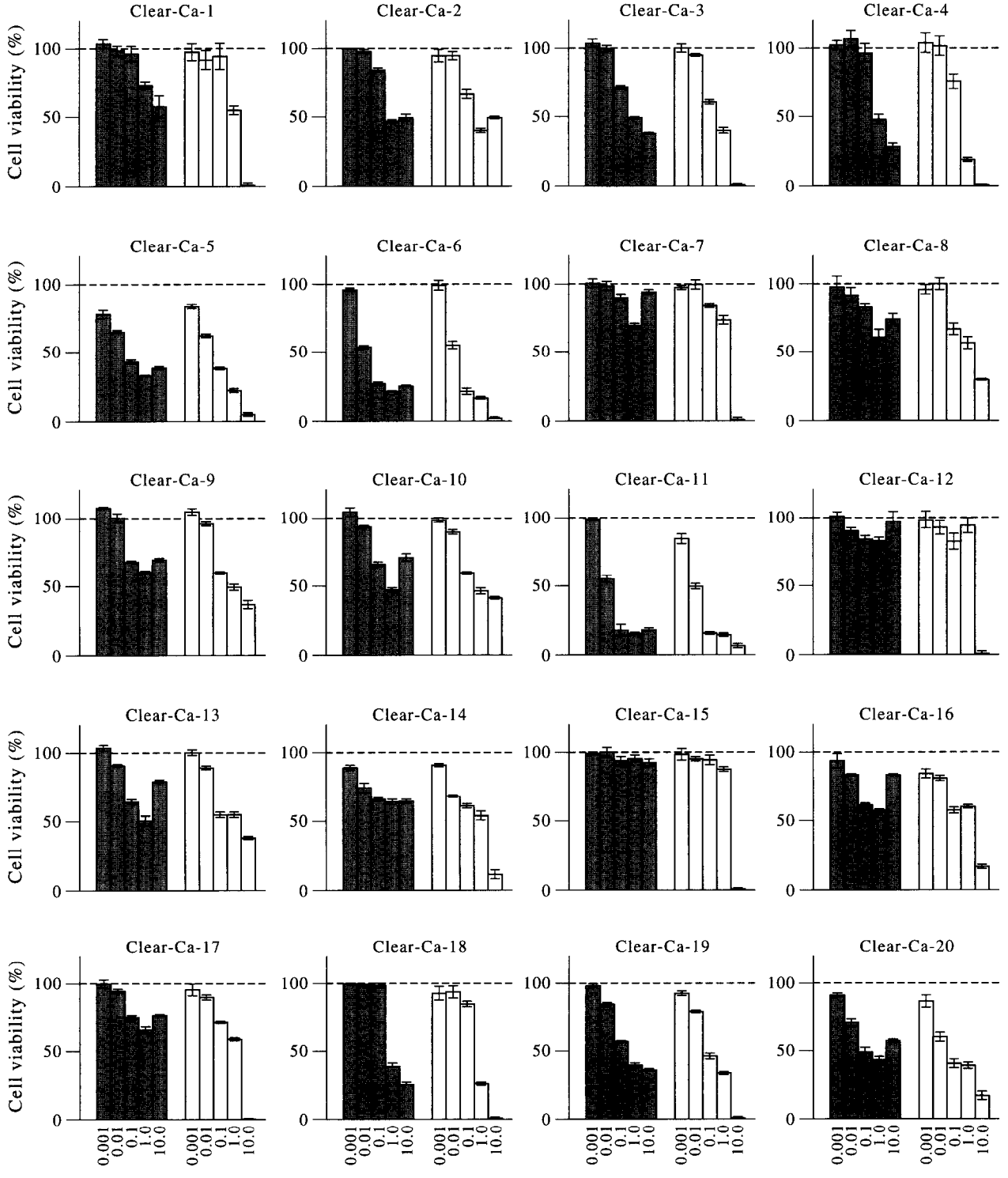

Figure 1. Growth inhibitory effects of paclitaxel dissolved either in DMSO (■) or in Cremophor® EL/ethanol (□) on 20 different human carcinoma cell lines of the clear cell type at concentrations of 0.001, 0.01, 0.1, 1.0 and 10.0 μM.

