# Characterization of the Interaction of TZT-1027, a Potent Antitumor Agent, with Tubulin

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**TZT-1027, a derivative of dolastatin 10 isolated from the Indian Ocean sea hare *Dolabella auricularia* in 1987 by Pettit *et al.*, is a potent antimicrotubule agent. We have compared the activity of TZT-1027 with that of dolastatin 10 as well as the *vinca* alkaloids vinblastine (VLB), vincristine (VCR) and vindesine (VDS). TZT-1027 and dolastatin 10 inhibited microtubule polymerization concentration-dependently at 1–100 $\mu M$ with $IC_{50}$ values of 2.2±0.6 and 2.3±0.7 $\mu M$, respectively. VLB, VCR and VDS inhibited microtubule polymerization at 1–3 $\mu M$ with $IC_{50}$ values of 2.7±0.6, 1.6±0.4 and 1.6±0.2 $\mu M$, respectively, but showed a slight decrease in inhibitory effect at concentrations of 10 $\mu M$ or more. TZT-1027 also inhibited monosodium glutamate-induced tubulin polymerization concentration-dependently at 0.3–10 $\mu M$, with an $IC_{50}$ of 1.2 $\mu M$, whereas VLB was only effective at 0.3–3 $\mu M$, with an $IC_{50}$ of 0.6 $\mu M$, and caused so-called "aggregation" of tubulin at 10 $\mu M$. Scatchard analysis of the binding data for [$^3$H]VLB suggested one binding site ($K_d$ 0.2±0.04 $\mu M$ and $B_{max}$ 6.0±0.26 n$M$/mg protein), while that for [$^3$H]TZT-1027 suggested two binding sites, one of high affinity ($K_d$ 0.2±0.01 $\mu M$ and $B_{max}$ 1.7±0.012 n$M$/mg protein) and the other of low affinity ($K_d$ 10.3±1.46 $\mu M$ and $B_{max}$ 11.6±0.83 n$M$/mg protein). [$^3$H]TZT-1027 was completely displaced by dolastatin 10 but only incompletely by VLB. [$^3$H]VLB was completely displaced by dolastatin 10 and TZT-1027. Furthermore, TZT-1027 prevented [$^3$H]VLB from binding to tubulin in a non-competitive manner according to Lineweaver-Burk analysis. TZT-1027 concentration-dependently inhibited both [$^3$H]guanosine 5′-triphosphate (GTP) binding to and GTP hydrolysis on tubulin. VLB inhibited the hydrolysis of GTP on tubulin concentration-dependently to a lesser extent than TZT-1027, but no inhibitory effect of VLB on [$^3$H]GTP binding to tubulin was evident even at 100 $\mu M$. Thus, TZT-1027 affected the binding of VLB to tubulin, but its binding site was not completely identical to that of VLB. TZT-1027 had a potent inhibitory effect on tubulin polymerization and differed from *vinca* alkaloids in its mode of action against tubulin polymerization.**

Key words: TZT-1027—Microtubule polymerization—Tubulin binding—Antimicrotubule agent—Dolastatin 10

Dolastatin 10 was isolated from the Indian Ocean sea hare *Dolabella auricularia* by Pettit *et al.* in 1987.[1] It exhibits cytotoxicity *in vitro* and is effective *in vivo* against transplantable tumors in mice.[2–4] As with *vinca* alkaloids,[5–8] these activities of dolastatin 10 are due to its binding to tubulin and inhibition of the assembly of microtubules in cells.[9–11] TZT-1027, a derivative of dolastatin 10, has greater antitumor activity and less toxicity than its mother compound.[12] The chemical structure of TZT-1027 is shown in Fig. 1. The i.v. injection of TZT-1027 was found to increase life span and to inhibit remarkably the growth of P388 leukemia and three solid tumors (colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma) in mice, with an efficacy superior or comparable to those of several reference agents; dolastatin 10, cisplatin, vincristine (VCR) and 5-fluorouracil.[13] TZT-1027 was also effective against human xenografts, that is, tumor regression

was observed in mice bearing MX-1 breast and LX-1 lung carcinomas.[13] Because of its good preclinical activity, TZT-1027 has been entered into phase I clinical trials in Japan.

Microtubule proteins are important in mitosis, but in addition, their main components, tubulins, are critical to many facets of cellular function. They provide support for organelles and membranes, resist compressive forces, provide tracks for vesicular transport and sorting, and contribute motile force for cell locomotion in conjunction with actin filaments.[14, 15]

Antimicrotubule agents interfere with the dynamics of microtubules and act as inhibitors of cell division. Indeed, several such agents, including paclitaxel (PTX), are clinically useful antitumor agents.

Here we describe a detailed investigation of the interaction of TZT-1027 with tubulin. TZT-1027 potently inhibited the polymerization of microtubule proteins and also purified tubulin, greatly inhibited the binding of radiolabeled *vinca* alkaloids and radiolabeled guanosine 5′-triphosphate (GTP) to tubulin, and inhibited GTP hydroly-

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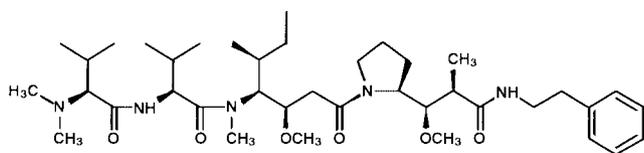E-mail: kobayashi-m1@kw.teikoku-hormone.co.jp

Fig. 1.    The chemical structure of TZT-1027.

sis on tubulin. TZT-1027 has different modes of action against microtubules and tubulin.

## MATERIALS AND METHODS

**Materials**    TZT-1027, dolastatin 10 and dolastatin 15 were synthesized in our laboratories. The chemical structure of TZT-1027 is shown in Fig. 1. VCR sulfate and vinblastine (VLB) sulfate were purchased from WAKO Pure Chemical Co., Ltd. (Osaka), vindesine (VDS) from Shionogi Pharmaceutical Co., Ltd. (Osaka), PTX from Sigma Chemical Co. (St. Louis, MO), and colchicine from Nacalai Tesque Inc. (Kyoto).

[3H]TZT-1027 was synthesized in our laboratories with a relative activity of 2123.8 GBq/mmol. [3H]VLB was purchased from Amersham International (Buckinghamshire, UK) with a relative activity of 414 GBq/mmol. [3H]Colchicine was obtained from Daiichi Kagaku Pharmaceutical Co., Ltd. (Tokyo) with a relative activity of 2590.0 GBq/mmol. [8-5′-3H]GTP was purchased from Amersham International with a relative activity of 362.6 GBq/mmol.

