Comparison of the antivascular and cytotoxic activities of TZT-1027 (Soblidotin) with those of other anticancer agents

Junichi Watanabe, Tsugitaka Natsume and Motohiro Kobayashi

TZT-1027 (Soblidotin), a microtubule-depolymerizing agent exerts both a direct cytotoxic activity against cancer cells and an indirect antivascular activity against tumor vascular endothelial cells. We compared both activities of TZT-1027 with those of various anticancer agents having different mechanisms of action, including vinca alkaloids, a vascular targeting agent, a taxane and nonmicrotubule-binding agents. In the MTT assay, TZT-1027 most potently inhibited the growth of both murine colon C26 cancer cells and human umbilical vein endothelial cells, implying its potent antivascular activity against tumor vasculature in addition to its cytotoxic activity against cancer cells. Treatment with 0.1 μg/ml TZT-1027 significantly enhanced vascular permeability in human umbilical vein endothelial cell monolayers and a single intravenous administration of 2 mg/kg TZT-1027 significantly reduced the perfusion of Colon26 tumors implanted into mice, with efficacies superior to vinca alkaloids and comparable to a known vascular targeting agent. These results strongly suggest that TZT-1027 exerts marked antivascular activity. Next, to clarify the mechanism of the antivascular activity, we have taken a novel approach, and analyzed the relationships among human umbilical vein endothelial cells cytotoxicity, vascular permeability and tumor perfusion, on the basis of efficacies of each agent. Analyses revealed strong and significant correlations, and indicated that the vascular endothelial cell damage leads to endothelial barrier dysfunction and, thereby, tumor vascular shutdown. In summary, TZT-1027 was verified to have not only an excellent cytotoxic activity, but also an attractive antivascular activity through the induction of damage to vascular endothelial cells. We believe that these dual activities may make TZT-1027 useful for treating solid tumors. *Anti-Cancer Drugs* 18:905–911 © 2007 Lippincott Williams & Wilkins.

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Introduction

TZT-1027 (Soblidotin) is a microtubule (MT)-depolymerizing agent synthesized to have enhanced antitumor activity and reduced toxicity compared with dolastatin 10 [1,2]. The chemical structures of TZT-1027 and dolastatin 10 are shown in Fig. 1. Inhibition of MT assembly and prevention of GTP hydrolysis by TZT-1027 resulted in cell cycle arrest at the G_2/M phases and induction of apoptosis in cancer cells [2,3]. *In vitro*, TZT-1027 showed potent cytotoxic activities in a variety of human cancer cells, including lung, breast, prostate, stomach, leukemia and colorectal cancer cells, with 50% inhibitory concentration (IC_{50}) values of 1 pg/ml to 1 ng/ml [3,4]. *In vivo*, TZT-1027 exhibited broad-spectrum antitumor activity against murine syngeneic transplantable tumors, including P388 leukemia, Colon26 (C26) adenocarcinoma, B16 melanoma and M5076 sarcoma, with efficacy superior or comparable to those of reference agents [dolastatin 10, cisplatin (CDDP), vincristine (VCR) and 5-fluorouracil (5-FU)] [5]. In addition, TZT-1027 exerted antitumor activity against human tumor xenografts, including LX-1 lung and MX-1 breast carcinomas, and caused almost complete tumor regression in rats that spontaneously developed breast tumors induced by dimethylbenz[*a*]anthracene, without causing serious body weight loss [5,6]. Currently, TZT-1027 is undergoing clinical evaluation [7,8].

Tumor vasculature has a crucial role in providing the oxygen and nutrient supply necessary for the survival and proliferation of solid tumors [9]; therefore, it is one of the most promising targets in the treatment of solid tumors [10,11]. Thus, a number of vascular targeting agents (VTAs), which selectively disrupt existing tumor vasculature, have been developed recently [12] and the antivascular activity of TZT-1027 has already been assessed, as most VTAs inhibit MT assembly, like TZT-1027 [12]. Consequently, we obtained results characteristic of actions closely involved in antivascular effects – disruption of the MT cytoskeleton in vascular endothelial cells and enhanced vascular permeability in human umbilical vein endothelial cell (HUVEC) monolayers *in vitro*, as well as enhanced vascular permeability in tumor, thrombus formation, vascular shutdown and marked hemorrhage tumor necrosis *in vivo* [13,14]. Therefore, we could have confirmed that TZT-1027 exactly possessed antivascular activity.

