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Antitumor Activity of TZT-1027, a Novel Dolastatin 10 Derivative

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Dolastatin 10, a pentapeptide isolated from the marine mollusk *Dolabella auricularia*, has antitumor activity. TZT-1027, a dolastatin 10 derivative, is a newly synthesized antitumor compound. We evaluated its antitumor activity against a variety of transplantable tumors in mice. Intermittent injections of TZT-1027 were more effective than single or repeated injections in mice with P388 leukemia and B16 melanoma. Consequently, TZT-1027 shows schedule dependency. TZT-1027 was effective against P388 leukemia not only when administered i.p., but also when given i.v. However, although TZT-1027 given i.v. was active against murine solid tumors, TZT-1027 administered i.p. was ineffective against all the tumors tested with the exception of colon 26 adenocarcinoma. The i.v. injection of TZT-1027 at a dose of 2.0 mg/kg remarkably inhibited the growth of three murine solid tumors; colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma, with T/C values of less than 6%. The antitumor activities of TZT-1027 against these tumors were superior or comparable to those of the reference agents; dolastatin 10, cisplatin, vincristine, 5-fluorouracil (5-FU) and E7010. In experiments with drug-resistant P388 leukemia, TZT-1027 showed good activity against cisplatin-resistant P388 and moderate activity against vincristine- and 5-fluorouracil-resistant P388, but no activity against adriamycin-resistant P388. 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. TZT-1027 at 10 μM almost completely inhibited the assembly of porcine brain microtubules. Therefore, its mechanism of antitumor action seems to be, at least in part, ascribable to the inhibition of microtubule assembly. Because of its good preclinical activity, TZT-1027 has been entered into phase I clinical trials.

Key words: TZT-1027 — Antitumor activity — Tubulin binder — Dolastatin 10 — Microtubule

Dolastatin 10, isolated by Pettit *et al.* from the Indian Ocean sea hare *Dolabella auricularia* in 1987, has shown cytotoxicity *in vitro* and efficacy *in vivo* against transplantable tumors in mice.[1–4] It is one of the most potent known anticancer agents *in vitro* with an IC_{50} for P388 leukemia cell growth of $5 \times 10^{-11} M$.[1] Dolastatin 10 has a unique structure consisting of five units; dolavaline, valine, dolaisoleuine, dolaproine and dolaphenine. The agent is a mitotic spindle poison, that inhibits microtubule assembly by interacting with tubulin in the *Vinca* alkaloid-binding domain.[5–8] Navelbine, a new *Vinca* alkaloid, was demonstrated to be a clinically effective antitumor drug against non-small cell lung cancer and advanced breast cancer,[9–11] and consequently, attention has recently been directed toward tubulin binders, including taxol and taxotere. In addition to studying the unique structure and interesting biological activity of dolastatin 10, our efforts have been directed towards the design of new dolastatin 10 derivatives with higher antitumor activity and less toxicity. We synthesized derivatives with some modifications of each subunit and evaluated their *in vivo* activity.[12] Among them, TZT-1027, N^2-(*N*,*N*-dimethyl-L-valyl)-*N*-[(1*S*,2*R*)-2-methoxy-4-[(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-[(2-phenylethyl)amino]propyl]-1-pyrrolidinyl]-1-[(*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, was found to have potent antitumor activity against P388 leukemia with low toxicity.

In this study we evaluated the antitumor activity of TZT-1027 against various murine tumors and human xenografts, and compared it with that of dolastatin 10 and reference drugs including VCR, CDDP and 5-FU. We also examined the mechanism of action of TZT-1027. The drug showed potent antitumor activities against all murine tumors and human xenografts tested. Therefore, it has been entered into phase I clinical trials in Japan.

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The abbreviations used are: MST, median survival time; ILS, increase in life span; Mes, 2-morpholinoethanesulfonic acid; DTT, dithiothreitol; VCR, vincristine; CDDP, cisplatin; 5-FU, 5-fluorouracil; ADM, adriamycin; VBL, vinblastine; Pgp, P-glycoprotein.

MATERIALS AND METHODS

Drugs TZT-1027, dolastatin 10 and E7010, a novel sulfonamide with antitubulin activity, were synthesized in our laboratories.[12, 13] The chemical structure of TZT-1027 is shown in Fig. 1. VCR was purchased from Shionogi Pharmaceutical Co., Ltd. (Osaka), CDDP from Bristol-Meyers Squibb Co., Ltd. (Tokyo), and 5-FU and ADM from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). TZT-1027 and dolastatin 10 were dissolved in and diluted with 0.05 *M* lactate buffer (pH 4.5) and administered i.v. E7010 was suspended in 0.5% methylcellulose and administered p.o. VCR, CDDP, and ADM were dissolved in and diluted with saline and administered i.v. 5-FU was diluted with saline, and administered i.v. or i.p. The control group was given 0.05 *M* lactate buffer.

Animals Female DBA/2, BALB/c, C57BL/6, BALB/c $\times$DBA/2 (hereafter called CDF_1), C57BL/6$\times$DBA/2 (hereafter called BDF_1), and BALB/c *nu/nu* athymic nude mice were purchased from Charles River Japan Inc. (Kanagawa). All mice, with the exception of the athymic nude mice, were fed on a pellet diet (MM-3; Funabashi Farm, Chiba) with UV-irradiated water *ad libitum*. The diet for the athymic nude mice consisted of γ-ray-irradiated food (CL-2; Nihon Clea Co., Ltd., Tokyo) and autoclaved water *ad libitum*.

Tumor cells P388 leukemia, colon 26 adenocarcinoma, B16 melanoma, M5076 sarcoma, MX-1 breast carcinoma and LX-1 lung carcinoma, were kindly supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. The P388/VCR,[14] P388/ADM[15] and P388/CDDP leukemia sublines[16, 17] were supplied by Dr. Tashiro, while the P388/5-FU leukemia subline[18, 19] was supplied by Dr. Inaba, both of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. The murine tumors were maintained in the respective syngeneic mice according to the protocol of the National Cancer Institute.[20] The human xenografts were maintained in female BALB/c *nu/nu* mice.

