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ORIGINAL ARTICLE

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# Assessment of microtubule stabilizers by semiautomated in vitro microtubule protein polymerization and mitotic block assays

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**Abstract** Paclitaxel (Taxol) a clinically active anticancer agent, exerts its cytotoxicity by inducing tubulin polymerization, leading to cellular mitotic block. In contrast, other antimitotic drugs, such as colchicine, podophyllotoxin, and vinblastine, act by depolymerizing microtubules. We report here (a) a semiautomated assay which measures the tubulin-polymerizing activity of paclitaxel analogs and (b) a cellular assay to measure the potential of these compounds to block cells in mitosis. The microtubule-polymerizing assay measured the turbidity of bovine brain microtubule protein (MTP) polymerized by the test compound in a 96-well plate. We maximized the sensitivity of this assay by conducting the polymerization reaction at 20 °C, at which temperature the baseline reaction, i.e. the basic ability of the untreated MTP control to polymerize, was minimal. At 20 °C, the effect of 0.05 μg/ml of paclitaxel on MTP could be detected, whereas at 37 °C, >1 μg/ml of paclitaxel was required to detect a significant effect relative to untreated MTP. We describe the analysis of the complex curves of MTP polymerization with varying concentrations of test compounds. The polymerization of microtubules leads to cells being blocked in mitosis. This mitotic blocking effect in intact cells was determined using a cell settling chamber which allowed eight samples to be deposited on a slide. This method required a smaller number of cells ($10^3$–$10^5$), maintained cell morphology, and allowed for rapid screening of samples. The activity of several new paclitaxel analogs is reported.

**Key words** Microtubule stabilizer · Microtubule polymerization · Mitotic block · Automation

**Abbreviations** *DMSO* dimethyl sulfoxide · *EDTA* ethylene-diamine-tetraacetic acid · *EGTA* ethylene-bis (oxyethylenenitrilo) tetraacetic acid · *MAP* microtubule-associated proteins · *MEM* minimum essential medium · *MTP* microtubule protein · *PBS* phosphate-buffered saline · *PEM* 0.1 M PIPES – NaOH, pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$ · *PIPES* piperazine-*N*,*N*′-bis(2-ethane sulphonic acid)

## Introduction

Paclitaxel, a diterpenoid plant product, belongs to a new class of antimicrotubule agents used in cancer chemotherapy [13, 32, 37] and has shown promising activity in refractory ovarian carcinoma, where some of the remissions have occurred in women with cancer resistant to cisplatin [7, 26, 30, 34]. Paclitaxel, and a semisynthetic analog docetaxel (Taxotere), have also shown activity in other tumors including non-small-cell lung and breast carcinoma [10, 11, 30]. Paclitaxel acts by inhibiting cell division by blocking cells in the $G_2$/M phase of the cell cycle. Cells escape the mitotic block with prolonged exposure to paclitaxel, without cytokinesis and enter the next round of DNA synthesis to form multinucleated polyploid cells [2, 23–25].

Paclitaxel-induced programmed cell death or apoptosis has been studied extensively [3, 22, 27, 38]. The target of the drug appears to be the microtubule, and unlike other antimicrotubule agents (colchicine, vincristine, vinblastine etc.) which act by inducing microtubule disassembly [6, 14, 32], paclitaxel promotes the assembly of microtubules and stabilizes tubulin polymers by preventing their depolymerization [25, 29, 33,

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35]. Paclitaxel reduces the critical concentration of tubulin required for polymerization and enhances the apparent rate of microtubule assembly. Microtubule assembly can be monitored by the rate of increase in turbidity in isolated tubulin [36]. Since assembled tubules scatter light as essentially infinite rods, the kinetic and thermodynamic properties of assembly can be obtained from measurements of the amount of polymerized protein [9].

Microtubules are known to assemble and disassemble in response to a wide variety of chemical agents and physical conditions, such as microtubule associated proteins (MAPs), glycerol, DMSO, $Mg^{2+}$, polycations, $Zn^{2+}$, $Co^{2+}$, paclitaxel, organic sulphonate and polyanionic compounds [1]. The paclitaxel binding site has been found to be distinct from that of podophyllotoxin, vinblastine sulfate and colchicine [19]. Kinetic analysis of microtubule assembly demonstrates that there is a nucleation step followed by elongation of microtubules [15]. Schiff et al. [36] found that bovine brain microtubules assembled at 37 °C in the presence of 10 μ*M* paclitaxel are completely resistant, and those assembled in the presence of 5 μ*M* are partially resistant to depolymerization by 4 m*M* $CaCl_2$. Docetaxel has been found to be 2.5 times more potent than paclitaxel in inhibiting the replication of J774.2 (mouse macrophage) and P388 (mouse leukemia) cells and at least five times more potent in taxol-resistant cells. It also appears to be a better promoter of tubulin polymerization than paclitaxel [31].

We report here the development of a screening system which enabled us to analyze and select potent paclitaxel analogs for further study, based on their ability to polymerize microtubule protein (MTP) in a 96-well plate. This in vitro assay which measured the interaction of MTP and drug was supplemented by an intact cell assay based on the ability of paclitaxel to block cells in mitosis. The intact cell assay was modified from the standard procedure [4] to screen for a large number of samples by employing a cell-settling chamber [21]. These methods would be valuable as secondary assays for identification and synthesis of microtubule stabilizers.

## Materials and methods

### Chemicals and reagents

Paclitaxel was obtained from Polysciences (Warrington, Pa.) and docetaxel and analogs (PNU compounds) from Medicinal Chemistry, Pharmacia & Upjohn, Kalamazoo, Mich. [16, 18]. The compounds were dissolved in DMSO at 5 mg/ml for MTP polymerization and 1 mg/ml for the mitotic block assay. Structures and molecular weights for these compounds are shown in Fig. 1. Bovine brain MTP was obtained from the laboratory of Dr. Leslie Wilson (University of California, Santa Barbara). MTP consisting of 70% tubulin and 30% MAPs was isolated according to the modified method of Farrell and Wilson [8]. The MTP obtained was depolymerized in PEM buffer (0.1 *M*-PIPES-NaOH, pH 6.8, containing 1 m*M* EGTA, and 1 m*M* $MgSO_4$) containing 1 m*M* GTP and stored frozen at −70 °C until use. The protein assay kit was from Bio-Rad Laboratories (Hercules, Calif.). Cell-settling chambers were obtained from Neuroprobe (Cabin John, Md.). Orcein stain was obtained from Sigma Chemical Company (St. Louis, Mo.). A 2% orcein solution was prepared by dissolving orcein in a 1:1 mixture of glacial acetic acid and boiling distilled water, which was then filtered. The fixative used was ethanol/glacial acetic acid (3:1).

