Antitumor Activity of TZT-1027 (Soblidotin)

JUNICHI WATANABE[1], MEGUMI MINAMI[2] and MOTOHIRO KOBAYASHI[1]

[1]ASKA Pharmaceutical Co., Ltd., R&D Administration, 1604 Shimosakunobe, Kawasaki-shi, Kanagawa 213-8522 Takatsu-ku;
[2]Daiichi Pharmaceutical Co., Ltd., R&D Division, New Product Research Laboratories III, 16-13 Kita-Kasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan

Abstract. *Background: TZT-1027 (Soblidotin), a newly synthesized dolastatin 10 derivative that depolymerizes microtubules, has potent antitumor activity. Materials and Methods: Cell-killing kinetic analysis was performed by the colony-forming assay and the kinetics of TZT-1027 were compared with those of neocarzinostatin, adriamycin and vincristine, known to be typical concentration-, AUC- and time-dependent agents, respectively. DNA fragmentation was detectable by electrophoresis, cytotoxicity was evaluated by MTT assay and antitumor activity was examined by measuring the tumor weight after treatment. Results: TZT-1027 exhibited its cytocidal and apoptosis-inducing activity in a time-dependent manner. Its growth-inhibitory effect was less affected by overexpression of P-glycoprotein than that of other tubulin inhibitors and was not affected by the overexpression of breast cancer resistance protein or multidrug resistance-associated protein. TZT-1027 exhibited potent antitumor activities in an in vivo tumor model in which vincristine and docetaxel failed to show effectiveness. Conclusion: Because its growth-inhibitory and antitumor activities were superior to those of the other drugs tested, including the tubulin inhibitors paclitaxel, docetaxel and vincristine, TZT-1027 should be useful in the chemotherapy of tumors that are not responsive to other tubulin inhibitors.*

TZT-1027 (Soblidotin) is a newly synthesized (1) derivative of dolastatin 10, which was isolated by Pettit *et al.* (2) from the Indian Ocean sea hare, *Dolabella auricularia*, in 1987. TZT-1027 (1-10 μM) inhibited the polymerization of microtubule protein, with an IC_{50} value of 2.2 μM (3) and monosodium glutamate-induced tubulin polymerization, with an IC_{50} value of 1.2 μM (4). TZT-1027 has a broad spectrum of antitumor activity against various murine tumors – P388 leukemia, Colon26 and LLC carcinomas, B16 melanoma and M5076 sarcoma – as well as human tumor xenografts, MX-1, LX-1 and SBC-3 carcinomas (3, 5). Our previous results suggested an association of the growth-inhibitory effect of TZT-1027 with the induction of apoptosis and indicated that TZT-1027 induced apoptosis following G_2/M arrest, even in the absence of caspase-3 or Bcl-2 (6). Furthermore, TZT-1027 displayed a potent antivascular effect against advanced-stage Colon26 adenocarcinoma (7, 8). TZT-1027 is currently undergoing clinical evaluation and, in phase I clinical trials, its major dose-limiting toxicity was neutropenia (9-14).

In this study, the cell-killing kinetics of TZT-1027 were compared with those of positive control drugs with typical mechanisms of action on WiDr human colon cancer cells and were then analyzed to classify TZT-1027 as a concentration-, AUC-, or time-dependent drug (15). Next, the *in vitro* growth-inhibitory effects of TZT-1027 against human tumor cell lines that overexpress the multidrug-efflux pumps, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) were assessed and were compared with those of several anticancer agents currently in clinical use. Finally, not only the *in vitro* cytotoxicity, but also the *in vivo* antitumor activity of TZT-1027 were evaluated using the murine fibrosarcoma cell line Meth A.

Materials and Methods

Agents. TZT-1027, its metabolites and docetaxel (DTX) were synthesized in the laboratories of ASKA Pharmaceutical Co., Ltd. (Kawasaki, Japan) and Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Adriamycin (ADM) and vinorelbine (VNB) were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Cisplatin (CDDP) and etoposide (VP-16) were purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Paclitaxel (PTX) was purchased from Indena S.p.A. (Milan, Italy). Vincristine (VCR) was purchased from Shionogi & Co. (Osaka, Japan) and neocarzinostatin (NCS) was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Dilutions were performed just prior to the addition of the agents.

Correspondence to: Junichi Watanabe, ASKA Pharmaceutical Co., Ltd., R&D Administration, 1604 Shimosakunobe, Takatsu-ku, Kawasaki-shi, Kanagawa 213-8522, Japan. Tel: +81-44-812-8632, Fax: +81-44-833-5310, e-mail: watanabe-j@aska-pharma.co.jp

Key Words: TZT-1027, Soblidotin, tubulin inhibitor, cell-killing kinetics, multidrug resistance, fibrosarcoma Meth A.

Table I. *Human cancer cell lines used in these experiments.*

Cell line	Cancer type	Type of drug resistance	Drug for selection and maintenance	Drug concentration required for maintenance (ng/mL)	Resistance to other drugs	Efflux-pump overexpression[a]	Ref.
HCT116	Human colon cancer (carcinoma)	(Parent cell line)	–	–	–	–	17
HCT116/tere-1-1		Acquired multidrug resistance	Docetaxel	10	Paclitaxel	P-gp ++	
PC-6		(Parent cell line)	–	–	–	MRP+/–	
PC-6/Tax1-1		Acquired multidrug resistance	Paclitaxel	8	Adriamycin, Vincristine	MRP+/–, P-gp +	
PC-6/ADM2-1	Human lung cancer (oat cell carcinoma)	Acquired multidrug resistance	Adriamycin	10	Paclitaxel, Vincristine	MRP+/–, P-gp ++	
PC-6/VCR29-9		Acquired multidrug resistance	Vincristine	10	Paclitaxel, Adriamycin	MRP+/–, P-gp ++	16
PC-6/VP1-1		Acquired multidrug resistance	VP-16	1000	Paclitaxel, Adriamycin, Vincristine, CPT-11	MRP+/–, P-gp +++	
PC-6/SN2-5		Acquired multidrug resistance	SN-38	2	Mitoxantrone, Topotecan	MRP+/–, BCRP+	
PANC-1	Human pancreatic cancer (epithelioid carcinoma)	Intrinsic multidrug resistance	–	–	Cisplatin, Adriamycin, Vincristine	MRP++	19, 20
NCI-H460	Human lung cancer (large cell carcinoma)	(Parent cell line)	–	–	–	–	18
NCI-H460/PTX-13		Acquired multidrug resistance	Paclitaxel	20	Cisplatin, Adriamycin, Vincristine	P-gp ++	

[a]–, negative; +/–, weakly positive; +, positive; ++ and +++, strongly positive in order of ++ < +++.

