Cancer Chemother Pharmacol (1997) 40: 228–232

ORIGINAL ARTICLE

Dan L. Sackett · Paraskevi Giannakakou
Marianne Poruchynsky · Antonio Fojo

Tubulin from paclitaxel-resistant cells as a probe for novel antimicrotubule agents

Received: 30 October 1996 / Accepted: 9 December 1996

Abstract *Purpose*: Treatment with paclitaxel (PTX) can lead to the appearance of drug resistance with accompanying changes in tubulin. The purpose of this study was to develop an assay for microtubule-active agents that are able to circumvent changes in tubulin that result in acquired resistance to paclitaxel. *Methods*: The assay measured the promotion of microtubule polymerization when target agents were added to solutions containing tubulin purified from cultured cells. Tubulin was prepared from PTX-sensitive 1A9 ovarian carcinoma cells and from a PTX-resistant clone. Polymerization was monitored spectrophotometrically and validated by electron microscopy. *Results*: Exposure of tubulin isolated from PTX-sensitive 1A9 ovarian carcinoma cells to substoichiometric PTX resulted in polymerization equivalent to that observed with brain tubulin. In contrast, tubulin from a PTX-resistant 1A9 clone failed to polymerize under identical conditions. If a C-2-modified analog of PTX (2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel) was substituted for PTX in the same experiment, the tubulins from both sensitive and resistant cells polymerized as well as brain tubulin. As predicted from these results, the PTX analog was nearly as cytotoxic to the PTX-resistant cells as it was to the parental cells: the relative resistance of the resistant cells compared to the parental is only 3–5-fold for the PTX analog versus 25–30-fold for PTX. *Conclusion*: Polymerization of purified tubulin from the paclitaxel-resistant cells provided an assay for agents able to circumvent the tubulin alterations that result in acquired paclitaxel resistance.

D.L. Sackett · P. Giannakakou · M. Poruchynsky · A. Fojo
Medicine Branch, National Cancer Institute,
NIH, Bethesda,
MD 20892, USA

D.L. Sackett (✉)
NIH, Bldg 8, Rm 2A-23,
8 Center Dr, MSC 0830,
Bethesda, MD 20892-0830, USA
Tel.: 301-496-4033; Fax: 301-402-0240;
email: dsackett@helix.nih.gov

Key words Tubulin · Taxol · Paclitaxel · Drug resistance · Microtubules

Introduction

Antimicrotubule (anti-MT) drugs are important therapeutic agents in several areas of human medicine as well as having important roles in veterinary medicine and in agriculture. In treating human disease, anti-MT agents have been particularly important as anticancer drugs. Notable examples are the vinca alkaloids and the taxanes, and members of both of these classes of drugs are in wide clinical use [1, 2]. Many other anti-MT agents are under study as anticancer drugs.

Evaluating the usefulness of new anti-MT agents must eventually be based on experience in clinical trials, but long before that stage is reached, laboratory methods are needed to determine potency and to assess possible unintended effects on cellular metabolism. These laboratory methods include assays of cytotoxicity, cell cycle studies, measures of binding to the MT subunit protein, tubulin, and polymerization assays that measure the ability of the test agent to alter the assembly of tubulin into microtubules [3–6].

A problem with treatment with anti-MT agents, as with other agents, is the development of resistance. One mechanism for resistance is overexpression of the drug efflux pump, *MDR-1*. Where overexpression of *MDR-1* does not occur, other mechanisms must exist, including alterations in the target, tubulin. These changes could be point mutations that alter the affinity of tubulin for the drug, alter the conformational changes that follow drug binding, or alter the dynamic equilibrium between soluble and polymerized tubulin that exists in the absence of drug. Resistance might also alter the mixture of tubulin isotypes expressed in the target cells, or possibly alter the many posttranslational modifications that tubulin undergoes.

Is it possible to identify agents that overcome the development of resistance? Using in vitro polymerization

assays with brain tubulin is likely to yield information on the relative potencies of different agents, but will not address the issue of resistance. We show here that polymerization assays using tubulin isolated from cells that are resistant to paclitaxel (PTX) provide a viable means of addressing this important point.

Materials and methods

Cell culture

The cultured cells used were 1A9 cells, which is a clonal line derived from A2780 ovarian carcinoma cells [7]. These cells are sensitive to PTX (IC_{50} 2 n*M*). Cells were grown in RPMI medium with 10% fetal calf serum at 37 °C in an atmosphere containing 5% CO_2. The PTX-resistant cell line was isolated in a single-step by exposure to PTX in the presence of verapamil, to prevent development of *MDR-1*-mediated resistance. The resistant cell lines are described in detail in separate communications (Giannakakou et al., submitted for publication[1], [8]). The PTX-resistant cells used here were clone #22. These cells are 20–25-fold resistant to PTX compared with the parental line. They are not PTX-dependent but do show collateral sensitivity to microtubule destabilizing drugs such as colchicine.