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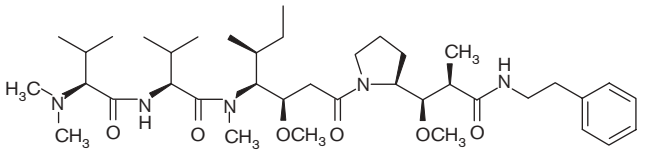
Fig. 1



The chemical structure of TZT-1027.

Despite having similar activity in terms of disrupting the MT dynamics, whereas vinca alkaloids and VTAs act by inhibiting MT assembly [12,15], the taxanes act conversely by stabilizing MT [16]. In addition, although both vinca alkaloids and VTAs have antivascular activity, vinca alkaloids exert antivascular activity at near-toxic doses [17–19], whereas VTAs do so at doses that are less than 1/10 of the maximum tolerated dose [20–22]. Thus, the agents with selectivity against tumor vascular endothelial cells have been regarded as VTAs. In spite of having a unique chemical structure that differs from the vinca alkaloids and VTAs, TZT-1027 also exerts an antivascular activity at a tolerable dose [1,2,13,14]. At present, however, the level of the TZT-1027 antivascular activity remains unclear. The aim of this study was to compare both the antivascular and cytotoxic activities of TZT-1027 with those of various anticancer agents having different mechanisms of action, including vinca alkaloids [VCR and vinorelbine (VNB)], a VTA [combretastatin A4 disodium phosphate (CA4DP)], a taxane [docetaxel (DTX)] and non-MT-binding agents (5-FU and CDDP). For that purpose, the cytotoxic activities against murine colon adenocarcinoma C26 cells and HUVECs were measured by MTT assay after both 24 and 72 h of treatment. Meanwhile, the antivascular activities were assessed in both an in-vitro and an in-vivo study, by measuring the diffusion of fluorescein isothiocyanate (FITC)–dextran passing through the HUVEC monolayer and by measuring the remaining Evans blue dye in the tumor tissue, respectively. Both the vascular permeability study [23,24] and the tumor perfusion study [18,20] were performed frequently so as to confirm each agent's antivascular activity.

Methods

Experimental animals and cell lines

Five-week-old female BALB/c and CDF1 mice were purchased from Japan Charles River (Kanagawa, Japan). A murine adenocarcinoma, the Colon26 solid tumor, and its cell line for culture, C26 cancer cells, were kindly supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo, Japan). The C26 solid tumor was maintained in syngeneic BALB/c mice in our laboratory, and all animal experiments were conducted according to the Rules for the Care and Use of Laboratory Animals of ASKA Pharmaceutical. The C26 cancer cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. HUVECs, endothelial basal medium (EBM) and endothelial cell medium-2 (EGM-2) were purchased from Cambrex (Walkersville, Maryland, USA). HUVECs were cultured in EGM-2 medium and used for the experiments until passage 5. All cells were maintained at 37°C in the presence of 5% CO_2.

Reagents

TZT-1027 and CA4DP were synthesized in our laboratory. VCR was purchased from Shionogi (Osaka, Japan), VNB and 5-FU from Kyowa Hakko Kogyo (Tokyo, Japan), DTX from Aventis Pharma (Tokyo, Japan) and CDDP from Bristol Myers-Squibb (Princeton, New Jersey, USA). TZT-1027 was dissolved in 0.05 mol/l lactate buffer (LB, pH 4.5), and VCR and CA4DP were dissolved in a vehicle appropriate for each experiment. In the in-vivo study, TZT-1027 was diluted with LB and the other agents were diluted with saline. In the in-vitro study, all agents were diluted with a medium appropriate for each experiment. FITC–dextran (70 kDa) was purchased from Sigma (St Louis, Missouri, USA), Evans blue from Nacalai Tesque, (Kyoto, Japan), MTT reagent from Funakoshi (Tokyo, Japan) and Biocoat Cell Culture Inserts (fibronectin-coated, pore size: 3 μm) from Becton Dickinson (Bedford, Massachusetts, USA).