### Cell culture

B16/F10 (mouse melanoma) cells were obtained from Dr. I.J. Fidler (MD Anderson Hospital and Tumor Institute, University of Texas, Houston, Tx.). The cells were maintained in MEM medium, supplemented with MEM nonessential amino acids (10 ml of 100× concentration/liter), 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, essential MEM vitamins (10 ml of 100× concentration/liter) and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, Utah). Trypsin-EDTA (containing 0.5 mg/ml of trypsin and 0.2 mg/ml of EDTA) was from Irvine Scientific (Santa Ana, Calif.). The doubling time of the cells was 12 h.

### Microtubule protein polymerization assay

The constituents of the assay (MTP, GTP, buffer) were kept cold on ice. Various concentrations of paclitaxel, docetaxel and PNU compounds were diluted with PEM buffer containing 1 m*M* GTP, keeping the DMSO concentration the same (0.04%) in all samples. The 96-well plates were always kept at the temperature (20 °C, 25 °C, 37 °C) at which the experiment was conducted. The buffer with or without test compound was added in duplicate to the wells of the 96-well plate. Polymerization of MTP by the test compound resulting in increased turbidity was measured using a Biotek reader EL 340. For experiments conducted at 20 °C, the Biotek reader was placed in a refrigerated incubator. However, for the initial assays conducted at 25 °C and 37 °C, the electronically controlled chamber of the Biotek reader was adjusted to obtain the required temperature. The assay was initiated by adding cold MTP at 3 mg/ml to each well (to a total volume of 200 μl). The plate was incubated at the respective temperatures in the Biotek reader and optical density (OD) readings taken every 3 min for a duration of 60 min. These readings were saved on disk and further processed using an IBM PC program which subtracted the initial buffer reading obtained before MTP was added to the 96-well plate. Kinetic curves were constructed using a program developed in the RS/1 language. A second degree polynomial was then fitted to the data. The polynomial coefficients and goodness of fit parameters are outputs of the program. RS/1 is a product of Bolt Beranek and Newman Inc., Cambridge, Mass.

### Protein assay for polymerized MTP

MTP was polymerized with the test compound for 60 min at 20 °C (as described above) and centrifuged in a TLA 100 rotor (Beckman centrifuge) at 225 000 *g* for 30 min at 20 °C. The supernatant was aspirated and the pellet was solubilized in PEM buffer by overnight incubation in the refrigerator. The amount of protein was determined by the BIO-RAD method described on the package insert.

### Electron microscopy studies

MTP was incubated with test compound or DMSO in a 96-well plate at 20 °C according to the protocol described previously. Once the MTP was assembled for 60 min, a 10-μl sample was fixed with 2% glutaraldehyde and then applied to a copper grid coated with parlodion. The sample was stained with 1.5% uranyl acetate as described by Jordan et al. [17]. The grid was dried overnight and photomicrographs taken at suitable magnifications with a Philips CM10 photomicroscope.

### Mitotic block assay

This assay was conducted using a multiwell cell-settling chamber (Neuroprobe) which allowed the preparation of randomly