Experimental animals and cell lines. Male BALB/c mice (6 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained under specific pathogen-free conditions. All the animal experiments were conducted in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd. A human colorectal adenocarcinoma cell line, WiDr, a human colorectal carcinoma cell line, HCT116, a human non-small cell lung cancer cell line, NCI-H460 and a human pancreatic epithelioid carcinoma cell line, PANC-1, were purchased from the American Type Culture Collection (Rockville, MD, USA). A human oat cell lung cancer cell line, PC-6, was purchased from Immuno-Biological Laboratories (Gunma, Japan) and a murine fibrosarcoma Meth A cell line was obtained from the Institute of Immunological Science, Hokkaido University (Sapporo, Japan). Drug-resistant sublines were established in the Daiichi laboratories by stepwise exposure to DTX for HCT116/tere1-1, PTX for PC-6/Tax1-1 and NCI-H460/PTX-13, SN-38 for PC-6/SN2-5, ADM for PC-6/ADM2-1, VCR for PC-6/VCR29-9 and VP-16 for PC-6/VP1-1 (16). The sublines, HCT116/tere1-1, PC-6/Tax1-1, PC-6/ADM2-1, PC-6/VCR29-9, PC-6/VP1-1 and NCI-H460/PTX-13 overexpressed P-gp, and PC-6/SN2-5 over-expressed BCRP (16-18). Additionally, PANC-1 overexpressed a significant amount of MRP spontaneously (19, 20). All the cell lines, except for PANC-1 and WiDr, were cultured in RPMI 1640 medium supplemented with 10% FBS. The PANC-1 and WiDr cell lines were cultured in Eagle's MEM alpha medium supplemented with 10% FBS and MEM medium supplemented with 10% FBS, respectively. The drug-resistant sublines derived from HCT116, PC-6 and NCI-H460 were maintained in the presence of the drugs used to select them. The concentrations of the drugs used for maintenance are indicated in Table I. The cultures were grown at 37˚C under 5% CO_2.

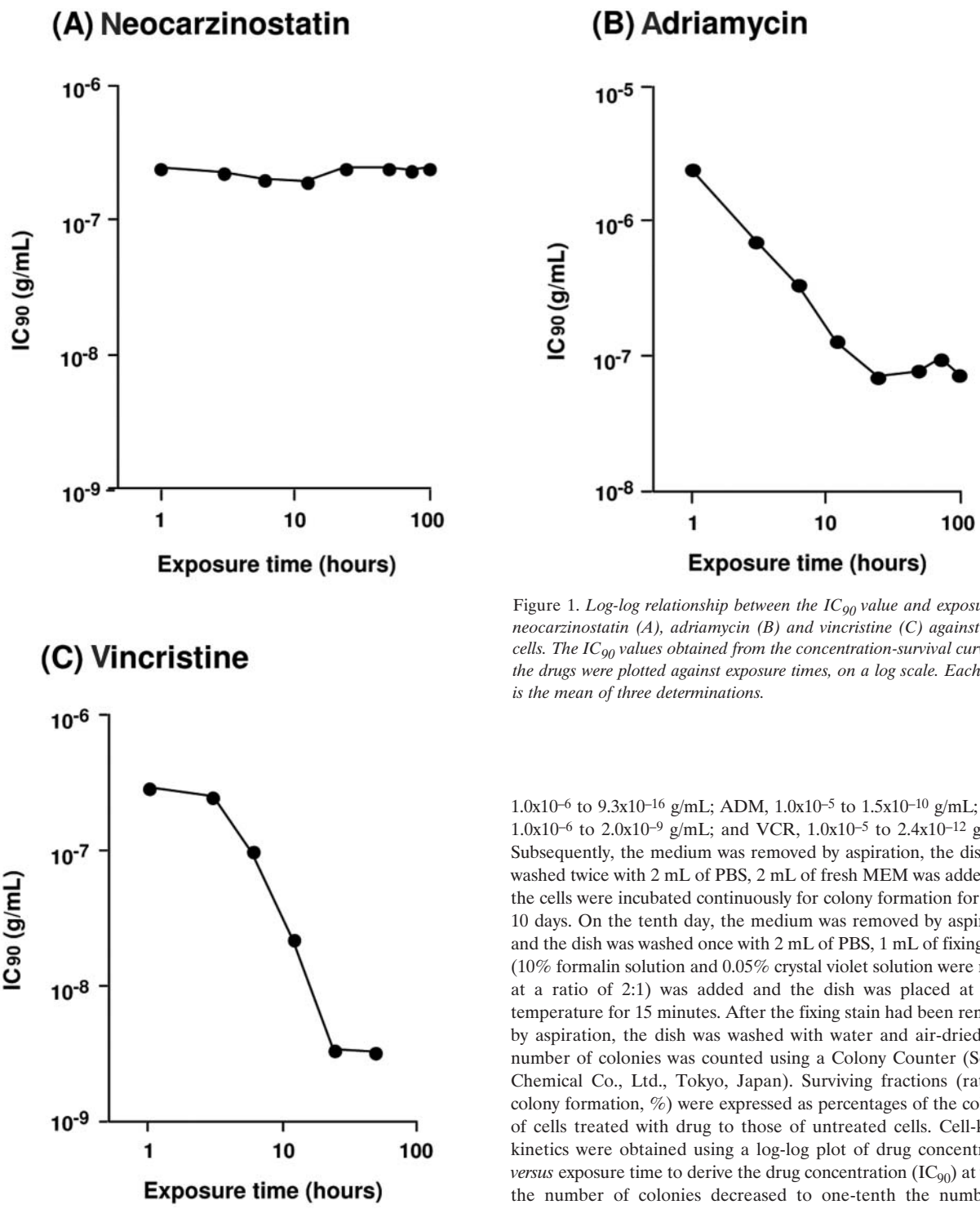

Figure 1. *Log-log relationship between the IC_{90} value and exposure for neocarzinostatin (A), adriamycin (B) and vincristine (C) against WiDr cells. The IC_{90} values obtained from the concentration-survival curves for the drugs were plotted against exposure times, on a log scale. Each value is the mean of three determinations.*