MTT assay

Cytotoxic activities against C26 cells and HUVECs were determined using the MTT assay [25]. Briefly, C26 cells and HUVECs were plated on a 96-well plate at 1×10^4 or 0.2×10^4 cells/well for a 24- or 72-h treatment, respectively. After overnight incubation, each agent was added to each well (final concentrations: 1×10^{-5} to 10^{-12} g/ml for TZT-1027, VCR, VNB, CA4DP and DTX; 1×10^{-5} to 10^{-8} g/ml for 5-FU and CDDP) and the cells were treated for 24 or 72 h. After treatment, 5 mg/ml MTT solution (Funakoshi) was added to each well and the cells were further incubated for 4 h. Thereafter, the absorbance of formazan eluted in dimethylsulfoxide was measured at 570 nm using a 96-well plate reader (Nippon InterMed, Tokyo, Japan). The inhibition rate (IR, %) of cell proliferation was calculated from the absorbance in control cells (C) and that in agent-treated cells (T) using the following formula: $IR = (1 - T/C) \times 100$, and the IC_{50} values were calculated from the IRs and drug concentrations. Three independent experiments were performed in sextuplicate determinations and the results are expressed as the mean ± SD of the IC_{50} values.

Human umbilical vein endothelial cell monolayer permeability study

Diffusion of FITC–dextran (70 kDa; Sigma) passing through the HUVEC monolayer was determined as described previously [13,23,26]. Briefly, HUVECs were

cultured on fibronectin-coated culture inserts (upper compartment pore size, 3 μm) set on 24-well companion plates (lower compartment; Becton Dickinson) containing EGM-2 medium at 2×10^5 cells/well. When confluent monolayers were obtained after 2 days of culture, the two compartments were washed twice with phosphate-buffered saline, and the agent in 0.15 ml diluted with 1 mg/ml FITC–dextran in EBM medium and the same concentration of the agent in 0.75 ml diluted with EBM medium were added to the upper and lower compartment, respectively. After 1 h of treatment, a 50-μl aliquot was sampled from the lower compartment and its fluorescence intensity was measured (excitation, 490 nm; emission, 530 nm) using a 96-well microplate reader (Corona Electric, Ibaragi, Japan).

Tumor perfusion study

Tumor blood volume was measured by the Evans blue dye perfusion technique, as described previously [13,27,28]. Briefly, tumor fragments (2 mm^3) of C26 tumors were inoculated subcutaneously into the right flank of female CDF1 mice and a single dose of various agents or vehicle (LB) was administered intravenously or intraperitoneally at 10 ml/kg when the tumor volume reached approximately 400–600 mm^3. At 6 h after drug administration, 1% Evans blue was injected intravenously at 10 ml/kg. After 2 min, the mice were exsanguinated and killed, and the tumor tissues were extirpated, weighed and homogenized in a five-fold volume of digestive solution (0.5% sodium sulfate–acetone, 2 : 3). After incubation for 48 h at room temperature to extract the Evans blue dye, the suspensions were centrifuged at 1700*g* for 10 min, and the amount of Evans blue in the supernatant was measured using a 96-well microplate reader (Nippon InterMed) with absorbance set at 620 nm.

Statistical analyses

Statistical analyses in the HUVEC monolayer permeability study and the tumor perfusion study were performed by parametric Student's *t*-tests using SAS-system Release 8.2 software (SAS Institute, Tokyo, Japan). The correlation analyses and linear regression analyses were performed using Graphpad Prism 4.0 (San Diego, California, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Cytotoxic activities against C26 cancer cells and human umbilical vein endothelial cells

