Tryprostatin A, a specific and novel inhibitor of microtubule assembly

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We have investigated the cell cycle inhibition mechanism and primary target of tryprostatin A (TPS-A) purified from *Aspergillus fumigatus*. TPS-A inhibited cell cycle progression of asynchronously cultured 3Y1 cells in the M phase in a dose- and time-dependent manner. In contrast, TPS-B (the demethoxy analogue of TPS-A) showed cell-cycle non-specific inhibition on cell growth even though it inhibited cell growth at lower concentrations than TPS-A. TPS-A treatment induced the reversible disruption of the cytoplasmic microtubules of 3Y1 cells as observed by indirect immunofluorescence microscopy in the range of concentrations that specifically inhibited M-phase progression. TPS-A inhibited the assembly *in vitro* of microtubules purified from bovine brains (40 % inhibition at 250 μM); however, there was little or no effect on the self-assembly of purified tubulin when polymerization was induced by glutamate even at 250 μM TPS-A. TPS-A did not inhibit assembly promoted by taxol or by digestion of the C-terminal domain of tubulin. However, TPS-A blocked the tubulin assembly induced by inducers interacting with the C-terminal domain, microtubule-associated protein 2 (MAP2), tau and poly-(L-lysine). These results indicate that TPS-A is a novel inhibitor of MAP-dependent microtubule assembly and, through the disruption of the microtubule spindle, specifically inhibits cell cycle progression at the M phase.

INTRODUCTION

Mitosis is the process by which eukaryotic cells ensure the distribution of their chromosomes at cell division. In this process the cytoplasmic microtubule is disrupted and reformed as a spindle consisting of large numbers of short microtubules that surround each centrosome. As mitosis proceeds, the elongating ends of the microtubules attach to the chromosomes at each kinetochore and align the chromosomes on the metaphase plate. If this alignment is disrupted by irregular microtubules, mitosis is arrested. Thus a drug that disrupts the microtubule array is useful in the treatment of malignant tumours showing rapid and abnormal cell proliferation. Indeed, some of the most useful cancer therapeutic agents are microtubule inhibitors such as vinblastine and taxol [1]. The primary target molecule of most of these inhibitors is β-tubulin, and such compounds can be placed in one of three categories on the basis of the binding kinetics to β-tubulin: (1) colchicine, colcemid and podophyllotoxin, which bind to the colchicine-binding site, including Cys-354, of β-tubulin [2–4]; (2) vinblastine and vincristine, which bind to the vinblastine-binding site [5,6]; and (3) rhizoxin and maytansine, which bind to the maytansine–rhizoxin-binding site [7–9]. All these agents bind directly to β-tubulin itself and inhibit the self-assembly of purified tubulin irrespective of the species of the assembly inducers *in vitro*. The following compounds with different inhibitory actions have been reported. Estramustine phosphate binds to both microtubule-associated protein 2 (MAP2) and tubulin [10], and inhibits microtubule assembly [11–13]. 5,5′-Bis[8-(phenylamino)-1-naphthalenesulphonate] (bis-ANS) interacts with the C-terminal domain of the tubulin heterodimer and specifically inhibits MAP-dependent microtubule assembly [14,15]. These compounds might also be useful cancer therapeutic agents. Indeed, estramustine in combination with other antimicrotubule agents exhibits synergistic cytotoxicity *in vitro* [16,17] and *in vivo* [18,19].

We have established a convenient bioassay method utilizing the synchronous culture of the temperature-sensitive $p34^{cdc2}$ mutant, tsFT210, derived from a mouse mammary carcinoma FM3A cell line [20,21] to detect cell cycle inhibitors [22]. During the screening of natural products that interfere with cell cycle progression, we purified tryprostatins A and B (TPS-A and TPS-B) from the culture broth of *Aspergillus fumigatus* BM939 [23–25]. Both compounds arrested cell cycle progression at the G_2/M phase at final concentrations of 125 μM and 62.5 μM respectively, but the primary target molecule is unclear.

We have investigated whether tubulin could be a target of the compounds *in vivo* and *in vitro*. Our results indicate an interesting mode of action of TPS-A that inhibits microtubule assembly, i.e. MAP2- and tau-dependent assembly.

