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 µM with IC₅₀ values of 2.2±0.6 and 2.3±0.7 µM, respectively. VLB, VCR and VDS inhibited microtubule polymerization at 1–3 µM with IC₅₀ values of 2.7±0.6, 1.6±0.4 and 1.6±0.2 µM, respectively, but showed a slight decrease in inhibitory effect at concentrations of 10 µM or more. TZT-1027 also inhibited monosodium glutamate-induced tubulin polymerization concentration-dependently at 0.3–10 µM, with an IC₅₀ of 1.2 µM, whereas VLB was only effective at 0.3–3 µM, with an IC₅₀ of 0.6 µM, and caused so-called “aggregation” of tubulin at 10 µM. Scatchard analysis of the binding data for [³H]VLB suggested one binding site (Kₐ 0.2±0.04 µM and Bₘₐₓ 6.0±0.26 nM/mg protein), while that for [³H]TZT-1027 suggested two binding sites, one of high affinity (Kₐ 0.2±0.01 µM and Bₘₐₓ 1.7±0.012 nM/mg protein) and the other of low affinity (Kₐ 10.3±1.46 µM and Bₘₐₓ 11.6±0.83 nM/mg protein). [³H]TZT-1027 was completely displaced by dolastatin 10 but only incompletely by VLB. [³H]VLB was completely displaced by dolastatin 10 and TZT-1027. Furthermore, TZT-1027 prevented [³H]VLB from binding to tubulin in a non-competitive manner according to Lineweaver-Burk analysis. TZT-1027 concentration-dependently inhibited both [³H]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 [³H]GTP binding to tubulin was evident even at 100 µM. 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 hydrolysis.
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. 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 mM 2-morpholinoethanesulfonic acid (Mes) (pH 6.9), 2 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 1 mM MgSO₄·7H₂O and 2 mM dithiothreitol (DTT). Purification of tubulin from microtubule proteins was carried out by the method of Williams and Lee with some modifications. 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 mM GTP. The pure tubulin was eluted from the column with assembly buffer containing 0.1 mM 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 (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 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. Briefly, the polymerizing reaction mixtures were composed of 3.0 mg of homogeneous porcine brain microtubule proteins, 100 mM Mes (pH 6.9), 0.5 mM MgCl₂, 0.5 mM 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. IC₅₀ 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. with minor modifications. Briefly, the polymerizing reaction mixtures were composed of 1.8 mg of bovine brain tubulin (dissolved in assembly buffer), 1.0 mM monosodium glutamate (pH 6.8), 1 mM MgCl₂, 1 mM EGTA, 0.5 mM 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 IC₅₀ 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. and Borisy et al. with slight modifications. Reaction mixtures containing 0.02 mg of porcine brain tubulin, non-radiolabeled 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 µl) 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 µM [3H]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 DMSO and diluted with DW. VCR and colchicine were 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, and non-radiolabeled agents and 10 µM [3H]GTP and 1 µM 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 µl of 20% acetic acid to aliquots (10 µl) of the reaction mixtures. Then, 10-µl aliquots of the stopped reaction mixtures were spotted upon PEI-cellulose thin-layer chromatography sheets (Merck, Frankfurt, Germany) and developed with 1.0 M KH₂PO₄. 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 CaCl₂.* The polymerizing reaction mixtures were composed of 3.0 mg of homogeneous porcine brain microtubule protein, 100 mM Mes (pH 6.9), 0.5 mM MgCl₂, 0.5 mM 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 (UVlDEC-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 µl of 400 mM CaCl₂ at 30 min, depolymerization was carried out for an additional 15 min. Experiments were conducted twice. The IC₅₀ values were determined as the concentration of PTX required to suppress the depolymerization induced by 4 mM CaCl₂ 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 mM Mes (pH 6.9), 0.5 mM MgCl₂, 0.5 mM GTP and 3 µM TZT-1027 (or 3 µM VLB) and 1 µM 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 (ΔA₅₃₂) 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 (A₅₃₂) 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 absorbance at 350 µM (A₅₃₂) versus 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 µM with IC₅₀ values of 2.2±0.6 and 2.3±0.7 µM, 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 µM with IC₅₀ values of 2.7±0.6, 1.6±0.4 and 1.6±0.2 µM, respectively, and at concentrations of 10 µM or more, showed a slight decrease in inhibitory effect. Thus, tubulin binding by TZT-1027 and vincristine alkaloids may be different.

