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A convenient tubulin-based quantitative assay for paclitaxel (Taxol) derivatives more effective in inducing assembly than the parent compound

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Abstract A room temperature biochemical assay, based on centrifugal removal of tubulin polymer, was developed to permit ready detection of paclitaxel analogs more active than the parent compound and to permit reliable quantification of differences in activity relative to paclitaxel in terms of drug concentration. The assay was validated by comparing paclitaxel to two compounds (docetaxel and 2-debenzoyl-2-*meta*-azidobenzoylpaclitaxel) known to be more active under multiple reaction conditions. The assay was designed to yield a relatively high EC_{50} (23 μM) for paclitaxel. This was possible because paclitaxel only weakly induced tubulin assembly at room temperature in 0.4 *M* glutamate without exogenous GTP. Under these same reaction conditions 50% assembly occurred with 4.7 μM 2-debenzoyl-2-*meta*-azidobenzoylpaclitaxel and 11 μM docetaxel. These biochemical EC_{50} values were in agreement with the relative cytotoxicity of the three compounds for human Burkitt lymphoma CA46 cells (IC_{50} values for paclitaxel, docetaxel, and 2-debenzoyl-2-*meta*-azidobenzoylpaclitaxel were 40, 10, and 3 n*M*, respectively).

Key words Paclitaxel · Tubulin-based assay · Docetaxel · 2-Debenzoyl-2-*meta*-azidobenzoylpaclizaxel · Glutamate

Introduction

The growing therapeutic roles of paclitaxel (Taxol; Fig. 1, compound 1) and docetaxel (Taxotere; Fig. 1, compound 2) and their poor aqueous solubility, which makes their clinical use more difficult, have stimulated extensive synthetic efforts to produce more effective and/or convenient analogs [8, 11, 14]. The taxoids, unlike most antimitotic drugs, stimulate rather than inhibit microtubule assembly, and polymer formed in their presence is more stable than control polymer [15]. These properties have complicated ready evaluation of paclitaxel derivatives in terms of inhibitory or effective drug concentrations (e.g. IC_{50} or EC_{50} values), particularly for rapid biochemical analysis of newly synthesized compounds.

The problem is less severe for analogs with biochemical activity comparable to that of paclitaxel or with reduced activity, and assays based both on stabilization of polymer [9] and on relative stimulation of assembly, as measured by turbidity development [3], have been employed. In our own work we have found these measurements unsatisfactory for compounds we were evaluating, for these new agents had biochemical properties that were quite distinct from those of paclitaxel.

A number of analogs derivatized at C-7, for example 7-benzoylpaclitaxel (Fig. 1, compound 3), differ little from paclitaxel in their ability to stabilize polymer. In the disassembly assay of Lataste et al. [9] we obtained IC_{50} values of 0.42 and 0.65 μM for paclitaxel and compound 3, respectively. These analogs are similar to paclitaxel in their ability to induce more extensive assembly than occurs without drug at 37°C, provided both GTP and either microtubule-associated proteins (MAPs) or a high concentration of glutamate (an alternative to MAPs as an inducer of assembly) are in the reaction mixture [4, 13]. However, at low temperatures, without GTP or MAPs, compound 3 has much reduced activity relative to paclitaxel in promoting assembly.

The opposite problem arises with 2-debenzoyl-2-*meta*-azidobenzoylpaclitaxel (Fig. 1, compound 4) and several related analogs [1, 4], for they are dramatically

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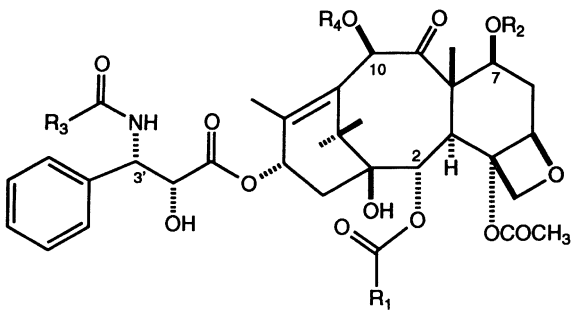
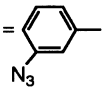
Fig. 1 Structures of paclitaxel and paclitaxel analogs



1 Paclitaxel:
$R_1 = R_3 = Ph$, $R_2 = H$, $R_4 = Ac$

2 Docetaxel:
$R_1 = Ph$, $R_2 = R_4 = H$, $R_3 = t\text{-BuO}$

3 $R_1 = R_3 = Ph$, $R_2 = PhCO$, $R_4 = Ac$

4 $R_1 =$  $R_2 = H$, $R_3 = Ph$, $R_4 = Ac$

more active than paclitaxel in inducing assembly at low temperatures (even at 0°C) and in the absence of GTP and/or MAPs. Yet in the disassembly assay compound 4 yields an IC_{50} value of 0.31 μM, little different from that of paclitaxel. Moreover, under most reaction conditions we have studied, compound 4 is also substantially more potent than docetaxel in promoting tubulin polymerization. (In the disassembly assay we obtained an IC_{50} value of 0.28 μM for docetaxel; and the ratio of the disassembly IC_{50} value of docetaxel to that of paclitaxel that we obtained was 0.67, within the 0.5–0.7 range reported by others [5, 12]).