EXPERIMENTAL

Materials

TPS-A and TPS-B were purified from the culture broth of *A. fumigatus* BM939 (FERM P-15067), which was originally isolated from a sea sediment sample collected from the sea bed (760 m deep) at the mouth of the Oi river, Shizuoka prefecture, Japan, as described by Cui et al. [24]. [^{3}H]Colchicine and [^{3}H]vinblastine were purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.) and NEN Life Science Products respectively. Compounds were dissolved in DMSO.

Cell culture and flow cytometry

Rat normal fibroblast 3Y1 cells [26] were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum in a humidified air/CO_2 (19:1) atmosphere.

Flow cytometry was used to analyse the distribution of DNA content in the cell populations. The cells were fixed with cold (−20 °C) 70 % (v/v) ethanol and stained with propidium iodide (Sigma Chemical Co., St. Louis, MO, U.S.A.). Total fluorescence

Abbreviations used: bis-ANS, 5,5′-bis[8-(phenylamino)-1-naphthalenesulphonate]; MAP, microtubule-associated protein; TPS, tryprostatin.

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intensities were determined by quantitative flow cytometry with an Epics System (Coulter Electronics, Hialeah, FL, U.S.A.) equipped with a 5 W argon-ion laser operated at a wavelength of 488 nm.

Immunofluorescence procedures

For immunofluorescence observation, 3Y1 cells were plated on 24 mm × 24 mm glass coverslips at a low density and cultured for 1 day. After treatment with compounds for 3 h, the coverslips were washed with PBS and fixed with 3.7 % (v/v) formaldehyde in PBS for 5 min then permeabilized for 5 min with PBS containing 0.2 % (v/v) Triton X-100. After being washed with PBS-B [PBS containing 1 % (w/v) BSA and 0.02 % sodium azide], the coverslips were overlaid with anti-(α-tubulin) antibody (N-356; Amersham, Little Chalfont, Bucks., U.K.) in PBS-B then placed in a humidified container at 37 °C and incubated for 1 h. After being washed twice with PBS-B, the coverslips were overlaid with FITC-conjugated anti-(mouse IgG) (Kirkegaard and Perry, Gaithersburg, MD, U.S.A.), incubated for 45 min, washed with PBS-B and mounted. The cytoskeletons were photographed with a cooled charge-coupled device camera (PROVIS AX70; Olympus, Tokyo, Japan).

Preparation of tubulin, MAP2, tau and tubulin S

Microtubule proteins were prepared from bovine brain by the polymerization–depolymerization method of Shelanski et al. [27], with modifications. Microtubule protein, which was purified by two cycles of polymerization–depolymerization, was dissolved in Mes buffer [100 mM Mes/0.5 mM $MgCl_2$/1 mM EGTA (pH 6.8)] and stored at −80 °C. Tubulin was prepared by phosphocellulose (P11; Whatman, Maidstone, Kent, U.K.) column chromatography of microtubule proteins as the void fraction [28]. MAP2 and tau were fractionated by Mono Q column chromatography from the heat-stable fraction of microtubule proteins [29] with a linear KCl gradient from 50 to 300 mM. After column chromatography the proteins were desalted and concentrated to 5–10 mg/ml with Centricon 30 (Amicon, Beverly, MA, U.S.A.), and stored at −80 °C. Protein concentrations were determined with the Protein Assay® (Bio-Rad, Hercules, CA, U.S.A.).

Tubulin S was prepared from phosphocellulose-purified tubulin by digestion with subtilisin BPN′ (Sigma) at a concentration of 1 % (w/w) of tubulin, as described previously [30–32].

Microtubule assembly assay

Turbidimetric assays of microtubule and tubulin were performed by incubating microtubule protein (2.0 mg/ml in Mes buffer) or purified tubulin in cuvettes at 37 °C in a thermostatically controlled Pharmacia Ultrospec 2000 spectrophotometer and measuring the change in attenuance at 350 nm with time. To examine the effect of the drugs and MAPs on polymerization, the microtubule protein was preincubated with the respective drug and protein at 0 °C and polymerization was initiated with the addition of 1 mM GTP; the mixture was heated to 37 °C. For inhibition studies, tubulin assembly was induced by 0.45 mg/ml MAP2, 0.22 mg/ml tau or 0.05 mg/ml poly-(L-lysine) (molecular mass 150–300 kDa), with 15 μM tubulin.

