TZT-1027 Elucidates Antitumor Activity through Direct Cytotoxicity and Selective Blockade of Blood Supply

NAOKO HASHIGUCHI1, TETSURO KUBOTA1, JUN-ICHI KOH2, YOSHINORI YAMADA3, YOSHIRO SAIKAWA1, YOSHIHIDE OTANI1, MASAIKO WATANABE1, KOICHIRO KUMAI1, MASAKI KITAJIMA1, JUN-ICHI WATANABE3 and MOTOHIRO KOBAYASHI1

1Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582; 2Department of Surgery, Saitama Social Insurance Hospital, Saitama 336-0002; 3Department of Surgery, Kitasato Institute Hospital, Tokyo 108-8642; 4Pharmacological Research Department, Teikoku Hormone MFG, Co., Ltd., Kanagawa 213-8522, Japan

Abstract. Background: TZT-1027 is a newly developed antitumor agent derived from dolastatin 10. Materials and Methods: The in vitro activity of TZT-1027 on MCF-7 and R-27 cells was evaluated by MTT assay. TZT-1027 1 mg/kg/week was administered i.v. for 4 weeks into nude mice bearing MCF-7 and R-27. Subsequently, primary cultured cells from xenografts were also used for CD-DST. Two mg of TZT-1027 or 40 mg docetaxel per kg were injected i.v. into nude mice bearing R-27. 0.2% Evans blue was injected to assess the blood flow. Results: TZT-1027 suppressed the in vitro growth of MCF-7 cells, while R-27 cells were resistant to TZT-1027, although its in vivo antitumor activity was remarkable. TZT-1027 blocked R-27 tumor blood flow immediately after injection; blood flow was not affected by docetaxel. Conclusion: TZT-1027 exerts its antitumor activity through direct cytotoxicity against MCF-7 cells and through selective blockade of tumor blood flow against R-27 cells.

Since the introduction of colchicine derivatives to medical treatment for gout, tubulin has been considered a molecular target of antitumor agents. Plant alkaloids, such as vincristine and vinblastine, inhibit the polymerization of tubulin by binding microtubules, while newly developed taxanes (paclitaxel and docetaxel) exert their antitumor activity through the stabilization of microtubules. TZT-1027 (1) is a newly synthesized derivative of dolastatin 10, a compound isolated by Pettit and colleagues from the Indian Ocean sea hare, Dolabella auricularia, in 1987 (2). A preclinical study indicated that TZT-1027 had antitumor activity against several murine tumors, including P388 leukemia, colon 26, B16 melanoma, M5076 sarcoma and human tumor xenografts, MX-1 and LX-1 (3). Watanabe and colleagues investigated whether TZT-1027 inhibited the growth of various human cancer cells and whether the cell death caused by TZT-1027 was due to apoptosis in human leukemia HL-60 cells, human breast cancer MCF-7 cells and human prostate cancer DU145 cells, suggesting an association of the growth-inhibitory effect of TZT-1027 with the induction of apoptosis and indicating that the apoptosis induced by TZT-1027 was followed by G2/M arrest (4). In the present study, we further investigated the mode of action of TZT-1027 in terms of direct and indirect cytotoxicity through vascular blockade, using the human breast cancer cell lines MCF-7 and R-27.

Materials and Methods

Drugs. TZT-1027 was synthesized in the Pharmacological Research Department of Teikoku Hormone Mfg. Co., Ltd., Japan, and was dissolved in 0.05 M lactate buffer (pH 4.5), which was further diluted before experimental use. The chemical structure of TZT-1027 is shown in Figure 1. Docetaxel was purchased from Aventis Pharma. Ltd., Tokyo, Japan.