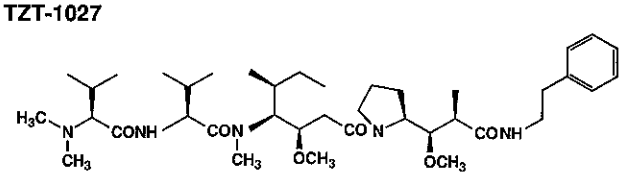
Evaluation of antitumor effects against murine tumors This study was carried out according to the standards of the National Cancer Institute.[20] P388 leukemia, P388/VCR, P388/ADM, P388/CDDP or P388/5-FU (1×10^6 cells) was implanted i.p. into CDF_1 mice on day 0. Colon 26 adenocarcinoma (2×10^6 cells) was implanted s.c. into CDF_1 mice on day 0. B16 melanoma (2×10^6 cells) and M5076 sarcoma (2×10^6 cells) were implanted s.c. into BDF_1 mice on day 0. TZT-1027 and the reference compounds (volume 0.1 ml/10 g body weight) were injected i.v., i.p. or p.o. into the tumor-bearing mice. The median survival time (MST, days) of the treated (T) and control (C) groups was determined, and the increase in life span (ILS; %) was calculated using the following equation: ILS (%) = [(MST treated)/(MST control) $-$ 1] $\times$ 100. Solid tumor volume (mm^3) was estimated from two dimensional measurements (mm): Solid tumor volume (mm^3) = length (mm) $\times$ [width (mm)]^2/2. Tumor growth inhibition (T/C value) was calculated using the following equation: T/C (%) = [(solid tumor volume treated)/(solid tumor volume control)] $\times$ 100.

Evaluation of antitumor effects against human xenografts The antitumor effects against human xenografts were evaluated by methods previously reported.[21] Briefly, tumor fragments (2 mm^3) of LX-1 lung and MX-1 breast carcinomas were implanted s.c. in the right flank of female BALB/c *nu/nu* mice, and drug administration was started when the tumor volume reached about 100 mm^3. TZT-1027 and CDDP were given i.v. once, or twice with a 7-day interval. Each tumor was measured twice a week with a sliding caliper. Tumor weights were measured on day 21. Antitumor evaluation was based on the tumor growth rate calculated for each group.

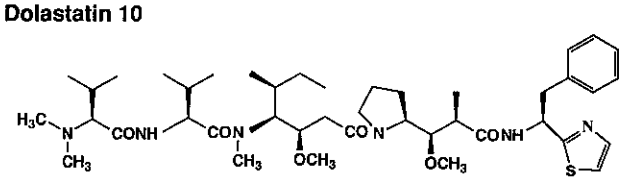
Criteria for activity In this study, the criteria for estimating activity in *in vivo* tumor models were the same as those of the National Cancer Institute.[20] For solid tumors, a T/C value of $\leq$42% was considered moderately active (+), while $\leq$10% was regarded as good activity (++). Statistical significance was determined by the Mann-Whitney U test ($P<0.05$). For ascitic tumors, an ILS value of $\geq$20% was considered moderately active (+), while $\geq$75% was regarded as good activity (++).

Effect on the assembly/disassembly of microtubules Microtubule protein was prepared from porcine brain by the method of Shelanski *et al.* with some modifications.[22] Briefly, porcine microtubule proteins were isolated by two cycles of polymerization and depolymerization, and stored as pellets frozen at -80°C in reassembly buffer

TZT-1027



Dolastatin 10



dolavaline valine dolaisoleuine dolaproine dolaphenine

Fig. 1. Chemical structures of TZT-1027 and dolastatin 10.

containing 100 m*M* 2-morpholinoethanesulfonic acid (Mes; pH 6.9), 2 m*M* EGTA, 1 m*M* $MgSO_4 \cdot 7\ H_2O$ and 2 m*M* DTT. Protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay) using bovine serum albumin as a standard.

Assays were performed as described by Bai *et al.*[5] Briefly, the 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 drugs 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, reaction mixtures were transferred to a spectrophotometer equipped with electronic temperature controllers (UVIDEC-610C; JASCO Corporation, Tokyo). Baselines were established with the cuvettes at 0°C, and reactions were initiated by a temperature jump to 37°C. Polymerization and depolymerization were followed by turbidity measurements for 30 min at 37°C. Experiments were repeated three times. The IC_{50} values were defined as the concentration of drugs required to suppress polymerization by 50% after 30 min.

RESULTS

Influence of route of administration In the P388 murine leukemia model (Fig. 2), the tumor was inoculated i.p. and TZT-1027 was given i.p. or i.v. twice with a 4-day interval. TZT-1027 exhibited potent activity when administered by either route, with ILS values of $\geq 70\%$. In murine solid tumor models inoculated s.c. (Fig. 2), TZT-1027 given i.v. twice with a 4-day interval caused tumor regression and/or growth inhibition as follows: against colon 26 adenocarcinoma, a T/C value of 29% at a dose of 2.0 mg/kg; against B16 melanoma, a T/C value of 16% at a dose of 1.0 mg/kg; and against M5076 sarcoma, T/C values of 21% and 10% at doses of 1.0 mg/kg and 2.0 mg/kg, respectively. However, similarly administered TZT-1027 given i.p. was ineffective against all tumors tested, except colon 26 adenocarcinoma.