Dolastatin 15 also inhibited the polymerization of microtubule proteins concentration-dependently at 10–100 µM with an IC₅₀ of 23.7±1.9 µM (Fig. 3d), being the weakest inhibitor investigated here. Almost the same IC₅₀ 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,
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 \textit{vinca} alkaloids were different.
Interaction of TZT-1027 with Tubulin

Inhibition of binding of radiolabeled VLB by TZT-1027. To identify an appropriate incubation time for binding, 1 µM [3H]TZT-1027 was added to the tubulin fraction 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).

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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.

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741
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 $0.2 \pm 0.01 \mu M$ and $B_{\text{max}}$ value of $1.7 \pm 0.012 \text{nM/mg}$ protein for the high-affinity site and values of $10.3 \pm 1.46 \mu M$ and $11.6 \pm 0.83 \text{nM/mg}$ protein for the low-affinity site, whereas VLB has only one binding site (Fig. 5b), with a $K_d$ value of $0.2 \pm 0.04 \mu M$ and $B_{\text{max}}$ value of $6.0 \pm 0.26 \text{nM/mg}$ 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 GTP hydrolysis on tubulin.
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 mM CaCl₂ had a depolymerizing effect of 85.2%. However, PTX concentration-dependently inhibited the depolymerizing effect induced by 4 mM CaCl₂. In the 10 µM PTX-treated fraction, the depolymerization amounted to only 12.9%. The IC₅₀ value for the inhibitory effect of PTX on depolymerization was 1.3 µM.

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 µM, respectively, because, as shown in Figs. 2a, 3a, and 10, the IC₅₀ values of these agents were calculated to be 2.2, 2.7 and 1.3 µM, respectively.

As shown in Fig. 11, when only 3 µM 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 µM 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. The intracellular target of dolastatin 10, the mother compound of TZT-1027, was reported to be tubulin, the major component of microtubule proteins. 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 µM with an IC₅₀ value of 2.2 µM. On the other hand, VLB, VCR and VDS inhibited it only at 1–3 µM with IC₅₀ values of 2.7, 1.6 and 1.6 µM, respectively, and at concentrations of 10 µM or more, a decrease in inhibitory effect was observed with all three vinca alkaloids. In a previous study, 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 µM 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. 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 µM with an IC₅₀ value of 1.2 µM. Dolastatin 10 inhibited it concentration-dependently at 1–3 µM with an IC₅₀ of 2.8 µM, but no further increase in inhibition was observed at concentrations of 10 µM 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 µM with an IC₅₀ of 1.2 µM in 4% DMSO. Hence, we carried out the same experiment with dolastatin 10 in 4% DMSO. We found that 3 µM 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 µM 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 µM with an IC₅₀ value of 0.6 µM, and it caused so-called “aggregation” of tubulin at 10 µM. This phenomenon has been reported in many papers, 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 Kᵣ and B_max of [³H]VLB were similar to previously reported values. Iwasaki et al. suggested that dolastatin 10 has two or more binding sites on tubulin from Scatchard analysis. Bai et al. reported a similar finding. 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 µM. 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. 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. 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. Huang et al. reported that VLB had an inhibitory effect on the binding of GTP to tubulin only above 100 µM. 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, epothilones and discodermolides 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.\textsuperscript{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\textsubscript{2}. PTX had a concentration-dependent stabilizing effect with an IC\textsubscript{50} value of 1.3 \(\mu\text{M}\). Schiff et al. reported that 0.1–5 \(\mu\text{M}\) PTX had a stabilizing effect against depolymerization of microtubules induced by 4 mM CaCl\textsubscript{2}.\textsuperscript{29} Our value is similar to theirs. We used 4 mM CaCl\textsubscript{2}, which is hardly a physiological intracellular concentration of Ca\textsuperscript{2+}, for depolymerization because microtubules have been reported to lose their sensitivity for Ca\textsuperscript{2+} as they are purified.\textsuperscript{43} Hence a higher concentration of Ca\textsuperscript{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.\textsuperscript{13} PTX can polymerize already-polymerized microtubules as well as pre-polymerized microtubules.\textsuperscript{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 polymerization occurs in a cell-free system is far higher than that at which cell proliferation is inhibited. TZT-1027 showed \textit{in vitro} cytotoxicity at a concentration of about several hundred \(\mu\text{M}\) (data not shown), while it inhibited polymerization of microtubule proteins and purified tubulin at a concentration of several \(\mu\text{M}\). 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 \textit{in vitro}.\textsuperscript{25, 44} As we have reported,\textsuperscript{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 \textit{vincra} 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.