Bovine brain tubulin was from Cytoskelton Co., Ltd. (Denver, CO). TZT-1027, dolastatin 10, dolastatin 15, VDS, VCR, and VLB, were dissolved in and diluted with 0.05 $M$ lactate buffer (pH 4.5) unless otherwise described. PTX was dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water (DW). The final concentration of DMSO used in experiments was 0.5%. Colchicine was dissolved in and diluted with DW.

**Preparation of microtubule proteins and purification of tubulin from porcine brain**    Microtubule proteins were prepared from porcine brain by the method of Shelanski *et al.* with some modifications.[16] Briefly, porcine microtubule proteins were isolated by two cycles of polymerization and depolymerization, and stored as pellets frozen at −80°C in assembly buffer containing 100 m$M$ 2-morpholinoethanesulfonic acid (Mes) (pH 6.9), 2 m$M$ ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA), 1 m$M$ MgSO$_4$·7H$_2$O and 2 m$M$ dithiothreitol (DTT). Purification of tubulin from microtubule proteins was carried out by the method of Williams and Lee with some modifications.[17] Tubulin was separated from microtubule associated proteins (MAPs) by phosphocellulose (Whatman P11;

Whatman, Maidstone, UK) chromatography at 4°C. Samples of microtubule proteins in assembly buffer were slowly loaded on a 2.5×35 cm column which had previously been extensively equilibrated with assembly buffer containing 0.1 m$M$ GTP. The pure tubulin was eluted from the column with assembly buffer containing 0.1 m$M$ GTP at a flow rate of 15 ml/h. The protein was stored in aliquots (2 ml) at −80°C. Protein concentrations were determined by the method of Bradford[18] (Bio-Rad protein assay; Bio-Rad, Hercules, CA) using bovine serum albumin as a protein standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously[19] to confirm that a single band with a molecular weight of 50 kD was obtained.

**Effects on polymerization of microtubules**    Assays were performed as described by Bai *et al.*[8] Briefly, the polymerizing reaction mixtures were composed of 3.0 mg of homogeneous porcine brain microtubule proteins, 100 m$M$ Mes (pH 6.9), 0.5 m$M$ MgCl$_2$, 0.5 m$M$ GTP and various concentrations of agents in a volume of 2.0 ml. All components except GTP were mixed and chilled on ice for 10 min. Immediately after addition of GTP, the polymerizing reaction mixtures were transferred to a spectrophotometer equipped with an electronic temperature controller (UVIDEC-610C; JASCO Corp., Tokyo). Baselines were established with the cuvettes at 0°C and reactions were initiated by a temperature jump up to 37°C. Polymerization was followed by turbidity measurements for 30 min at 37°C. Experiments were carried out more than three times. IC$_{50}$ values were determined as the concentration of agents required to suppress polymerization by 50% after 30-min incubation.

**Effects on polymerization of purified tubulin**    Assays were also performed as described by Bai *et al.*[8] with minor modifications. Briefly, the polymerizing reaction mixtures were composed of 1.8 mg of bovine brain tubulin (dissolved in assembly buffer), 1.0 $M$ monosodium glutamate (pH 6.8), 1 m$M$ MgCl$_2$, 1 m$M$ EGTA, 0.5 m$M$ GTP and various concentrations of agents in a volume of 1.8 ml. The reactions were initiated by the addition of GTP and the mixtures were immediately transferred to a spectrophotometer equipped with an electronic temperature controller (UVIDEC-610C; JASCO Corp.), which was prewarmed to 37°C. Baselines were established with the cuvettes at 0°C, and reactions were monitored for 30 min at 37°C. The IC$_{50}$ values were determined as the concentration of agents required to suppress polymerization by 50% after 30-min incubation.

**Effects of radiolabeled agents on tubulin**    Assays were performed as described by Mandelbaum-Shavit *et al.*[20] and Borisy *et al.*[21] with slight modifications. Reaction mixtures containing 0.02 mg of porcine brain tubulin, nonradiolabeled agents and radiolabeled agents ([3H]TZT-1027, [3H]VLB or [3H]colchicine) in a volume of 0.2 ml

were incubated for various periods at 37°C, and chilled on ice for 10 min. Aliquots (100 $\mu$l) were dropped onto DEAE-cellulose filters (DE81; Whatman), which were washed twice with the ice-cold assembly buffer described above, and the radioactivity on filters was measured using a liquid scintillation counter (LSC-900; ALOKA, Tokyo) after applying Clear-sol I (Nacalai Tesque Inc.). TZT-1027 was dissolved in ethanol and diluted with DW. Dolastatin 10 was dissolved in DMSO and diluted with DW. VLB, VCR and colchicine were dissolved in and diluted with DW.

**Effects on GTP binding to tubulin** The reaction mixtures containing 0.05 mg of porcine brain tubulin dissolved in assembly buffer, and non-radiolabeled agents were applied to membrane filter plates (IP filter; Millipore Corp., Bedford, MA) and incubated for 30 min at 37°C. Then 1 $\mu$M [$^3$H]GTP in a volume of 0.25 ml was applied to the plates and incubation was continued for an additional 15 min at 0°C. The plates were washed twice with ice-cold assembly buffer and the radioactivity on filters was measured with a liquid scintillation counter (LSC-900; ALOKA) after applying Clear-sol I (Nacalai Tesque Inc.). TZT-1027 and dolastatin 15 were dissolved in ethanol and diluted with DW. Dolastatin 10 was dissolved in DMSO and diluted with DW. VLB was dissolved in and diluted with DW.

**Effects against GTP hydrolysis on tubulin** Assays were performed as described by Hamel and Lin.[22] The reaction mixtures containing 0.2 mg of porcine brain tubulin dissolved in assembly buffer, non-radiolabeled agents and 10 $\mu$M [$^3$H]GTP and 1 $\mu$M monosodium glutamate (pH 6.8) in a volume of 0.1 ml were incubated for 15 min at 37°C and the reactions were stopped by adding 10 $\mu$l of 20% acetic acid to aliquots (10 $\mu$l) of the reaction mixtures. Then, 10-$\mu$l aliquots of the stopped reaction mixtures were spotted upon PEI-cellulose thin-layer chromatography sheets (Merck, Frankfurt, Germany) and developed with 1.0 $M$ KH$_2$PO$_4$. After searching for bands of GDP by exposing the filters to ultraviolet, the radioactivity was measured with a liquid scintillation counter (ALOKA ; LSC-900) after applying Clear-sol I (Nacalai Tesque Inc.). Treatment with agents was performed as described above.