Brains

Brains were obtained from female Sprague-Dawley rats weighing approximately 200 g. Animals were sacrificed by decapitation and the brains were removed, frozen on dry ice, and stored at −80 °C until use [9].

Tubulin purification

Tubulin was purified from cultured cells using a sequential combination of rapid ion exchange on a membrane absorber and temperature-dependent polymerization and depolymerization, as described previously [10]. Briefly, the procedure was as follows. Cells were lysed by sonication in Mes assembly buffer (0.1 *M* Mes, 1 m*M* EGTA, 1 m*M* $MgCl_2$, pH 6.9; MEM) supplemented with 1 m*M* DTT and 0.5 m*M* GTP. A 100 000 *g* supernatant was prepared and loaded into a DEAE membrane absorber (MemSep from Millipore Corporation). Unadsorbed proteins were eluted with MEM and loosely adsorbed protein eluted with MEM + 0.3 *M* sodium glutamate. Tubulin was eluted with 0.8 *M* sodium glutamate. Tubulin was polymerized at 37 °C following adjustment of the solution to 1 *M* sodium glutamate, 10% DMSO and 1 m*M* GTP. Polymers were collected by warm centrifugation, depolymerized in MEM on ice and centrifuged in the cold to eliminate any material that did not depolymerize. The final solution, usually 1–3 mg/ml, was stored frozen in aliquots in liquid nitrogen. The yield of tubulin from the parental and PTX-resistant cells was essentially the same, 0.5–0.6% of total protein.

Paclitaxel and *m*-azido analog

PTX and the C-2 analog 2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel were obtained from the Drug Synthesis and Chemistry Branch, NCI, and by synthesis as described previously [11].

Tubulin folding and stability

The native form and stability of the different tubulin isolates were compared by analyzing the proteolytic susceptibility of the native proteins. Time courses of cleavage by trypsin, chymotrypsin and subtilisin were analyzed by SDS polyacrylamide gel electrophoresis, basically as described previously [12].

Drug-induced tubulin polymerization

Tubulin polymerization assays were followed by light scattering-induced increases in turbidity. Optical density at 350 nm was monitored with a thermostated Cary Model 219 spectrophotometer. Typically tubulin was diluted to 7.5 μ*M* in MEM + 1 m*M* GTP at 37 °C and the optical density monitored for 5 min. Tubulin does not polymerize under these conditions and no changes in optical density were observed in the absence of added drug. PTX or other agents were then added, typically to 1 μ*M*, and changes in optical density were recorded for the following 12–20 min.

Electron microscopy

Electron microscopy was performed on unfixed samples applied to carbon/Formvar-coated grids. Samples were allowed to adsorb for 30 s and then negative stained with 1% uranyl acetate for 30 s, and air dried.

Results and discussion

We developed a protocol for evaluating the tubulin assembly-promoting activity of various agents. This protocol compared the drug-driven assembly of tubulin from PTX-sensitive cells with that of tubulin from a PTX-resistant clone, and with a brain tubulin standard. The tubulin was isolated from a human ovarian carcinoma parental cell line and a resistant clone which are described in Materials and methods. Tubulin was isolated from rat brains and from cultured cells using a combination of membrane ion exchange and temperature-dependent polymerization and depolymerization, as described in Materials and methods. To illustrate the progress of purification from the cultured cells, Fig. 1A shows an SDS gel separation of the proteins present at sequential steps in the purification. The ion exchange step provided a substantial initial purification (compare lane 2 with the total soluble protein fraction in lane 1). The subsequent steps of polymerization (lane 3) and depolymerization (lane 4) yielded a final tubulin preparation that was substantially free of other proteins. This material was used for the polymerization assays. The yield of polymerization-competent tubulin was the same for the parental and PTX-resistant cells.