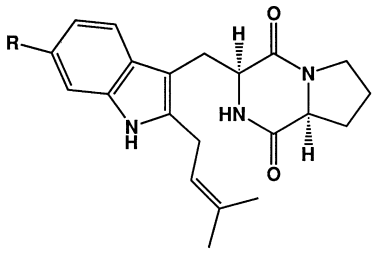
Binding measurement

The binding of radiolabelled vinblastine or colchicine to tubulin was monitored by the centrifugal gel-filtration method described by Hamel and Lin [29]; 1 ml columns of Sephadex G-50 (superfine) were used.

RESULTS

TPS-A and TPS-B inhibit cell cycle progression of rat normal fibroblast 3Y1 cells

TPS-A and TPS-B (Figure 1) inhibited G_2/M cell cycle progression of temperature-sensitive mutant cells, tsFT210. To clarify the mechanism of TPS-A and TPS-B on the cell cycle, we investigated the effects of drugs on the cell cycle in exponentially growing rat normal fibroblast (3Y1) cells. The results obtained 24 h after the addition of the agents are summarized in Table 1. A potent protein kinase inhibitor, staurosporine, arrested cell cycle progression at G_1 phase at a low concentration [33]. Colchicine induced M-phase arrest as indicated by the 4*C* DNA content cell population (Table 1). As a result of TPS-A treatment, 3Y1 cells were also arrested in the G_2/M phase as observed in G_2/M synchronous tsFT210 cells (Table 1). The cells were arrested in the M phase as judged from the DNA images stained with Hoechst 33258, and M-phase-specific inhibition was also observed in an early S synchronous culture of 3Y1 cells (results not shown). TPS-B also arrested cell cycle progression but there was no cell-cycle-specific arrest (Table 1). Most TPS-B-treated cells showed less than 2*C* DNA contents (above 25 %; results not shown), suggesting that TPS-B was highly toxic and most cells had degraded by 24 h after addition of the drug. Therefore we focused on the effects of TPS-A on the cell cycle and tried to determine the primary target of TPS-A *in situ*.



Tryprostatin A : R = OCH_3
Tryprostatin B : R = H

Figure 1 Structures of TPS-A and TPS-B

Table 1 Distribution of DNA content in asynchronous culture of 3Y1 cells treated with various drugs

Exponentially growing 3Y1 cells were treated with various compounds for 24 h and the distribution of DNA content and relative cell numbers were determined. The cell number is the ratio of the number of cells at 24 h to that at 0 h, expressed as a percentage.

		DNA content			
Drug	Concentration (μM)	2*C*	2*C*–4*C*	4*C*	Cell number (%)
Control	0.00	65.4	12.3	22.3	161.8
Staurosporine	0.02	76.9	8.5	11.1	143.1
Colchicine	1.00	9.4	11.5	65.9	69.4
TPS-A	25.00	29.2	16.2	49.4	89.6
	50.00	9.0	15.2	70.5	68.7
TPS-B	25.00	28.6	19.1	24.8	57.4
	50.00	31.8	20.4	15.2	57.8