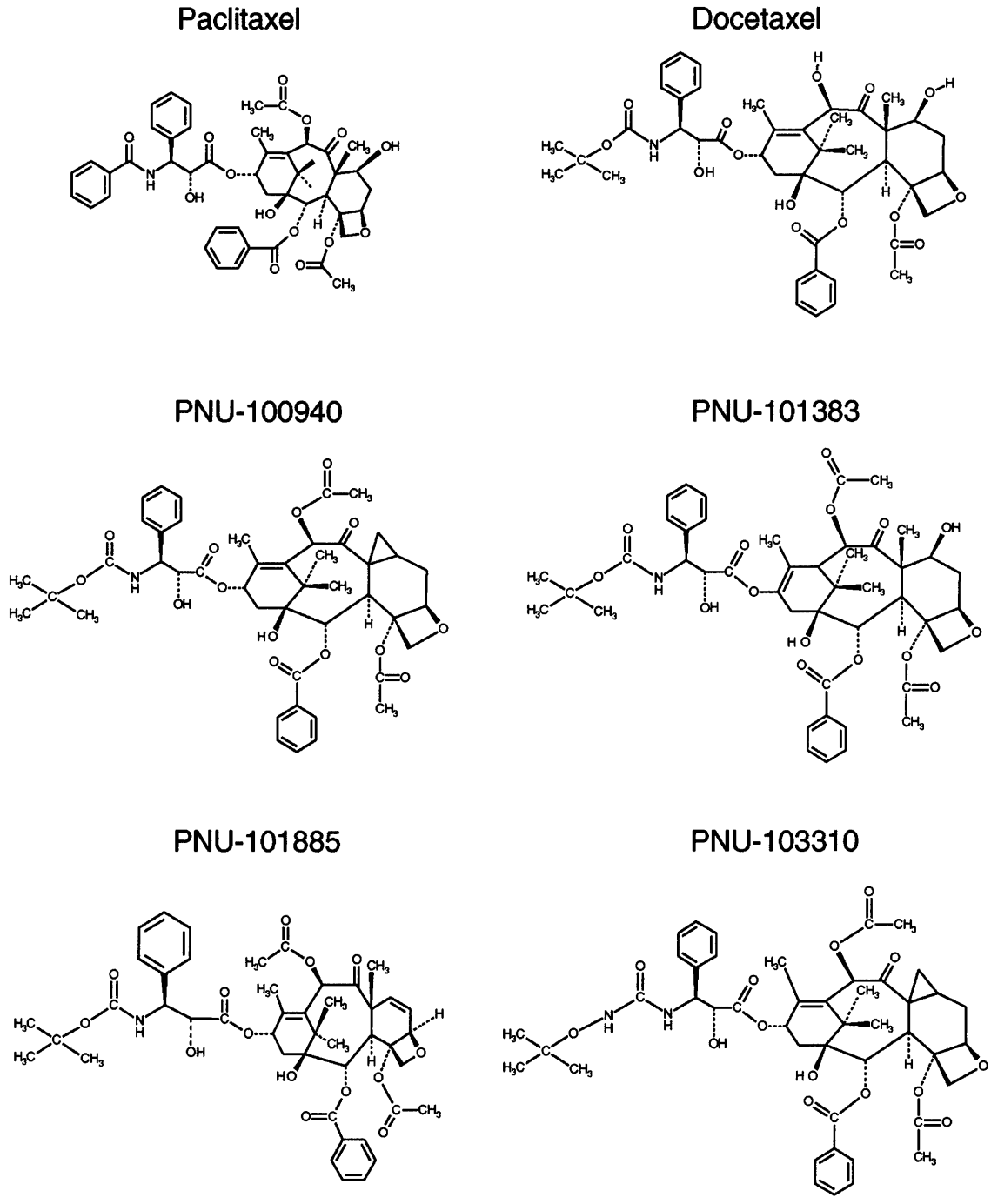


**Fig. 1** Structures of paclitaxel, docetaxel, PNU-100940, PNU-101383, PNU-101885 and PNU-103310 as reported by Johnson et al. [16] and Kelly et al. [18]. Molecular weights are 853.98; 807.86; 831.92; 849.94; 831.92 and 830.94, respectively

distributed cells on a microscope slide, suitable for morphological identification and counting [21]. The cell-settling chamber consists of a base plate, microscope slide, filter paper, silicone gasket, acrylic top plate and knurled assembly nuts. The chamber has eight wells and works on the principle that when 50 µl of a cell suspension at concentrations of $10^3$–$10^6$ cells/ml is added to the wells of the chamber, the cells settle on the glass slide. The fluid is slowly wicked away by the damp filter paper sandwiched between the microscope slide and the acrylic top plate of the multiwell chamber. Within 20–40 min, the cell monolayer on the slide was dry and the slides could be removed from the chamber and fixed in ethanol/acetic acid (3:1) for 10 min. The slides were air-dried and stained with aceto-orcein prior to the counting of mitotic cells. The results obtained using the multiwell cell settling chamber were often compared to the standard method used previously in this laboratory [4].

B16/F10 cells were planted in T/25-cm$^2$ flasks 2 days before the experiment to ensure exponential growth. After exposure to paclitaxel, test drug or DMSO for 5 h, the medium was saved in centrifuge tubes to collect detached mitotic cells. The attached cells were then harvested with trypsin and added to their respective tubes. Cells were centrifuged at 130 *g* for 5 min. The medium was aspirated and the cell pellet resuspended in 1 ml PBS. A cell suspension of 50 µl in duplicate was then added to the wells of the cell-settling chamber. The slides were fixed for 10 min prior to staining

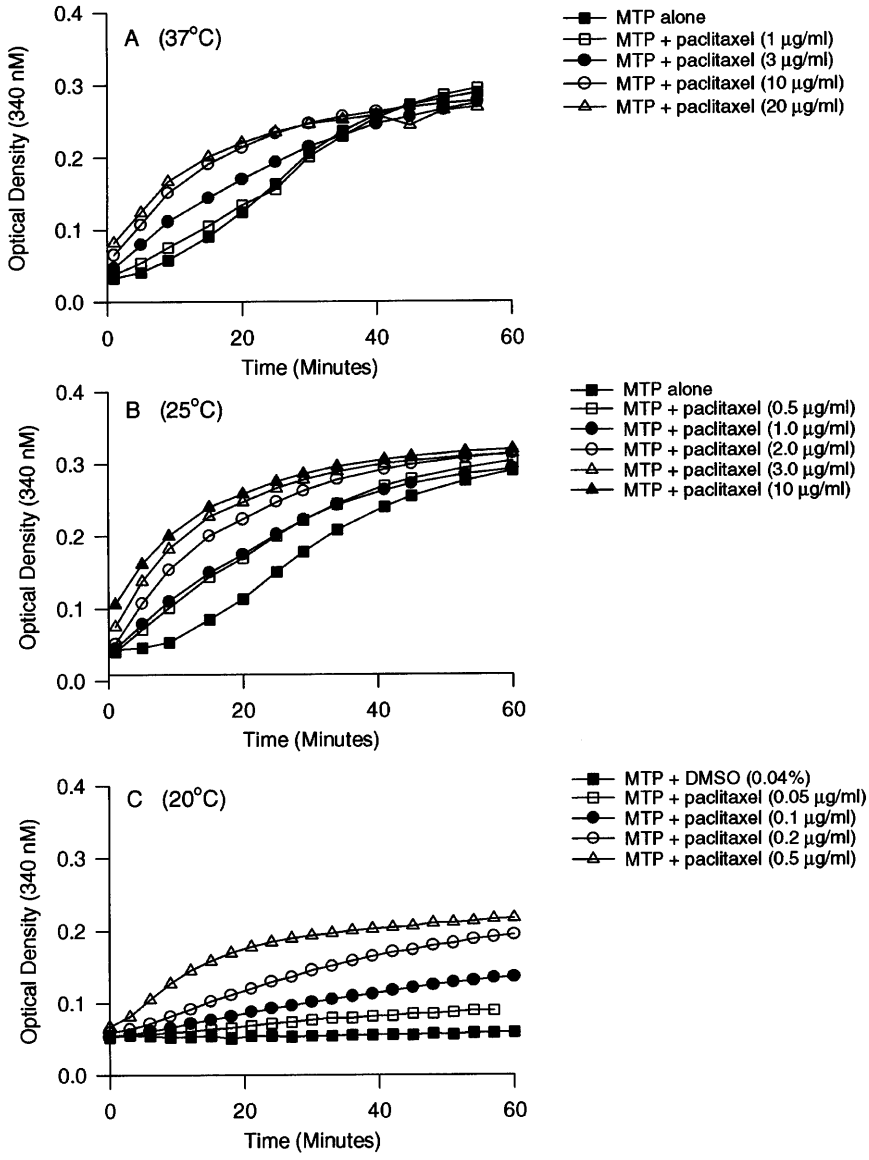


**Fig. 2A–C** MTP (3 mg/ml) was polymerized with various amounts of paclitaxel in a 96-well plate with 1 m*M* GTP and PEM buffer, pH 6.8, at 37 °C (**A**), 25 °C (**B**) or 20 °C (**C**)