Colony-forming assay. The experiment was performed according to the method of Inaba *et al.* (21). That is, WiDr cells (200 cells/1.8 mL) were seeded in a 35-mm dish containing MEM. On the following day, 0.2 mL of the drug at each concentration was added to the medium and incubated at 37˚C for 1 to 96 hours under 5% CO_2 (TZT-1027, $1.0x10^{-6}$ to $9.3x10^{-16}$ g/mL; ADM, $1.0x10^{-5}$ to $1.5x10^{-10}$ g/mL; NCS, $1.0x10^{-6}$ to $2.0x10^{-9}$ g/mL; and VCR, $1.0x10^{-5}$ to $2.4x10^{-12}$ g/mL). Subsequently, the medium was removed by aspiration, the dish was washed twice with 2 mL of PBS, 2 mL of fresh MEM was added and the cells were incubated continuously for colony formation for up to 10 days. On the tenth day, the medium was removed by aspiration and the dish was washed once with 2 mL of PBS, 1 mL of fixing stain (10% formalin solution and 0.05% crystal violet solution were mixed at a ratio of 2:1) was added and the dish was placed at room temperature for 15 minutes. After the fixing stain had been removed by aspiration, the dish was washed with water and air-dried. The number of colonies was counted using a Colony Counter (Sekisui Chemical Co., Ltd., Tokyo, Japan). Surviving fractions (rates of colony formation, %) were expressed as percentages of the colonies of cells treated with drug to those of untreated cells. Cell-killing kinetics were obtained using a log-log plot of drug concentration *versus* exposure time to derive the drug concentration (IC_{90}) at which the number of colonies decreased to one-tenth the number of colonies present in the absence of drug. As a result, when the IC_{90} value was constant, the drug was classified as a concentration-dependent drug; when the gradient was –1, the drug was classified as an AUC-dependent drug; and when the gradient was steeper than –1, the drug was classified as a time-dependent drug. Based on these results, the cell-killing kinetics of TZT-1027 were compared to those of the positive control drugs and the drugs which had cell-killing kinetics similar to those of TZT-1027 were identified.

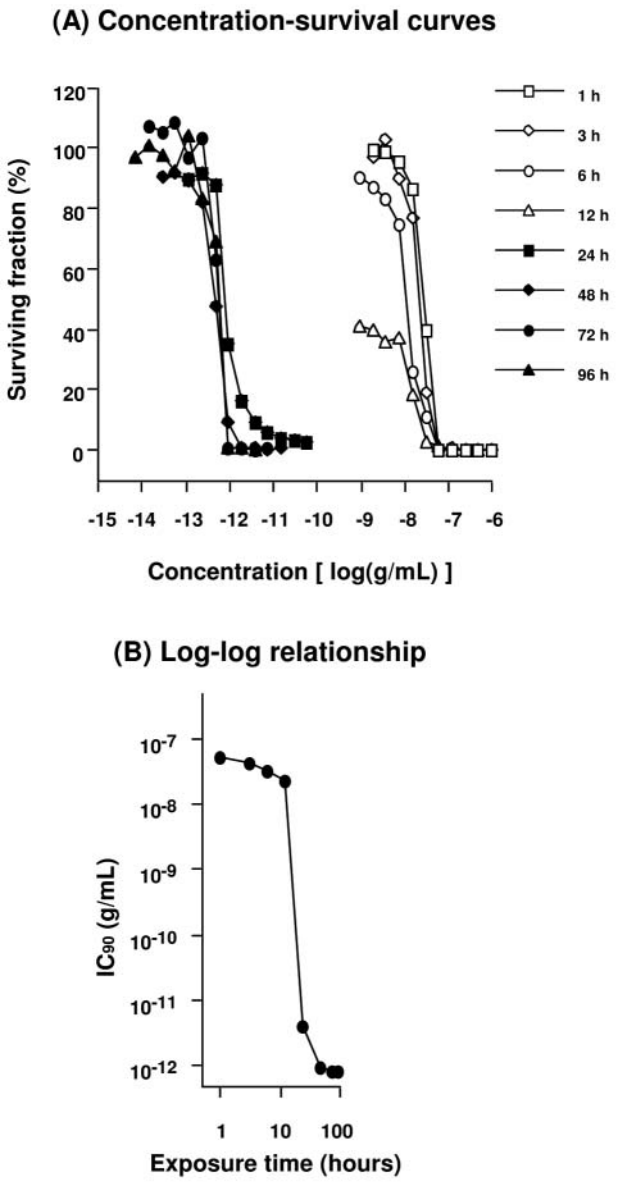

Figure 2. *Concentration-survival curves for various lengths of exposure time to TZT-1027 (A). WiDr cells were exposed to various concentrations of TZT-1027 for various time-periods and the surviving fractions were evaluated by a colony assay. Log-log relationship between the IC_{90} value and exposure time for TZT-1027 against WiDr cells (B). The IC_{90} values obtained from the concentration-survival curves for TZT-1027 were plotted against exposure times on a log scale. Each value is the mean of three determinations.*