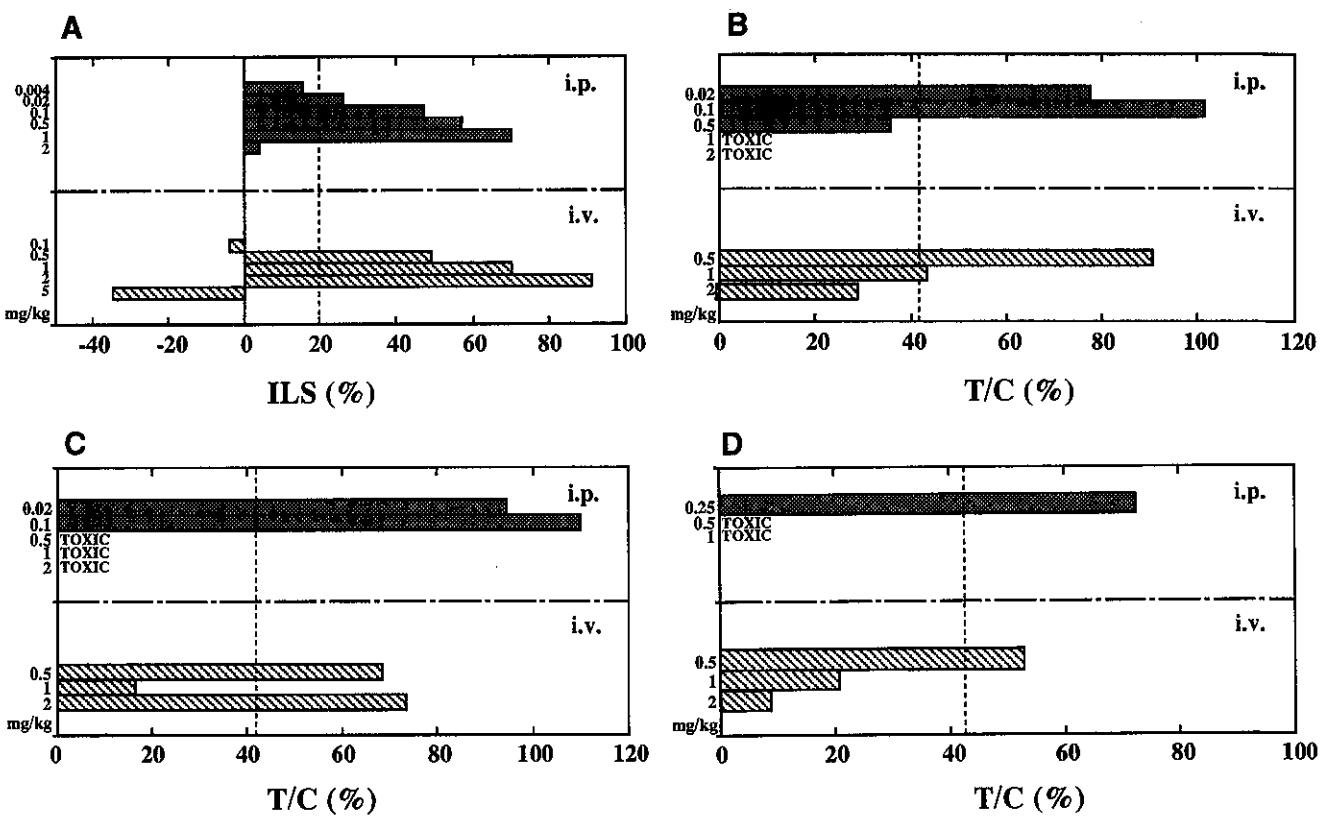


Fig. 2. Effect of route of administration on TZT-1027 activity against murine tumors. P388 leukemia was inoculated i.p. into mice on day 0. Solid tumors were inoculated s.c. into mice on day 0. TZT-1027 was given i.p. or i.v. to mice on days 1 and 5. ILS% was calculated as (median survival time in treated group/median survival time in control group $-1) \times 100$. T/C% was calculated as (mean tumor volume in treated group/mean tumor volume in control group) $\times 100$. "TOXIC" indicates toxicity on the evaluation day. A, P388 leukemia; B, Colon 26 adenocarcinoma; C, B16 melanoma; D, M5076 sarcoma.

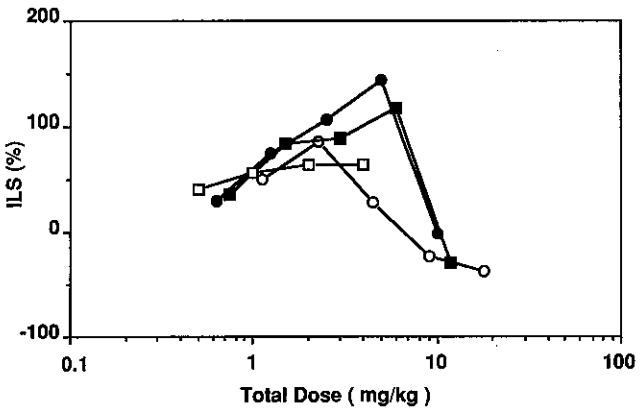


Fig. 3. Schedule dependency of the antitumor effect of TZT-1027 against P388 leukemia in mice. P388 leukemia was inoculated i.p. into mice on day 0. The schedule of TZT-1027 administration was varied. ILS% was calculated as (median survival time in treated group/median survival time in control group$-1)\times100$. □, Day 1; ■, Q4d×3; ●, Q2d×5; ○, Q1d×9.

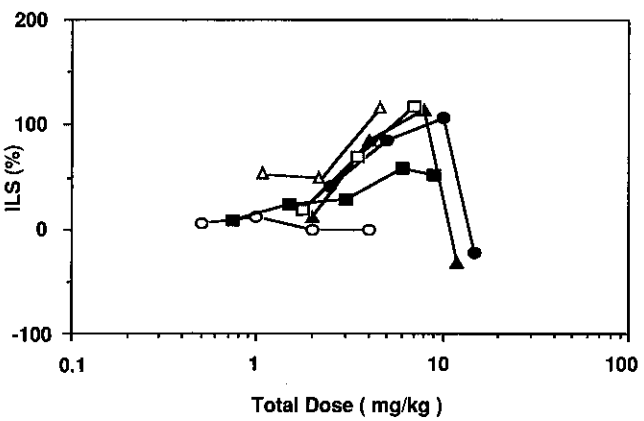


Fig. 4. Schedule dependency of the antitumor effect of TZT-1027 against B16 melanoma in mice. B16 melanoma was inoculated s.c. into mice on day 0. The schedule of TZT-1027 administration was varied. ILS% was calculated as (median survival time in treated group/median survival time in control group$-1)\times100$. ○, Day 1; ■, Q7d×3; ▲, Q5d×4; ●, Q4d×5; □, Q3d×7; △, Q1d×9.

Schedule dependency We examined the extent to which the antitumor effect of TZT-1027 depends on treatment schedule using the i.p.-i.v. P388 leukemia and the s.c.-i.v. B16 melanoma models. In the P388 leukemia model (Fig. 3), TZT-1027 was administered i.v. according to the following four schedules; Q1d×1, Q4d×3, Q2d×5 and Q1d×9. Among these, Q4d×3 and Q2d×5 were found to maximize the effect of the drug (ILSmax, 117% and 144%, respectively). In the B16 melanoma model (Fig. 4), TZT-1027 was administered i.v. according to the following six schedules; Q1d×1, Q7d×3, Q5d×4, Q4d×5, Q3d×7 and Q1d×9. Q3d×7 and Q1d×9 caused loss of body weight in mice and seemed to be toxic (data not shown). Among these, Q5d×4 and Q4d×5 maximized the effect of the drug. Administration schedule-dependency of TZT-1027 was observed in both P388 leukemia and B16 melanoma models.