with aceto-orcein. The cells were evenly spread and stained mitotic cells could be easily differentiated from nonmitotic cells [4]. Approximately 400 cells were counted in two different sections of each well and the percentage of mitotic cells determined. In the standard method [4], about $10^6$ cells treated with the test drug were centrifuged at 130 *g* for 5 min. The cell pellet was then fixed by slow drop-wise addition of fixative without disturbing the pellet and left standing for 10 min. The fixative was poured off and the cell pellet resuspended with 5 ml fixative and centrifuged at 130 *g* for 5 min. The fixative was poured off, leaving about 0.2–0.3 ml with the cell pellet. The cell pellet was tapped and a drop of the cell suspension added to a clean slide. Cells were spread by blowing gently, and air dried. The slides were stained with aceto-orcein and mitotic cells counted.

### Cytotoxicity assay

Exponentially growing B16/F10 cells were planted in T/25-$cm^2$ flasks at $2 \times 10^4$ cells/flask. After exposure to test compounds for 60 h (five cell doublings), the cells were harvested with trypsin-EDTA and counted in a Coulter Counter ZM (Coulter Electronics, Hialeah, Fl.). A dose response curve was constructed after calculating percent cellular growth relative to control, and $IC_{50}$ values determined (drug concentration required to kill 50% of cells).

## Results

### Parameters defined for the MTP polymerization assay

#### *Effect of temperature*

Figure 2A shows the effect of paclitaxel (1–20 μg/ml) on MTP polymerization at 37 °C. MTP treated with 3–20 μg/ml of paclitaxel polymerized at a faster rate than untreated MTP. However, the rate of polymerization with 1 μg/ml paclitaxel was similar to untreated MTP, i.e the assay could detect only effects of paclitaxel $>1$ μg/ml (Fig. 2A). When the assay was conducted at

25 °C, the initial rate of polymerization of MTP treated with 0.5 μg/ml of paclitaxel was significantly different from untreated control (Fig. 2B). When this reaction was carried out at 10 °C, only MTP treated with 10 μg/ml paclitaxel showed an increased rate of polymerization relative to untreated control (data not shown). These results suggested that the assay could be made more sensitive by conducting it at a constant temperature above 10 °C and below 25 °C. This was achieved by installing the Biotek reader in a refrigerated incubator. Figure 2C shows that at 20 °C there was minimal polymerization of vehicle-treated MTP, and the effect of even 0.05 μg/ml paclitaxel could be detected.

### *Effect of DMSO*

Algaier and Himes [1] have reported that in the presence of DMSO (10%), the critical MTP concentration is lowered eight- to tenfold at 37 °C, leading to rapid tubulin polymerization. Since paclitaxel was dissolved in DMSO for our in vitro studies, we determined the effect of this solvent on the rates of MTP polymerization at 20 °C. Figure 3A shows that even 0.1% DMSO gave a higher rate of polymerization than MTP polymerization at 20 °C, whereas with 0.05% DMSO (Fig. 3B), the rate of polymerization of DMSO-treated MTP was similar to the untreated MTP. In subsequent assays, all samples (treated and untreated) contained 0.04% DMSO.

### *Sensitivity and reproducibility*

The in vitro MTP polymerization assay conducted at 20 °C (Fig. 2C), with 3 mg/ml MTP and 0.04% DMSO, was 10–20 times more sensitive in detecting paclitaxel activity than that conducted at 37 °C (Fig. 2A). Thus, at 20 °C the rate of polymerization of MTP treated with 0.05 μg/ml of paclitaxel was significantly higher than the DMSO control (Fig. 2C). Figure 4 shows the average rate of polymerization with paclitaxel in three different experiments. The assay was adequately reproducible for our purposes. However, it must be realized that different batches of MTP could polymerize at different rates. Therefore paclitaxel-treated MTP was run in every assay as the positive control and the rate of polymerization with the test compound is reported as relative to that with paclitaxel.

## Analysis of data

Once the parameters for the assay were established, we analyzed the MTP polymerization curves to determine the average rate of polymerization at different paclitaxel and analog concentrations. The kinetic data for assembly reaction yielded complex curves with initiation steps and growth steps, making a detailed analysis very

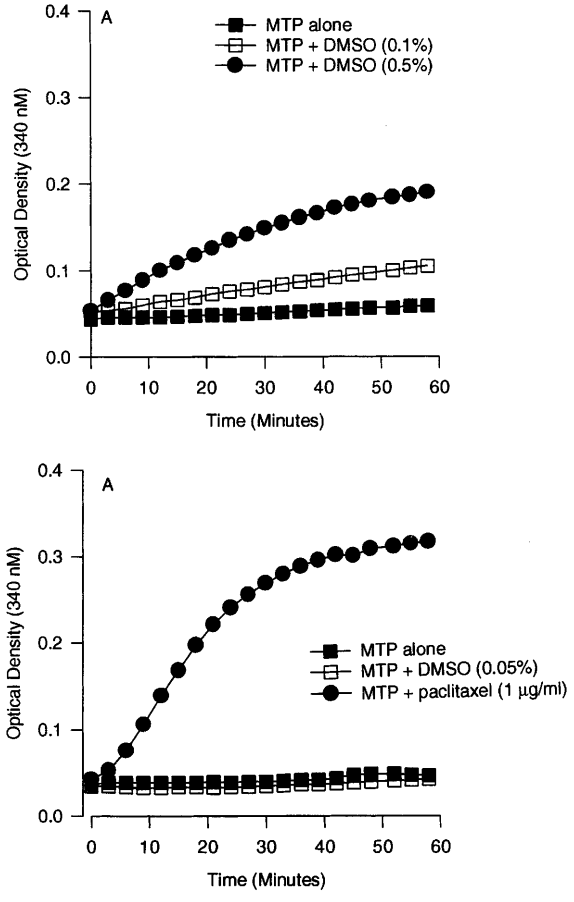


**Fig. 3A–B** The rate of MTP polymerized is enhanced with DMSO (>0.05%) at 20 °C when the reaction mixture contains PEM buffer, pH 6.8, and 1 m*M* GTP