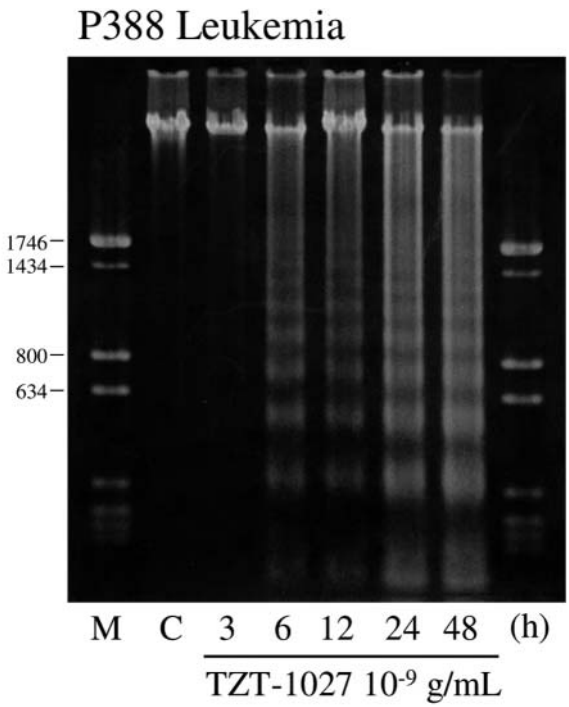

Figure 3. *Agarose gel electrophoresis of DNA extracted from P388 leukemia cells treated with TZT-1027. The P388 leukemia cell line was treated for the indicated times in the presence or absence of TZT-1027 ($1x10^{-9}$ g/mL). M: molecular size marker, C: untreated control.*

DNA fragmentation. Untreated and drug-treated cells were harvested, the cell pellets were washed with ice-cold PBS three times and cellular DNA was extracted and purified using Sepa Gene (Sanko Junyaku Co., Ltd., Tokyo, Japan). The samples were next dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and incubated with RNase A (20 μg/mL) at 37˚C for 30 minutes. The DNA was purified once again and the purity was determined spectrophotometrically. DNA (2 μg) was electrophoresed at 50 V for 30 min on a 2% agarose gel using a mini-gel electrophoresis system and the gels were stained with ethidium bromide (0.5 μg/mL). The DNA bands were visualized under UV-light and photographed with Polaroid type 667 film. A Hind III digest of lambda DNA served as a wt. standard.

MTT assay. On day 0, the cells (except for Meth A cells) were plated into 96-well plates at a density of approximately 500 cells/well (NCI-H460 and NCI-H460/PTX-13), 1000 cells/well (HCT116 and its sublines), 2000 cells/well (PANC-1), or 5000 cells/well (PC-6 and its sublines) in RPMI 1640 supplemented with 10% FBS. The Meth A cells were plated on day 1 at a density of 1000 cells/well. On day 1, each drug-diluted solution was added to individual wells. After 3 days of culture, viable cells were quantified by means of the MTT assay (22). T/C (%) was calculated by the following equation from the OD value of each well:

T/C (%) = [(SV on day 4 – SV on day 1)/(CV on day 4 – CV on day 1)]x100

SV = OD value of treated well, CV = OD value of untreated control well.

The concentrations of a compound and T/C (%) values were plotted on a graph and the concentration that produced 50% growth inhibition (GI_{50} value) was calculated automatically from the graph. The resistance factor (R_f), which represents the degree of resistance to drug treatment for each resistant subline, was determined by the following formula: R_f = GI_{50} value for each drug for the resistant cell line / GI_{50} value for the parental cell line.

Table II. *In vitro growth inhibition caused by various anticancer agents against multidrug-resistant cell lines that overexpress P-gp.*

Drug	HCT116	HCT116/tere1-1		PC-6	PC-6/ADM2-1		PC-6/TAX1-1		PC-6/VCR29-9		PC-6/VP1-1	
	GI_{50}	GI_{50}	R_f	GI_{50}	GI_{50}	R_f	GI_{50}	R_f	GI_{50}	R_f	GI_{50}	R_f
TZT-1027	0.026	1.08	42	0.023	0.917	39	0.327	14	0.686	29	9.89	417
Paclitaxel	0.073	493	6772	0.923	529	573	263	285	541	586	>10000	>10834
Docetaxel	0.160	68.5	428	0.419	97.9	234	42.7	102	93.7	224	1150	2745
Adriamycin	19.6	270	13.8	6.21	238	38	52.5	8.5	143	23	2510	404
Vincristine	1.71	198	116	0.423	224	530	56.7	134	122	288	877	2073
Etoposide	463	1990	4.3	162	868	5.4	286	1.8	531	3.3	3080	19
Cisplatin	776	373	0.48	182	494	2.7	119	0.65	99.3	0.55	267	1.5

GI_{50}: ng/mL.
The resistance factor (Rf) was determined from the following formula:
R_f = GI_{50} value for each drug for the resistant cell line / the GI_{50} value for the parental cell line.

Antitumor activity against murine fibrosarcoma Meth A. The Meth A cells maintained *in vivo* were harvested and washed with Hank's Balanced Saline Solution (HBSS). The washed cells were re-suspended in HBSS and were inoculated subcutaneously (*s.c.*) into BALB/c mice ($1x10^6$ cells/0.1 mL/mouse) on day 0. When the mean tumor volume had reached approximately 120 mm^3 on day 7 after tumor inoculation, the drugs were administered. The volume administered to each mouse was calculated from the body weight measured prior to each administration (10 mL/kg). TZT-1027, DTX, or VCR and ADM were administered on a q7dx2 schedule (once every 7 days for a total of two administrations), on a q2dx3 schedule (once every 2 days, for a total of three administrations), or on a qdx1 schedule (single administration), respectively. On the twenty-first day after the tumor inoculation, the animals were sacrificed by cervical vertebrae dislocation and the tumors were excised and weighed. The antitumor activity of each treatment was evaluated using tumor weight. The tumor growth inhibition ratio (IR) was calculated by means of the following formula: IR (%) = $[1 - (TW_t/TW_c)]x100$ (TW_t: the mean tumor weight of a treated group, TW_c: the mean tumor weight of the control group). When the IR was 58% or higher, the therapy was considered effective (23). The rate of body weight loss (BWL) was calculated by the following formula: BWL (%) = $(1 - BW_n/BW_s)x100$ (BW_n, the mean body weight of mice on day n; BW_s, the mean body weight of mice on the day administration started). BWL_{max} = the maximum value of BWL caused by administration of the test compound. A BWL_{max} <0 indicated no body weight loss. Groups with BWL_{max} values of greater than 20% or with death caused by toxicity were defined as "toxic groups". The statistical analysis of tumor weight was conducted with the EXSAS version 6.10 (Arm Corp.; Osaka, Japan), based on SAS release 8.2 (SAS Institute Japan, Tokyo, Japan). Dunnett's test was used to assess differences in mean tumor weight, and *p* values less than 5% were considered statistically significant with regard to tumor weight.