Comparison of antitumor activity of TZT-1027 with those of other anticancer agents against murine tumors We compared the antitumor effects of TZT-1027 with those of dolastatin 10, CDDP, VCR, 5-FU and E7010, against P388 leukemia, colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma in mice (Table I).

Against P388 leukemia, TZT-1027, given i.v. twice with a 4-day interval, showed moderate activity with ILS values of 54%, 57%, 57% and 70% at doses of 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg and 3.0 mg/kg, respectively. TZT-1027 was highly active over a wide range of doses. Dolastatin 10, VCR and E7010, similarly administered, showed good activity with ILS values of 81% for dolastatin 10 at a dose of 0.5 mg/kg, i.v., 87% for VCR at a dose of 2.0 mg/kg, i.v., and 96% for E7010 at a dose of 400 mg/kg, p.o.

In the experiment on colon 26 adenocarcinoma, the drugs were given i.v. or p.o. four times a 4-day interval, and the tumor volumes were measured on day 13. TZT-1027 showed good activity against the tumor with T/C values of 11% and 5% at doses of 1.0 mg/kg and 2.0 mg/kg, i.v., respectively. In particular, the drug prolonged the life of mice (ILS value 77%) at a dose of 1.0 mg/kg (data not shown). A dose of 3.0 mg/kg seemed to be toxic. CDDP (5.0 mg/kg, i.v.) and 5-FU (50 mg/kg, i.v.) showed good activity with T/C values of 2% and 0%, respectively, but dolastatin 10 and VCR were ineffective. TZT-1027 was more effective than E7010 when administered at Q4d×4.

In the experiment on B16 melanoma, the drugs were given i.v. or p.o. six times a 4-day interval, and the tumor volumes were measured on day 21. TZT-1027 showed good activity against the tumor, with T/C values of 34%, 1% and 1% at doses of 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg, i.v., respectively. In particular the drug prolonged the life of mice; ILS values were 80% and 110% at doses of 1.0 mg/kg and 2.0 mg/kg, respectively (data not shown). CDDP (2.5 mg/kg, i.v.), VCR (2.0 mg/kg, i.v.) and E7010 (400 mg/kg, p.o.) were highly active, with T/C values of 12%, 1% and 2%, respectively, but 5-FU was ineffective.

In the experiment on M5076 sarcoma, the drugs were given i.v. or p.o. six times every 4th day, and the tumor volumes were measured on day 20. TZT-1027 showed good activity against the tumor, with T/C values of 42%,

Table I. Antitumor Activity of TZT-1027 and Other Anticancer Drugs against Murine Tumors

Drug (route)	Dose	P388	M5076 (Q4d×6)	Colon26 (Q4d×4)	B16 (Q4d×6)
	mg/kg	ILS %[a]	T/C % (Day 20)[b]	T/C % (Day 13)[b]	T/C % (Day 21)[b]
TZT-1027 (i.v.)	0.125	2	108	90	74
	0.25	15	96	87	44
	0.5	54	42[d]	59	34[d]
	1	57	1[d]	11[d]	1[d]
	2	57	0[d]	5[d]	1[d]
	3	70	Toxic[c]	Toxic[c]	Toxic[c]
D10 (i.v.)	0.0625	2	95	79	52
	0.125	6	66	89	4[d]
	0.25	40	25[d]	82	0[d]
	0.5	81	Toxic[c]	Toxic[c]	Toxic[c]
	1	−33	Toxic[c]	Toxic[c]	Toxic[c]
CDDP (i.v.)	0.625	8	39[d]	97	73
	1.25	4	12[d]	41[d]	37[d]
	2.5	15	0[d]	45	12[d]
	5	45	Toxic[c]	2[d]	Toxic
VCR (i.v.)	0.125	4	113	98	55
	0.25	12	103	76	43
	0.5	35	81	88	22[d]
	1	57	71	62	2[d]
	2	87	51	Toxic[c]	1[d]
5-FU (i.v.)	12.5	8	148	85	146
	25	24	82	49	85
	50	39	131	0[d]	59
	100	44	Toxic[c]	0[d]	Toxic[c]
E7010 (p.o.)	50	5	74	87	86
	100	19	36[d]	63	Toxic[c]
	200	67	1[d]	33[d]	4[d]
	400	96	0[d]	Toxic[c]	2[d]
	600	−2	Toxic[c]	Toxic[c]	Toxic[c]

P388 leukemia was inoculated i.p. into mice on day 0. The drugs were given i.v. or p.o. to mice on day 1 and day 5. Solid tumors were inoculated s.c. into mice on day 0. The drugs were given i.v. or p.o. to mice at Q4d×4 or Q4d×6.

a) ILS % was calculated as (median survival time in treated group/median survival time in control group − 1) × 100.

b) T/C % was calculated as (mean tumor volume in treated group/mean tumor volume in control group) × 100.

c) "Toxic" indicates toxicity on the evaluation day.

d) T/C % ≤ 50 and $P < 0.01$ by the Mann-Whitney U test, as compared with the untreated group.

1% and 0% at doses of 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg, i.v., respectively. In particular the drug prolonged the life of mice; ILS values were 57% and 48% at doses of 1.0 mg/kg and 2.0 mg/kg, respectively (data not shown). CDDP (2.5 mg/kg, i.v.) and E7010 (400 mg/kg, p.o.) showed good activity with the T/C values of 0% and 0%, respectively, whereas dolastatin 10 was moderately active with T/C value of 25% at a dose of 0.25 mg/kg. In contrast, 5-FU was ineffective.

Antitumor effects on human xenografts The antitumor effects of TZT-1027 against MX-1 breast and LX-1 lung carcinomas inoculated s.c. into BALB/c nude mice were compared with those of CDDP (Table II and Fig. 5). In this experiment, the drugs were given i.v. to the mice either once or twice with a 7-day interval. Tumor weights were measured on day 21 after tumor implantation.