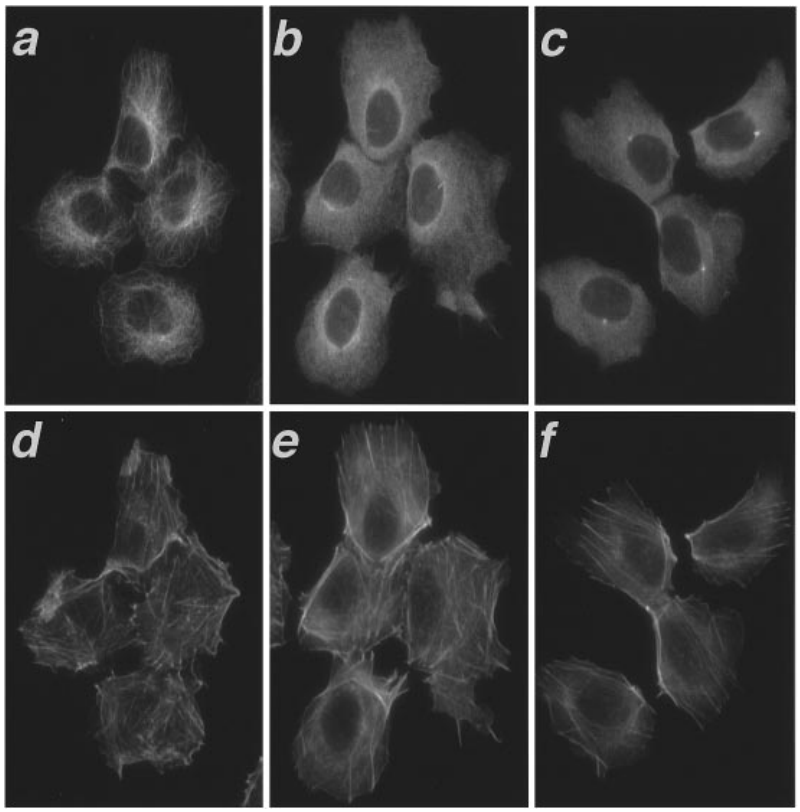


Figure 2 Depolymerization of cellular microtubules by TPS-A

3Y1 cells were incubated with 1 μg/ml colchicine (**b**, **e**), 20 μM TPS-A (**c**, **f**) or in the absence of either compound (**a**, **d**) for 6 h. Microtubules (**a**–**c**) and actin filaments (**d**–**f**) were observed under a fluorescence microscope.

TPS-A disrupted the microtubule network *in situ*

TPS-A arrested cell cycle progression at the M phase. It is known that many M-phase inhibitors affect the function of the spindle apparatus via microtubule disassembly. Thus, to analyse the effect of TPS-A on tubulin, we observed the cytoplasmic microtubule network *in situ* by indirect fluorescence microscopy (Figure 2). The cells treated with colchicine, a potent inhibitor of microtubule assembly, lost their microtubule network (Figure 2b). The microtubule network was completely disrupted at 20 μM TPS-A (Figure 2c), as in the cells treated with colchicine, but the actin filaments were not affected (Figure 2f). This effect of TPS-A was dose-dependent and a few microtubules were observable at low concentrations. The effect of TPS-A was reversible: fast reorganization of microtubules was observed after removal of the drug. Treatment with 50 μM TPS-A disrupted the microtubule network within 3 h; after withdrawal of the inhibitor the microtubule reassembly was complete within 3 h (results not shown).

TPS-A induced microtubule disassembly *in vitro*

Next we determined whether TPS-A inhibited microtubule assembly *in vitro*. As shown in Figure 3(a), the effect of TPS-A on the turbidimetric time course of microtubule assembly was measured. With microtubule proteins at a concentration of 2 mg/ml, the assembly was clearly inhibited by TPS-A in a dose-dependent manner (40 % inhibition at 250 μM TPS-A). When 250 μM TPS-A was added to a microtubule preparation at an equilibrium point (Figure 3b, left-hand vertical arrow), a slow decrease in turbidity was recorded and the reaction reached a new equilibrium. The new equilibrium was higher than the equilibrium observed for microtubules treated with TPS-A at zero time (Figure 3b, ●). The microtubules obtained could be disassembled by cooling to 0 °C (Figure 3b, right-hand vertical arrow); rewarming to 37 °C induced assembly as in the control (results not shown).

To investigate the binding site of TPS-A on tubulin, we conducted binding competition experiments with [^{3}H]colchicine and [^{3}H]vinblastine. Whereas unlabelled colchicine and vinblastine decreased the binding of radiolabelled colchicine and vinblastine respectively (Table 2), TPS-A did not inhibit the binding of either [^{3}H]colchicine or [^{3}H]vinblastine at all. These results suggest that TPS-A interacts at a site other than the colchicine- and vinblastine-binding sites of tubulin.