Results

Cell-killing kinetics. The cell-killing kinetics are shown in Figures 1 and 2B. The concentration-survival curve of TZT-1027 is shown in Figure 2A. NCS, an antitumor antibiotic, showed no difference in effective doses, regardless of the length of exposure (data not shown) and the cell-killing kinetics also showed a constant IC_{90} value for exposure times of 1 to 96 hours (Figure 1A). These results confirmed that this drug is a concentration-dependent drug. In the case of ADM, an antitumor antibiotic, a line with the gradient of –1 (Y=–1.138X–5.614, r=0.990) was obtained from the results of the cell-killing kinetics (Figure 1B), showing the typical pattern of concentration and time dependence, that is, an AUC-dependent drug. The results of the cell-killing kinetics for VCR, an antimicrotubule agent like TZT-1027, indicated that the gradient of the line (Y=–2.429X–5.091, r=0.998) for the exposure times of 3 to 24 hours was steeper than –1, indicating the typical pattern of a time-dependent drug (Figure 1C). Based on the mechanisms classified by Ozawa *et al.* (15), the cell-killing kinetics of TZT-1027 were studied.

Table III. *In vitro growth inhibition caused by various anticancer agents against a human lung cancer cell line, PC-6, its camptothecin-resistant subline that overexpresses BCRP and a human pancreatic cancer cell line, PANC-1, that spontaneously overexpresses MRP.*

Drug	PC-6	PC-6/SN2-5[a]		PANC-1[b]
	GI_{50}	GI_{50}	R_f	GI_{50}
TZT-1027	0.010	0.006	0.63	0.029
Paclitaxel	0.307	0.321	1.05	1.38
Docetaxel	0.331	0.280	0.85	1.90
Adriamycin	5.22	6.35	1.22	109
Vincristine	0.257	0.193	0.75	6.23
Etoposide	88.6	82.6	0.93	5630
Cisplatin	177	87.8	0.50	846

[a]MRP+/–, BCRP+, [b]MRP+++.
GI_{50}: ng/mL.
The resistance factor (R_f) was determined from the following formula:
R_f = GI_{50} value for each drug for the resistant cell line / the GI_{50} value for the parental cell line.

Table IV. *In vitro cytotoxicity of TZT-1027 and its metabolites against human cancer cell lines.*

Drug	HCT116 [Colon]		NCI-H460 [Lung]		NCI-H460/PTX-13 [Lung]	
	P-gp (–)		P-gp (–)		P-gp (+)	
	GI_{50}	Ratio	GI_{50}	Ratio	GI_{50}	Ratio
TZT-1027	0.172±0.045	1.0	0.227±0.069	1.0	1.69±0.41	1.0
Ma	0.181±0.029	1.1	0.271±0.038	1.2	9.04±0.79	5.4
Ma(R)	0.194±0.010	1.1	0.285±0.002	1.3	12.9±0.58	7.6
Ma(S)	0.162±0.013	0.9	0.230±0.016	1.0	11.8±1.50	7.0
Mb	0.262±0.029	1.5	0.512±0.085	2.3	24.0±1.73	14
Mc	0.488±0.030	2.8	0.928±0.061	4.1	25.5±0.88	15
Md	0.446±0.042	2.6	1.04±0.153	4.6	36.4±3.51	22
Mg	0.172±0.045	1.0	0.982±0.075	4.3	48.4±5.62	29

GI_{50}: ng/mL
Ratio = GI_{50} for each metabolite/that for TZT-1027.

TZT-1027 showed low cell-killing effects to cancer cells at exposure times of 1 to 12 hours. However, this drug showed dramatically enhanced cell-killing effects at exposure times of 12 hours or longer, demonstrating high time-dependence (Figure 2A). TZT-1027 exerted its cell-killing effect, regardless of time, at exposure times of 1 to 12 hours, indicating a fast-acting pattern (concentration-dependent pattern) at first glance. However, the gradient of the line (Y=–7.273X–0.347, r=0.919) at exposure times of 12 to 24 hours was far steeper than –1 (Figure 2B), and was greater than that of VCR. Therefore, it was concluded that TZT-1027 was a time-dependent antitumor agent, with a higher time dependence than VCR.

Analysis of DNA fragmentation. DNA fragmentation of the P388 leukemia cell line was induced by TZT-1027 at a concentration of $1x10^{-9}$ g/mL within 6 hours and this DNA fragmentation, the hallmark of apoptosis, increased time-dependently (Figure 3). After 24 hours of treatment with TZT-1027, DNA fragmentation was maximally increased.

In vitro growth inhibition against various multidrug-resistant human cancer cell lines that overexpress P-gp, BCRP, or MRP. The *in vitro* growth-inhibitory effects of the drugs were examined using five P-gp-overexpressing cell lines that were derived from two parental cell lines: the human colon cell line, HCT116, and the human lung cancer cell line, PC-6 (Table I). These variants showed cross-resistance to TZT-1027, as well as to VCR, ADM, PTX and DTX, which are known substrates of P-gp. For these sublines, the degrees of resistance to these drugs correlated well with the level of P-gp which each subline expressed (Table II). The R_f values for TZT-1027 ranged from 14- to 417-fold; this range is higher than the range for VP-16 (1.8- to 19-fold), equivalent to the range for ADM (8.5- to 404-fold), but significantly lower than the ranges for the tubulin inhibitors, VCR (116- to 2073-fold), DTX (102- to 2745-fold) and PTX (285- to >10000-fold). PC-6/SN2-5 overexpressed BCRP, which conferred the subline with resistance to camptothecin derivatives and mitoxantrone (Table I). However, it showed no cross resistance to TZT-1027 or other anticancer agents, including other tubulin inhibitors (Table III). PANC-1, the human pancreatic epithelioid carcinoma cell line that spontaneously overexpresses MRP, was used to examine the *in vitro* growth-inhibitory effects more fully. Compared with the parental HCT116 and PC-6 cell lines, this cell line showed a similar high susceptibility to TZT-1027, but was less sensitive to ADM, VCR and VP-16 (Table III).