A single injection of TZT-1027 inhibited the growth of MX-1 carcinoma with T/C values of 7% and 8% at doses of 1.0 mg/kg and 2.0 mg/kg, respectively, while two injections of TZT-1027 or CDDP at the same doses almost completely inhibited its growth. Furthermore, 7 of 8 mice given 2.0 mg/kg of TZT-1027 had no palpable tumors on day 21. Against LX-1 carcinoma, TZT-1027 showed remarkable activity with T/C values of 16% and 1% at single doses of 1.0 mg/kg and 2.0 mg/kg, respectively, and exhibited good activity with T/C values of less than 2% at repeated doses. Thus, the antitumor activity of TZT-1027 against LX-1 carcinoma was more potent

Table II. Antitumor Activity of TZT-1027 and CDDP against Human Tumor Xenografts

Tumor	Drug	Schedule	Dose (mg/kg)	Tumor weight (g, mean±SD)	T/C %	Body weight[a] change (g)
MX-1	Control	day 7		1.91±0.23	100	4.2
	TZT-1027	day 7	0.5	1.12±0.13	59	3.9
		day 7	1	0.14±0.04	7	3.2
		day 7	2	0.15±0.41	8	3.5
	CDDP	day 7	5	0.00±0.00	0	3.1
		day 7	10	0.00±0.00	0	2.4
	Control	days 7 and 14		1.70±0.13	100	4.3
	TZT-1207	days 7 and 14	0.5	0.81±0.12	48	3.6
		days 7 and 14	1	0.00±0.00	0	2.5
		days 7 and 14	2	0.00±0.00	0	1.5
	CDDP	days 7 and 14	5	0.00±0.00	0	2.7
		days 7 and 14	10	0.00±0.00	0	−3.4
LX-1	Control	day 7		1.76±0.18	100	−2.5
	TZT-1027	day 7	0.5	0.83±0.11	47	−1.6
		day 7	1	0.28±0.04	16	0.6
		day 7	2	0.02±0.00	1	2.7
	CDDP	day 7	5	0.90±0.09	51	−2.7
		day 7	10	0.30±0.05	17	−1.2
	Control	days 7 and 14		1.39±0.09	100	−3.0
	TZT-1027	days 7 and 14	0.5	0.63±0.09	45	−2.9
		days 7 and 14	1	0.03±0.01	2	1.3
		days 7 and 14	2	0.02±0.00	1	0.9
	CDDP	days 7 and 14	5	0.67±0.07	48	−4.5
		days 7 and 14	10	0.23±0.05	17	−5.3

Nude BALB/c mice were implanted s.c. with human tumors. Drugs were injected i.v. once (day 7) or twice (days 7 and 14), when the tumor volume reached about 100 mm^3. Tumor weights were measured on day 21 after tumor implantation.
a) Body weight change from day 7 to 21.

than that of CDDP, as shown in Table II. Although a dose of 4.0 mg/kg of TZT-1027 seemed to be toxic (data not shown), little decrease in body weight was observed at effective doses. As a result of the growth of LX-1 carcinoma, control and CDDP-treated mice lost body weight during the experimental period. In contrast, TZT-1027-treatment induced weight gain (Table II).

Antitumor effects on drug-resistant P388 leukemia The antitumor effects of TZT-1027 against drug-resistant tumors are of particular interest from a clinical viewpoint. Thus, TZT-1027 was evaluated against a variety of drug-resistant P388 sublines, including P388/VCR, P388/ADM, P388/5-FU and P388/CDDP (Table III). Mice were inoculated i.p. with drug-resistant P388 leukemia, and the drugs were given i.v. or i.p. twice with a 4-day interval. VCR (0.25 to 4.0 mg/kg i.v.) was ineffective against P388/VCR, as was ADM (0.625 to 10 mg/kg i.v.) against P388/ADM, 5-FU (25 to 200 mg/kg given i.p.) against P388/5-FU and CDDP (1.25 to 10 mg/kg i.v.) against P388/CDDP. TZT-1027 increased the survival time against P388/VCR, P388/5-FU and P388/CDDP, but not against P388/ADM, though its range of effective doses was limited. Thus, TZT-1027 was active in a narrower range of doses against P388/VCR, P388/5-FU and P388/CDDP than against the parent cell line.

Effect on assembly/disassembly of microtubules Because dolastatin 10 was reported to inhibit tubulin polymerization by binding to the *Vinca* alkaloid-binding domain in tubulin,[5-8] the action of TZT-1027 on microtubule assembly was examined. Evidence for the interaction of TZT-1027, as well as dolastatin 10 and VBL, with microtubules was obtained using a microtubule protein polymerization assay. Fig. 6 shows a typical experiment in which microtubule protein polymerization was induced by a rapid rise in temperature from 0°C to 37°C, and then inhibited by the drugs. The IC_{50} values of TZT-1027, dolastatin 10 and VBL were 2.2±0.6 μM, 2.3±0.7 μM and 3.0±0.5 μM, respectively.

When the drugs were added after a 10-min incubation of microtubule proteins at 37°C, they also depolymerized the polymerized microtubule proteins, as shown in Fig. 7. Adding 1.0 μM and 10 μM of TZT-1027 induced approximately 10% and 80% depolymerization, respectively, after a further 20-min incubation.