The cytotoxic activities against C26 cancer cells and HUVECs after the 24- and 72-h treatments were determined using the MTT assay (Table 1). Although the MTT assay is generally performed after a 72-h treatment, as it needs more than the cell doubling time so as to estimate growth inhibition, we further investigated the efficacies after a 24-h treatment, because the antivascular activity occurred rapidly, within 1 h [13], and the results with short-term treatment would provide an indication of not only cytotoxicity but also dysfunction in vascular endothelial cells, such as cell shape change and cell detachment. Against C26 cancer cells, all of the anticancer agents (TZT-1027, VCR, VNB, CA4DP, DTX, 5-FU and CDDP) showed cytotoxic activity after the 72-h treatment, with IC_{50} values of 0.014–100 ng/ml. Notably, TZT-1027 most strongly inhibited the growth of C26 cancer cells, with an efficacy 100 times higher than that of the other agents. None of the agents, however, was effective after the 24-h treatment, indicating that long-term treatment is necessary for evaluating the cytotoxic activity. On the other hand, against HUVECs, only TZT-1027, the vinca alkaloids (VCR, VNB) and the VTA (CA4DP), which had in common the ability to inhibit MT assembly, showed cytotoxic activity even after a 24-h treatment, with IC_{50} values of 0.098–3.5 ng/ml, respectively. In contrast, the taxane (DTX) showed cytotoxic activity only after the 72-h treatment, and none of the non-MT-binding agents (5-FU and CDDP) was effective after either the 24- or 72-h treatment. Therefore, it was indicated that only the agents with the ability to inhibit MT assembly could cause distinctive damage leading to the antivascular activity against vascular endothelial cells in the early phase, but an MT-stabilizing agent (taxane) and non-MT-binding agents could not. In summary, the most potent cytotoxic activities of TZT-1027 against both C26 cancer cells and HUVECs implied that it had marked antivascular activity against tumor vasculature in addition to strong cytotoxic activity against cancer cells.

Table 1 Cytotoxic activities against C26 cancer cells and HUVECs

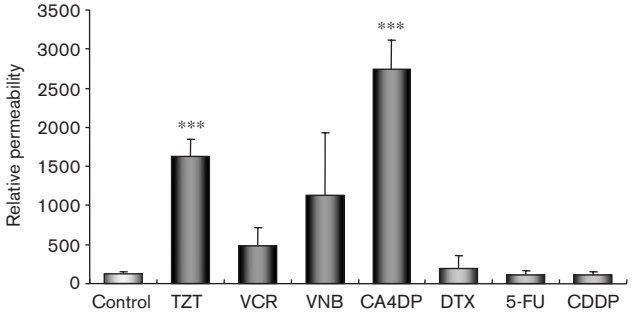
	C26 cancer cells		HUVECs	
Treatment	24 h	72 h	24 h	72 h
TZT	>1000	0.014 ± 0.013	0.098 ± 0.11	0.0046 ± 0.0036
VCR	>1000	2.6 ± 0.5	1.0 ± 1.9	0.43 ± 0.35
VNB	>1000	4.5 ± 2.4	9.2 ± 5.4	1.5 ± 0.7
CA4DP	>1000	4.4 ± 0.3	3.5 ± 2.6	3.0 ± 1.4
DTX	>1000	2.4 ± 1.1	4400 ± 630	0.86 ± 0.67
5-FU	>1000	51 ± 6.8	>10000	>1000
CDDP	>1000	100 ± 35	>10000	>1000

IC_{50} values (ng/ml) were obtained from three independent experiments in sextuplicate determinations.
CA4DP, combretastatin A4 disodium phosphate; CDDP, cisplatin; DTX, docetaxel; 5-FU, 5-fluorouracil; HUVEC, human umbilical vein endothelial cell; TZT, TZT-1027; VCR, vincristine; VNB, vinorelbine.

Human umbilical vein endothelial cell monolayer permeability study

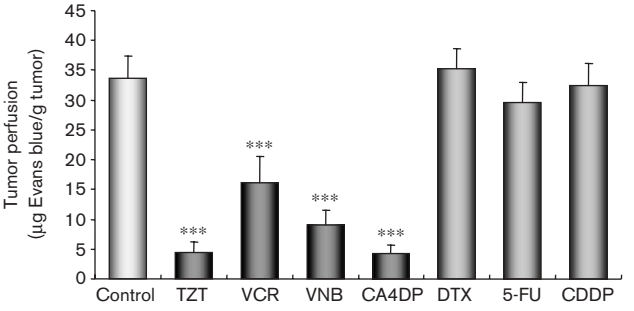
The ability of each agent to enhance vascular permeability in the HUVEC monolayers after a 1-h treatment was evaluated to compare the antivascular activity in an in-vitro study (Fig. 2). In this study, the maximum or effective blood concentration of each agent after administration of a clinical dose was employed [VCR, 0.5 μg/ml; VNB, 1 μg/ml; DTX, 2 μg/ml; 5-FU, 15 μg/ml; and CDDP, 1.5 μg/ml (refer to the package inserts for the agents) and CA4DP, 3.3 μg/ml] [29], because TZT-1027 could have

