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# Anti-tumour activity of a panel of taxanes toward a cellular model of human cervical cancer

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**Abstract** Using a model of human cervical cancer (ME-180 cells), the anti-tumour activity of paclitaxel was compared to that of docetaxel and IDN5109, a newly developed taxane. The growth inhibition effect of taxanes was assessed after 3 days of exposure. DNA analysis, the taxane-dependent modulation of the expression of the α and β subunits of tubulin and DNA fragmentation were assessed by flow cytometry. The presence of apoptosis was confirmed by morphological analysis using a laser scan cytometer. For the evaluation of "in vivo" anti-tumour activity, taxanes were administered to nude mice intravenously once daily, according to a q3/4d × 4 schedule. Docetaxel, IDN5109 and paclitaxel obtained "in vitro" $IC_{50}$ values of 0.86, 1.4 and 2.4 nM, respectively. DNA analysis demonstrated a transient block at the $G_2$/M phase of the cell cycle only after 12 h of culture in the presence of taxanes and an increase of nuclear fragmentation suggestive for apoptosis after additional 12 and 60 h of exposure. Morphological analysis confirmed the presence of apoptosis. Taxanes induced a down-modulation of the α subunit of tubulin in the $G_{0/1}$ phase of the cell cycle, and an overexpression of the β subunit in the $G_2$/M phase. A strong anti-tumour activity was obtained "in vivo" for nude mice xenografted using ME-180 cells (T/C = 0% for all drugs). These data indicate that the three taxanes are strongly active both "in vitro" and "in vivo" toward ME-180 cells. Clinical studies are now needed to ascertain if the higher anti-tumour activity observed "in vitro" using docetaxel and IDN5109 yields a better clinical response in advanced cervical carcinoma with respect to paclitaxel.

**Key words** Taxanes · Cervical cancer · Apoptosis · Tubulin

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## Introduction

Despite the increasing success of early diagnosis and technological advancements with consequent significant reduction of the incidence, carcinoma of the uterine cervix in its advanced stages is still a widely prevalent disease with a severe prognosis and remains a major health problem for women. Radiotherapy and traditional chemotherapies often fail to obtain a stable response, and relapsing disease is frequently resistant to additional treatments. Over the last decade, however, intense research has yielded a large number of new molecules endowed with potent anti-tumour activity, increasing the chances for a better outcome for advanced cervical cancer patients. Among these newly developed compounds, taxanes represent a class of promising anticancer drugs able to inhibit tubulin disassembly with the consequent arrest of the cell cycle at the M checkpoint [19]. Concomitantly, microtubule disorganisation leads to the triggering of apoptotic cell death [2], probably by the functional inactivation of the anti-apoptotic bcl-2 protein [11].

Paclitaxel and its semi-synthetic derivative docetaxel are the only two taxanes currently in use for gynaecological malignancies and they obtain a high response rate in both ovarian and breast cancer. One of the main advantages of taxanes is their ability to be active also in cancer cell lines lacking p53 function [22], whereas an important clinical limitation is presented by the expression of the multi-drug resistance (MDR) phenotype in cancer cells [12]. This restriction has been resolved by the evolution of a new series of taxanes modified by intro-

ducing a carbonate group into the C1-C14 sites [15]. These newly developed taxanes can induce cell cycle block and apoptosis, showing a mechanism of action similar to that of both paclitaxel and docetaxel [5]. Remarkably, this activity also remains considerable in cells expressing the MDR phenotype [5, 18].

Despite the fact that preclinical studies have shown that paclitaxel does not produce a consistent radio-sensitisation effect in human cervical cancer cells [6], at least two phase I clinical studies have been conducted (albeit with poor results) in cervical cancer patients using low amounts of paclitaxel as a radiation-sensitising agent either in a single regimen [24] or in combination with cisplatin [3]. Few clinical studies have been performed using paclitaxel at full dosage, either alone [13] or in combination with cisplatin [17]. To our knowledge, other taxanes have never been tested in the treatment of this disease. Here, we compared the "in vitro" and "in vivo" anti-tumour properties of paclitaxel with respect to docetaxel and IDN5109 (a MDR-active taxane) in a human cervical cancer cell line.