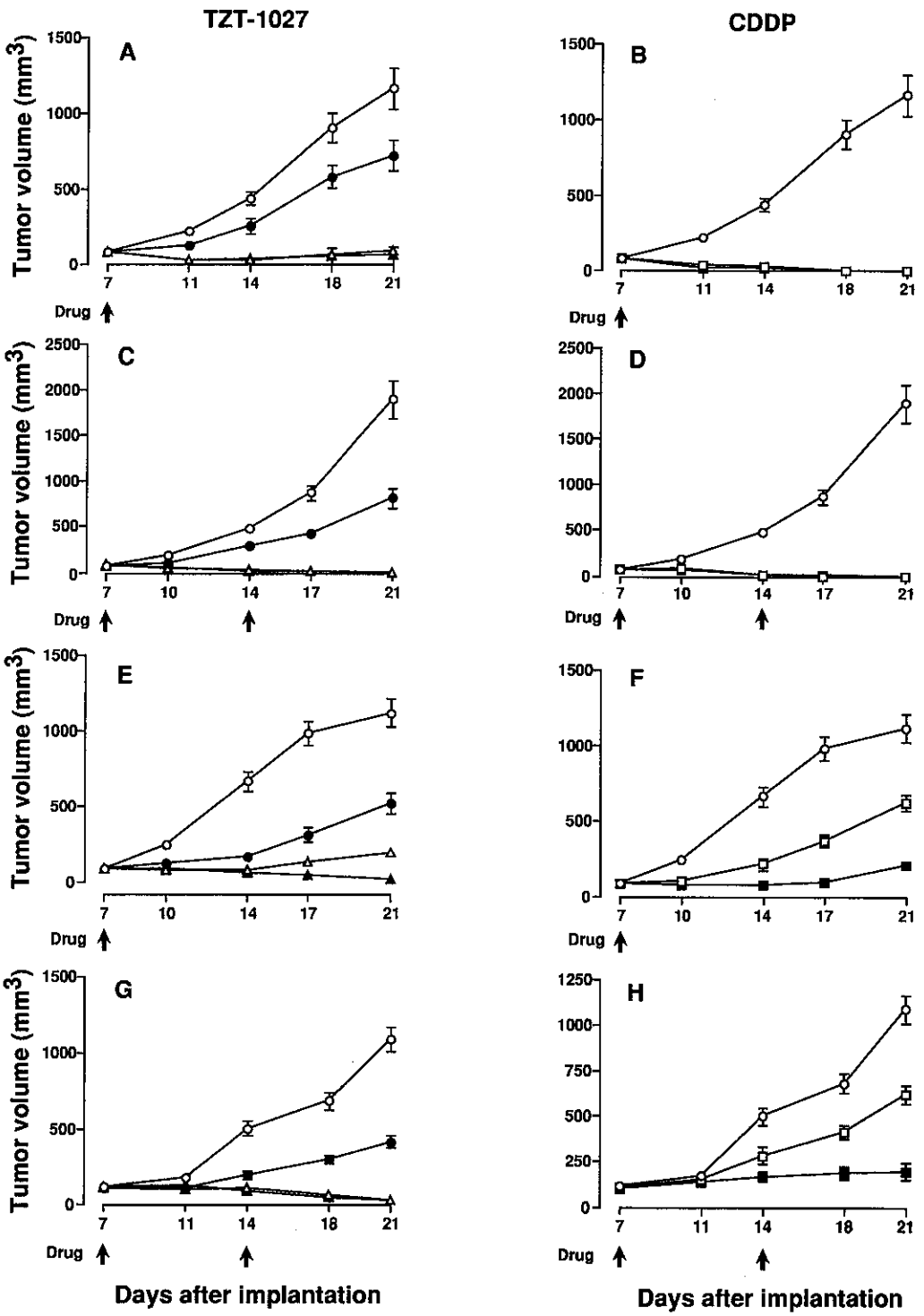


Fig. 5. Growth of human xenografts treated with TZT-1027 or CDDP. Human MX-1 breast (A, B, C and D) and LX-1 lung (E, F, G and H) carcinomas were transplanted into female BALB/c nude mice. Drugs were injected i.v. once (A, B, E and F), or twice (C, D, G and H) with a 7-day interval, when the tumor volume reached about 100 mm^3. Arrows indicate day of drug injection. ○, Control; ●, TZT-1027 (0.5 mg/kg); △, TZT-1027 (1.0 mg/kg); ▲, TZT-1027 (2.0 mg/kg); □, CDDP (5.0 mg/kg); ■, CDDP (10.0 mg/kg).

Table III. Antitumor Activity of TZT-1027 against Drug-resistant P388 Leukemias

Drug (route)	Dose (mg/kg)	P388 ILS (%)	P388/VCR ILS (%)	P388/ADM ILS (%)	P388/5-FU ILS (%)	P388/CDDP ILS (%)
TZT-1027 (i.v.)	0.25	24	−2	1	5	4
	0.5	64	7	1	25	26
	1	85	19	7	44	102
	2	82	47	13	−28	−33
	3	42	−42	−40	−60	−44
VCR (i.v.)	0.25		0			
	0.5	30	−1			
	1	66	−3			
	2	80	−21			
	4	−10	−51			
ADM (i.v.)	0.625	3		0		
	1.25	10		0		
	2.5	16		−3		
	5	57		−3		
	10	130		−2		
5-FU (i.p.)	25	48			5	
	50	101			−3	
	100	115			−22	
	150	59			−40	
	200	38			−36	
CDDP (i.v.)	1.25	12				2
	2.5	18				2
	5	19				−6
	10	62				−25

P388 leukemia was inoculated i.p. into mice on day 0. The drugs were given i.v. or i.p. to mice on days 1 and 5. ILS % was calculated as (median survival time in treated group/median survival time in control group − 1) × 100.

DISCUSSION

A dolastatin 10 derivative, TZT-1027, is a newly synthesized antitumor agent.[12] Dolastatin 10 was isolated from the Indian Ocean sea hare *Dolabella auricularia*, in 1987 and has been found to possess antitumor activity against various murine tumors and NCI human xenografts.[1-4] We have been searching for more potent and effective derivatives, evaluating their activity using *in vitro* and *in vivo* P388 leukemia models. TZT-1027 has been found to be potent in both *in vitro* and *in vivo* screening systems.[12] In the present study, TZT-1027 was tested against a variety of murine tumors and human xenografts to evaluate its breadth of activity.

TZT-1027 was effective against P388 leukemia not only by local administration (i.p.-i.p. system), but also by systemic administration (i.p.-i.v.). Although TZT-1027 given i.v. was found to be active against s.c. implanted tumors, it was less effective when administered i.p. The reason why the i.p. administration of TZT-1027 was ineffective against solid tumors is unclear, but the i.v. route may prove to be more suitable for clinical use. The influence that the schedule of administration of the drug has on antitumor efficacy was then investigated using the P388 leukemia and the B16 melanoma models. The antitumor effect of TZT-1027 was confirmed to be schedule-dependent; intermittent injections of TZT-1027 had a stronger antitumor effect than single or repeated injections in mice with P388 leukemia and B16 melanoma.