In our analysis of the effects of compound 4 and related derivatives on tubulin assembly we have been hampered by an inability to compare these agents on a quantitative basis in terms of drug concentration [1]. We obtained unsatisfactory results in attempts to employ competition with radiolabeled paclitaxel in binding to tubulin polymer, and we were concerned that turbidimetric assays might yield inaccurate results. Morphology can substantially alter the turbidity reading obtained with the same amount of polymer formed on a weight basis [2, 16], and paclitaxel and its analogs have been demonstrated to yield polymers with both microtubule and non-microtubule morphology [4, 10, 15]. The present report describes one approach to obtaining useful quantitative comparative values in terms of drug concentration with analogs that are more potent than paclitaxel in inducing tubulin assembly.

Materials and methods

Materials

Electrophoretically homogeneous bovine brain tubulin [6] was prepared as described previously, as were docetaxel [4] and compound 3 [13]. The preparation of compound 4 has been outlined previously [1], and its synthesis and characterization will be described in detail elsewhere. Paclitaxel was generously provided by the Drug Synthesis & Chemistry Branch, National Cancer Institute. Monosodium glutamate was obtained from United States Biochemical (Cleveland, Ohio), and 2 *M* stock solutions were adjusted to pH 6.6 with HCl.

Methods

Tubulin polymerization was measured turbidimetrically in a Gilford model 250 recording spectrophotometer equipped with an electronic temperature controller. With this device the temperature in the cuvettes rose at about 0.5°C/s once a higher temperature was set and fell at about 0.1°C/s once a lower temperature was set on the control device. Assay volume was 0.25 ml, with reaction components as described for the individual experiments.

In the centrifugal polymerization assay, reaction components were as described for the individual experiments. Reaction volume was 0.1 ml, with incubation for 15 min at room temperature. Samples were centrifuged at 14 000 rpm (maximum setting) for 10 min in an Eppendorf model 5415C microcentrifuge. The supernatants were carefully removed and their protein content determined by the Lowry procedure. Two control samples without drug were used in each experiment (90–95% of total protein in the control reaction mixtures remained in the supernatants, and no visible pellets were observed following centrifugation). The EC_{50} value, determined by linear interpolation, was defined as the drug concentration that yielded a supernatant with 50% of the average protein content of the control supernatants.

Results and Discussion

We decided to explore a centrifugal assay for polymer formation to achieve our goal of obtaining a substantial difference in the effective concentrations of compound 4 and paclitaxel. For convenience,we wished to use a room temperature incubation if possible.

In earlier work with paclitaxel effects on the polymerization of purified tubulin we had found that

increasing concentrations of glutamate (in the absence of GTP) lead to a progressively more vigorous assembly reaction [7]. In the controls without drug there was no assembly with or without GTP at lower glutamate concentrations, while assembly at the higher glutamate concentrations required the addition of GTP. GTP also substantially enhanced assembly reactions with paclitaxel. We therefore focused our attention on the GTP-independent reactions.

Figure 2 presents a turbidimetric analysis at four progressively higher glutamate concentrations (panel A, 0.1 *M*; panel B, 0.4 *M*; panel C, 0.7 *M*; panel D, 1.0 *M*), with assembly followed sequentially at 0°C, 10°C, 20°C, and 37°C. The four compounds shown in Fig. 1 were compared with a drug-free control (curve 0) under each condition. (Note that the curves are labeled with the compound numbers shown in Fig. 1.) In these experiments both tubulin and drugs were present at 10 μ*M*. Without GTP there was no significant assembly reaction, and, as anticipated based on previous studies [1, 13], compound 3 had little effect. With paclitaxel (1), docetaxel (2), and compound 4 the assembly reactions shifted progressively to lower temperatures as the glutamate concentration increased, and there was a clear qualitative difference between the three compounds, with compound 4 most active and paclitaxel least active. With 0.7 *M* and 1.0 *M* glutamate the differences between compound 4 and docetaxel were less dramatic than at the lower glutamate concentrations. Although compound 4 appeared more active at the lowest temperature at which assembly occurred (10°C at 0.7 *M* glutamate, 0°C at 1.0 *M* glutamate), at the next temperature step substantially higher turbidity readings were obtained with docetaxel than with compound 4.