from a questionable effect of doubtful biological significance in clearCa-15 to a pronounced reduction of cell viability down to 19% of the control in clearCa-11 after exposure to 10 μM paclitaxel. In 11 out of 20 cell lines, the maximum growth inhibitory effect was observed at a concentration of 1 μM paclitaxel. A further 10-fold increase in paclitaxel concentration yielded a further decline of cell viability in only 5 out of 20 cell lines (clearCa-1, 3, 4, 18, 19). In contrast, the effects of 10 μM paclitaxel proved to be significantly ($P < 0.05$) less pronounced than the effects of 1 μM paclitaxel in 11 out of 20 cell lines (clearCa-5, 7, 8, 9, 10, 11, 12, 13, 16, 17, 20). The heterogeneity of response between the different cell lines was also evident from differences in the IC_{50}s (Table 1). In 3 cell lines (clearCa-5, 6, 11), the IC_{50} did not exceed 0.1 μM, whereas in 7 cell lines the IC_{50} exceeded 10 μM paclitaxel.

Control experiments using DMSO at a concentration equivalent to that present in a 10 μM solution of paclitaxel in DMSO did not reveal growth inhibitory effects (data not shown).

Effects of paclitaxel dissolved in Cremophor® EL/ethanol

The extent of response to paclitaxel in Cremophor® EL/ethanol markedly varied between the different cell lines with a marked reduction of cell viability down to 1–2% of the control in 9 cell lines (clearCa-1, 3, 4, 6, 12, 15, 17, 18, 19) after exposure to 10 μM paclitaxel. In 19 out of 20 cell lines, the maximum effects were observed after exposure to 10 μM paclitaxel. In clearCa-2, the reduction of cell viability was most pronounced after exposure to 1 μM, whereas a further 10-fold increase in the paclitaxel concentration resulted in an increase of cell viability. ClearCa-12 and clearCa-15 responded with a modest decrease in cell viability up to a concentration of 1 μM and only exposure to 10 μM