**Effects of the combination of TZT-1027 and PTX on polymerization of microtubule proteins**

*Stabilizing effects of PTX against depolymerization induced by CaCl$_2$*: The polymerizing reaction mixtures were composed of 3.0 mg of homogeneous porcine brain microtubule protein, 100 m$M$ Mes (pH 6.9), 0.5 m$M$ MgCl$_2$, 0.5 m$M$ GTP and various concentrations of PTX in a volume of 2.0 ml. All components except GTP were mixed and chilled on ice for 10 min. Immediately after addition of GTP, the polymerizing reaction mixtures were transferred to a spectrophotometer equipped with an electronic temperature controller (UVIDEC-610C; JASCO

Corp.). Baselines were established with the cuvettes at 0°C, and reactions were initiated by a temperature jump up to 37°C. Polymerization was followed by turbidity measurements for 30 min at 37°C. After addition of 20 $\mu$l of 400 m$M$ CaCl$_2$ at 30 min, depolymerization was carried out for an additional 15 min. Experiments were conducted twice. The IC$_{50}$ values were determined as the concentration of PTX required to suppress the depolymerization induced by 4 m$M$ CaCl$_2$ by 50% after 15-min incubation. *The combination effects of TZT-1027 or VLB and PTX*: The polymerizing reaction mixtures were composed of 3.0 mg of homogeneous porcine brain microtubule protein, 100 m$M$ Mes (pH 6.9), 0.5 m$M$ MgCl$_2$, 0.5 m$M$ GTP and 3 $\mu$M TZT-1027 (or 3 $\mu$M VLB) and 1 $\mu$M PTX in a volume of 2.0 ml. The combinations of agents were: 1) simultaneous addition of TZT-1027 (or VLB) and PTX and preincubation for 10 min, 2) addition of TZT-1027 (or VLB), preincubation for 5 min, then addition of PTX and preincubation for 5 min, and 3) as in 2), but with opposite order of addition. The polymerization was initiated by immediately adding GTP and transferring to the device at 37°C as described above. Experiments were carried out three times. The effects of combination were determined as the decrease in turbidity ($\Delta A_{350}$) versus the agent-untreated control at 30 min.

**RESULTS**

**Inhibition of polymerization of microtubule proteins by TZT-1027** To monitor the effects of antimicrotubule agents, we used the turbidity method and monitored the absorbance at 350 nm ($A_{350}$) for 30 min after the addition of GTP and the temperature jump to 37°C. The inhibitory effect was identified from the ratio of the increase of $A_{350}$ ($\Delta A_{350}$) in the agent-treated compared to the untreated fraction. As is shown in Fig. 2, a and b, TZT-1027 and dolastatin 10 inhibited the polymerization of microtubule proteins concentration-dependently at 1–100 $\mu$M with IC$_{50}$ values of 2.2±0.6 and 2.3±0.7 $\mu$M, respectively.

On the other hand, as shown in Fig. 3, a, b, and c, VLB, VCR and VDS inhibited the polymerization only at 1–3 $\mu$M with IC$_{50}$ values of 2.7±0.6, 1.6±0.4 and 1.6±0.2 $\mu$M, respectively, and at concentrations of 10 $\mu$M or more, showed a slight decrease in inhibitory effect. Thus, tubulin binding by TZT-1027 and *vinca* alkaloids may be different.

Dolastatin 15 also inhibited the polymerization of microtubule proteins concentration-dependently at 10–100 $\mu$M with an IC$_{50}$ of 23.7±1.9 $\mu$M (Fig. 3d), being the weakest inhibitor investigated here. Almost the same IC$_{50}$ value was obtained by Bai *et al.*[23]