Tubulin is well known for its lability and for its sensitivity to subtle denaturation. Polymerization competence is quickly compromised by such changes, and it is partly for this reason that the last step in the isolation procedure selected for tubulin that was polymerization- and depolymerization-competent. Nonetheless, we wished additionally to assess the native character of the tubulin by a method independent of polymerization. We examined the sensitivity to proteolysis of the tubulin isolated from the PTX-sensitive parental cells, and from the PTX-resistant clones and compared the results with those obtained using rat brain tubulin as reference. Figure 1B shows the results of comparative time courses of digestion using chymotrypsin, trypsin, and subtilisin, each of which cleaves at distinct sites on the tubulin dimer. This technique has been shown to provide a

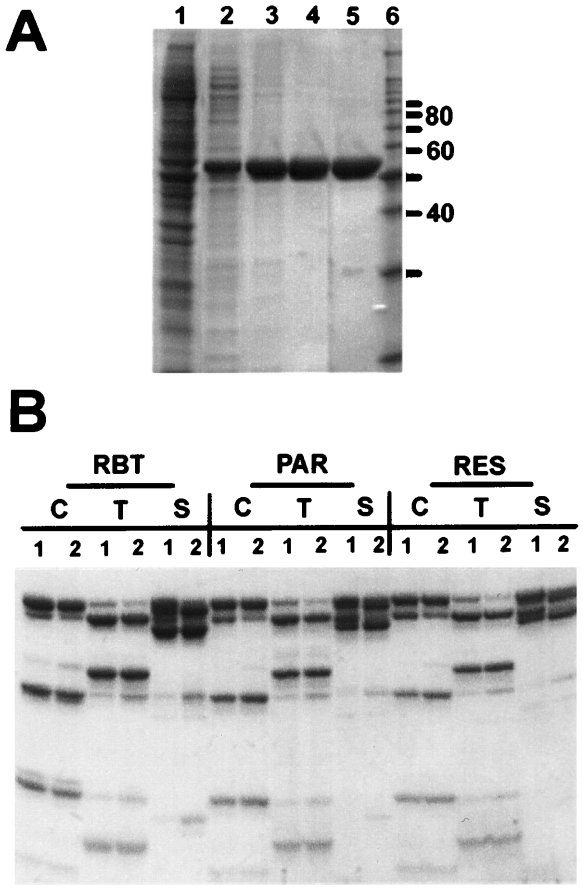

Fig. 1 **A** Purification of tubulin from cultured cells. An SDS gel showing samples of subsequent steps in the purification is shown (*lane 1* high-speed soluble fraction, *lane 2* 0.8 *M* sodium glutamate fraction from the ion exchange step, *lane 3* polymer produced by GTP-induced assembly at 37 °C and collected by centrifugation, *lane 4* final sample, the supernatant resulting from cold-induced depolymerization of the polymer in *lane 3*, *lane 5* rat brain tubulin standard, *lane 6* 10 kDa ladder of protein molecular weight standards). **B** Proteolytic susceptibility of tubulins. SDS gel separation of proteolysis products of the different tubulin samples is shown to demonstrate the normal folded state of the cultured cell tubulins. Tubulins from brain (*RBT*), parental (*PAR*), and PTX-resistant (*RES*) cells were analyzed. All tubulins were adjusted to 1 mg/ml in MEM + 0.1 m*M* GDP at 30 °C and digested with 0.033 mg/ml chymotrypsin (*C*), 0.033 mg/ml trypsin (*T*) or 0.02 mg/ml subtilisin (*S*). Samples were removed after 7 and 25 min of digestion (*lanes 1* and *2*, respectively) and digestion stopped by the addition of PMSF to *C* and *S*, or leupeptin to *T*. Samples were then boiled in SDS loading solution

sensitive probe of the folded conformation of native tubulin [12, 13]. It is clear that the digestion patterns obtained with all three tubulin samples were essentially the same. The bands obtained were those expected from published studies [12, 13], and the time-dependence of digestion was essentially the same for tubulin from the parental cells, the resistant clones, and the rat brain. We conclude that the protein obtained from the cultured cells was normal, native tubulin (from the experiments shown in Fig. 1B), that is polymerization-competent (from the final step in purification).

Clearly, we do not mean that the tubulin from the PTX-resistant cells was identical with that from the