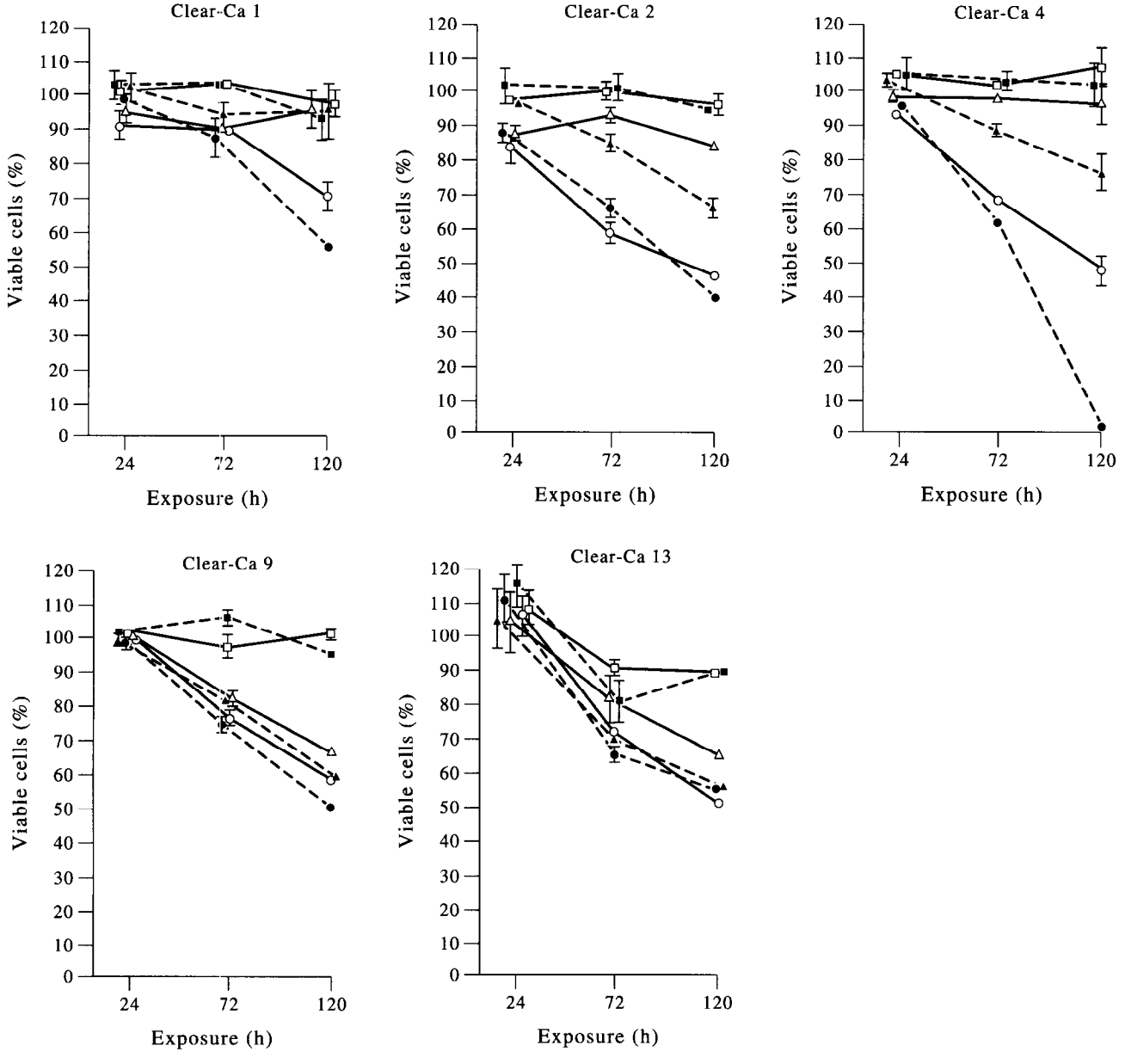

Figure 2. Time course of growth inhibition after exposure to paclitaxel dissolved in DMSO (□, ▲, ○) or Cremophor® EL/ethanol (■, ▲, ●) at concentrations of 0.01 (□, ■), 0.1 (△, △) and 1 μM (○, ●).

resulted in a marked reduction of cell viability down to 1–2% of the control. The heterogeneity of response between the different cell lines was also shown by differences in the IC_{50} (Table 1). In 4 cell lines (clearCa-5, -6, -11, -20), the IC_{50} did not exceed 0.1 μM and in no cell line did the IC_{50} exceed 10 μM. Control experiments using ethanol in a concentration equivalent to that present in a 10 μM solution did not reveal growth inhibitory effects (data not shown). In contrast, control experiments with the diluent Cremophor® EL/ethanol revealed reduction of cell viability down to 70% of the control.

Time course of growth inhibition after exposure to paclitaxel

The time course of growth inhibition after exposure to paclitaxel dissolved in DMSO or Cremophor® EL/ethanol was further analysed in five randomly selected cell lines (Figure 2) showing a time-dependent decrease in cell viability. No significant ($P < 0.05$) decrease in cell viability became evident 24 h after exposure to paclitaxel.

Morphological aspects of growth inhibition by paclitaxel

Two cell lines were selected for analysis of morphological alterations induced by paclitaxel: clearCa-15 cells had been shown to be largely resistant to the growth inhibitory effects of paclitaxel and clearCa-11 cells had shown pronounced growth inhibition.