**Inhibition of tubulin polymerization by TZT-1027** We examined the effect of TZT-1027 on tubulin polymerization to verify that its target is tubulin. As shown in Fig. 4a,
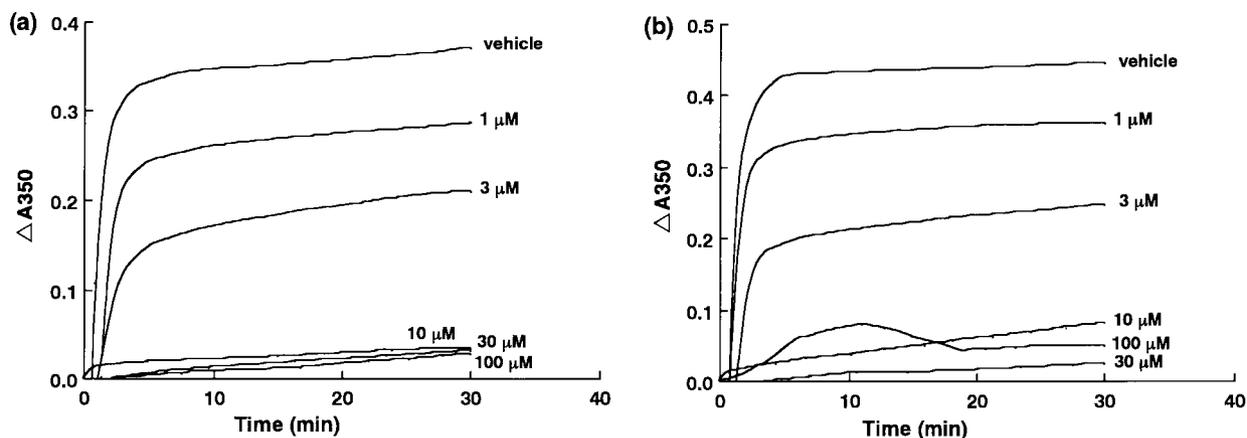
Fig. 2. Inhibitory effects of (a) TZT-1027 and (b) dolastatin 10 on the polymerization of microtubule proteins.
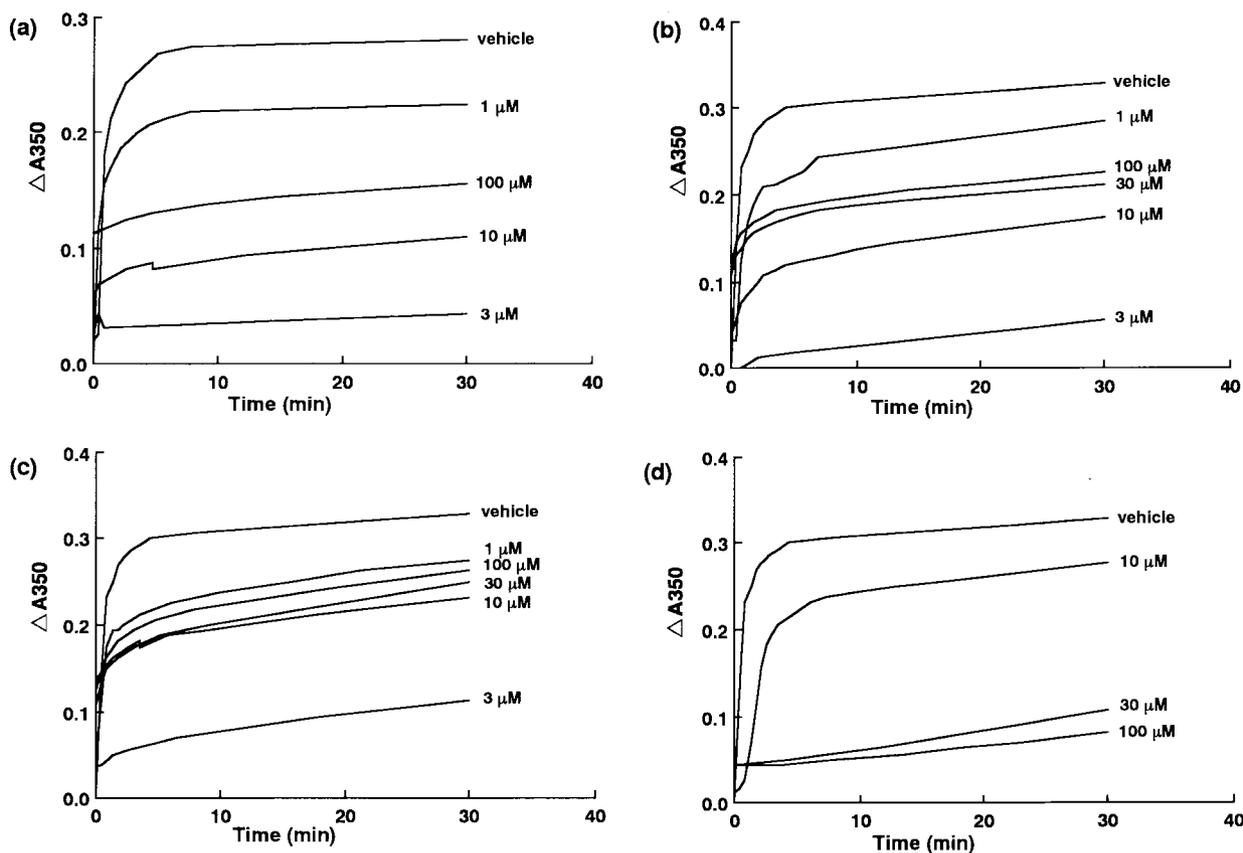


Fig. 3. Inhibitory effects of (a) VLB, (b) VCR, (c) VDS and (d) dolastatin 15 on the polymerization of microtubule proteins.