The turbidity patterns observed at 20°C clearly indicated that it was worth exploring a room temperature centrifugal assay to distinguish compounds in terms of an effective drug concentration. The data of Fig. 2 strongly indicated that the most substantial differences would be observed at lower glutamate concentrations.

Nevertheless, we first determined whether the concentration of paclitaxel required to lead to a 50% reduction in protein remaining in the supernatant changed if the glutamate concentration was varied (Fig. 3). As in the experiments shown in Fig. 2, the tubulin concentration was 10 μ*M*. In this assay we decided to define the 'EC_{50}' as the effective drug concentration for reducing the protein content of the supernatant, presumably representing unpolymerized tubulin, by 50% compared to control reaction mixtures without drug. Incubation (15 min) and centrifugation (10 min) were at room temperature. We found that this value fell as the glutamate concentration increased. The data of Fig. 3 yield EC_{50} values for paclitaxel of 2.6 μ*M* at 1 *M* glutamate, 3.5 μ*M* at 0.7 *M* glutamate, 11 μ*M* at 0.5 *M* glutamate, 23 μ*M* at 0.4 *M* glutamate, and > 40 μ*M* at 0.1 *M* glutamate.

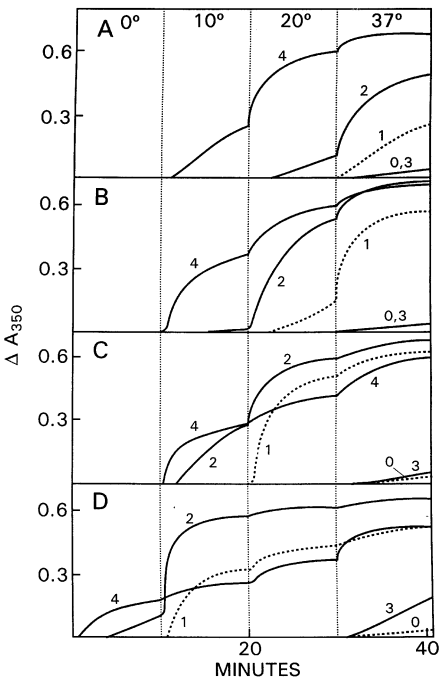


Fig. 2 Tubulin assembly induced by paclitaxel and paclitaxel analogs at different monosodium glutamate concentrations. **A** 0.1 *M* glutamate. **B** 0.4 *M* glutamate, **C** 0.7 *M* glutamate **D** 1.0 *M* glutamate. Each 0.25 ml reaction mixture contained 1.0 mg/ml (10 μ*M*) tubulin, 4% (v/v) dimethyl sulfoxide (the drug solvent), and, if present, 10 μ*M* drug. Baselines were established in the spectrophotometer with the samples at 0°C prior to drug addition, with drug the last component added to each reaction mixture. Temperature changes set on the control unit as indicated. Turbidity development was followed at 350 nm. *Curves 0,* no drug, *curves 1* paclitaxel, *curves 2* docetaxel, *curves 3*, compound 3; *curves 4*, compound 4

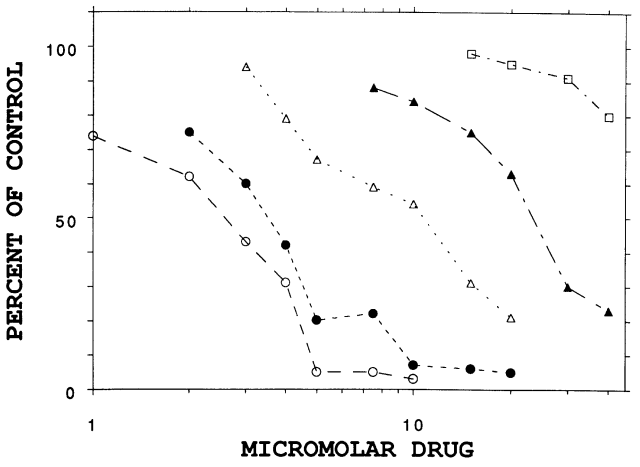


Fig. 3 Increasing effectiveness of paclitaxel on tubulin assembly at room temperature with increasing glutamate concentration. Each 100 μl reaction mixture contained 1.0 mg/ml (10 μ*M*) tubulin, 4% (v/v) dimethyl sulfoxide, and the indicated concentrations of monosodium glutamate and paclitaxel. Samples were incubated, centrifuged, and processed at room temperature as described in the text. The control values were obtained from the protein content of supernatants of samples containing dimethyl sulfoxide but no drug (□ 0.1 *M* glutamate, ▲ 0.4 *M* glutamate, △ 0.5 *M* glutamate, ● 0.7 *M* glutamate, ○ 1.0 *M* glutamate)