Human breast cancer cell lines. Human breast cancer cell lines MCF-7 and R-27 were used for the experiments. MCF-7 was initially established by Soule and coworkers (5) as a cultured cell line of hormone-dependent human breast cancer and we transplanted it into a nude mouse treated with estrogen and progesterone (6). R-27 was established as a tamoxifen-resistant variant of MCF-7 by Nawata and colleagues (7) and was also transplanted into nude mice by the same procedure as for MCF-7 (8). Both cell lines were maintained in RPMI 1640 (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; CSL Limited, Australia), 100 IU. penicillin, 100 μg streptomycin and 0.25 μg amphotericin B per ml (RPMI 1640 medium). The xenografts were maintained by serial subcutaneous
inoculation into BALB/c female nude mice (CLEA, Tokyo, Japan) that were treated with 5 mg/kg 17α-estradiol dipropionate and 250 mg/kg 17β-progesterone caproate, which accounts for 0.1 ml EP hormone depot™ (Teikoku Hormone Mfg, Co. Ltd., Tokyo, Japan) per mouse. These two cell lines and xenografts are estrogen and progesterone receptor-positive in vitro and in vivo.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. We evaluated the in vitro cytotoxicity of TZT-1027 against MCF-7 and R-27 cells in culture using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay as reported by Mosmann (9), with some modifications (10-13).

After the cells had reached confluency, they were harvested, centrifuged once, suspended in RPMI 1640 medium and diluted to 1 x 10^5 cells/ml. Aliquots (100 μl) plated into 96-well microplates (GIBCO) resulted in approximately 10^4 cells per well. Drug solutions were dissolved in RPMI 1640 medium and 100-μl aliquots were added to each well, giving final concentrations of 0.002, 0.02, 0.2, 2, 20, 200, 2,000, 20,000 pg of TZT-1027 per ml. Control wells contained 100 μg cell suspension and 100 μl RPMI 1640 medium, while 200 μl RPMI 1640 medium was used as a blank. Plates were incubated for 48 or 72 hours at 37°C in a humidified atmosphere containing 95% air and 5% CO2. A mixture of 0.4% MTT (Sigma) and 0.1 M sodium succinate (Wako Pure Chemical Ind. Ltd., Osaka, Japan), each dissolved in 10 μl phosphate-buffered saline and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), was then added and the plates were incubated for an additional 3 hours at 37°C. After the final incubation, 150 μl dimethyl sulfoxide (Nacalai Tesuque, Kyoto, Japan) was added to each well to dissolve the MTT-formazan salt and the plates were shaken mechanically for 10 minutes on a mixer (Model 250, Sonifier, Branson, MO, USA). The optical densities of each well were determined using the following formula: (1 - A/B) x 100 (percentage), where A and B represent the mean absorbance of the treated and control wells, respectively.

Antitumor activity of TZT-1027 against human breast carcinoma xenografts in nude mice. Tumor tissue fragments, each measuring approximately 3 x 3 x 3 mm, were inoculated bilaterally into subcutaneous tissues of the dorsum of ether-anesthetized nude mice, using a trocar needle. EP hormone depot™ was administered once intramuscularly into the thigh of the mice on the inoculation day and repeated on Days 7 and 14 for MCF-7. Implanted tumors were measured (length and width) with a sliding caliper three times weekly by the same observer. Tumor weight was calculated according to the method of Geran and colleagues (14) from linear measurements using the following formula:

\[
\text{tumor weight (mg)} = \text{length (mm)} \times \left(\text{width (mm)}\right)^2/2.
\]

When tumors reached 100 to 300 mg, tumor-bearing mice were allocated randomly to test groups each consisting of 6 mice and treatment was initiated. One mg of TZT-1027 per kg suspended in 0.1 ml sterile water per mouse was administered intravenously once weekly for four weeks.

Relative mean tumor weight (RW) was calculated as RW = Wi/Wo, where Wi is the mean tumor weight at any given time and Wo is the mean tumor weight at the time of initial treatment. The antitumor activity of TZT-1027 was evaluated as the lowest T/C value (%) during the experiment, where T is the RW of the treated group and C the RW of the control group at any given time. Tumors were characterized as sensitive when the lowest T/C value was equal to or less than 42%, which was calculated from (0.75)^3, corresponding to a 25% reduction of each dimension (15). At the end of the experiments, all the tumors were weighed in mg, mean and standard deviation were calculated, and the differences between control and treated groups were assessed by Student's t-test.