## Materials and methods

### Drugs

Paclitaxel, docetaxel and IDN5109 were provided by Dr. Bombardelli, Indena, Italy. To study the "in vitro" antiproliferative activity, taxanes were solubilised in dimethyl sulphoxide (DMSO; 10 mM stock solution) and used within 7 days. Control cells were treated with the same amount of vehicle alone. The final DMSO concentration never exceeded 0.1% (v/v) in either control or treated samples.

### Cell cultures

The cervical cancer cell line ME-180 (kindly provided by Dr. E. Proietti from Istituto Superiore di Sanità, Rome, Italy) was used. ME-180 cells were grown in minimum essential medium (MEM) complemented with 10% foetal calf serum (FCS) and 200 U/ml penicillin (Sigma, St. Louis, Mo.). Cells were routinely propagated as monolayer cultures in 75-cm$^2$ tissue culture flasks and trypsinised twice a week at a density of $1 \times 10^5$ cells/ml. Cells were incubated at 37 °C in a 95% air, 5% $CO_2$, high-humidity atmosphere.

### Growth experiments

Cells were plated in six-well flat-bottom plates (Falcon, Lincoln Park, N.J.) at a density of $8 \times 10^4$ cells/ml in complete medium. After 24 h, the medium was replaced with fresh medium containing drugs and incubated for an additional 72 h. Control cells were treated with vehicle alone. After 3 days of exposure to the drugs, cell counts were performed only on adherent trypsinised cells, while dead cells floating in the medium were discarded. Triplicate counts of viable cells in triplicate cultures were performed.

### Flow cytometry

Cells were plated at a concentration of $10^5$ cells/ml in the medium supplemented as above. After 24 h, the medium was replaced with a fresh one containing the compounds to be tested or vehicle alone. After various lengths of culture (from 12 to 72 h) cells were harvested and nuclei were isolated and stained using a solution containing 0.1% sodium citrate, 0.1% NP40, 4 mM EDTA and 50 μg/ml propidium iodide (PI) as DNA dye [8]. Incubation of the cells with staining solution lasted a minimum of 12 h at 4 °C. Flow cytometric DNA analysis was performed by acquiring a minimum of 20,000 nuclei with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). DNA fluorescence was collected in linear mode and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using a Multicycle software package (Phoenix, San Diego, Calif.). DNA fragmentation was computed in the subG$_1$-region and was used to quantify the amount of apoptosis [7].

In other experiments, DNA analysis was performed in whole cells with dual staining of microtubules. Briefly, cells were fixed in cold methanol for 10 min at −20 °C. After the first washing pellets were resuspended in cold acetone for 20 s. After the second washings, cells were labelled either with a fluorescein isothiocyanate-conjugated monoclonal antibody anti-α (diluted 1:50) or anti-β (diluted 1:25) tubulin (Sigma). After 1-h incubation at 4 °C samples were collected in phosphate-buffered saline containing 50 U/ml RNase A (Sigma) and incubated at 37 °C for 20 min. Finally, PI at a concentration of 50 μg/ml was added and samples were incubated for a minimum of 12 h at 4 °C.

To ascertain the presence of apoptosis morphologically, cells were prepared as above indicated. They were then deposited onto microscope slides by cytocentrifugation at 1000 rpm for 6 min (Shandon Co., Pittsburgh, Pa.). The specimens were fixed in 70% cold ethanol and then processed following the same steps as indicated for DNA analysis for whole cells. A laser scan cytometer (LSC, CompuCyte Inc., Cambridge, Mass.) with standard collection optics and excitation (488-nm laser line) was used. After fluorescence acquisition, slides were stained with May Grünwald-Giemsa and morphological analysis (on a minimum of 500 cells) was performed using the software provided with the LSC. Apoptotic nuclei were identified according to previously described standard criteria [7].

### Mice and tumour xenografts

Female athymic mice of approximately 6 weeks of age were used in the study. The animals (obtained from Charles River, Calco, Italy) were housed in clear solid-bottom polycarbonate cages with sterile sawdust bedding material. Animals were handled in a sterile manner in a laminar down-flow hood and they were given free access to sterilised food and water. The mice were checked daily for any adverse clinical reactions and weighed at weekly intervals throughout the study.

For xenograft solid tumour trial, a suspension of $4 \times 10^6$ ME-180 cells was injected subcutaneously in the right flank of each animal (0.2 ml/mice). The day of inoculation was considered day 0 of the study. A total of four groups (one vehicle control and three treated), with eight animals each, were used in this study.