\textbf{REFERENCES}

1) Pettit, G. R., Kamano, Y., Herald, C. L., Tuinman, A. A., Boettner, F. E., Kizu, H., Schmidt, J. M., Baczynskyj, L., Tomer, K. B. and Bontems, R. J. The isolation and structure of a remarkable marine animal antineoplastic constituent: dolastatin 10. \textit{J. Am. Chem. Soc.}, \textbf{109}, 6883–6885 (1987).

2) Pettit, G. R., Kamano, Y., Herald, C. L., Fujii, Y., Kizu, H., Boyd, M. R., Boettner, F. E., Doubek, D. L., Schmidt, J. M., Chapuis, J. C. and Michel, C. Isolation of dolastatin 10–15 from the marine mollusk \textit{Dolabella auricularia}.

3) Pettit, G. R., Singh, S. B., Hogan, F., Lloyd-Williams, P., Herald, D. L., Burkett, D. D. and Clewlow, P. J. The absolute configuration and synthesis of natural (–)-dolastatin 10. \textit{J. Am. Chem. Soc.}, \textbf{111}, 5463–5465 (1987).

4) Hamada, Y., Hayashi, K. and Shioiri, T. Efficient stereoselective synthesis of dolastatin 10, an antineoplastic peptide from a sea hare. \textit{Tetrahedron Lett.}, \textbf{32}, 931–934 (1991).

5) Owellen, R. J., Hartke, C. A., Dickerson, R. M. and Hains, F. O. Inhibition of tubulin-microtubule polymerization by deutério-dolastatin 10. \textit{Tetrahedron}, \textbf{49}, 9151–9170 (1993).