TZT-1027 inhibited monosodium glutamate-induced tubulin polymerization concentration-dependently at 0.3–10 $\mu M$ with an $IC_{50}$ of 1.2 $\mu M$. Dolastatin 10 inhibited it concentration-dependently at 1–3 $\mu M$ with an $IC_{50}$ of 2.8 $\mu M$ (Fig. 4b), but at concentrations of 10 $\mu M$ or more, no further increase in inhibition was observed in 0.005 $M$ lactate buffer. VLB was inhibitory only at 0.3–3 $\mu M$ with an $IC_{50}$ of 0.6 $\mu M$, and caused so-called "aggregation" of tubulin at 10 $\mu M$ (Fig. 4c). Thus, the tubulin binding properties of TZT-1027 and *vinca* alkaloids were different.
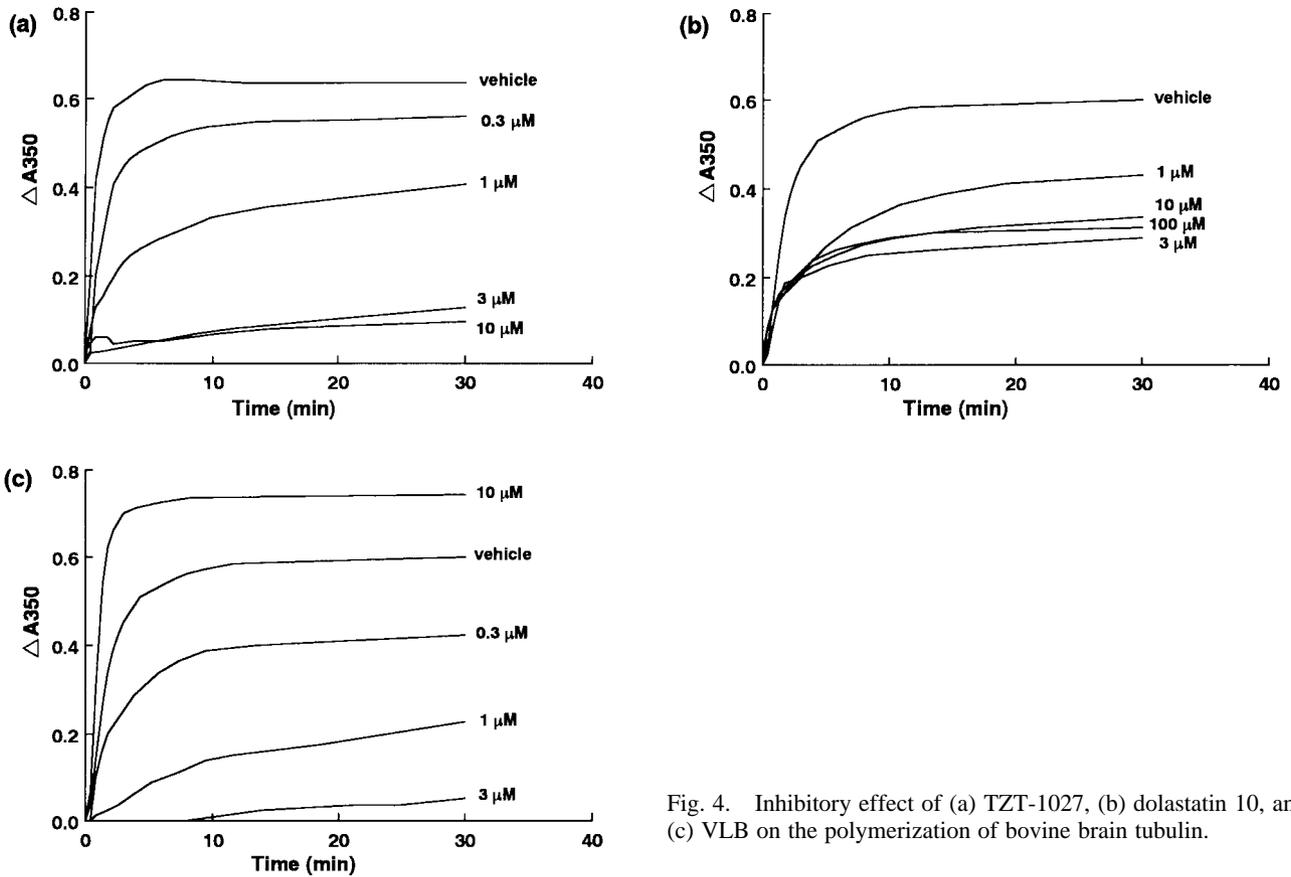
Fig. 4. Inhibitory effect of (a) TZT-1027, (b) dolastatin 10, and (c) VLB on the polymerization of bovine brain tubulin.
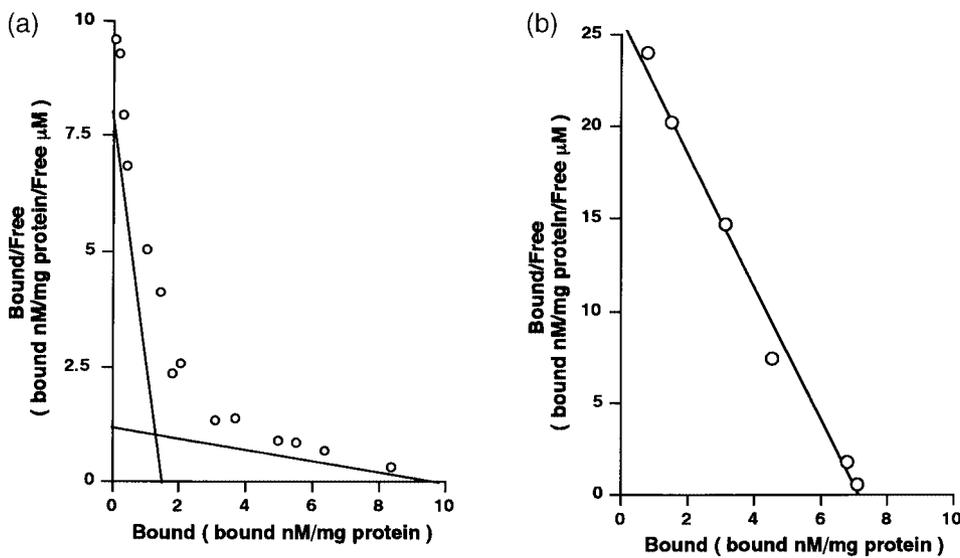


Fig. 5. Scatchard analysis of the binding of (a) [³H]TZT-1027 and (b) [³H]VLB to tubulin.

**Inhibition of binding of radiolabeled VLB by TZT-1027** To identify an appropriate incubation time for binding, 1 $\mu M$ [³H]TZT-1027 was added to the tubulin frac-

tion and incubated for various periods. We found that [³H]TZT-1027 bound to tubulin time-dependently and the reaction reached a plateau in 20 min (data not shown).

Therefore, we fixed the incubation time for [³H]TZT-1027 at 30 min.

Scatchard analysis of [³H]TZT-1027 and [³H]VLB binding suggested that TZT-1027 has two different binding sites on tubulin (Fig. 5a), with a dissociation constant ($K_d$ value) of 0.2±0.01 $\mu M$ and $B_{max}$ value of 1.7±0.012 n$M$/mg protein for the high-affinity site and values of 10.3±1.46 $\mu M$ and 11.6±0.83 n$M$/mg protein for the low-affinity site, whereas VLB has only one binding site (Fig. 5b), with a $K_d$ value of 0.2±0.04 $\mu M$ and $B_{max}$ value of 6.0±0.26 n$M$/mg protein.

We examined the displacement curves of non-radiolabeled TZT-1027, dolastatin 10, VLB, VCR and colchicine with [³H]TZT-1027 and [³H]VLB on tubulin. As shown in Fig. 6, TZT-1027 and dolastatin 10 concentration-dependently displaced [³H]VLB on tubulin. Dolastatin 10 concentration-dependently displaced [³H]TZT-1027 on tubulin. On the other hand, while VLB and VCR could displace it, the displacement was not complete even at 100 $\mu M$. Colchicine had no effect on either [³H]TZT-1027 or [³H]VLB binding on tubulin. Lineweaver-Burk plot analysis of TZT-1027 and [³H]VLB on tubulin revealed the binding to be non-competitive (Fig. 7).

**Inhibition of GTP binding on tubulin by TZT-1027**
Ordinarily, 2 mol of GTP binds to 1 mol of tubulin and is easily exchangeable with GTP or GDP. In order to polymerize microtubules from tubulin, it is necessary for GTP to bind at this exchangeable GTP binding site, where it is hydrolyzed to GDP. As shown in Figs. 5a and 6, TZT-1027 binds to tubulin, so we investigated its effect on GTP binding and hydrolysis on tubulin.

Inhibition of the binding of [³H]GTP on tubulin by various agents is illustrated in Fig. 8. TZT-1027 and dolastatin 10 showed concentration-dependent inhibition of the binding of GTP to tubulin. This may explain the inhibitory effects on the polymerization of purified tubulin of these two agents. Further, the binding sites of these agents on tubulin are close to that of GTP. On the other hand, dolastatin 15 and VLB showed only slight inhibitory effects at higher concentrations.