### Dosing

Drugs were dissolved in equal volumes of absolute ethanol and Cremophor EL, as a stock solution of 10 mg/ml. The solutions were sonicated for 1 min and then stored at 4 °C for up to 1 week. On each injection day, the stock solutions were diluted with sterile physiological saline to yield appropriate concentrations immediately before administration. All drugs were administered intravenously at a dose volume of 10 ml/kg on the basis of the individual body weight measured prior to dosing. Control animals received the vehicle alone at the same dose volume.

Since the xenograft model used in this study gives a 100% take rate, early treatments were performed. Chemotherapy was started 1 day after tumour inoculation. The taxanes were administered once daily, according to a q3/4d × 4 schedule. The same schedule treatment was repeated 35 days after the first administration (i.e., day 36 of the study). IDN5109 and paclitaxel were administered at

a dosage of 20 mg/kg per injection (total dose of 160 mg/kg). Docetaxel was administered at a dose of 10 mg/kg per injection (total dose of 80 mg/kg). These dosages were selected on the basis of previous results obtained in our laboratory (unpublished data).

### Evaluation of anti-tumour activity

Tumours were measured with a caliper twice weekly, until the tumours reached 2000 mg. Solid tumour weights (mg) were estimated from two dimensional tumour measurements (mm):

$$\text{Tumor weight} = \frac{\text{length} \times \text{width}}{2}$$

When the median tumour weight of the control (C) reaches approximately 1000 mg, the median tumour weight of each treated group (T) was determined. The T/C value in percentage was calculated as follows:

$$\text{T/C (\%)} = \frac{\text{median tumor weight of treated}}{\text{median tumor weight of control}} \times 100$$

According to NCI standards we used a T/C value $<42\%$ as the minimum level acceptable for a drug having anti-tumour activity. A T/C value $<10\%$ is considered as a high anti-tumour activity level.

### Statistical analysis

In some experiments multivariate analysis of variance (Manova) was performed and used to detect possible significant differences induced by drug treatment. Post-hoc comparisons were evaluated using the Tukey-HSD test to detect significant interactions. A *P* value lower than 0.05 was considered significant. All the analyses were performed using the Statistica 5.0 software package (Statsoft, Tulsa, Okla.).

## Results

The growth inhibition effect of paclitaxel, docetaxel and IDN5109 was assessed in the human cervical cancer cell line ME-180 after 3 days of exposure to the drugs (Fig. 1). The concentrations resulting in the half-maximal growth inhibition ($IC_{50}$) were then calculated to rank the potency of the three drugs. Docetaxel, IDN5109 and paclitaxel obtained $IC_{50}$ values of 0.86, 1.4 and 2.4 nM, respectively. To establish if the growth inhibition effect was dependent on either a block in the cell cycle or the induction of cell death, we performed flow cytometric DNA analysis using a concentration range around the $IC_{50}$ value for each test compound. A concentration-dependent increase of cells blocked at the $G_2/M$ phase of the cell cycle was clearly evident after 12 h of culture in the presence of taxanes (Table 1). After additional 12 h (Table 1), the cell cycle block rapidly disappeared and concomitantly, the increase in DNA fragments as visualised in the sub-$G_{0/1}$ region suggested that apoptosis was occurring in this cellular system. Interestingly, at the highest drug concentrations some DNA histograms had such an irregular appearance that it was impossible to reliably fit a model to assess the various phases of the cell cycle. After 72 h of culture, the amount of DNA fragmentation further increased (Table 1).

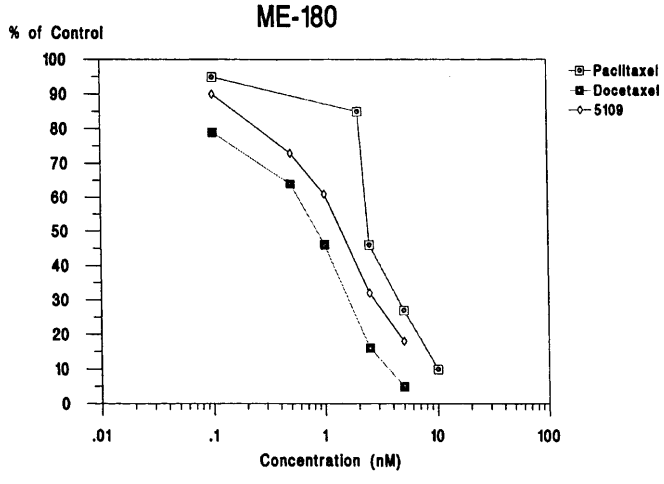


**Fig. 1** Tumour cell growth inhibition of ME-180 cells treated for 72 h with paclitaxel, docetaxel and IDN5109. Standard deviations (less than 10%) have been omitted to add clarity to the graph