(Received February 9, 2000/Revised April 20, 2000/Accepted April 28, 2000)
drugs of the Vinca alkaloid class. Cancer Res., 36, 1499–1502 (1976).
6) Himes, R. H., Kersey, R. N., Heller-Bettinger, I. and Samson, F. E. Action of Vinca alkaloids vincristine, vinblastine and desacetylvinblastine amide on microtubules in vitro. Cancer Res., 36, 3798–3802 (1976).
7) Safa, A. R., Hamel, E. and Felsted, R. L. Photoaffinity labeling of tubulin subunits with a photosensitive analogue of vinblastine. Biochemistry, 26, 97–102 (1987).
8) Bai, R., Pettit, G. R. and Hamel, E. Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Biochim. Biophys. Acta, 439, 1941–1949 (1990).
9) Bai, R., Pettit, G. R. and Hamel, E. Binding of dolastatin 10 to tubulin at a distinct site for peptide antagonistic agents near the exchangeable nucleotide and Vinca alkaloid sites. J. Biol. Chem., 265, 17141–17149 (1990).
10) Ludueña, R. F., Roach, M. C., Prasad, V. and Pettit, G. R. Interaction of dolastatin 10 with bovine brain tubulin. Biochim. Biophys. Acta, 439, 539–543 (1992).
11) Li, Y., Kobayashi, Y., Hashimoto, Y., Shirai, R., Hirata, A., Hayashi, K., Hamada, Y., Shioiri, T. and Iwasaki, S. Interaction of marine toxin dolastatin 10 with porcine brain tubulin: competitive inhibition of rhizoxin and phomopsin A binding. Chem. Biol. Interact., 93, 9175–9183 (1994).
12) Miyazaki, K., Kobayashi, M., Natsume, T., Gondo, M., Mikami, T., Sakakibara, K. and Tsukagoshi, S. Synthesis of antitumor activity of novel dolastatin 10 analogs. Chem. Pharm. Bull., 43, 1706–1718 (1995).
13) Kobayashi, M., Natsume, T., Tamaoki, S., Watanabe, J., Asano, H., Mikami, T., Miyazaki, K., Miyazaki, K., Gondo, M., Sakakibara, K. and Tsukagoshi, S. Antitumor activity of TZT-1027, a novel dolastatin 10 derivative. Jpn. J. Cancer Res., 88, 316–327 (1997).
14) Hyams, J. S. and Lloyd, C. W. “Microtubules,” pp. 1–644 (1994). Wiley-Liss, New York.
15) Hamm-Alvarez, S. F. Microtubule-based motor proteins: new targets for enhancing drug delivery? Pharm. Res., 13, 489–496 (1996).
16) Shelanski, M. L., Gaskin, F. and Cantor, C. R. Microtubule assembly in the absence of added nucleotides. Proc. Natl. Acad. Sci. USA, 70, 765–768 (1973).
17) Williams, R. C., Jr. and Lee, J. C. Preparation of tubulin from brain. Methods Enzymol., 85, 376–385 (1982).
18) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, 248–254 (1976).
19) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685 (1970).
20) Mandelbaum-Shavit, F., Wolpert-DeFilippes, M. K. and Johns, D. G. Binding of maytansine to rat brain tubulin. Biochem. Biophys. Res. Commun., 72, 47–54 (1976).
21) Borisy, G. G. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. Anal. Biochem., 50, 373–385 (1972).
22) Hamel, E. and Lin, C. M. Glutamate-induced polymerization of tubulin: characteristics of the reaction and application to the large-scale purification of tubulin. Arch. Biochem. Biophys., 209, 29–40 (1981).
23) Bai, R., Friedman, S. J., Pettit, G. R. and Hamel, E. Dolastatin 15, a potent antimitotic depsipeptide derived from Dolabella auriculata. Biochem. Pharmacol., 43, 2637–2645 (1992).
24) Takahashi, M., Iwasaki, S., Kobayashi, H., Okuda, S., Murai, T. and Sato, Y. Rhizoxin binding to tubulin at the maytansine-binding site. Biochim. Biophys. Acta, 926, 215–223 (1987).
25) Li, Y., Kobayashi, H., Tokiwa, Y., Hashimoto, Y. and Iwasaki, S. Interaction of phomopsin A with porcine brain tubulin. Inhibition of tubulin polymerization and binding at a rhizoxin binding site. Biochem. Pharmacol., 43, 219–224 (1992).
26) Bai, R., Paull, K. D., Herald, C. L., Malspeis, L., Pettit, G. R. and Hamel, E. Halichondrin B and homohalichondrin B, marine natural products binding in the vinca domain of tubulin. Discovery of tubulin-based mechanism of action by analysis of differential cytotoxicity data. J. Biol. Chem., 266, 15882–15889 (1991).
27) Bai, R., Taylor, G. F., Schimidt, J. M., Williams, M. D., Kepler, J. A., Pettit, G. R. and Hamel, E. Interaction of dolastatin 10 with tubulin: induction of aggregation and binding and association reactions. Mol. Pharmacol., 47, 965–976 (1995).
28) Huang, A. B., Lin, C. M. and Hamel, E. Maytansine inhibits its nucleotide binding at the exchangeable site of tubulin. Biochim. Biophys. Acta, 128, 1239–1246 (1985).
29) Schiff, P. B., Fant, J. and Horwitz, S. B. Promotion of microtubule assembly in vitro by taxol. Nature, 277, 665–671 (1979).
30) Parness, J. and Horwitz, S. B. Taxol binds to polymerized tubulin in vitro. J. Cell Biol., 91, 479–487 (1981).
31) Manfredi, J. J., Parness, J. and Horwitz, S. B. Taxol binds to cellular microtubules. J. Cell Biol., 94, 688–696 (1982).
32) Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., Goetz, M., Lazarides, E. and Woods, C. M. Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. Cancer Res., 55, 2325–2333 (1995).
33) Mühlradt, P. F. and Sasse, E. Epothilone B stabilizes microtubuli of macrophages like taxol without showing taxol-like endotoxin activity. Cancer Res., 57, 3344–3346 (1997).
34) Kowalski, R. J., Giannakakou, P. and Hamel, E. Activities of microtubule-stabilizing agents epothilone A and B with purified tubulin and in cells resistant to paclitaxel (taxol). J. Biol. Chem., 272, 2534–2541 (1997).
35) Haar, E., Kowalski, R. J., Hamel, E., Lin, C. M., Longley, R. E., Gunasekera, S. P., Rosenkranz, H. S. and Day, B. W. Docetaxel, a cytotoxic marine agent that stabilizes microtubules more potently than taxol. Biochemistry, 35, 243–250 (1996).
Interaction of TZT-1027 with Tubulin