**Inhibition of GTP hydrolysis on tubulin by TZT-1027**
As is shown in Fig. 9, TZT-1027, dolastatin 10, dolastatin 15 and VLB showed concentration-dependent inhibition of
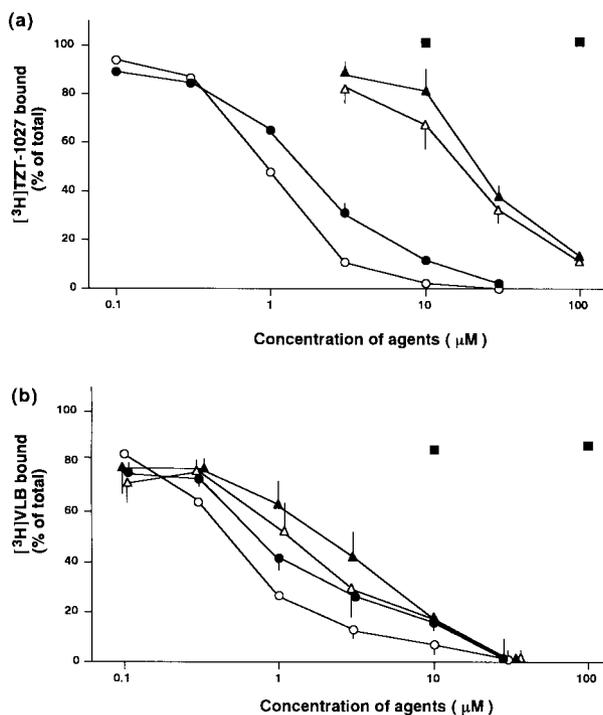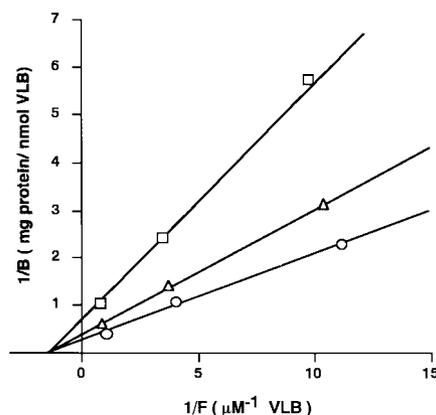


Fig. 7. Lineweaver-Burk plot demonstrating the effect of TZT-1027 on [³H]VLB binding to tubulin. The reciprocal of bound [³H]VLB is plotted against the reciprocal of the free concentration of [³H]VLB at several concentrations of TZT-1027: ○ none, △ 0.3 $\mu M$ and □ 1 $\mu M$.



Fig. 6. Effects of various antimicrotubule agents on the binding of (a) [³H]TZT-1027 and (b) [³H]VLB to tubulin. ● TZT-1027, ○ dolastatin 10, △ VLB, ▲ VCR and ■ colchicine.
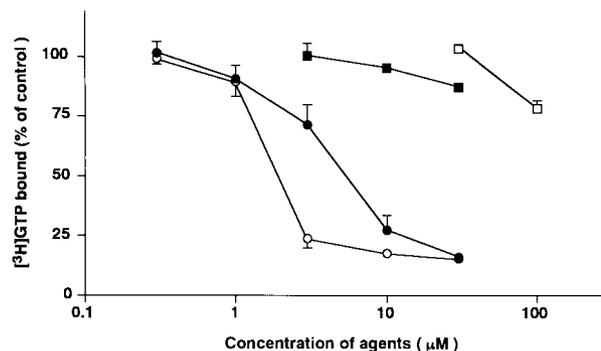


Fig. 8. Effects of various antimicrotubule agents on the binding of [³H]GTP to tubulin. ● TZT-1027, ○ dolastatin 10, ■ dolastatin 15 and □ VLB.
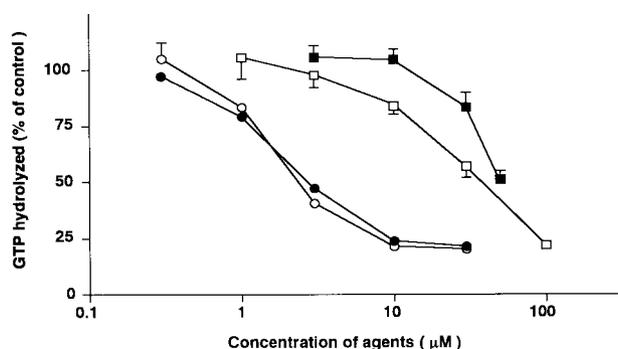
Fig. 9. Effects of various antimicrotubule agents on tubulin-dependent GTP hydrolysis. ● TZT-1027, ○ dolastatin 10, ■ dolastatin 15 and □ VLB.
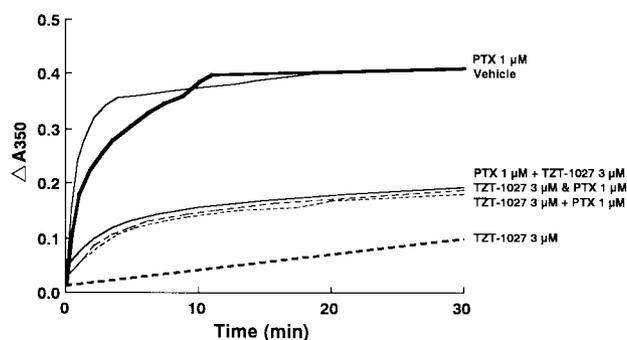


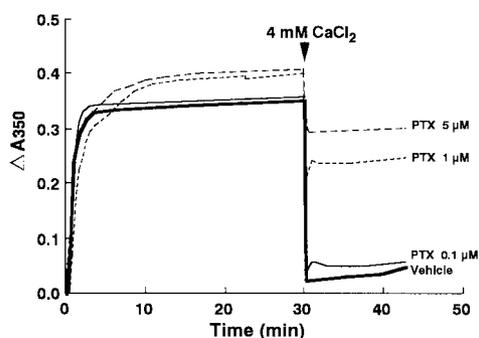Fig. 11. The effect of PTX upon the inhibitory effect of TZT-1027 on the polymerization of microtubule proteins.



Fig. 10. Stabilizing effect of PTX against depolymerization of the microtubule proteins induced by $CaCl_2$ (4 m$M$).