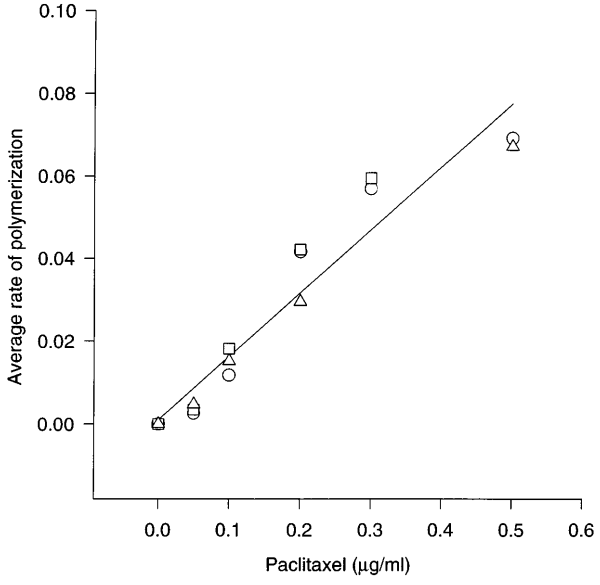


**Fig. 4** Reproducibility of the assay and linear dose response as evaluated by the effect of paclitaxel concentration on the average rate of polymerization over 20 min: average rate = $Bt/2 + Ct^2/3$, where t = 20 min

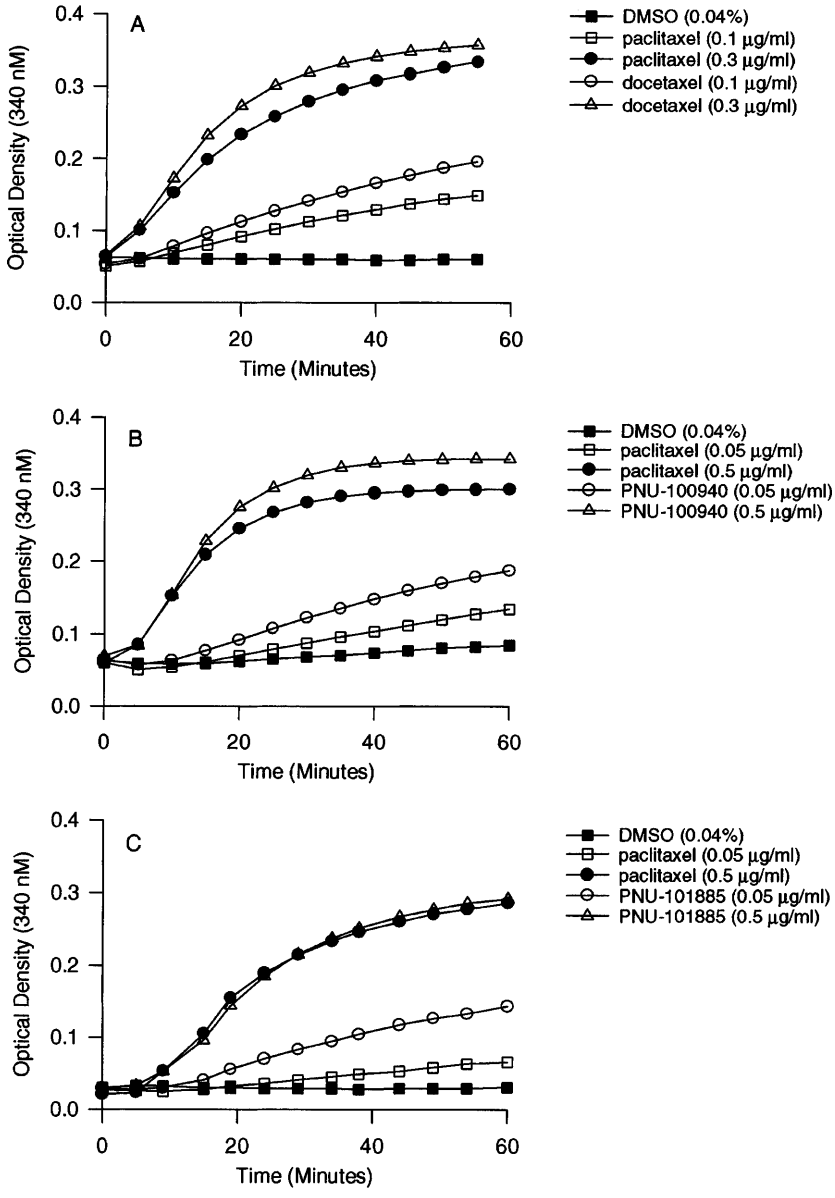


**Fig. 5A–C** MTP (3 mg/ml) was polymerized at 20 °C with paclitaxel or docetaxel (**A**), with paclitaxel or PNU-100940 (**B**), or with paclitaxel or PNU-101885 (**C**); all tested in the assay at 20 °C with 1 m*M* GTP and PEM buffer, pH 6.8

complex. Although every effort was made to keep solutions cold in our assay to initiate the polymerization reaction at the time of the first OD measurement, there were practical limitations to this approach. At first glance, the polymerization curves appeared to be sigmoidal in shape. However, with either high concentrations or very low concentrations of paclitaxel, sigmoidal curves were not obtained. A sigmoidal function fit to the curves would therefore yield large uncertainties in the parameters fitted, owing to lack of information about the first region of the curve as happens with high paclitaxel concentrations, and similarly lack of information about $E_{max}$ with low paclitaxel concentrations (owing to the longer lag phase). Since the kinetics of assembly showed a drug concentration dependence of a second-order reaction, we modeled the curves to a second order polynomial to calculate the 'average rate of polymerization'. This analysis also disregarded the sources of variance beyond the control of the investigator.