Collagen-droplet drug sensitivity test. The collagen gel droplet-embedded culture drug sensitivity test (CD-DST) was performed as reported previously (16). Briefly, "Cellmatrix" type CD, Ham’s F-12 medium at 10-fold concentration, and a reconstitution buffer (all from Nitta Gelatin Inc., Yao City, Osaka, Japan) were mixed at a ratio of 8:1:1 to homogeneity in an ice-cold bath and the collagen solution was prepared. The cell suspension prepared above was added to this solution to a final density of 1 x 10^5
cells/ml, affording a collagen mixture. This collagen mixture was placed into 6-well multi-plates (Nalge-NUNC Inc., Rochester, NY, USA) at a volume of 30 µl per collagen gel droplet using a micropipette, and subjected to gelation in a CO₂ incubator at 37°C for 1 hour. In each well, 3 ml of DF medium (DF (10)), to which 10% FBS had been added, was overlaid. After overnight incubation, TZT-1027 was added at different concentrations (0.0001-20 µg/ml) and docetaxel was added from 0.01 to 10 µg/ml. After 24 hours, each well was washed with Hank’s solution twice, 4 ml/well of PCM-2 (with FBS from removed PCM-1) medium was overlaid and the cells were cultured for 7 days. On the 8th day of culture, 40 µg/well of neutral red solution (5 mg/ml) was added and incubation was continued for 2 hours. After removal of the solution, the cells were fixed with 10% formalin buffered at neutral pH. The plates were immersed in a tray of water for 10 minutes without agitation and then air-dried and subjected to analysis. To obtain the total volume quantification, binary images of proliferating cells in collagen gel drops were obtained with an image analyzing apparatus (VIDAS-plus, Carl Zeiss Inc., Tokyo, Japan), and the upper and lower limits were set. Next, an image with light covered (BC) was obtained and an image with the light covered (TC) was calculated and used. The cumulative density of the test sample image was calculated, and the image optical density (A) was obtained according to the following formula:

\[ A = \log_{10}\left(\frac{\Sigma WC - \Sigma BC}{\Sigma TC - \Sigma BC}\right) \]

where \(\Sigma WC\) is the cumulative density value of the blank image, \(\Sigma BC\) is the cumulative density value with the light covered and \(\Sigma TC\) is the cumulative density of the test sample image. For this processing, a personal computer (Apple Power Macintosh G-3) with grayscale image digitizer (LG-3 Image ONE, Tokyo) and a macroprogram, were used.

**Anti-vascular activity of tzt-1027 against R-27 tumors.** The anti-vascular activity of TZT-1027 against R-27 tumors was measured according to the method of Otani and colleagues (17).

Tumor fragments (2 x 2 x 2 mm in size) of R-27 were implanted subcutaneously into the right flank of female BALB/c nude mice supplemented with 5 mg/kg 17β-estradiol dipropionate and 250 mg/kg l7α-progestosterone caproate. When the estimated tumor weight reached 100-300 mg, TZT-1027 and docetaxel were administered intravenously at doses of 1 and 2 mg/kg for TZT-1027 and 40 mg/kg for docetaxel. At 0, 1, 6 and 24 hours after drug injection, 0.2% Evans blue was given intravenously at 0.2 ml/head and the mice were killed 2 minutes after Evans blue injection. Immediately after the mice had been sacrificed, tumors were isolated and frozen in liquid nitrogen. The tumor fraction was suspended in 1-3 ml of formamide and incubated for 48 hours at 60°C to extract the Evans blue (18). Then, the suspension was filtered on a 0.5-µm membrane filter and the absorbance at 620 nm (reference at 690 nm) was measured. The intra-tumoral amount of Evans blue was calculated as mg/g.

**Results**

The antitumor activity of TZT-1027 against MCF-7 and R-27 cells is shown in Figure 2. TZT-1027 showed a concentration-dependent cytotoxicity against MCF-7 cells after 48 and 72 hours of incubation. A time-dependent effect was not obvious in this experiment. In contrast, R-27 was resistant to TZT-1027 and no 50% inhibitory concentrations were obtained for either the 48- or 72-hour incubation until the highest concentration tested (20 ng/ml), which is extremely high.

The in vivo antitumor activity of TZT-1027 is shown in Figure 3A (MCF-7) and Figure 3B (R-27). Both strains were sensitive to TZT-1027. The lowest T/C value and RW of MCF-7 were 25.5% and 0.312, respectively and there was a statistically significant difference between the actual tumor weights in the control (249.2±151.0 mg) and treated (96.8±53.1 mg) groups (p<0.002). R-27 was also highly sensitive to TZT-1027 and the lowest T/C value and RW were 28.6% and 0.8, respectively. There was also a statistically significant difference between the control (647±301.7 mg) and treated (247.4±131.8 mg) tumors (p<0.05).