TPS-A inhibited the MAP-dependent tubulin assembly

To determine the effect of TPS-A on the assembly of purified tubulin, we investigated assembly under several conditions. We used both intrinsic and artificial inducers of assembly. MAP2 and tau are major components of MAPs and stimulate tubulin assembly *in vitro*. Glutamate, taxol and poly-(L-lysine) are artificial inducers that promote the assembly of purified tubulin

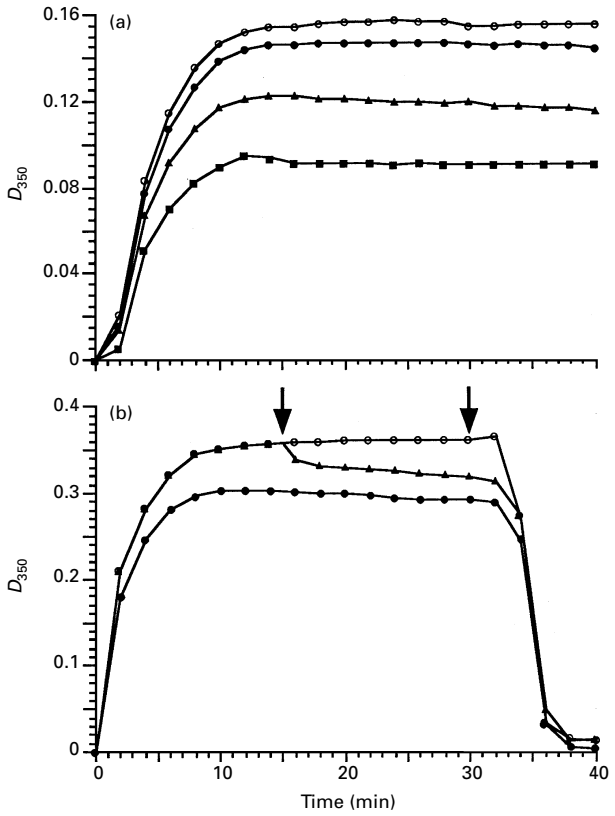


Figure 3 Effects of TPS-A on microtubule assembly

(**a**) Time course of microtubule assembly *in vitro* is shown as increase in attenuance. Microtubule proteins (2 mg/ml) were incubated with 1% DMSO containing various concentrations of TPS-A. The final concentrations of TPS-A were 0 μM (○), 50 μM (●), 125 μM (▲) and 250 μM (■). (**b**) Effect of TPS-A on assembled microtubules. Microtubule proteins were assembled in the absence of TPS-A; DMSO containing TPS-A (final concentration 250 μM; ▲) or not (○) was added at 15 min after the assembly (left-hand vertical arrow), and samples were cooled at 0 °C for 30 min (right-hand vertical arrow); ●, sample incubated with 250 μM TPS-A at 0 min.

Table 2 Inhibitory effects of TPS-A on the binding of colchicine and vinblastine to tubulin

The reaction mixture (0.35 ml) contained 0.5 mg/ml (approx. 5 μM) tubulin, 50 nM [^{3}H]colchicine or [^{3}H]vinblastine, 5% (v/v) DMSO and a mitotic inhibitor (50 μM colchicine, 50 μM vinblastine or 250 μM TPS-A), which was incubated for 5 min at room temperature. Aliquots (0.1 ml) were applied to triplicate columns of Sephadex G-50 and processed by centrifugal gel filtration. Results are means ± S.D. ($n = 3$ experiments).

	Radiolabelled drug bound (% of control)	
Drug added	[^{3}H]Colchicine	[^{3}H]Vinblastine
Colchicine	30.0 ± 0.7	119.7 ± 5.1
Vinblastine	160.4 ± 9.2	19.7 ± 2.5
TPS-A	112.7 ± 4.7	97.1 ± 4.8

in vitro. Apart from these inducers, tubulin S, which is tubulin heterodimer from which the C-terminal domain has been removed enzymically, underwent assembly in the absence of MAPs and inducers. The effects of TPS-A and colchicine on the assembly of tubulin under several conditions are shown in Table 3. Although colchicine inhibited tubulin assembly irrespective of the species of inducers, 250 μM TPS-A did not inhibit the glutamate- or taxol-induced assembly, nor tubulin S assembly (Table 3). However, MAP2-, tau- and poly-(L-lysine)-induced assembly were blocked by the same concentration of TPS-A (Figures 4b, 4c and 4d). Poly-(L-lysine) induced tubulin assembly through binding to the C-terminal domain of tubulin in the same way as the microtubule-associated proteins MAP2 and tau. Therefore these results strongly suggest that TPS-A inhibits the C-terminal domain-mediated microtubule assembly.