The antitumor effects of TZT-1027 against four kinds of murine tumors, P388 leukemia, colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma, were examined and compared with those of dolastatin 10, CDDP, VCR, 5-FU and E7010. The antitumor activity of TZT-1027 was dose-dependent between 0.5 mg/kg and 2.0 mg/kg, against all four tumors studied. TZT-1027 was highly active over a wide range of doses against these murine tumors, and demonstrated a broad spectrum of activity. TZT-1027 seemed to be toxic at a dose of 3.0 mg/kg against all tumors except P388 leukemia. In contrast, dolastatin 10 was ineffective against colon 26 adenocarcinoma, and was only active against M5076 sarcoma at one dose, 0.25 mg/kg. The effective dose range of TZT-1027 was much wider than that of CDDP against all four tumors. VCR was ineffective against both colon 26 adenocarcinoma and M5076 sarcoma. 5-FU was in-

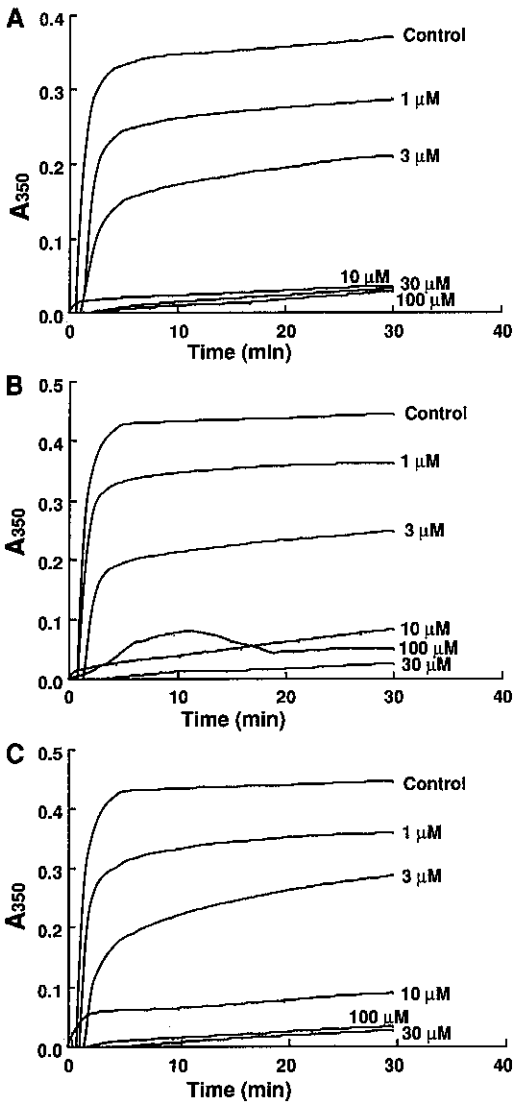


Fig. 6. Inhibition of microtubule assembly by TZT-1027 (A), dolastatin 10 (B) and VBL (C). Microtubule proteins (1.5 mg/ml) were incubated at 37°C in the absence or presence of various concentrations of drugs, and the turbidity was monitored at 350 nm. A, absorbance units.

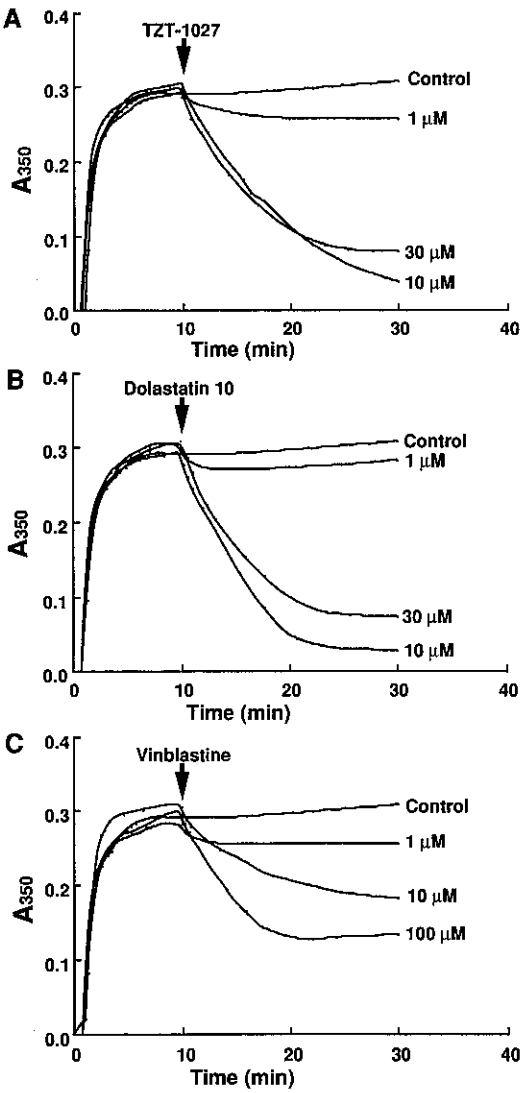


Fig. 7. Microtubule disassembly by TZT-1027 (A), dolastatin 10 (B) and VBL (C). Microtubule proteins (1.5 mg/ml) were incubated at 37°C. Various concentrations of drugs were added 10 min later, and the turbidity was monitored at 350 nm. A, absorbance units.

effective against both B16 melanoma and M5076 sarcoma. E7010 was only active against colon 26 adenocarcinoma at one dose, 200 mg/kg. Therefore, the antitumor activities of TZT-1027 against these tumors were superior or comparable to those of the other anticancer drugs studied, although it is possible that we underestimated the activities of the other drugs, as the schedule was designed to optimize the effect of TZT-1027.

TZT-1027 also showed remarkable activity against human tumors inoculated into BALB/c nude mice, without causing serious body weight reduction, that is, it exhibited remarkable antitumor effects against both MX-1 breast and LX-1 lung carcinomas, giving T/C values of less than 2%, which resulted in tumor regression. The antitumor activity of TZT-1027 against MX-1 carcinoma was almost comparable to that of CDDP. Furthermore, it should be noted that the antitumor activity of TZT-1027 against LX-1 carcinoma, insensitive to most chemotherapeutic drugs, was distinctly superior to that of CDDP. TZT-1027 should prove valuable in

clinical chemotherapy, as it causes regression of tumor nodules.