36) Hung, D. T., Chen, J. and Schreiber, S. L. (+)-Discodermolide binds to microtubules in stoichiometric ratio to tubulin dimers, blocks taxol binding and results in mitotic arrest. *Chem. Biol.*, 3, 287–293 (1996).

37) Kowalski, R. J., Giannakakou, P., Gunasekera, S. P., Longley, R. E., Day, B. W. and Hamel, E. The microtubule-stabilizing agent discodermolide competitively inhibits the binding of paclitaxel (taxol) to tubulin polymers, enhances tubulin nucleation reactions more potently than paclitaxel, and inhibits the growth of paclitaxel-resistant cells. *Mol. Pharmacol.*, 52, 613–622 (1997).

38) Knick, V. C., Eberwein, D. L. and Miller, C. G. Vinorelbine tartrate and paclitaxel combinations: enhanced activity against *in vivo* P388 murine leukemia cells. *J. Natl. Cancer Inst.*, 87, 1072–1077 (1995).

39) Photiou, A., Shah, P., Leong, L. K., Moss, J. and Retsas, S. *In vitro* synergy of paclitaxel (taxol) and vinorelbine (navelbine) against human melanoma cell lines. *Eur. J. Cancer*, 33, 463–470 (1997).

40) Chang, A. Y. and Garrow, G. C. Pilot study of vinorelbine (navelbine) and paclitaxel (taxol) in patients with refractory breast cancer and lung cancer. *Semin. Oncol.*, 22, 66–71 (1995).

41) Retsas, S., Mohith, A. and Mackenzie, H. Taxol and vinorelbine: a new active combination for disseminated malignant melanoma. *Anticancer Drugs*, 7, 161–165 (1996).

42) Speicher, L. A., Barone, L. and Tew, K. D. Combined antimicrotubule activity of estramustine and taxol in human prostatic carcinoma cell lines. *Cancer Res.*, 52, 4433–4440 (1992).

43) Nishida, E. and Sakai, H. Calcium-sensitivity of the microtubule reassembly system. Difference between crude brain extract and purified microtubular proteins. *J. Biochem.*, 82, 303–306 (1977).

44) de Arruda, M., Cocchiaro, C. A., Nelson, C. M., Grinnell, C. M., Janssen, B., Haupt, A. and Barlozzari, T. LU103793 (NSC D-669356): a synthetic peptide that interacts with microtubules and inhibits mitosis. *Cancer Res.*, 55, 3085–3092 (1995).