Micronucleus formation by light microscopy

Untreated controls consisted largely of singly nucleated cells and no cells with multiple micronuclei were seen (Figure 3(a)). Paclitaxel treatment resulted in the emer-

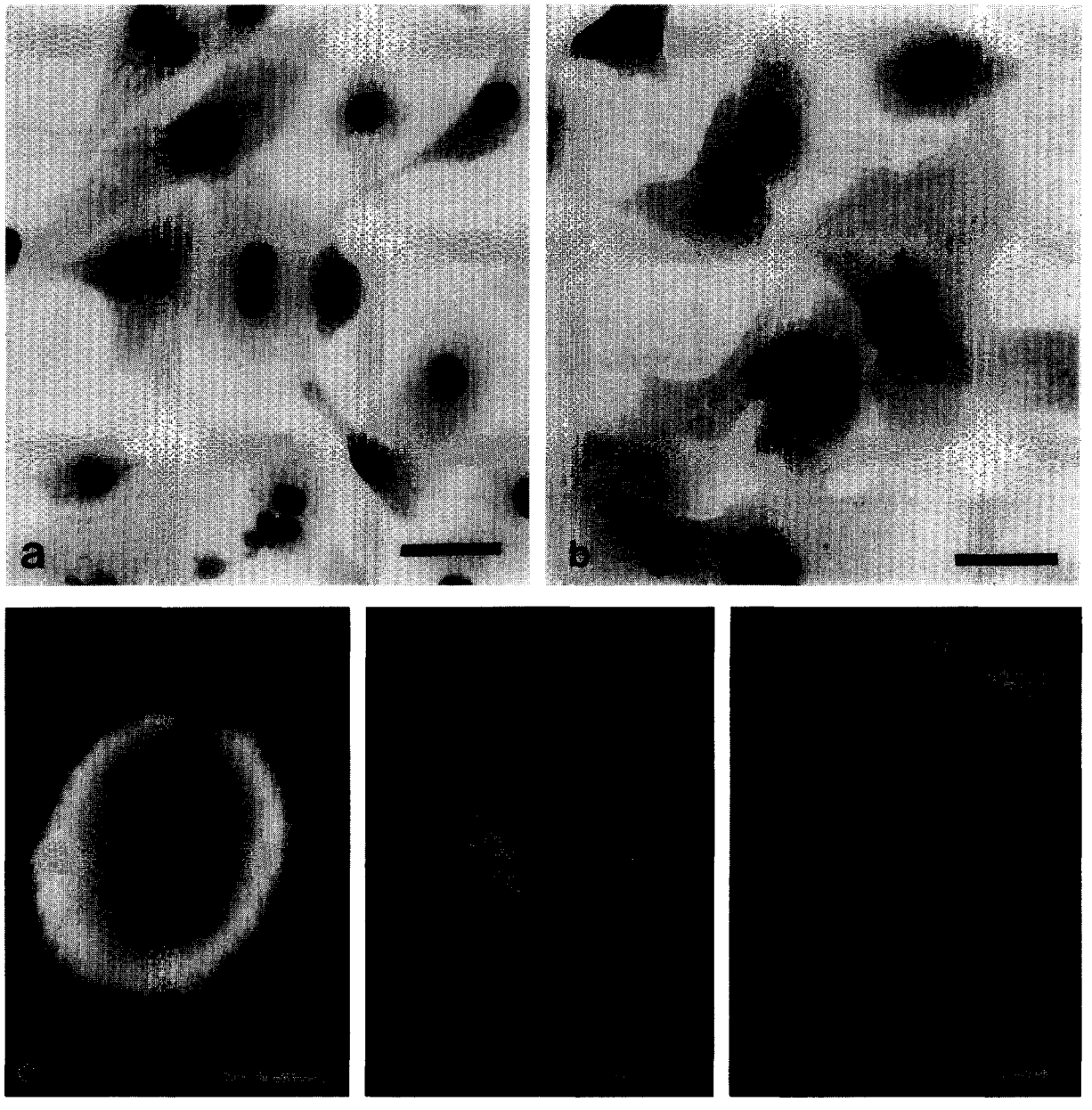

Figure 3. Morphological effects of paclitaxel. (a) Untreated control cells without micronuclei. (b) Multiple micronuclei in paclitaxel-treated cells. (c) Untreated control cells showing a radial distribution pattern of α-tubulin. (d) Paclitaxel-induced abnormal microtubule bundle around the nucleus (star) after exposure for 120 h. (e) Paclitaxel-induced coarse meshworks of microtubule: (a,b) bar = 50 μm; (c,d) bar = 20 μm; (e) bar = μm.