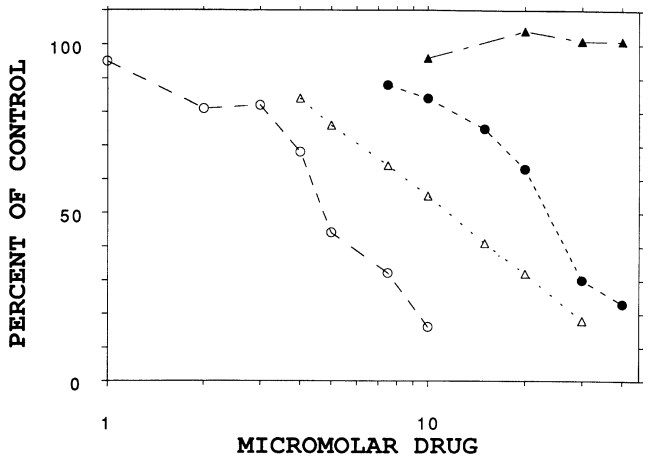


Fig. 4 EC_{50} values consistent with differences in qualitative drug efficacies obtained in 0.4 *M* glutamate at room temperature. Each 100 µl reaction mixture contained 1.0 mg/ml (10 µ*M*) tubulin, 0.4 *M* monosodium glutamate, 4% (v/v) dimethyl sulfoxide, and the indicated concentrations of compound **4** (○), docetaxel (△), paclitaxel (●), or compound **3** (▲). Samples were incubated, centrifuged, and processed at room temperature as described in the text. The control values were obtained from the protein content of supernatants of samples containing dimethyl sulfoxide but no drug

The above experiments led us to focus our attention for drug comparisons on the 0.4 *M* glutamate condition, with 10 µ*M* tubulin, and an experiment comparing paclitaxel with docetaxel and compounds 3 and 4 is shown in Fig. 4. Compound 3 remained unable to induce tubulin assembly at room temperature at concentrations as high as 40 µ*M*, but, in accord with the turbidity patterns shown in Fig. 2B, EC_{50} values for both docetaxel and compound 4 were significantly lower than that of paclitaxel, with compound 4 more potent than docetaxel, in accord with the qualitative data presented here and previously [4].

The patterns shown in Fig. 4 were adequately reproducible, and the following average EC_{50} values were obtained ($\pm$ SE) in nine experiments with paclitaxel and docetaxel and five with compound 4: paclitaxel, 23 ($\pm$ 2) µ*M*;[1] docetaxel, 11 ($\pm$ 0.9) µ*M*; and compound 4, 4.7 ($\pm$ 0.1) µ*M*.

[1]This EC_{50} value was obtained with 10 µ*M* tubulin. In less extensive experiments (two repetitions) with different amounts of tubulin very different EC_{50} values were obtained. With 5 µ*M* tubulin, the EC_{50} value was $>$ 100 µ*M* paclitaxel, while with 20 µ*M* tubulin the EC_{50} value was 12 ± 1 µ*M* paclitaxel. Interestingly, this is the opposite of our experience with inhibitors of assembly, where the IC_{50} values always increase as the tubulin concentration increases. These results with paclitaxel indicate that the critical concentration of tubulin for drug-dependent assembly in 0.4 *M* glutamate at room temperature is between 0.5 and 1.0 mg/ml. The lower EC_{50} for paclitaxel with 20 µ*M* tubulin is consistent with our previous observation that the tubulin critical concentration varies with the paclitaxel concentration [4] and probably indicates that polymer with a substoichiometric paclitaxel content readily forms in glutamate

Human Burkitt lymphoma CA46 cells treated with paclitaxel and other antitubulin agents yield large numbers of cells in apparent mitotic arrest (condensed chromosomes), and we used this line to compare docetaxel and compound 4 with paclitaxel for their inhibitory effects on cell growth. The following IC_{50} values were obtained: paclitaxel, 40 n*M*; docetaxel, 10 n*M*; compound 4, 3 n*M*. Thus, with this limited group of agents the tubulin-based assay and the Burkitt cell cytotoxicity assay are in accord.

In summary, we have developed a tubulin-based biochemical assay that appears suitable for quantitative comparison (in terms of drug concentration) of taxoids more potent than paclitaxel in inducing tubulin assembly. Initial data indicate that the order of activity in the biochemical assay will be in accord with relative cytotoxicity. The biochemical assay can be performed rapidly and entirely at room temperature.[2] We are presently determining whether it will yield satisfactory quantitative results for an extensive series of C-2-modified paclitaxel derivatives.

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[2]The room temperature incubation and centrifugation, as opposed to 37°C, is required to obtain a significant difference between the EC_{50} values for compound 4 and paclitaxel (cf. Fig. 2 and reference 4). Initial experiments (two assays) at 37°C yielded EC_{50} values of 4.4 $\pm$ 0.04 µ*M* for compound 4 and 4.5 $\pm$ 0.07 µ*M* for paclitaxel.

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