Since, in principle, the presence of DNA fragments in the sub-$G_{0/1}$ region is not sufficient to ascertain the occurrence of apoptosis in a cellular system [4], we used an LSC to perform morphological analysis of the cells present in the sub-$G_{0/1}$ region. The LSC is a newly developed instrument that performs the acquisition of the signal fluorescence on microscope slides using an approach similar to that of a flow cytometer. Along with data on fluorescence signals, acquisition on slides allows tracking of each cell in the x-y positions. This makes it possible to relocate cells on the slide after restaining for the visual inspection of each event generating a given fluorescence signal. Using this methodology, we first acquired DNA fluorescence of cells treated for 24 h with the taxane concentration capable of inducing the maximal amount of DNA fragmentation (i.e. 5 nM for paclitaxel and 2.5 nM for both IDN5109 and docetaxel). Then, the same slides were restained for optical analysis. Cell morphology in the sub-$G_{0/1}$ region was analysed and compared with that of untreated cells in the other phases of the cell cycle. A representative gallery as obtained with the LSC is shown in Fig. 2. Results demonstrated the vast majority (87–94%) of the cells in this region exhibited apoptotic features, namely nuclear shrinkage, blebbing and chromatin condensation, without morphological differences between the drug treatments. Taken together these findings, we concluded that taxanes induce apoptosis in this system and that IDN5109 and docetaxel did this to a higher extent than paclitaxel.

To assess the interaction of taxanes with tubulin in ME-180 cells, we performed a dual staining for DNA and tubulin. We used the three drugs at equitoxic concentrations ($IC_{50}$ values) to observe the modulation in the expression of the tubulin subunits after 12 h of exposure to the drugs. The three drugs were able to slightly, but significantly, down-modulate the expression of the $\alpha$ subunit of tubulin only in the $G_{0/1}$ phase of the

**Table 1** Cell cycle analysis of ME-180 human cancer cells treated with paclitaxel, docetaxel and IDN5109 for 12 h, 24 h and 72 h (*nf* not fittable)

| | 12 h | | | | 24 h | | | | 72 h | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound (nM) | $G_1$ | S | $G_2$ | % of DNA fragmentation | $G_1$ | S | $G_2$ | % of DNA fragmentation | $G_1$ | S | $G_2$ | % of DNA fragmentation |
| Control | 27.1 | 24 | 48.9 | 1.5 | 31.2 | 36 | 32.8 | 3 | 62 | 12.7 | 25.2 | 1.2 |
| DMSO 0.1% | 30 | 21.4 | 48.6 | 2.0 | 32.2 | 35.4 | 32.4 | 4.5 | 60.8 | 11.7 | 27.5 | 2.1 |
| Paclitaxel 1 | 27.2 | 24.6 | 40.1 | 1.4 | 25.2 | 43.2 | 31.5 | 2.7 | 64.3 | 15.1 | 20.6 | 2.3 |
| Paclitaxel 2 | 26.2 | 24.4 | 49.7 | 2.0 | 28.3 | 42.9 | 28.8 | 5.2 | 65.3 | 13.9 | 20.8 | 2.4 |
| Paclitaxel 2.5 | 21.1 | 31.3 | 47.5 | 4.0 | 23.9 | 59.6 | 16.5 | 14.4 | 50.6 | 43.4 | 6 | 16.6 |
| Paclitaxel 5 | 12 | 33.8 | 54.2 | 7.4 | nf | nf | nf | 24.9 | 34.6 | 59.3 | 6 | 29.3 |
| Docetaxel 0.1 | 28.9 | 22.4 | 48.8 | 1.6 | 30.9 | 32.5 | 36.5 | 1.4 | 63.9 | 10.7 | 25.4 | 1.3 |
| Docetaxel 0.5 | 23.1 | 30.3 | 46.5 | 3.8 | 29.4 | 41.9 | 28.6 | 6 | 58.7 | 33.4 | 7.9 | 9.3 |
| Docetaxel 1 | 14.9 | 33.9 | 51.2 | 9.9 | 30.9 | 32.5 | 36.5 | 12 | nf | nf | nf | 37.2 |
| Docetaxel 2.5 | 6.9 | 20.8 | 72.3 | 2.7 | nf | nf | nf | 28.3 | nf | nf | nf | 46.9 |
| IDN5109 0.5 | 20.6 | 31.9 | 47.5 | 8.0 | 26.6 | 40.6 | 32.8 | 3.3 | 61.8 | 21.1 | 17.1 | 3.9 |
| IDN5109 1 | 18.7 | 34 | 47.4 | 11.6 | nf | nf | nf | 20.6 | 43.2 | 52.3 | 4.5 | 22.3 |
| IDN5109 2.5 | 8.3 | 25.3 | 66.3 | 6.2 | nf | nf | nf | 29.3 | nf | nf | nf | 39.9 |
| IDN5109 5 | 8 | 23.2 | 68.8 | 2.5 | nf | nf | nf | 28.6 | nf | nf | nf | 43.8 |