Table V. *In vitro cytotoxic activity of tubulin inhibitors against the murine fibrosarcoma cell line Meth A and the human lung cancer cell line PC-6.*

Drug	Meth A		PC-6	
	GI_{50}	Ratio	GI_{50}	Ratio
TZT-1027	0.012	1	0.024	1
Vincristine	2.72	221	0.423	18
Vinorelbine	2.15	175	0.055	2
Docetaxel	1.1	89	0.419	18
Paclitaxel	6.59	536	0.923	39

GI_{50}: ng/mL.
Ratio = (GI_{50} of each agent for Meth A or PC-6)/(GI_{50} of TZT-1027 for Meth A or PC-6).

In vitro cytotoxicity of TZT-1027 and its metabolites against human cancer cell lines. The *in vitro* cytotoxicities of TZT-1027 and its metabolites were examined against the human colon cancer cell line, HCT116, the human lung cancer cell line, NCI-H460 and its P-gp-overexpressing subline, NCI-H460/PTX-13

Table VI. *In vivo antitumor effect TZT-1027 against murine fibrosarcoma Meth A solid tumors subcutaneously implanted into male BALB/c mice.*

Drug	Route	Treatment schedule	Total dose (mg/kg)	Tumor weight			BWLmax[b] (%)[day]	Toxic death[c]
				Mean±SE (g)	Sig.	IR(%)[a]		
Experiment 1								
Control	-	-	-	3.02±0.30		0	<0	0/6
TZT-1027	*i.v.*	q7d x 2	4	0.08±0.01	***	98	11.7[9]	0/6
			2	0.69±0.17	***	77	4.5[9]	0/6
Experiment 2								
Control	-	-	-	4.34±0.38		0	<0	0/6
Docetaxel	*i.v.*	q2d x 3	80	1.19±0.30	***	73	34.4[21]	0/6
			65	1.55±0.22	***	64	24.7[18]	0/6
			50	2.11±0.30	***	51	16.1[18]	0/6
			31	2.94±0.40	*	32	3.2[16]	0/6
Vincristine	*i.v.*	qd x 1	2	4.10±0.27	ns	6	16.7[11]	0/6
Adriamycin	*i.v.*	qd x 1	12	1.17±0.33	***	73	4.8[16]	0/6

[a]IR%: Tumor growth inhibition rate = [1–(mean tumor weight of treated group)/ (that of the untreated control group)] x 100. When the IR value was 58% or higher, the drug was considered effective.
[b]BWLmax: Maximum value for rate of body weight loss (%); numbers in parentheses denote the day. <0: no loss.
[c]Number of mice that died/number of mice used.
Asterisks (***) and (***, *) indicate statistically significant differences from the untreated control indicated by Dunnett's test, $p < 0.001$ and by Student's *t*-test, $p<0.001$ and $p<0.05$, respectively; ns, no significance.

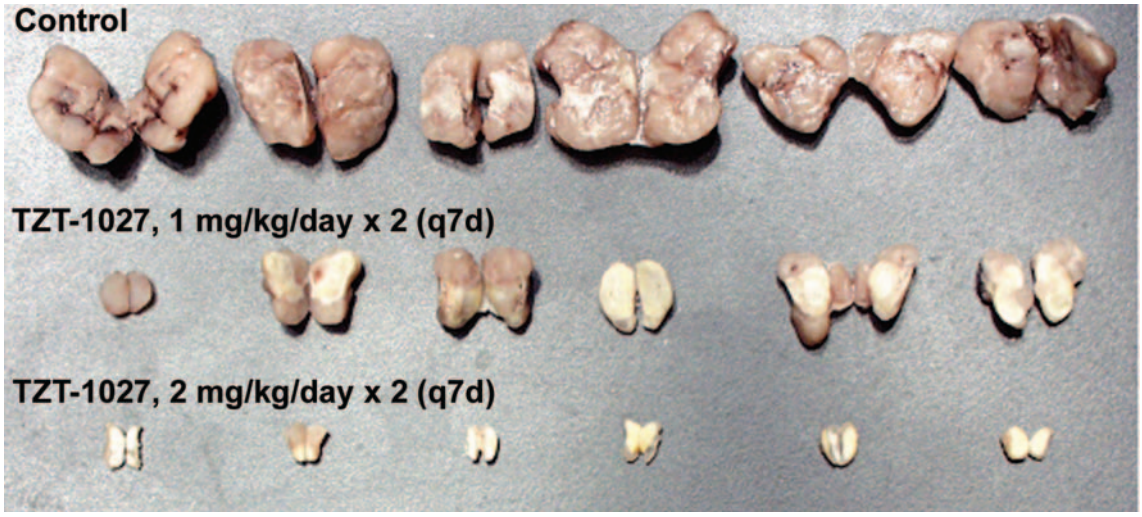

Figure 4. *Meth A tumors excised on day 21 (median sagittal plane views). TZT-1027 dose-dependently inhibited the growth of Meth A tumors subcutaneously implanted into BALB/c mice. The antitumor activity was characteristically accompanied by ischemic necrosis of the tumors.*

(Table IV). The seven metabolites of TZT-1027, Ma, Ma(R), Ma(S), Mb, Mc, Md and Mg, showed equivalent or somewhat weaker cytotoxicity against HCT116 and NCI-H460 compared to that shown by TZT-1027 (ratio: 0.9 to 4.6). They also showed weaker cytotoxicity against NCI-H460/PTX-13 compared with that of TZT-1027 (ratio: 5.4 to 29). Furthermore, there was no difference in the cytotoxicities of Ma, Ma(R) and Ma(S).

In vitro cytotoxic activity of TZT-1027, VCR, VNB, DTX and PTX against the Meth A and PC-6 cell lines. The GI_{50} values of all five drugs against the murine fibrosarcoma cell line Meth A and the human lung cancer cell line PC-6 are shown in Table V. TZT-1027 exhibited the strongest cytotoxic activity among the drugs tested: the GI_{50} values for TZT-1027 were 0.012 against Meth A cells and 0.024 ng/mL against PC-6 cells. Other tubulin-polymerization inhibitors,

VCR and VNB, and the tubulin-depolymerization inhibitors, DTX and PTX, also showed similar potent activity against PC-6 cells, with GI_{50} values below 1 ng/mL, but all failed to do so against Meth A. VCR and VNB were 175-fold or less effective, DTX was about 90-fold less effective and PTX was about 500-fold less effective than TZT-1027 against the Meth A cells.