Table 3 Effects of TPS-A and colchicine on the assembly of tubulin under several assembly conditions

Percentage activity of tubulin assembly *in vitro* induced under several conditions with TPS-A or colchicine. The concentration of TPS-A was 250 μM in all cases and that of colchicine was the same as the tubulin concentration in each case. In each case the turbidity of purified tubulin without drug at 37 °C was taken as 100%.
Results are means ± S.D. ($n = 3$ experiments).

		Tubulin assembly (% of control)	
Assembly inducer	[Tubulin] (μM)	TPS-A	Colchicine
Glutamate	5	86.0 ± 2.7	22.8 ± 0.0
Taxol	10	86.2 ± 0.7	6.0 ± 1.9
S-Tubulin	15	105.8 ± 0.4	61.1 ± 5.0

DISCUSSION

We previously reported that TPS-A and TPS-B inhibited cell proliferation in a temperature-sensitive p34^{cdc2} mutant cell line from the mouse, tsFT210 [22–24]. In the present paper we show that TPS-A inhibited cell cycle progression of rat normal fibroblast 3Y1 cells specifically in the M phase. TPS-B also arrested cell cycle progression at lower concentrations than TPS-A, but the inhibition was non-specific. The concentrations of TPS-A that arrested cell cycle progression in the M phase (20–50 μM) corresponded to those inducing a marked depolymerization *in situ* of the microtubules containing both cytoplasmic network and spindle apparatus. The results suggest that the M-phase-specific inhibitory effects of TPS-A are due to interference with the function of the spindle apparatus via the disassembly of microtubules.

The inhibition of microtubule assembly by TPS-A was also observed *in vitro*. The IC_{50} for microtubules (2.0 mg/ml) was more than 250 μM. This concentration was much higher than the IC_{50} of the other antimitotic agents colchicine and vinblastine (for which in this study the IC_{50} values were 5.0 and 12.3 μM respectively). It seems that there is a discrepancy between the concentration of TPS-A required to inhibit microtubule assembly *in vitro* and and that required *in situ*. However, the disassembly of the microtubule network *in situ* was caused equally by colcemid and TPS-A at concentrations requiring 20% inhibition *in vitro* (results not shown); the discrepancy between the concentrations *in vitro* and *in situ* might be due to the different concentrations of microtubule proteins *in vitro* and *in situ*. TPS-A disrupted preformed microtubules in a dose-dependent manner *in vitro*, as did vinblastine and rhizoxin [34,35]. However, the microtubules obtained were depolymerized by cooling to 0 °C, similarly to the control. Whereas *Vinca* alkaloids, such as vincristine and vinblastine, induce irregular aggregates of microtubules at high concentrations and the aggregates were not depolymerized by