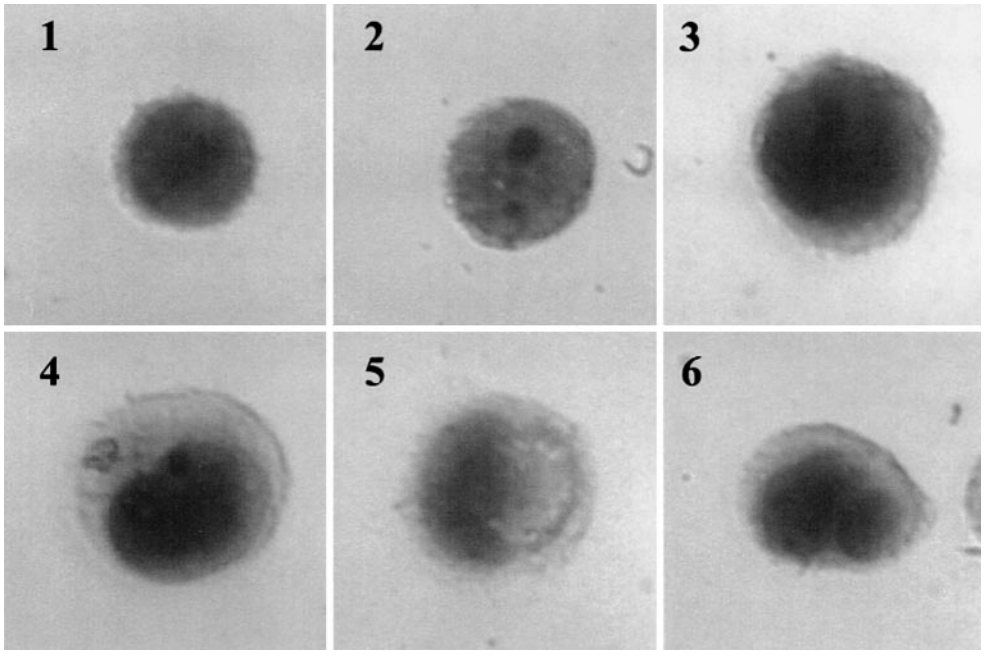


**Fig. 2** LSC-generated gallery of cell morphology in $G_{0/1}$, S and $G_2$/M phase of untreated cells (from *1* to *3*) and in the sub-$G_{0/1}$ region of treated cells (from *4* to *6*). In *1–3* nuclear morphology is regular with minimal presence of condensed chromatin. In *4–6* cells show typical apoptotic features such as chromatin condensation and nuclear shrinkage and marginalisation (*LSC* laser scan cytometer). ×400

cell cycle (Fig. 3). However, there was no significant difference between the three drugs. In contrast, the expression of the β subunit was enhanced by all drugs in the $G_2$/M phase, but docetaxel and IDN5109 produced a statistically significantly higher accumulation of these subunits than paclitaxel (Fig. 3).