Table 2. Frequency of cells with micronuclei in clearCa-11 and clearCa-15 after exposure to paclitaxel for 120 h

	ClearCa-11 Paclitaxel		ClearCa-15 Paclitaxel	
	in DMSO	in Cremophor EL/ethanol	in DMSO	in Cremophor EL/ethanol
Control	0%	0%	0%	0%
0.01 μM	10%	22%	21%	46%
0.1 μM	52%	61%	59%	71%
1.0 μM	63%	61%	59%	70%

gence of micronuclei, which was dose-dependent (Figure 3(b) and Table 2). The number of cells with micronuclei only slightly differed between clearCa-11 and 15.

Microtubule cytoskeleton by immunofluorescence microscopy

Multiple mitotic spindle-like asters were not seen after exposure to paclitaxel for 120 h. In untreated control cells (Figure 3(c)), a finely radial distribution pattern of the microtubules could be observed. Thick microtubule bundles (Figure 3(d)) or an alteration of the microtubule cytoskeleton with irregular coarse meshworks of microtubules (Figure 3(e)) were exclusively observed in paclitaxel-treated cells, but not in control cells. A dose-dependent increase of abnormal microtubule bundles and a dose-dependent decrease of irregular meshworks were observed (Table 3). No differences in the frequency of microtubule alterations became evident between clearCa-11 and clearCa-15, although both cell lines differed in their growth inhibitory response. The frequency of abnormal microtubules did not differ between paclitaxel dissolved in DMSO or in Cremophor® EL/ethanol.

DISCUSSION

The results of our study clearly demonstrate that paclitaxel effectively inhibits the proliferation of human RCCC *in vitro*. In 19 out of 20 cell lines, a significant ($P < 0.05$) dose-dependent inhibition of proliferation was observed after exposure to paclitaxel, dissolved in either DMSO or Cremophor® EL/ethanol. The concentrations of paclitaxel used in our study had previously been achieved in clinical trials [4] showing that 6 h short-term infusions of paclitaxel resulted in peak plasma concentrations of 1–10 μM. Therefore, clinically relevant concentrations of paclitaxel yielded antiproliferative effects against the majority of our human RCCC cell lines.

However, the extent of response to paclitaxel markedly varied between the different cell lines of our study, ranging from modest effects of doubtful biological significance in one cell line (clearCa-15) to surviving fractions of less than 2% after exposure to 10 μM paclitaxel in Cremophor EL/ethanol. This heterogeneity of response cannot be explained by differences in histogenetic derivation, since all our cell lines originated from RCCCs [29, 30]. Although there is still limited information on the mechanisms that confer resistance to paclitaxel [4, 32], the heterogeneity of response observed in our study might at least in part be related to differences in the multidrug resistant (MDR) phenotype. Thus, RCCCs are known to express the *MDR 1* gene and its gene product, i.e. the *p*-glycoprotein, which functions as an efflux pump for hydrophobic compounds such as paclitaxel [33]. Further investigations, therefore, will have to show whether the heterogeneity of response observed between our RCCC cell lines can satisfactorily be explained by differences in *MDR* 1 gene expression.

The effects of paclitaxel dissolved in Cremophor® EL/ethanol proved to be more pronounced than the effects of paclitaxel dissolved in DMSO. Corresponding observations have been reported for other tumour types [24]. Control experiments using DMSO or ethanol in concentrations equivalent to those present in a 10 μM solution of paclitaxel did not reveal any effect on the proliferation of our cell lines. In contrast, control experiments with Cremophor® EL/ethanol demonstrated growth inhibitory effects of the diluent itself. The increased efficacy of the Taxol® formulation, therefore, is probably related to the growth inhibitory effects of the diluent Cremophor® EL itself. The growth inhibitory effect of Cremophor® EL might even be further enforced by its ability to inhibit the *p*-glycoprotein efflux pump [27, 34–36] which further increases the intracellular concentration of paclitaxel. Therefore, the Taxol® formulation efficiently combines the growth inhibitory agent paclitaxel with a compound capable of reversing the multidrug resistance phenotype and thus reducing resistance to paclitaxel.