In vivo antitumor activity of TZT-1027 against Meth A solid tumors subcutaneously implanted into BALB/c mice. The *in vivo* antitumor activity of TZT-1027 against Meth A solid tumors subcutaneously implanted into mice is shown in Table VI. TZT-1027 induced significant antitumor activity at doses of 2 mg/kg/day (maximum tolerated dose, [MTD]) and 1 mg/kg/day ($^1/_2$ MTD), with IR values of 98% and 77%, respectively. There were no toxic deaths or severe body weight loss (<20%) associated with treatment with TZT-1027. The antitumor activity of TZT-1027 was accompanied by ischemic necrosis of the tumors (Figure 4), probably related to the induction of local circulatory disturbances, as reported elsewhere (7). Single intravenous administration of VCR resulted in no antitumor activity, with an IR of 6%, even though considerable body weight loss occurred (BWL_{max}: 16.7%). DTX was administered intravenously on a schedule of q2d x 3 (every other day for a total of three times). DTX showed antitumor activities at total doses of 65 and 80 mg/kg, with IRs of 64% to 73%, but because the BWL_{max} values exceeded 20%, these were defined as toxic doses. At total doses of 50 mg/kg (defined as the MTD) and below, DTX failed to show significant antitumor activity. ADM, a positive control, exhibited significant antitumor activity, with an IR of 73% at a non-toxic dose.

Discussion

The cell-killing kinetics of TZT-1027 were first compared with those of positive control drugs that have typical mechanisms of action on human colon cancer (WiDr) cells and analyzed to determine which type, in the classification by Ozawa *et al.* (15), is consistent with the cell-killing kinetics of TZT-1027. These results revealed that TZT-1027 was a highly time-dependent antitumor agent. Furthermore, it was found that DNA fragmentation, induced by TZT-1027, was increased time-dependently. Previous studies have shown that TZT-1027 induced growth arrest at the G_2/M-phase of the cell cycle (6). Therefore, we confirmed that TZT-1027 exerted its effects in a time-dependent manner and, rather than a single, high-dose administration, divided administration of small amounts or intermittent administration was considered to be the optimum administration schedule.

The *in vitro* growth-inhibitory effects of TZT-1027, as well as of other tubulin inhibitors, declined depending on the amount of P-gp expressed by the tumor cells. The degree of resistance to TZT-1027 of the five P-gp-overexpressing sublines ranged from 14- to 417-fold of the parental cell lines. However, these values were lower than those of VCR (from 116- to 2073-fold), DTX (from 102- to 2475-fold), or PTX (from 285- to 10000-fold or more). These results indicated that TZT-1027 is 6-20 times less affected by P-gp than other tubulin inhibitors. TZT-1027, as well as three other tubulin inhibitors, showed potent *in vitro* growth-inhibitory effects on BCRP-overexpressing cells that are resistant to camptothecin derivatives and mitoxantrone. TZT-1027, PTX and DTX also potently inhibited the *in vitro* growth of a tumor cell line that spontaneously overexpresses MRP and shows low sensitivity to several antitumor agents, including VCR. These results suggest that the growth-inhibitory activity of TZT-1027 was less affected by the overexpression of P-gp in tumor cells than the growth-inhibitory activity of other tubulin inhibitors currently available clinically, and that the activity of TZT-1027 was not affected by the overexpression of BCRP or MRP.

The cytotoxicity of TZT-1027 metabolites was examined against a human colon cancer cell line (HCT116), a human lung cancer cell line (NCI-H460) and its P-gp-overexpressing subline, NCI-H460/PTX-13. Five metabolites (Ma, Mb, Mc, Md and Mg) and the enantiomers of Ma, a main metabolite, Ma(R) and Ma(S) showed equivalent or somewhat weaker cytotoxicity compared to that of TZT-1027 against HCT116 and NCI-H460 and were more affected by the overexpression of P-gp than was TZT-1027. Furthermore, there were no significant differences in the GI_{50} values of Ma, Ma(R) and Ma(S). Therefore, it may be assumed that the antitumor efficacy in clinical trials was exerted by the parent compound and not by its metabolites.

The *in vitro* cytotoxicity and *in vivo* antitumor activity of TZT-1027 were evaluated using the murine fibrosarcoma cell line Meth A. The *in vitro* cytotoxicity of TZT-1027 was compared with that of the vinca alkaloids (VCR and VNB) and the taxanes (PTX and DTX). When the GI_{50} values were compared, TZT-1027 showed much greater cytotoxicity than these other tubulin inhibitors. TZT-1027 was approximately 200-fold more cytotoxic than the vinca alkaloids and 90- to 500-fold more potent than the taxanes. Therefore, the *in vitro* cytotoxic effect of TZT-1027 was superior to that of clinically available tubulin-polymerization or -depolymerization inhibitors. The *in vivo* antitumor activity of TZT-1027 was investigated in BALB/c mice implanted with a Meth A solid tumor. TZT-1027 at the MTD and at $^1/_2$ MTD exhibited strong antitumor activities without death caused by toxicity or significant body weight loss, even though VCR and DTX failed to show effectiveness in the same tumor model. The antitumor activities were accompanied by ischemic necrosis of the tumors, probably related to the induction of local circulatory

disturbances. In summary, these results showed that TZT-1027 inhibited the growth of murine fibrosarcoma *in vivo* as well as *in vitro*.

In conclusion, the presented results show that TZT-1027 had strong cytotoxicity, not only against parental cancer cells, but also against various multidrug-resistant cancer cells and that its cytotoxic activity was superior to that of other anticancer agents tested, including the tubulin inhibitors PTX, DTX and VCR against murine fibrosarcoma *in vitro* and *in vivo*. Therefore, we anticipate that TZT-1027 should be useful for the chemotherapy of cancers that are not responsive to other tubulin inhibitors.