Fitting quadratic lines to each curve plotted with the start and end times specified, the RS/1 program prints line coefficients B and C for the equation:

$$Y = A + Bt + Ct^2$$

where A = intercept at time 0 (offset)

B = coefficient of 't'
C = coefficient of $t^2$
t = time period
Y = optical density

These coefficients are further described in *RS/1 users guide: statistical tools*, pages 5–22.

Correcting for the offset of the curve from the origin (coefficient of A) and integrating over time, the area

from time t = 0 to time $t_1$ (the time polymerization is measured) is given by:

$$\text{Area integrated between specified time} = \frac{Bt_1^2}{2} + \frac{Ct_1^3}{3}$$

This is the integrated change in OD with respect to time. Therefore, the average change in OD with respect to time, i.e. the average rate of polymerization, is given by dividing by time ($t_1$):

$$\text{Average rate} = \frac{Bt_1}{2} + \frac{Ct_1^2}{3}$$

The average rate of polymerization for the different concentrations of paclitaxel were then fitted to a linear regression, from which the slope and intercept were derived. The paclitaxel equivalence concentration of the test compound was calculated and the ratio of the average rate of polymerization of the test compound to paclitaxel determined. Figure 4 shows a typical linear increase in rate of MTP polymerization at different concentrations of paclitaxel.

## Paclitaxel, docetaxel and related analogs

Determination of the average rate of polymerization over 25 min showed that docetaxel, and most of the related analogs were more potent than paclitaxel (Fig. 5A–C; Fig. 6A,B; Table 1) in their ability to polymerize MTP. This enhanced activity of docetaxel has been reported by other researchers [20]. It is worth noting, that at 0.05 μg/ml, the rate of polymerization of PNU-101885 was clearly higher (2.2 × more potent) than paclitaxel. However, at the higher concentration of 0.5 μg/ml the rates of polymerization of both compounds were similar. We observed this phenomena with several other taxane analogs and are unable to explain it. We wondered whether this effect was due to some artifact in the formation of microtubules, resulting in incorrect OD readings. Therefore, we polymerized MTP to steady state (60 min) at both high and low concentrations of paclitaxel and PNU-101885 and measured the amount of protein polymerized. These measurements corresponded with the ODs obtained and confirmed that at low concentrations the amount of protein polymerized by PNU-101885 was greater than that obtained with a similar paclitaxel concentration. At high concentrations of PNU-101885 the amount of protein polymerized was equal to that obtained with paclitaxel (data not shown).

### *Electron microscopic studies*

We conducted electron microscopic studies to confirm that we were indeed measuring microtubule formation in this assay conducted at 20 °C, instead of nonspecific protein aggregates. MTP was polymerized by paclitaxel, docetaxel and PNU-101885 along with the DMSO

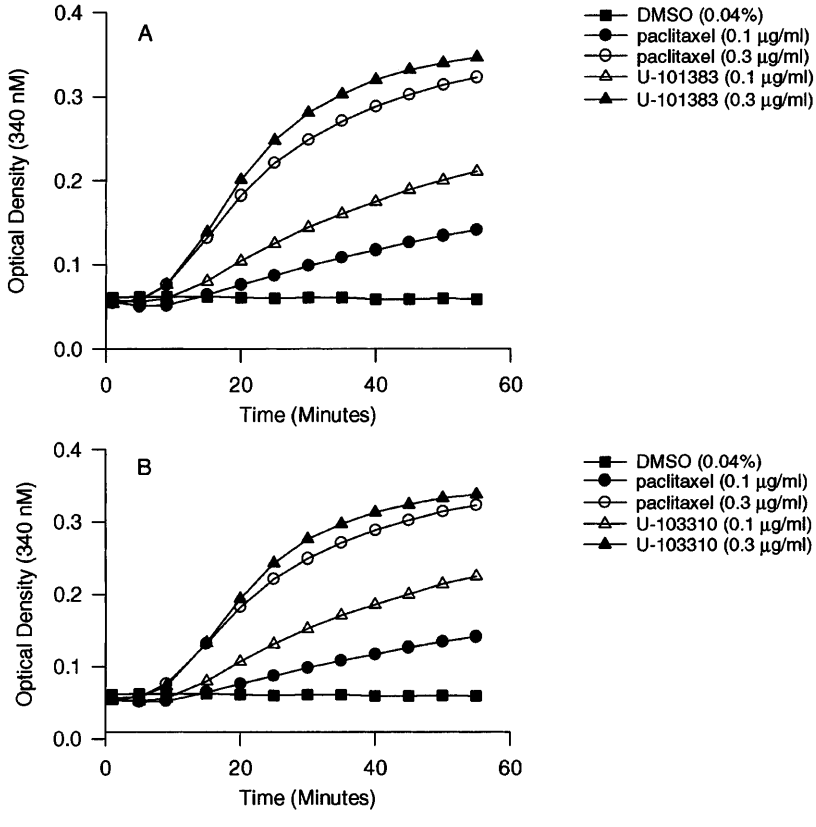


**Fig. 6A,B** MTP (3 mg/ml) was polymerized at 20 °C with paclitaxel or U-101383 (**A**), or with paclitaxel or PNU-103310 (**B**) with 1 m*M* GTP and PEM buffer, pH 6.8

**Table 1** Assessment of taxane analogs in MTP polymerization and mitotic block assays (*n* number of observations)

| Compound | MTP polymerization [a] | Mitotic block [b] | Cytotoxicity [c] |
|---|---|---|---|
| Paclitaxel | 1.0 | 1.0 | 1.0 |
| Docetaxel | 2.5 ± 0.6 ($n$ = 9) | 2.0 ± 0.4 ($n$ = 3) | 5.4 ± 0.8 ($n$ = 3) |
| PNU-100940 | 1.7 | 2.9 | 3.9 |
| PNU-101383 | 2.2 | 1.4 | 5.9 |
| PNU-101885 | 2.2 | 3.3 | 11.0 |
| PNU-103310 | 2.5 | 3.4 | 6.7 |