Interestingly in this context, high concentrations of Cremophor® EL have also been reported to antagonise the growth inhibitory effects of paclitaxel in some tumour cell lines [24]. A corresponding observation became evident in our RCCC cell line clearCa-2, in which the most pronounced growth inhibition was observed after exposure to 1 μM Taxol®, whereas a concentration of 10 μM Taxol® revealed less marked growth inhibitory effects. An even more pronounced paradoxical increase in cell viability was observed after exposure to paclitaxel dissolved in DMSO. With DMSO used as diluent, in 11 out of 20 cell lines the growth inhibitory effects of 10 μM paclitaxel were less pronounced than the effects of 1 μM paclitaxel. This increase

Table 3. Frequency of cells with abnormal microtubule bundles and irregular coarse meshworks of microtubules in clearCa-11 and clearCa-15 after exposure to paclitaxel

	ClearCa-11 Paclitaxel				ClearCa-15 Paclitaxel			
	in DMSO		in Cremophor EL/ethanol		in DMSO		in Cremophor EL/ethanol	
	Abnormal bundles	Irregular meshworks	Abnormal bundles	Irregular meshworks	Abnormal bundles	Irregular meshworks	Abnormal bundles	Irregular meshworks
Control	0%	0%	0%	0%	0%	0%	0%	0%
0.01 μM	55%	38%	51%	37%	50%	43%	52%	35%
0.1 μM	55%	38%	50%	40%	51%	40%	45%	45%
1.0 μM	79%	16%	74%	17%	79%	16%	74%	17%

in cell viability could not be attributed to the diluent DMSO as shown by control experiments in corresponding concentrations. Although a satisfactory explanation cannot yet be offered, this observation further emphasises the complexity of actions induced by paclitaxel treatment.

Abnormal microtubules and micronuclei are the most prominent morphological alterations that can be observed after exposure to paclitaxel [12, 37–39]. Since paclitaxel, *inter alia*, effectively impairs the function of the mitotic spindle, sister chromatids cannot segregate during mitosis [39]. Cytokinesis, however, does not occur prior to the tight clustering of segregated chromatids around individual spindle poles. Consequently, the progression of cycling cells from M to G1 is effectively blocked in the presence of paclitaxel and cells can only revert to a pseudo-G1 phase [39]. This reversion is morphologically indicated by the formation of micronuclei resulting from the reformation of nuclear membranes around random clusters of non-segregated tetraploid chromatids. Although the formation of micronuclei has been observed to precede cell death [40], the failure to demonstrate differences of micronucleus formation between paclitaxel-resistant clearCa-15 cells and paclitaxel-sensitive clearCa-11 cells argues against a causal relationship of micronuclei to growth inhibition in RCCCs.

Abnormal microtubule bundles have been observed in different cell types after exposure to paclitaxel [4, 12]. The functional significance of microtubule bundles observed in both paclitaxel-sensitive clearCa-11 cells and paclitaxel-resistant clear-Ca-15 cells remains to be further elucidated. Thus, it has been emphasised that microtubule bundling is reversible in cells resistant to paclitaxel-induced cytotoxicity, but irreversible in cells sensitive to paclitaxel-induced cytotoxicity [4].

In conclusion, our study demonstrates that paclitaxel effectively inhibits the proliferation of human RCCCs. Our data confirm recent *in vitro* observations on RCCC responsiveness to paclitaxel using short-term cultures of human RCCCs [41]. Nevertheless, *in vitro* observations are at variance with the only reported phase II trial on RCCC, which failed to demonstrate clinical response to paclitaxel [22, 23]. However, the 18 patients included in this clinical trial had presented with advanced, usually widespread metastatic disease. Therefore, further testing of paclitaxel and its integration in combined modality regimens will have to show whether paclitaxel might have a stronger impact in patients with less extensive disease. Moreover, further *in vitro* investigations are clearly needed to arrive at a better understanding of the mechanisms determining the responsiveness or non-responsiveness of human RCCCs to paclitaxel treatment.

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