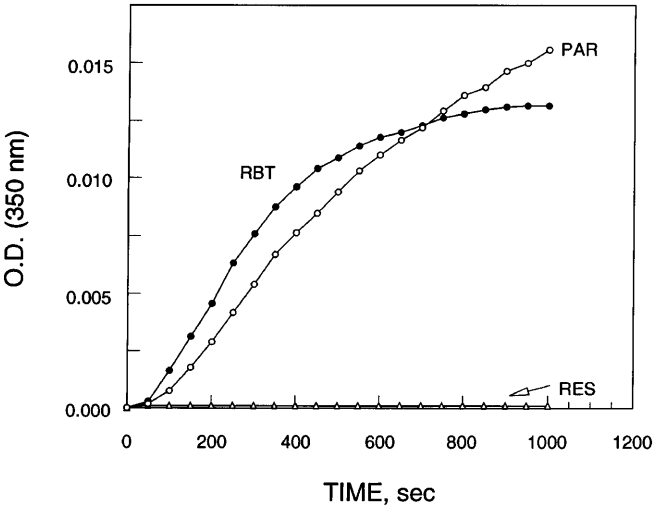

Fig. 2 Paclitaxel-induced polymerization of tubulins from brain, and from parental and resistant cells. Tubulin from brain (*RBT*), or from parental (*PAR*) or PTX-resistant (*RES*) cells was adjusted to 7.5 μ*M* in MEM + 0.1 m*M* GTP at 37 °C, and the optical density at 350 nm was followed with time. Following a period of 10 min during which no change was observed (not shown), PTX was added to a final concentration of 1 μ*M*. The increase in optical density with time is shown. Time of drug addition is 0 s

drug-sensitive parental cells. Indeed, we are actively seeking to define the differences that distinguish them, since these differences appear to underlie the acquired drug resistance[1]. However, the data presented show that the proteins did not differ from each other or from brain tubulin in terms of structural stability during a 30-min incubation. Therefore, the differences we showed subsequently in drug-promoted assembly reflected differences specifically in response to the drugs and were not a result of differences in stability or inherent polymerizabilty.

The experiments shown in Fig. 2 were performed to demonstrate the effect of PTX on the purified tubulins. PTX-induced polymerization was monitored by the increase in optical density caused by the assembly of tubulin dimers into microtubules. Experiments were performed with limiting PTX. Tubulin samples from rat brain, or from the parental or PTX-resistant cell lines were incubated at 37 °C in a GTP-containing assembly mix. No polymerization was observed with any of the samples before the addition of drug (data not shown). Furthermore, the addition of DMSO, the solvent for all of the drugs in this study, did not result in any polymerization even when added to a concentration of 5% (v/v), five times the final concentration in the experiments presented here (data not shown). Upon addition of PTX, polymerization was observed in the tubulin samples from rat brain and from the parental samples. The higher optical density obtained with the parental cell tubulin compared to the brain tubulin probably reflects the production of some open sheet polymers in addition to microtubules. Little or no polymerization occurred in the tubulin sample from the resistant cells.

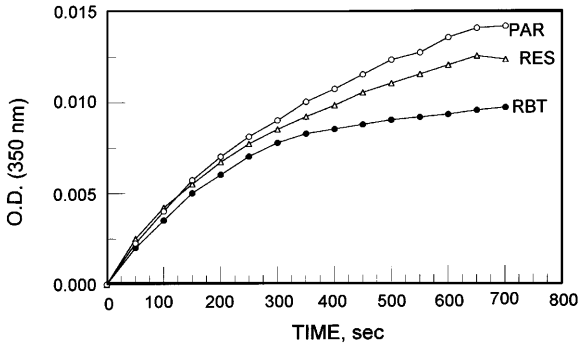

Fig. 3 Paclitaxel analog-induced polymerization of tubulins. The polymerization experiments were performed exactly as described for Fig. 2, except that at time 0, the *m*-azido-PTX analog was added to a final concentration of 1 μM

Thus the phenotype of PTX resistance is reflected in the behavior of the purified tubulin.

The polymerization experiments shown in Fig. 2 were repeated with other agents, and it was found that some other agents, in contrast to PTX, could polymerize tubulin from the resistant cells as well as tubulin from the parental cells or from brain. Such an experiment is shown in Fig. 3, using the PTX analog, 2-debenzoyl-2-(*m*-azidobenzoyl)paclitaxel. This agent, chosen because of its known increased potency [14], induced polymerization of brain and parental cell tubulin (as shown in Fig. 2 with PTX). Again the parental cell tubulin gave a higher optical density than the brain tubulin. Unlike the case with PTX, however, tubulin from the resistant cells polymerized as well as that from the parental cells. Thus, this in vitro assay allows identification of compounds able to circumvent the PTX resistance phenotype.

To be certain of the nature of the polymers that generated the optical density presented in Figs. 2 and 3, electron microscopy was performed. Samples were removed from cuvettes at the end of the polymerizations shown in Fig. 3 and examined by negative stain electron microscopy. Representative micrographs are presented in Fig. 4. The polymers found with parental and PTX-resistant cell tubulin contained both normal microtubules and some open sheet polymers, as expected from the optical properties.