The antitumor effects of TZT-1027 against drug-resistant, as well as drug-sensitive, tumor cells should further promote interest in its development. In this study, the antitumor effects of TZT-1027 against four types of drug-resistant P388 sublines; P388/VCR, P388/ADM, P388/CDDP and P388/5-FU, were examined. The acquired resistance of P388/VCR and P388/ADM is reported to be due to elevations in the level of Pgp,[14, 15, 23] that of P388/CDDP involves reduced uptake of the drug,[16, 17] and that of P388/5-FU involves reduced uridine kinase activity.[18, 19] TZT-1027 was active over a limited range of doses against P388/VCR, P388/5-FU and P388/CDDP, as compared with the parent cell line. Though the mechanism of action of TZT-1027 is mainly ascribed to the inhibition of microtubule assembly, being similar to that of VCR as described below, in contrast to VCR, TZT-1027 was active against P388/VCR. The acquired resistance of both P388/VCR and P388/ADM mainly arises from accelerated outward transport of the drugs owing to the elevation of Pgp.[14, 15, 23] Both P388/VCR and P388/ADM were markedly cross-resistant to taxol and taxotere.[24, 25] The involvement of the Pgp in dolastatin 10-resistance is suggested by the finding that cells expressing multidrug-resistance phenotypes and transfected with human mdr 1 cDNA are cross-resistant to dolastatin 10.[26] However, TZT-1027 was active against P388/VCR, but ineffective against P388/ADM. Navelbine, a new *Vinca* alkaloid analog, and rhizoxin have also been reported to show antitumor activity against P388/VCR.[27, 28] Although the reason why TZT-1027 activity against P388/VCR and P388/ADM differs is not clear, there are two possible explanations: (a) the amount of Pgp could differ between P388/VCR and P388/ADM; (b) acquired resistance of P388/VCR and/or P388/ADM could involve other unknown mechanisms in addition to Pgp elevation.

Dolastatin 10 causes the accumulation of cells arrested in mitosis and the disappearance of intracellular microtubules. Also, its intracellular target is tubulin, the major component of microtubules.[5, 29, 30] Therefore, we examined the inhibition of microtubule protein polymerization by TZT-1027, as well as the microtubule protein depolymerization effect. TZT-1027 inhibited microtubule protein polymerization in a concentration-dependent manner. Though TZT-1027 at 10 μM almost completely depolymerized the polymerized microtubule proteins, the microtubule depolymerization effect of VBL at 100 μM was less than 40%. Our results indicated that both the inhibition of microtubule protein polymerization and the microtubule protein depolymerization effect of TZT-1027 were as potent as those of dolastatin 10, and more potent than those of VBL. The structure of TZT-1027 is markedly different from those of other tubulin binders, and consequently the mechanism of the antitubulin activity of TZT-1027 is of interest. We studied the TZT-1027 binding site and obtained results similar to those reported recently for dolastatin 10 by Bai *et al.*[5, 30] and Li *et al.*[8]: (a) TZT-1027 binds to tubulin at two or more binding sites according to Scatchard analysis; (b) TZT-1027 inhibits the binding of [^{3}H]VBL to tubulin in a non-competitive manner according to Lineweaver-Burk analysis; and (c) TZT-1027 affects the binding of VBL to tubulin, but its binding site is not identical to the VBL binding site (data not shown). Consequently, the mechanism of TZT-1027 antitumor action seems to be, at least in part, ascribable to the inhibition of microtubule assembly.

It is noteworthy that *in vitro* cytotoxicity of TZT-1027 occurred at a concentration of several hundred pM (data not shown), while inhibition of microtubule polymerization occurred at a concentration of several μM. The concentration necessary to inhibit microtubule polymerization in a cell-free system is thus about 10,000-fold higher than the concentration necessary to inhibit cell proliferation. Likewise, the ability of other tubulin binders to inhibit cell proliferation does not correlate well with their ability to interact with microtubules *in vitro*.[31, 32] Several possible explanations for this discrepancy have been proposed: (a) the drug could penetrate into cells with high efficiency, increasing intracellular concentration to a level that disrupts microtubule polymerization; (b) the drug could act on other targets unrelated to microtubules; (c) the drug could bind with much higher affinity to spindle tubulin of tumor cell lines than to neuronal tubulin of porcine brain; (d) the drug could be metabolized intracellularly to a more active compound. Jordan *et al.* recently reported an excellent correlation between the concentration at which cell proliferation was inhibited and the concentration at which a 50% accumulation of cells at metaphase occurred, for each of the *Vinca* alkaloids they tested.[33] Thus, they came to the conclusion that the drugs inhibit cell proliferation by altering the dynamics of tubulin addition and loss at the ends of mitotic spindle microtubules, rather than by depolymerizing microtubules. However, as none of these explanations can account for the discrepancy, we propose the following. A tubulin binder inhibits microtubule polymerization at substoichiometric concentrations, that is, one molecule of the drug binds to one molecule of tubulin, resulting in inhibition of microtubule polymerization. Microtubules consist primarily of heterodimers (molecular weight; 100,000) of α- and β-tubulin, and microtubule-associated proteins (molecular weight; 300,000–350,000). The molar concentration of tubulin (1.5 mg/ml of microtubule proteins) used in the present experiment could, therefore, be estimated at several μM. The difference between the level of tubulin in cells and *in vitro* could account for the discrepancy.

The spectra of antitumor activity of TZT-1027 and VCR were found to differ *in vivo*, despite the fact that the mechanism of action of TZT-1027 appears to be similar to that of the *Vinca* alkaloids. In this study, VCR was highly active against P388 leukemia, had a limited spectrum of antitumor activity against solid tumors, and showed no activity against colon 26 and M5076 sarcoma. In contrast, TZT-1027 had a broader spectrum of antitumor activity than VCR and, furthermore, was effective against P388/VCR. Two possible explanations for this difference are that: (a) the mode of action of TZT-1027 on tubulin differs from that of VCR; (b) the antitumor activity of TZT-1027 could be attributable to other mechanisms of action in addition to the inhibition of microtubule polymerization. Further studies are in progress.

In conclusion, TZT-1027 was active against murine P388 leukemia, colon 26 adenocarcinoma, B16 melanoma and M5076 sarcoma, as well as human xenografts LX-1 lung and MX-1 breast carcinomas. Studies with P388 leukemia and B16 melanoma indicated that the efficacy of TZT-1027 is schedule-dependent. Despite having a mechanism of action similar to that of *Vinca* alkaloid, TZT-1027 was more active than VCR. In addition, unlike VCR, it has activity against P388/VCR. TZT-1027 possesses a unique chemical structure and a broad spectrum of antitumor activity different from those of other clinically available drugs. This compound is currently undergoing phase I clinical trials in Japan.

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