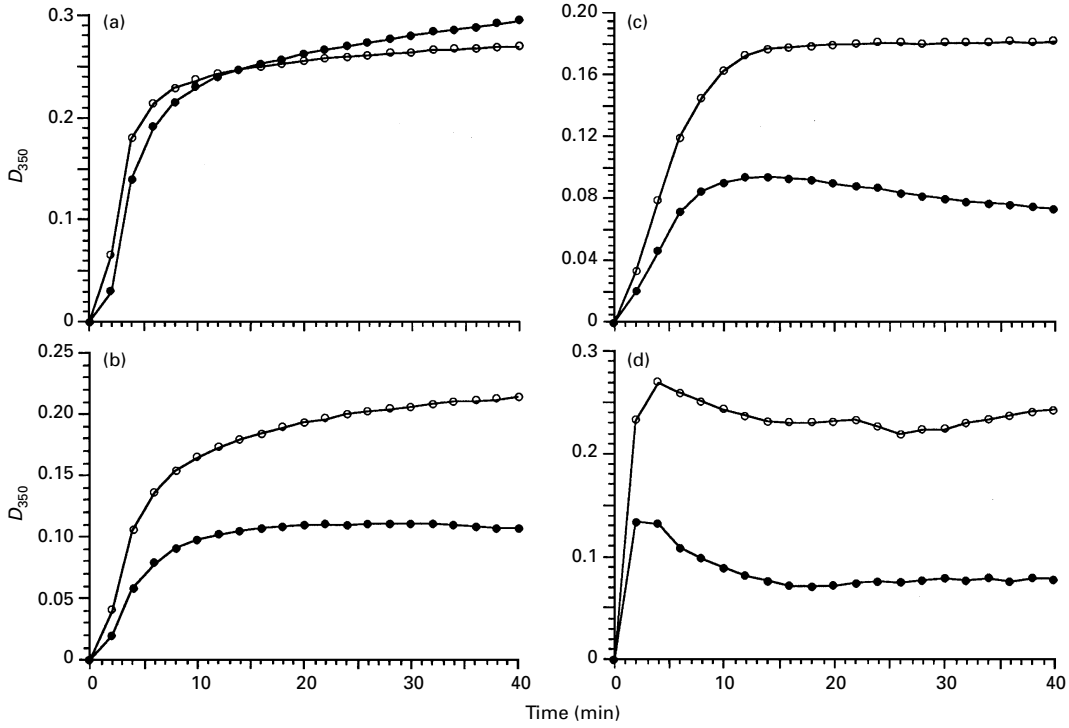


Figure 4 Effects of TPS-A on tubulin assembly in the presence of assembly inducers

Time course of tubulin assembly *in vitro* induced by glutamate (**a**), MAP2 (**b**), tau (**c**) and poly-(L-lysine) (**d**). Tubulin assembly was induced by 0.45 mg/ml MAP2, 0.22 mg/ml tau or 0.05 mg/ml poly-(L-lysine) (molecular mass 150–300 kDa), with 15 μM tubulin. DMSO containing TPS-A (final concentration 250 μM; ●) or not (○) was added at zero time.

cooling [34], TPS-A did not induce aggregate formation even at high concentrations (results not shown). These results suggest that TPS-A inhibits microtubule assembly by a mechanism different from that of *Vinca* alkaloids. Furthermore it is thought that TPS-A is a different type of inhibitor from colchicine, vinblastine and maytansine–rhizoxin because TPS-A did not inhibit the binding of [^{3}H]colchicine or [^{3}H]vinblastine to β-tubulin at all (Table 2).

Both intrinsic and non-physiological inducers that stimulate tubulin assembly have been reported. Intrinsic inducers, MAP2 and tau, induce tubulin assembly through interaction with the C-terminal domain of tubulin [36–39]. Low-molecular-mass agents such as glutamate, taxol and poly-(L-lysine) also promote the assembly of purified tubulin *in vitro* [40–42]. Apart from these inducers, tubulin S, which is a tubulin heterodimer with the C-terminal domain removed enzymically, autonomously assembles by itself in the absence of MAPs [30–32]. In the present study we showed that TPS-A exhibited inhibitory specificity with respect to tubulin assembly inducers, whereas colchicine inhibited tubulin assembly irrespective of the species of inducers and assembly conditions. TPS-A inhibited MAP2-, tau- and poly-(L-lysine)-induced microtubule assembly, but not glutamate- and taxol-promoted tubulin assembly (Table 3 and Figure 4). These results strongly suggest that the inhibition mechanism of TPS-A is different from that of colchicine and vinblastine.

It has been reported that bis-ANS inhibits MAP-dependent microtubule assembly but that the effect was very slight when polymerization was induced by glutamate and taxol [14,15]. Mazumdar et al. [14] also reported that bis-ANS inhibited MAP- and poly-(L-lysine)-induced microtubule assembly, which was regulated by interaction at the C-terminal domain of tubulin. Their observations are very similar to ours. Indeed, our results strongly suggest that TPS-A inhibits microtubule assembly by interfering with interaction between MAPs and the C-terminal domain of tubulin. This assumption is also supported by the result that TPS-A did not inhibit the self-assembly of tubulin S (Table 3), because tubulin S did not contain the putative target site of TPS-A. Our observations are consistent with those for bis-ANS.

TPS-A is the first natural compound that inhibits microtubule assembly by interfering with the interaction between MAPs and the C-terminal domain of tubulin. Many organic chemists aim at synthesizing cancer chemotherapeutic reagents modelled on TPS-A and TPS-B [43,44]. With tryprostatins as a basis it might be possible to develop new agents, useful both as experimental inhibitors in the study of microtubules and in cancer therapy.

We thank Dr. Ohmori for protein purification and useful discussion, and Professor Kobayashi for useful discussion. This work was supported by a grant for Multibioprobe (RIKEN) and by a grant from the Ministry of Education, Science, Sports and Culture, Japan.

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Received 20 October 1997/15 April 1998; accepted 22 April 1998