In addition to analogs of PTX, other structurally unrelated agents were also tested. The epothilones are a new group of antimicrotubule agents known to induce tubulin assembly and to stabilize microtubules [15]. Like the PTX analog in Fig. 3, epothilone B also induced assembly of tubulin from the resistant clones to an extent similar to that of the parental cell tubulin (data not shown).

The biological implication of these in vitro polymerization studies is that the PTX analog and epothilone B should be more effective agents against the PTX-resistant cells than is PTX. This was confirmed by determining the cytotoxicity of PTX, the PTX analog, and epothilone B against the parental cells and against the

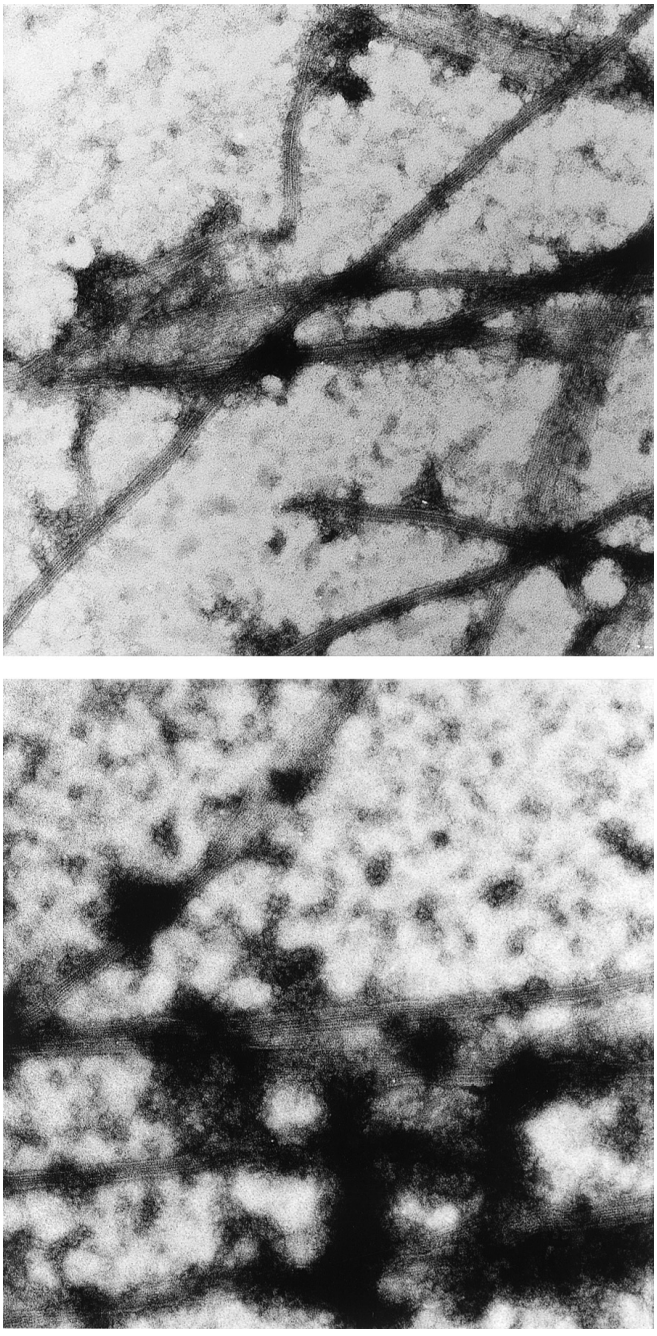

Fig. 4 Electron micrographs of polymers. Polymers produced with tubulin from parental (*upper*) and PTX-resistant (*lower*) cells are shown. Samples were taken at the end of the time-course shown in Fig. 3

PTX-resistant cells. Whereas the resistant cells showed 25–30-fold resistance to PTX compared with the parental cells, their relative resistance was only 3–5-fold for the PTX analog, and only about 2-fold for epothilone B (data not shown).

The results presented here demonstrate that in vitro polymerization of tubulin from PTX-resistant cultured cells provides a valid system for testing new microtubule-stabilizing or assembly-promoting agents. In addition to

assessing potency, this assay provides information on the ability of agents to circumvent the alterations in tubulin brought about by the cellular acquisition of resistance to PTX.

Acknowledgement We thank Prof. David G.I. Kingston and Dr. A.G. Chaudhary for the supply of 2-debenzoyl-a-(*m*-azidobenzoyl)paclitaxel.

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[1]**Note added in proof** Subsequent to submission of this manuscript, we obtained results demonstrating that the PTX-resistant cells differ from parental cells due to a point mutation in the predominant isotype of β-tubulin. A manuscript presenting these results has been accepted for publication in the Journal of Biological Chemistry (Giannakakou et al.).

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