Fig. 2



Enhanced vascular permeability in HUVEC monolayers. Confluent HUVEC monolayers were treated with vehicle or various agents for 1 h, and then diffusion of FITC–dextran passing through the HUVEC monolayer was measured using a 96-well microplate reader. Each bar represents the mean ± SD of three independent experiments. ***$P < 0.001$, significantly different from the vehicle control group (Student's *t*-test). FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; TZT, TZT-1027; VCR, vincristine; VNB, vinorelbine; CA4DP, combrestatin A4 disodium phosphate; DTX, docetaxel; 5-FU, 5-fluorouracil; CDDP, cisplatin.

Fig. 3



Reduction of tumor perfusion in C26 tumors implanted into syngeneic mice. Various agents were administered intravenously or intraperitoneally to mice bearing C26 tumors. Evans blue contents in tumors were measured at 6 h after administration. Each bar represents the mean ± SD of six animals. ***$P < 0.001$, significantly different from the vehicle control group (Student's-*t* test); TZT, TZT-1027; VCR, vincristine; VNB, vinorelbine; CA4DP, combrestatin A4 disodium phosphate; DTX, docetaxel; 5-FU, 5-fluorouracil; CDDP, cisplatin.

induced an increase in vascular permeability at a concentration of 0.1 μg/ml, which corresponded to near the maximum concentration (C_{max}) after administration of an optimal dose of TZT-1027 (2 mg/kg) [13]. Consequently, TZT-1027, the vinca alkaloids (VCR, VNB) and the VTA (CA4DP) markedly enhanced vascular permeability, with values 12.6- 3.8- 8.7- and 21.3-fold of the control value. In particular, TZT-1027 and CA4DP significantly enhanced vascular permeability in comparison to the control ($P < 0.001$). Neither the taxane (DTX) nor the non-MT-binding agents (5-FU, CDDP), however, was effective in enhancing the vascular permeability. These findings indicated that enhanced vascular permeability was also preferentially caused by the agents with the ability to inhibit MT assembly. Notably, TZT-1027 potently induced barrier dysfunction in vascular endothelial cells, with efficacy comparable to that of CA4DP, a representative VTA.