The anti-tumour activity of the panel of taxanes was tested "in vivo" on nude mice xenografted using ME-180 cells. Preliminary studies were performed to establish the maximum tolerated dose (i.e. no lethal toxicity and $< 20\%$ body weight loss). Such cumulative dose was 80 mg/kg for docetaxel and 160 mg/kg for both paclitaxel and IDN5109. The treatment schedule was as follows: drugs were administered at 10 mg/kg per injection for docetaxel and 20 mg/kg per injection for paclitaxel and IDN5109 on day 1 and repeated on days 4, 7 and 10. This scheme was repeated again after 35 days. No deaths in treated groups were recorded and the maximum body weight loss observed was comparable between the treated groups. In addition, at the end of the study, no differences in the mean body weight values were observed in treated and control animals (data not shown).

Drug treatment induced a strong anti-tumour activity, obtaining a significant reduction ($F = 162.6$; $P < 0.001$) in the development of the xenografts

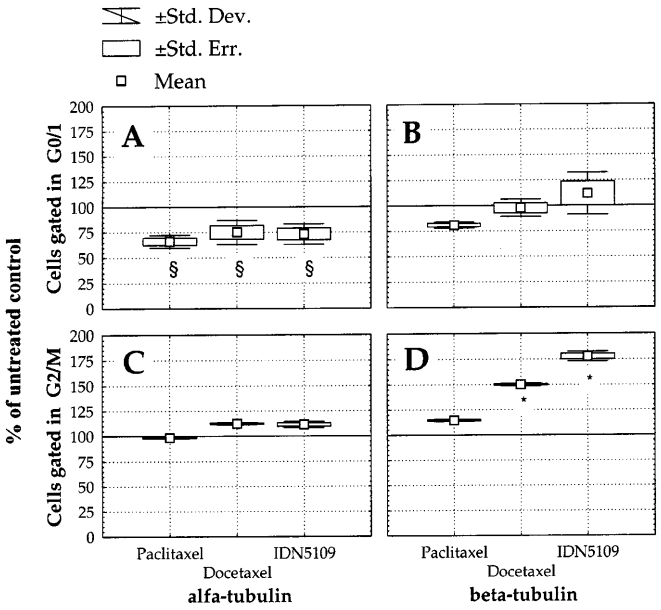


**Fig. 3A–D** Box whisker plots indicating the expression of tubulin subunits (α in **A**,**C**; β in **B**,**D**) in cells gated in $G_{0/1}$ (**A**,**B**) and in $G_2$/M phase (**C**,**D**) of the cell cycle. Experiments have been performed in triplicates and *squares*, *boxes* and *bars* correspond to the mean, standard error and standard deviation, respectively. In **A**, *§* indicates a statistically significant ($P < 0.01$) difference with respect to control. In **D**, *asterisk* indicates a statistically significant ($P < 0.01$) higher expression of β tubulin subunits with respect to paclitaxel. The latter showed a statistically significant ($P < 0.01$) difference with respect to control. This experiment was repeated twice with similar results

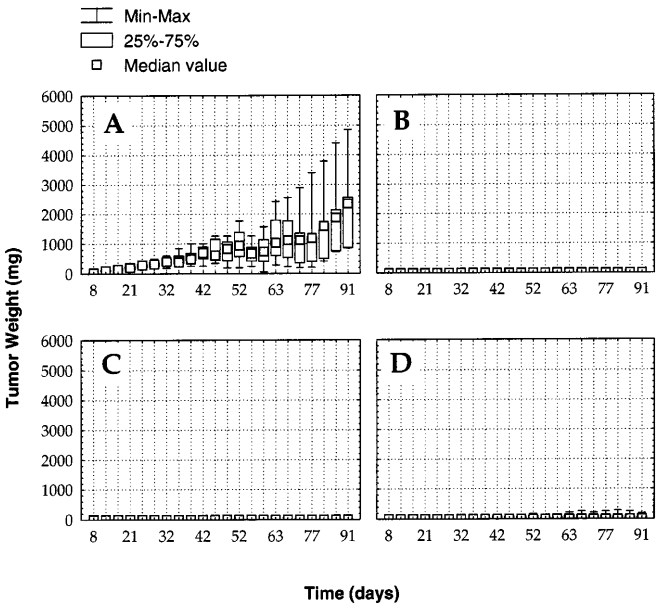


**Fig. 4A–D** Box whisker plots representing the growth of xenografts of ME-180 cells in nude mice treated with the vehicle alone (**A**), docetaxel (**B**), paclitaxel (**C**), and IDN5109 (**D**). *Squares*, *boxes* and *bars* represent the median value, the range of 25–75 percentiles and the absolute range (min-max), respectively. Although at the end of the study a minimal tumour growth was detected within the group treated with IDN5109, no statistically significant differences were observed between the three drugs that resulted very effective in inhibiting tumour growth

(Fig. 4). All the three taxanes were found to be highly active against with T/C of 0% (Table 2). Paclitaxel and docetaxel induced a 100% cure rate at the selected dosages, while for IDN5109 three out of eight animals treated had developed tumours approximately 60 days after tumour inoculation. However, these tumours did not achieve more than 300 mg of weight up to the end of the study (day 91 post-tumour inoculation).