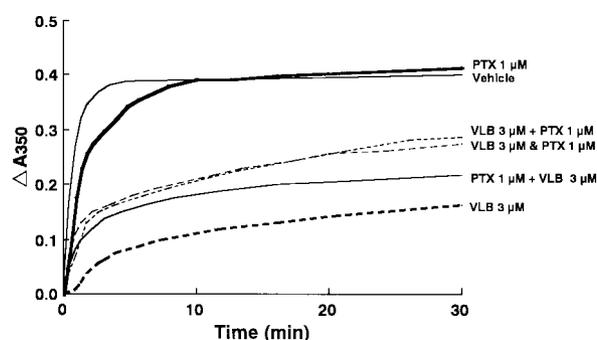

Fig. 12. The effect of PTX upon the inhibitory effect of VLB on the polymerization of microtubule proteins.

GTP hydrolysis on tubulin. TZT-1027 and dolastatin 10 were more effective than dolastatin 15 and VLB.

**The effect of the combination of TZT-1027 and PTX on the polymerization of microtubule proteins** First, we investigated the stabilizing effects of PTX on microtubules. As shown in Fig. 10, PTX increased the maximum amount of polymerized microtubules to some extent. In the PTX-untreated control fraction, in which the vehicle (0.5% DMSO) was applied to the polymerizing reaction mixture for 30 min, the addition of 4 m$M$ $CaCl_2$ had a depolymerizing effect of 85.2%. However, PTX concentration-dependently inhibited the depolymerizing effect induced by 4 m$M$ $CaCl_2$. In the 10 $\mu M$ PTX-treated fraction, the depolymerization amounted to only 12.9%. The $IC_{50}$ value for the inhibitory effect of PTX on depolymerization was 1.3 $\mu M$.

Next, we investigated the combination effect of TZT-1027 or VLB and PTX on microtubule polymerization. The concentrations of TZT-1027, VLB and PTX were fixed at 3, 3 and 1 $\mu M$, respectively, because, as shown in Figs. 2a, 3a, and 10, the $IC_{50}$ values of these agents were calculated to be 2.2, 2.7 and 1.3 $\mu M$, respectively.

As shown in Fig. 11, when only 3 $\mu M$ TZT-1027 was applied, a potent inhibition, 78.6%, of microtubule polymerization was observed. Cotreatment with TZT-1027 and PTX produced a modest inhibition of about 50%, regardless of the order in which these agents were added. The inhibition was less than with TZT-1027 alone.

Similar results were obtained with the combination of VLB and PTX. As shown in Fig. 12, when 3 $\mu M$ VLB alone was applied, microtubule polymerization was inhibited 64.7%, while cotreatment with VLB and PTX caused less than 50% inhibition, regardless of the order in which the agents were added.

**DISCUSSION**

We have reported the potent antitumor activity of TZT-1027 against a variety of transplantable tumors in mice.[13] The intracellular target of dolastatin 10, the mother compound of TZT-1027, was reported to be tubulin, the major component of microtubule proteins.[8–11] Therefore, we have investigated the activity of TZT-1027 on microtubule proteins, especially tubulin.

TZT-1027 inhibited the polymerization of microtubule proteins concentration-dependently at $1–100$ $\mu M$ with an $IC_{50}$ value of 2.2 $\mu M$. On the other hand, VLB, VCR and VDS inhibited it only at $1–3$ $\mu M$ with $IC_{50}$ values of 2.7, 1.6 and 1.6 $\mu M$, respectively, and at concentrations of 10 $\mu M$ or more, a decrease in inhibitory effect was observed with all three *vinca* alkaloids. In a previous study,[13] we found no such decrease in the inhibitory effect of VLB, even at relatively high concentrations, in the same experimental system. However, further study using additional lots of purified microtubule proteins revealed that VLB also showed a decrease in inhibitory effect at concentrations of 10 $\mu M$ or more. Therefore, the three *vinca* alkaloids appear to behave similarly. It is known that when *vinca* alkaloids are added to microtubule proteins, constructions different from normal microtubules are observed.[7, 8, 11] Thus, the modes of tubulin binding of TZT-1027 and *vinca* alkaloids may be different.

We investigated the activity of TZT-1027 for polymerization of purified tubulin to verify whether or not the target of TZT-1027 is tubulin. TZT-1027 inhibited monosodium glutamate-induced tubulin polymerization concentration-dependently at $0.3–10$ $\mu M$ with an $IC_{50}$ value of 1.2 $\mu M$. Dolastatin 10 inhibited it concentration-dependently at $1–3$ $\mu M$ with an $IC_{50}$ of 2.8 $\mu M$, but no further increase in inhibition was observed at concentrations of 10 $\mu M$ or more in 0.005 $M$ lactate buffer. Bai *et al.* reported that dolastatin 10 inhibited monosodium glutamate-induced tubulin polymerization concentration-dependently from $0.5–3$ $\mu M$ with an $IC_{50}$ of 1.2 $\mu M$ in 4% DMSO.[8] Hence, we carried out the same experiment with dolastatin 10 in 4% DMSO. We found that 3 $\mu M$ dolastatin 10 showed 88% inhibition of monosodium glutamate-induced tubulin polymerization (data not shown). The reason for the discrepancy in the activity levels of dolastatin 10 in the two solvents is unclear. At concentrations of 10 $\mu M$ or more, dolastatin 10 itself without tubulin was not precipitated in 0.005 $M$ lactate buffer as determined using a spectrophotometer. On the other hand, VLB inhibited monosodium glutamate-induced tubulin polymerization only at $0.3–3$ $\mu M$ with an $IC_{50}$ value of 0.6 $\mu M$, and it caused so-called "aggregation" of tubulin at 10 $\mu M$. This phenomenon has been reported in many papers,[7, 8, 11] and we suggest that constructions different from normal microtubules are formed. This confirms the idea that the modes of binding of TZT-1027 and *vinca* alkaloids to tubulin are different.

Scatchard analysis using [³H]TZT-1027 and [³H]VLB revealed TZT-1027 to have two different binding sites on tubulin, high and low affinity sites, and VLB to have only one. The $K_d$ and $B_{max}$ of [³H]VLB were similar to previously reported values.[24] Iwasaki *et al.* suggested that dolastatin 10 has two or more binding sites on tubulin from Scatchard analysis.[11, 25] Bai *et al.* reported a similar find-

ing.[26] Hence, it is reasonable to conclude that TZT-1027 has two binding sites on tubulin.