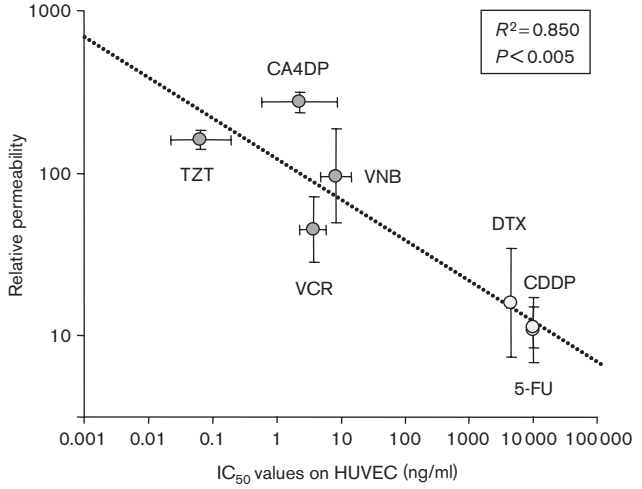
Tumor perfusion study

The ability of each agent to reduce the perfusion of C26 tumors implanted into syngeneic mice after a 6-h treatment was evaluated to compare the antivascular activity in an in-vivo study (Fig. 3). In this study, an optimal dose at which each agent may exert an antitumor activity after a single administration was employed (VCR, 2 mg/kg, intravenous [5]; VNB, 15 mg/kg, intravenous [30]; CA4DP, 500 mg/kg, intraperitoneal [31]; DTX, 40 mg/kg, intravenous; 5-FU, 50 mg/kg, intravenous [5]; and CDDP: 5 mg/kg, intravenous [5]), as TZT-1027 had induced acute vascular shutdown in tumors at a dose of 2 mg/kg, intravenous [13], at which it exhibits its most potent antitumor activity in animal models [5,13,14]. As in the in-vitro study, TZT-1027, the vinca alkaloids (VCR, VNB) and the VTA (CA4DP) significantly reduced tumor perfusion, with values 12.6- 3.8- 8.7- and 21.3-fold of the control value ($P < 0.001$), respectively. In addition, neither the taxane (DTX) nor the non-MT-binding agents (5-FU, CDDP) was effective in reducing the tumor perfusion. Therefore, the results of both an in-vitro and an in-vivo study conducted to evaluate the anti-vascular activities could confirm that TZT-1027 had a considerable antivascular activity, with efficacy superior to that of vinca alkaloids and comparable to that of a VTA.

Correlations among human umbilical vein endothelial cell cytotoxicity, vascular permeability and tumor perfusion

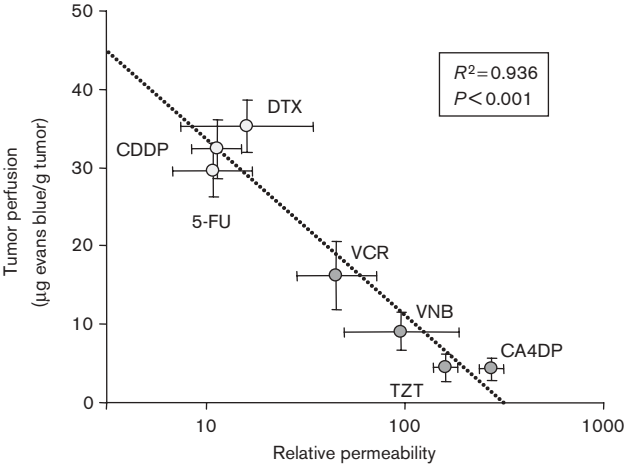
To clarify the mechanism of the antivascular activity, we have taken a novel approach, and analyzed the relationships among HUVEC cytotoxicity, vascular permeability and tumor perfusion, based on the efficacies of each agent. We first analyzed the relationship between the cytotoxicity against HUVECs and vascular permeability on HUVEC monolayers (Fig. 4). The IC_{50} values after the 24-h treatment against HUVECs and the values for vascular permeability after the 1-h treatment with each agent were plotted on the *x*- and *y*-axis, respectively. Analysis showed a phase I, inverse-proportion linearity ($R^2 = 0.850$), and a significant correlation between the cytotoxicity against HUVECs and enhanced vascular permeability in HUVEC monolayers ($P < 0.003$). This finding that a lower IC_{50} value correlated with a greater increase in vascular permeability indicated that the damage against vascular endothelial cells in the early phase would cause the endothelial barrier dysfunction. We next analyzed the relationship between vascular permeability and tumor perfusion (Fig. 5). The values for vascular permeability after the 1-h treatment and for tumor perfusion after the

Fig. 4



Correlation between the cytotoxic activity against HUVECs and vascular permeability. The IC_{50} values after the 24-h treatment against HUVECs and the values for vascular permeability in HUVEC monolayers after the 1-h treatment with each agent were plotted on the *x*- and *y*-axis, respectively. To calculate the correlation, linear regression analysis was performed using Graphpad Prism 4.0 and a *P*-value of less than 0.05 was considered statistically significant. HUVEC, human umbilical vein endothelial cell; TZT, TZT-1027; VCR, vincristine; VNB, vinorelbine; CA4DP, combrestatin A4 disodium phosphate; DTX, docetaxel; 5-FU, 5-fluorouracil; CDDP, cisplatin.