## Discussion

In the present study, we demonstrated that taxanes are active both "in vitro" and "in vivo" against the human cervical cancer cell line ME-180. When considering the growth inhibition effects of the three taxanes, we observed the following order of potency: docetaxel > IDN5109 > paclitaxel. The superior activity of docetaxel and IDN5109 was shown by DNA analysis and could be conceivably related to the observed higher inhibition (at equitoxic concentrations) of the β tubulin disassembly. This finding supports previous data showing that structural differences between taxanes considerably affect the mechanism of interaction between taxanes and tubulin [1, 23]. Moreover, in keeping with recent observations indicating that α tubulin also contributes to the binding of taxanes [14], we found that α tubulin was down-modulated in cells at the $G_{0/1}$ phase of the cell cycle. We concluded that "in vitro" docetaxel and IDN5109 have a higher cytotoxic activity toward ME-180 cells than that of paclitaxel.

Additional considerations arise from DNA analysis. In our model a cell cycle block at the M checkpoint was visible only after 12 h and then rapidly disappeared to be replaced by a large increase of the apoptotic index (quantified by DNA fragmentation). This result is different from that obtained in other human cancer cell lines, where the block at the M checkpoint of the cell

**Table 2** Anti-tumour activity of a panel of taxanes toward xenografts of ME-180 cells in nude mice. Schedule: 1, 4, 7, 10; 36, 39, 42, 45 days. Tumour growth inhibition was evaluated when the median tumour weight of the control (C) reached 1000 mg. (*C* weight control tumour, *T* weight treated tumour, *Max BWL* maximum body weight loss)

| Compound | Dosage (mg/kg per injection) | Total dose (mg) | T/C % | Max BWL % (day) | Drug death |
|---|---|---|---|---|---|
| Paclitaxel | 20 | 160 | 0 | 5 (45) | 0/8 |
| Docetaxel | 10 | 80 | 0 | 8 (45) | 0/8 |
| IDN5109 | 20 | 160 | 0 | 6 (45) | 0/8 |

cycle is stable and still visible after 48–72 h of culture in the presence of taxanes [5]. The rapid release from the block at the M phase of the cell cycle suggests that ME-180 cells have a defect in the mitotic checkpoint. A possible support for this interpretation is based upon the fact that specific strains of the human papillomavirus (HPV) are noticeable in approximately 85–90% of human cervical carcinomas (reviewed in [9] including ME-180 cells, where the genome of HPV-68 has been demonstrated [16]). The pathogenic role of HPV, determined by molecular analysis, involves at least three viral genes (E2, E6 and E7) [9] and one of the main effects of these v-oncogenes is to abrogate, via p53-dependent [10] and -independent pathways [21], the M checkpoint.

The potent anti-tumour activity of the three taxanes was confirmed in "in vivo" experiments using xenografts of ME-180 cells in nude mice. These experiments yielded excellent T/C values (0% for the three drugs). However, some differences were also detected in this case. Docetaxel exhibited more toxicity than IDN5109 and paclitaxel because the maximum non-lethal dose was 80 mg/kg for docetaxel compared to 160 mg/kg of paclitaxel and IDN5109. Additional studies are now needed to evaluate the activity of the three taxanes in late stages, by testing different schedules to identify the most active agent in advanced experimental tumours.

In conclusion, our data, albeit obtained in a single human cervical carcinoma cell line, support a possible use of taxanes in the treatment of this disease. The taxane concentration range (1–5 nM) needed to achieve the anti-tumour activity is within the range of the plasma and tumour concentrations obtained for paclitaxel and docetaxel [20]. Furthermore, this study confirms the presence of some relevant differences in biological activity of the three taxanes, prompting us to perform clinical trials to verify if either docetaxel or IDN5109 could induce a better clinical response in cancer of the uterine cervix with respect to paclitaxel.

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