References

1 Miyazaki K, Kobayashi M, Natsume T, Gondo M, Mikami T, Sakakibara K and Tsukagoshi S: Synthesis and antitumor activity of novel dolastatin 10 analogs. Chem Pharm Bull *43*: 1706-1718, 1995.

2 Pettit GR, Kamano Y, Herald CL *et al*: The isolation and structure of a remarkable marine animal antineoplastic constituent: dolastatin 10. J Am Chem Soc *109*: 6883-6885, 1987.

3 Kobayashi M, Natsume T, Tamaoki S, Watanabe J, Asano H, Mikami T, Miyasaka 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.

4 Natsume T, Watanabe J, Tamaoki S, Fujio N, Miyasaka K and Kobayashi M: Characterization of the interaction of TZT-1027, a potent antitumor agent, with tubulin. Jpn J Cancer Res *91*: 737-747, 2000.

5 Natsume T, Koh Y, Kobayashi M, Fukumoto H, Takahashi F, Nakamura T, Ohe Y, Saijo N and Nishio K: Enhanced antitumor activities of TZT-1027 against TNF-α or IL-6 secreting Lewis lung carcinoma *in vivo*. Cancer Chemother Pharmacol *49*: 35-47, 2002.

6 Watanabe J, Natsume T, Fujio N, Miyasaka K and Kobayashi M: Induction of apoptosis in human cancer cells by TZT-1027, an antimicrotubule agent. Apoptosis *5*: 345-353, 2000.

7 Otani M, Natsume T, Watanabe J, Kobayashi M, Murakoshi M, Mikami T and Nakayama T: TZT-1027, an antimicrotubule agent, attacks tumor vasculature and induces tumor cell death. Jpn J Cancer Res *91*: 837-844, 2000.

8 Kobayashi M, Natsume T, Watanabe J, Fujio N, Mikami T, Miyazaki K and Tsukagoshi S: Activity of a novel antitumor agent, TZT-1027. Nippon Yakurigaku Zasshi (Japanese) *114 Suppl*: 230-235, 1999.

9 Niitani H, Hasegawa K, Furuse K, Fukuoka M, Horikoshi N and Kudoh S: Phase I studies of TZT-1027, a novel inhibitor of tubulin polymerization [abstract 360]. Proc 10th NCI-EORTC Symposium on New Drug in Cancer Therapy, 1998.

10 Horti J, Juhasz E and Bodrogi I: Preliminary results of a phase I trial of TZT-1027, an inhibitor of tubulin polymerization, in patients with advanced non-small cell lung cancer [abstract 2744]. Proc Am Assoc Cancer Res, 2002.

11 Horti J, Juhasz E, Bodrogi I and Ikeda S: A phase I trial of TZT-1027, an inhibitor of tubulin polymerization, in patients with advanced non-small cell lung cancer (NSCLC) [abstract 256]. Proc AACR-NCI-EORTC International Conference, Molecular Targets and Cancer Therapeutics, 2003.

12 Yamamoto N, Andoh M, Kawahara M, Fukuoka M and Niitani H: Phase I study of TZT-1027, an inhibitor of tubulin polymerization, given weekly x 3 as a 1-hour intravenous infusion in patients (pts) with solid tumors [abstract 420]. Proc Am Soc Clin Oncol, 2002.

13 Scfoeffski P, Thate B, Beutel G, Bolte O, Otto D, Hofmann M, Ganser A, Jenner A, Cheverton P, Wanders J, Oguma T, Atsumi R and Satomi M: Phase I and pharmacokinetic study of TZT-1027, a novel synthetic dolastatin 10 derivative, administered as a 1-hour intravenous infusion every 3 weeks in patients with advanced refractory cancer. Ann Oncol *15*: 671-679, 2004.

14 De Jonge MJA, Van der Gaast A, Planting AST, Van Doorn L, Lems A, Boot I, Wanders J, Satomi M and Verweij J: Phase I and pharmacokinetic study of the dolastatin 10 analogue TZT-1027, given on days 1 and 8 of a 3-week cycle in patients with advanced solid tumors. Clin Cancer Res *11*: 3806-3813, 2005.

15 Ozawa S, Sugiyama Y, Mitsuhashi J and Inaba M: Kinetic analysis of cell killing effect induced by cytosine arabinoside and cisplatin in relation to cell cycle phase specificity in human colon cancer and Chinese hamster cells. Cancer Res *49*: 3823-3828, 1989.

16 Ishii M, Iwahana M, Mitsui I, Minami M, Imagawa S, Tohgo A and Ejima A: Growth inhibitory effect of a new camptothecin analog, DX-8951f, on various drug-resistant sublines including BCRP-mediated camptothecin derivative-resistant variants derived from the human lung cancer cell line PC-6. Anticancer Drugs *11*: 353-362, 2000.

17 Shionoya M, Jimbo T, Kitagawa M, Soga T and Tohgo A: DJ-927, a novel oral taxane, overcomes P-glycoprotein-mediated multidrug resistance *in vitro* and *in vivo*. Cancer Sci *94*: 459-466, 2003.

18 Ochi Y, Minami M and Tohgo A: The orally effective taxane DJ-927 has little ability to induce drug resistance in human non-small cell lung cancer cells. Eur J Cancer Suppl *2*: 161, 2004.

19 Miller DW, Fontain M, Kolar C and Lawson T: The expression of multidrug resistance-associated protein (MRP) in pancreatic adenocarcinoma cell lines. Cancer Lett *107*: 301-306, 1996.

20 Anderson KM, Alrefai WA, Anderson CA, Ho Y, Jadko S, Ou D, Wu YB and Harris JE: A response of Panc-1 cells to cisplatinum, assessed with a cDNA array. Anticancer Res *22*: 75-81, 2002.

21 Inaba M, Mitsuhashi J and Ozawa S: Kinetic analysis of 5-fluorouracil action against various cancer cells. Jpn J Cancer Res *85*: 187-193, 1994.

22 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65*: 55-63, 1983.

23 Fujita F, Fujita M, Taguchi T, Shimozuma K, Sakamoto Y, Kimoto Y *et al*: Multifactorial analysis of parameters influencing chemosensitivity of human cancer xenografts in nude mice. Int J Cancer *43*: 637-644, 1989.

Received January 30, 2006
Accepted March 20, 2006