Fig. 5



Correlation between vascular permeability and tumor perfusion. The values for vascular permeability on HUVEC monolayers after the 1-h treatment and for tumor perfusion in C26 tumors implanted into syngeneic mice after the 6-h treatment with each agent were plotted on the *x*- and *y*-axis, respectively. To calculate the correlation, linear regression analysis was performed using Graphpad Prism 4.0 and a *P*-value of less than 0.05 was considered statistically significant. HUVEC, human umbilical vein endothelial cell; TZT, TZT-1027; VCR, vincristine; VNB, vinorelbine; CA4DP, combrestatin A4 disodium phosphate; DTX, docetaxel; 5-FU, 5-fluorouracil; CDDP, cisplatin.

6-h treatment with each agent were plotted on the *x*- and *y*-axis, respectively. This analysis also showed a phase I, inverse-proportion linearity ($R^2 = 0.936$), and a significant correlation between enhanced vascular permeability in HUVEC monolayers and a reduction of tumor perfusion ($P < 0.0004$). Likewise, this finding that an increase in vascular permeability correlated with a greater reduction in tumor perfusion indicated that the barrier dysfunction in vascular endothelial cells led to the tumor perfusion shutdown. Taken together, these analyses suggested that the damage against vascular endothelial cells in the early phase could cause the endothelial barrier dysfunction, followed by tumor perfusion shutdown.

Discussion

TZT-1027 has been verified previously to exert antitumor activity and antivascular activity against advanced-stage C26 tumors [13] and its potent antitumor activity has been assumed to be due in part to its antivascular activity. In the MTT assay, we employed not only C26 cancer cells, but also HUVECs, to clarify the ability of TZT-1027 to exert both cytotoxic and antivascular activities, because the majority of the whole tumor mass was comprised of C26 cancer cells and the most important target in antivascular activity was the vascular endothelial cells. TZT-1027 most markedly inhibited the growth of C26 cancer cells among the reference agents, including VCR, VNB, CA4DP, DTX, 5-FU and CDDP (Table 1), and this result was consistent with a previous study that showed that the cytotoxic activity of TZT-1027 against various cancer cells was superior to those of VCR, CDDP and 5-FU [1]. Therefore, we suggested that TZT-1027 has a broad spectrum of activity, with a strong cytotoxic activity, and may be useful clinically, even if it would have no antivascular activity. TZT-1027 also showed growth-inhibitory activity against HUVECs even after a 24-h treatment, indicating that it would have an antivascular activity against tumor vasculature in addition to a cytotoxic activity against tumor cells. Several studies have reported that VTAs such as CA4P [32] and ZD6126 [33,34] disrupt the MT cytoskeleton in HUVECs 30–40 min after treatment, inducing morphological changes, membrane blebbing and cell detachment. Similarly, TZT-1027 achieved the disruption of the MT cytoskeleton throughout the cytoplasm within 15 min and the induction of marked cell contraction with membrane blebbing and cell detachment within 30 min, indicating that TZT-1027 acts not only on spindle MTs, but also on cytoskeletal MTs, in a cell cycle-independent manner. Taken together, the cytotoxic activity after a 24-h treatment in the MTT assay would be thought to represent the damage of HUVEC morphology within a few minutes. Therefore, the strongest cytotoxic activity of TZT-1027 against HUVECs would suggest its excellent antivascular activity.

To compare the antivascular activity of TZT-1027 with that of vinca alkaloids, a VTA, a taxane, and non-MT-binding agents in both an in-vitro and an in-vivo study, the ability of each agent to enhance vascular permeability and to reduce tumor perfusion, respectively was evaluated. A previous study has shown that TZT-1027 significantly enhanced vascular permeability more than 15 min after treatment, that it increased permeability in a time-dependent manner for at least 1 h, that it significantly reduced tumor perfusion at 1 h after administration, that it induced the maximum reduction at 6 h and that it had an effect that persisted for 24 h [13]. For these reasons, the ability of each agent in antivascular activity was evaluated after a 1- and a 6-h treatment in a vascular permeability study and tumor perfusion study, respectively. Consequently, all of the agents with the ability to inhibit MT assembly showed obvious antivascular activities to cause barrier dysfunction in vascular endothelial cells (Fig. 3) and to induce tumor vascular shutdown (Fig. 4). Therefore, it was indicated that the antivascular activity of an agent closely depended on whether it could cause MT assembly. Furthermore, it was especially noticeable that despite having a similar activity to inhibit MT assembly, TZT-1027 and CA4DP had marked antivascular activity, but the vinca alkaloids did not, suggesting that they act on vascular endothelial cells more selectively, and that TZT-1027 is definitely a VTA, as well as CA4DP.