[a]Rate of polymerization was determined by calculating the initial average rate of polymerization of test compound (0.05 or 1.0 μg/ml) over 20 min at 20 °C. The values are the rates of MTP polymerization with the test compounds divided by that with paclitaxel

[b]Mitotic block was obtained by exposing B16/F10 cells to test compound or paclitaxel for 5 h. The values are the concentration of paclitaxel needed to block 20% cells in mitosis divided by the concentration needed with the test compound

[c]Cytotoxicity was determined by exposing the B16/F10 cells to test compound or paclitaxel for 60 h (five cell doublings) and counting the cells on a Coulter counter. The values are the ratio of the $IC_{50}$ of paclitaxel to that of the test compound run in the same experiment. $IC_{50}$ value for paclitaxel is 7.3 n$M$ ± 0.8

control in the 96-well plate as described above. At low magnification (Fig. 7A,B) microtubules could be seen in the drug-treated samples but not in the DMSO control (not shown). High magnification showed that there were no sheet-like structures in these samples, but tube-like microtubules with protofilaments (Fig. 7C–E). The diameter of the microtubules polymerized with various drug concentrations was approximately 25 to 30 nm. These photomicrographs (Fig. 7) demonstrate that at the low experimental temperature (20 °C), we were indeed measuring microtubule formation. At higher concentrations (1.7 μg/ml) of paclitaxel, docetaxel and PNU-101885 a greater number of microtubules, but of shorter length, were seen (not shown).

### Cellular cytotoxicity and mitotic block assays

The cytotoxicity of paclitaxel and analogs for B16/F10 cells is shown in Table 1. Docetaxel and PNU compounds were much more cytotoxic (~4–11-fold) than paclitaxel. The potencies of taxane analogs in the mitotic block assay were evaluated on the basis of the concentration of compound needed to block 20% cells in mitosis in relation to that of paclitaxel after a 5-h exposure (Table 1). We chose a mitotic value of 20% since it was significantly higher than the value obtained for the untreated control (~3%), and the 5-h exposure time allowed us to complete the assay in one day. The potency ratios of some paclitaxel analogs are given in Table 1. An example of a dose-response obtained with paclitaxel and analogs is shown in Fig. 8. Docetaxel and PNU compounds were more potent than paclitaxel in this assay (Table 1).

Table 1 compares the potency of analogs with that of paclitaxel in two cell-based assays (cytotoxicity, mitotic block) and an in vitro biochemical assay. In all three assays, docetaxel and analogs were identified as being more potent than paclitaxel. However, the degree of potency as compared with paclitaxel differed in these three assays. This could be for several reasons, including the following. (a) In contrast to paclitaxel, the other compounds may have acted on multiple sites besides mitosis. This would have resulted in the potency ratios in the MTP polymerization assay being different from the cellular assays. (b) The cellular uptake of the compound was also a variable that differentiated the in vitro MTP polymerization assay from the cellular assays. (c) The difference in potency ratios between the two cellular assays would be related to measuring a single endpoint (mitotic block) versus multiple endpoints that result in killing of cells, e.g. mitotic block and apoptosis, which may have occurred in the cytotoxicity assay.

## Discussion

The ultimate goal of this work was to establish a sensitive and rapid in vitro MTP polymerization and cellular mitotic block assay capable of screening a large number of compounds. By using the low polymerization temperature of 20 °C, we abolished the basic ability of MTP to polymerize at 37 °C in the presence of MAPs and GTP, and thus made the assay more sensitive in detecting microtubule interacting agents. Photomicrographs also demonstrated that we were measuring formation of tubules rather than ribbons or sheets. MAPs, which stimulate the polymerization of microtubules, play a major role in the initiation of assembly. We also observed (data not shown) that the rate of polymerization was considerably lower when paclitaxel was added to phosphocellulose-purified (MAP-free) tubulin, as has been also noted by other researchers [17, 28].

Schiff et al. [35] originally showed that paclitaxel increases the rate of MTP polymerization at 37 °C in a dose-dependent manner. However, we noticed that at 37 °C, the differences in the rates of MTP polymerization at different paclitaxel concentrations were too small to allow us to construct meaningful dose-response curves in order to analyze the potency of paclitaxel analogs. Also, when the MTP polymerization assay was conducted at 37 °C, we were able to detect paclitaxel only at concentrations greater than 1 μg/ml. Parness et al. [29] compared the ability of several paclitaxel analogs to