From examination of the displacement curves of non-radiolabeled TZT-1027, dolastatin 10, VLB, VCR and colchicine against [³H]TZT-1027 and [³H]VLB on tubulin, TZT-1027 and dolastatin 10 displaced [³H]VLB concentration-dependently. Dolastatin 10 also displaced [³H]TZT-1027 concentration-dependently on tubulin, while VLB and VCR could also displace it, but not completely, even at a high concentration of 100 $\mu M$. Lineweaver-Burk plot analysis of the effect of TZT-1027 on [³H]VLB binding to tubulin revealed a non-competitive effect. Bai *et al.* reported that dolastatin 10 reacted with tubulin in the same fashion as VLB.[27] Our data suggest that TZT-1027 binds to tubulin at a site different from that of colchicine. Although TZT-1027 can interact with VLB with respect to tubulin binding, the binding sites of TZT-1027 and VLB are not completely identical with each other.

We have also investigated the effects of PTX and dolastatin 15 on the bindings of radiolabeled TZT-1027 and VLB to tubulin. PTX and dolastatin 15 did not have remarkable inhibitory effects (data not shown). It was reported that dolastatin 15 bound tubulin as efficiently as dolastatin 10, but did not interfere with the binding of dolastatin 10 or VLB.[27] Therefore, it is interesting that dolastatin 15 does not interfere with TZT-1027 binding. TZT-1027 seems to have a different binding site on tubulin from those of PTX and dolastatin 15.

TZT-1027, dolastatin 10, dolastatin 15 and VLB inhibited the hydrolysis of GTP on tubulin concentration-dependently. TZT-1027 and dolastatin 10 also inhibited the binding of GTP to tubulin, but dolastatin 15 and VLB had no such effect. Bai *et al.* reported similar results for dolastatin 10 and VLB.[26] Huang *et al.* reported that VLB had an inhibitory effect on the binding of GTP to tubulin only above 100 $\mu M$.[28] Therefore our findings are consistent with previous results. Consequently, the difference in the effect of the two agents on GTP binding to tubulin can be attributed to a difference of binding site. We speculate that the binding site for TZT-1027 on tubulin partly overlaps that for GTP.

Antimicrotubule agents can be divided into two groups, microtubule-disrupting agents and microtubule-stabilizing agents. The former group is thought to act against cancer cells by disrupting microtubules, namely inhibiting the polymerization of tubulin. TZT-1027, dolastatin 10 and *vinca* alkaloids are included in this group. In contrast, microtubule-stabilizing agents inhibit the depolymerization of polymerized tubulin. PTX,[29–31] epothilones[32–34] and discodermolides[35–37] are members of this group.

In the clinical field, the importance of combinations of these two kinds of agents is being recognized. Reports have appeared on their effects against cancer cell lines *in vitro*, transplantable cancer cells *in vivo* and various kinds

of cancer cells from patients.[38–42] However, few reports have dealt with combinations of the antimicrotubule agents having different effects on the polymerization of microtubules. Therefore, we carried out a basic study on combining two agents, TZT-1027 or VLB and PTX. First, we investigated the stabilizing effect of PTX itself on the depolymerization induced by $CaCl_2$. PTX had a concentration-dependent stabilizing effect with an $IC_{50}$ value of 1.3 $\mu M$. Schiff *et al.* reported that 0.1–5 $\mu M$ PTX had a stabilizing effect against depolymerization of microtubules induced by 4 m$M$ $CaCl_2$.[29] Our value is similar to theirs. We used 4 m$M$ $CaCl_2$, which is hardly a physiological intracellular concentration of $Ca^{2+}$, for depolymerization because microtubules have been reported to lose their sensitivity for $Ca^{2+}$ as they are purified.[43] Hence a higher concentration of $Ca^{2+}$ is needed to depolymerize purified microtubules.

We have found that the microtubule-disrupting agents TZT-1027 and VLB counteract the effect of a microtubule-stabilizing agent during polymerization. The molecular mechanism involved is unknown. However, our results show that TZT-1027 and VLB not only inhibit microtubule polymerization, but also depolymerize polymerized microtubules.[13] PTX can polymerize already-polymerized microtubules as well as pre-polymerized microtubules.[30] Hence, the polymerization and depolymerization (evident as an increase and decrease, respectively, in turbidity) can be attributed to the change in polymerization or depolymerization at both ends of the microtubule and also to the connecting or severing of intact microtubules. These two kinds of agents have been reported to have both synergistic and antagonistic effects on cultured cells. The discrepancy seems to be dependent on the cells used, the order in which the agents are applied, and the duration for which the cells are in contact with these agents. We also examined combinations utilizing TZT-1027. However, at least in the microtubule polymerization experiment, we did not obtain synergistic results regardless of the order in which the agents were applied.

It is of interest that the concentration at which depolymerization occurs in a cell-free system is far higher than that at which cell proliferation is inhibited. TZT-1027 showed *in vitro* cytotoxicity at a concentration of about several hundred p$M$ (data not shown), while it inhibited polymerization of microtubule proteins and purified tubulin at a concentration of several $\mu M$. Thus, the concentration required to inhibit microtubule polymerization in a cell-free system is about 10000-fold higher than that required for inhibiting cell proliferation. The ability of other antimicrotubule agents to inhibit cell proliferation does not correlate well with their ability to interact with microtubules *in vitro*.[23, 44] As we have reported,[13] a tubulin binder inhibits microtubule polymerization at substoichiometric concentrations, namely, one molecule of the agent binds to one molecule of tubulin, resulting in inhibition of microtubule polymerization. The difference between the levels of tubulin in cells and in a cell-free system could account for the discrepancy.

In conclusion, TZT-1027 possesses a unique chemical structure and a broad spectrum of antitumor activity different from that of other clinically available agents. TZT-1027 inhibits the polymerization of microtubule protein and purified tubulin. The differences between TZT-1027 and VLB as they relate to tubulin are as follows; 1) TZT-1027 has two binding sites, namely a high and a low affinity binding site, on tubulin, whereas VLB has only one, 2) on tubulin, the binding sites for TZT-1027 and VLB are not completely identical with each other, although TZT-1027 can interact with VLB, 3) TZT-1027 has an inhibitory effect on the binding of GTP to tubulin, whereas VLB does not, 4) in the polymerization of microtubule proteins, VLB (and other *vinca* alkaloids) at high concentrations show a decreased inhibitory effect, resulting in "aggregation," whereas TZT-1027 does not, 5) in the polymerization of purified tubulin, VLB at high concentrations causes so-called "aggregation" of purified tubulin, whereas TZT-1027 does not. Thus the binding profile of TZT-1027 is different from that of VLB.

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