Although having cytotoxic activity against vascular endothelial cells may raise the impact of side effects, TZT-1027 has been confirmed not to enhance the vascular permeability of normal vessels other than at nonphysiological concentrations using the skin reaction test of guinea pigs [13]. Reportedly, the structure of tumor vessels differs from that of normal vessels, as the tumor vessel is immature, and is not surrounded by a vessel wall of pericytes and smooth muscle cells [35]. Moreover, it was reported that DMXAA, one of the VTAs, showed no effect on the vascular endothelial cells of normal tissues, such as the spleen, liver, heart or brain, but it selectively induced apoptosis of tumor vessel endothelial cells [36]. Therefore, we believe that the cytotoxic activity of TZT-1027 against HUVECs corresponds to its antivascular activity against the tumor vasculature but not the normal vasculature.

Although many tumor vasculature targeting studies have denoted the importance of vascular targeting therapy to inhibit the growth and metastasis of solid tumors [10–12], the mechanism of the antivascular activity remains to be clarified. Some previous reports indicated a correlation between the cytotoxic activity (apoptosis) in HUVECs and reduction of tumor blood flow [36], and a correlation between the reduction of tumor blood flow and antitumor activity [37,38]. In addition, we have already provided sequential findings concerning the antivascular activity of TZT-1027. That is, TZT-1027 disrupted the MT cytoskeleton in vascular endothelial cells within 15 min and induced an increase in vascular permeability on HUVEC monolayers after 15 min, suggesting that disruption of the MT cytoskeletal tight junctions in vascular endothelial cells would cause enhanced vascular permeability. In histological analysis, enhanced tumor vascular permeability was confirmed at 1 h after treatment, followed by thrombus formation at 3 h, indicating that the contact of plasma components with the exposed basement membrane induces thrombosis formation. Subsequently, elicited vessel occlusion might cause vascular shutdown, the maximum reduction of which was observed at 6 h, and secondary ischemic tumor necrosis was observed at 24 h [13]. In this study, despite the treatment with each agent at only one dose, our analyses revealed strong and significant correlations among HUVEC cytotoxicity, vascular permeability and tumor perfusion. Thus, we suggest that this original approach, based on the efficacy of various anticancer agents with different mechanisms of action, makes those previous sequential findings clearer.

Although VTAs exert their antivascular activity at doses below the maximum tolerated dose with fewer side effects, they could cause necrosis only in the central region of the tumor, not at the peripheral region. As a result, because the tumor mass may be reassembled by the surviving rim of tumor cells, VTA showed weak antitumor activity when given as monotherapy [20,39,40]. Accordingly, VTA recently have been investigated in several combination therapies in xenograft models and in a phase I clinical trial [20,41–44]. In contrast, as TZT-1027 has strong cytotoxic activity against cancer cells in addition to potent antivascular activity, it induced marked hemorrhagic necrosis with scab formation at the peripheral rim of the tumor, and finally regression of tumor [13,14]. Furthermore, having dual activity would be thought to provide the surprising benefit of a potential synergistic effect. That is, rapid enhanced vascular permeability may allow TZT-1027 to invade tumor tissue efficiently and disruption of the tumor vasculature allows prevention of the diffusion of TZT-1027 from the tumor tissue. Therefore, it was suggested that prolonged residence time of TZT-1027 in the tumor tissue may enable it to exert its cytotoxic activity against tumor cells in a cell-cycle-dependent manner more efficiently.

In summary, these findings suggest that TZT-1027 has not only an excellent cytotoxic activity against cancer cells, but also an attractive antivascular activity through the induction of damage to vascular endothelial cells, with efficacies superior to that of vinca alkaloids and comparable to that of a VTA. We believe that its dual activities may make TZT-1027 useful for treating solid tumors in the clinical setting, even as monotherapy.

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