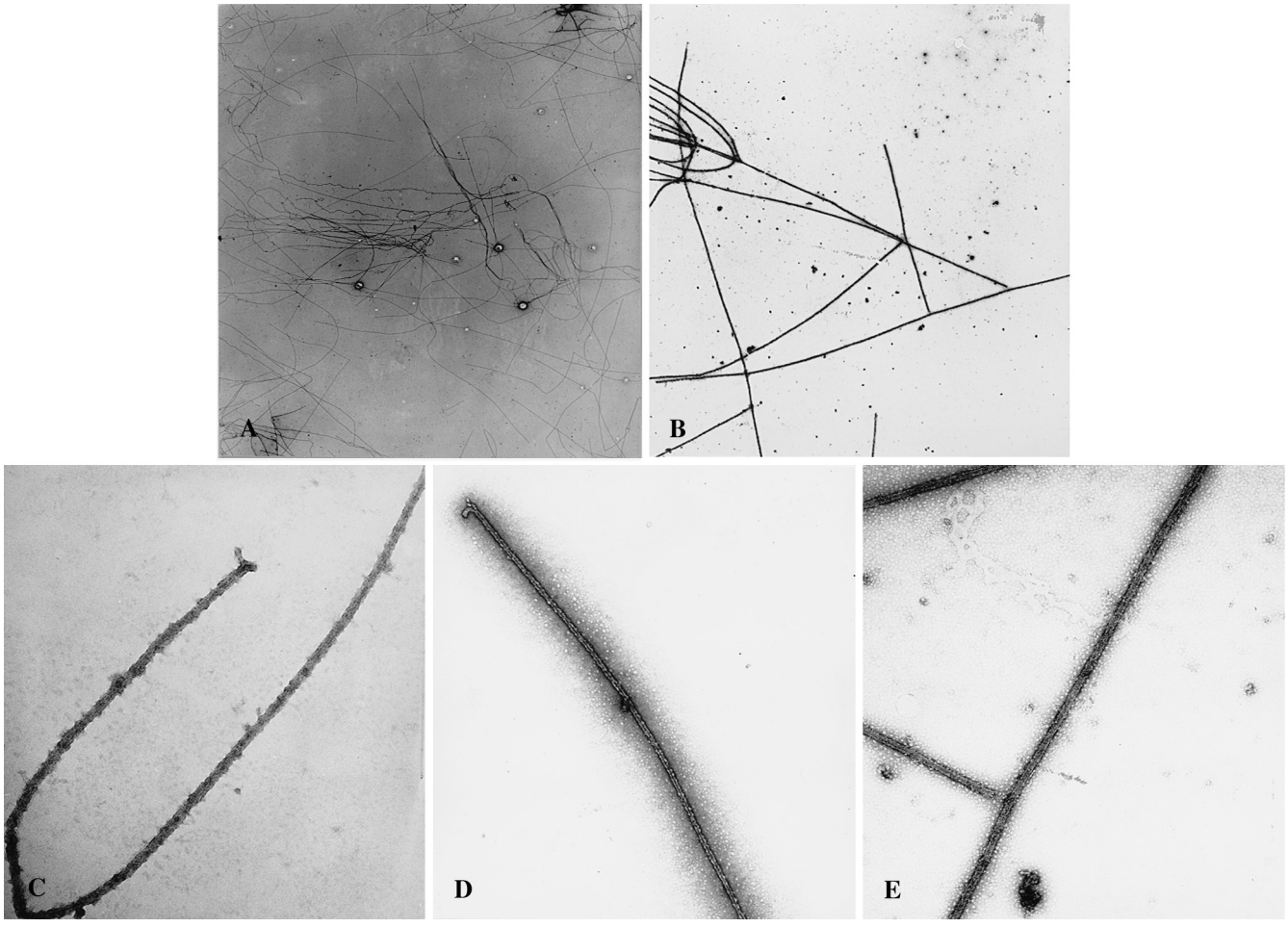


**Fig. 7A–E** **A**, **B** Negatively stained microtubules assembled with 1 m*M* GTP and 0.1 μg/ml paclitaxel (**A**) and PNU-101885 (**B**) at 20 °C ×1100 and ×5800, (respectively). **C–E** Microtubules assembled with 1 m*M* GTP and 0.2 μg/ml paclitaxel (**C**), docetaxel (**D**) and PNU-101885 (**E**) under similar conditions (×46 000, ×25 000, ×34 000, respectively)

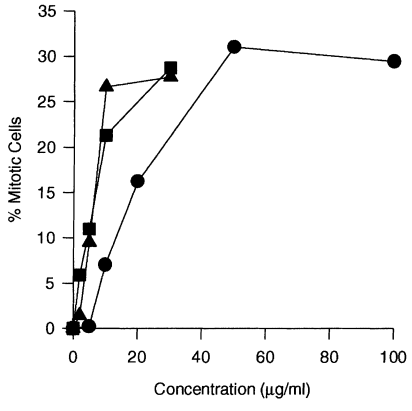


**Fig. 8** B16/F10 (mouse melanoma) cells were exposed to paclitaxel (●), PNU-100940 (■) and PNU-101885 (▲). Control mitotic values were subtracted from the values obtained with treated samples

polymerize MTP in the absence of GTP. The relative potencies of the analogs are presented as their relative initial rate of polymerization when all the compounds were compared at 15 μ*M* of drug. Our assay was basically similar to that described by Parness et al. [29], except that the use of a low temperature for the polymerization reaction made the assay considerably more sensitive and it was able to generate results on 96 samples (including controls) at the same time.

In contrast to these methods which use MTP polymerization, Lataste et al. [20] have based their assay on the disassembly of microtubules. Microtubules assembled in the presence of paclitaxel (10 μ*M*) are resistant to depolymerization at 0 °C. The ratio $V/V_0$ (V and $V_0$ being the rates of disassembly in the presence and absence of paclitaxel) decreased quasiexponentially with increasing drug concentration. Paclitaxel analogs were quantified based on 50% inhibition of the disassembly rate. We consider our method superior on two counts: (1) it was slightly more sensitive since we could detect 0.05 μg/ml paclitaxel compared with >0.1 μg/ml in the method of Lataste et al., and (2) the dose-response curve obtained by our method was linear (Fig. 4) compared to the quasiexponential curve of the earlier work [20]. Another assay for paclitaxel is based on the fact that

paclitaxel-dependent GTP hydrolysis is associated with MTP polymerization. Thus paclitaxel-induced MTP polymerization is associated with GTP hydrolysis under conditions where otherwise hydrolysis does not occur [12]. This assay is reported to be sensitive to 0.1 $\mu M$ (0.085 μg/ml) paclitaxel (actually probably 0.3 $\mu M$ is a reasonable limit) and has been used in measuring paclitaxel concentrations in serum. However, the assay is not amenable to high volume techniques and requires chromatographic separation of GTP and GDP.

Bollag et al. [5] have described a filtration-colorimetric assay to screen for nontaxane microtubule stabilizers by measuring the amount of protein polymerized using the amido-black reagent. Although this assay uses about the same amount of MTP as our assay and measures the amount of protein polymerized, it does not yield kinetic data although it was perhaps adequate for their purposes of identifying nontaxane microtubule stabilizers. Our assay, on the other hand, is a secondary assay and was primarily used to evaluate potency of new paclitaxel analogs. Finally, our results clearly show that we have developed an assay that is sensitive (could reproducibly detect 0.05 μg/ml of paclitaxel), and we could test 200 samples in a day when done in two sets with paclitaxel standard run in each set. This assay used small amounts of compounds and MTP.

The mitotic block assay using the cell-settling chamber can screen eight samples per slide and utilizes many fewer cells than the standard assay [4], enabling us to determine the ability of the test compound to block cells in mitosis. We were able to prepare 100 samples in a typical 8–9 h day (5-h drug exposure) with the morphology of the cells being better than that obtained by the standard method [4].

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