When the control tumors were used for CD-DST, only MCF-7 was sensitive to TZT-1027, with a 50% inhibitory concentration of 3,400 µg/ml, as shown in Figure 4. On the other hand, R-27 was resistant to TZT-1027 in CD-DST, where no 50% inhibitory concentration was observed until 20 µg/ml, while the control cells grew until they reached a density 2.3 times that of the initial seeding (Figure 5A). In contrast, docetaxel was cytotoxic against R-27, as detected by CD-DST at a concentration of 10 µg/ml (Figure 5B).

Figure 6 shows the Evans blue contents of R-27 tumors treated with TZT-1027 or docetaxel, representing the vascular blood flow of the tumors. TZT-1027 blocked tumor blood flow at 1 hour after TZT-1027-injection, when the Evans blue content decreased to almost half of that of the untreated control in both the 1- and 2-mg/kg TZT-1027 groups. This down-regulation continued until 24 hours after injection in both groups, while the 2 mg/kg TZT-1027 group had continuously suppressed blood flow until 8 mg at 6 hours and 9 mg at 24 hours of Evans blue per tumor weight in g after injection. In the 1-mg/kg TZT-1027 group, the Evans blue contents in the tumor were 14 mg/g at 6 and 24 hours after TZT-1027 injection. On the other hand, the suppression of blood flow by docetaxel was minimal, where the Evans blue content of the tumor was 16 and 18 mg/g tumor at 6 and 24 hours after TZT-1027 injection, respectively.

**Discussion**

Dolastatin 10 was initially isolated from the Indian Ocean sea hare, Dolabella auricularia, and TZT-1027 was newly synthesized as a derivative of dolastatin 10 with potent...
antitumor activity. Kobayashi and colleagues have evaluated its antitumor activity against a variety of murine tumors, including P388, B16 melanoma and colon 26 (19). 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 the experiments with drug-resistant P388 leukemia, TZT-1027 showed excellent antitumor 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, where tumor regression was observed in MX-1 and LX-1 strains (19).

In the present study, MCF-7 was sensitive to TZT-1027 in vitro, as detected by MTT assay and CD-DST, and in vivo in nude mice, while R-27 was resistant to TZT-1027 in vitro by both MTT assay and CD-DST. Although R-27 was established as a tamoxifen-resistant subline of MCF-7, its sensitivity to the conventionally available agents is known to be similar to that of MCF-7, compared with human breast carcinoma xenografts tested in our institute. Its different characteristics in vivo are less dependent on estradiol and less sensitive to hormonal agents compared with the wild-type, MCF-7. We have previously reported the chemosensitivity of MCF-7 and R-27 to conventionally available and new antitumor agents (8, 15, 20, 21). Figure 7 shows the chemosensitive pattern of MCF-7 (Figure 7A) and R-27 (Figure 7B) to several agents tested by their maximum tolerated doses. MCF-7 was sensitive to mitomycin C (MMC), doxorubicin (DXR) and UCN-01, and the effect of TZT-1027 followed from that of MMC and UCN-01, while its efficacy was more potent than that of DXR, UFT, cyclophosphamide (CPA) and tamoxifen (TAM), which are the standard drugs used to treat breast carcinoma. R-27 was more sensitive to MMC, CPA and TZT-1027, than to paclitaxel (PAC), DXR, hexamethylmelamine and UFT. This result was comparable to that in the report by Fujita and coworkers (22) who evaluated the antitumor effects of TZT-1027 against 16 human tumors xenografted in nude mice from gastric, breast, colon, lung, liver, renal cell and ovarian cancer lines. In their study, TZT-1027 was shown to be more potent in three cancer models than irinotecan, and to have markedly effective antitumor activity in two cancers in which vincristine was ineffective and in ovarian cancer in which irinotecan, cisplatin and DXR were ineffective. From these results, they suggested that TZT-1027 was an excellent candidate for clinical trials for the treatment of cancer. The direct cytotoxicity of TZT-1027 has been evaluated in several ways. Its main target is microtubulin polymerization, as for vinca alkaloids, including vinblastine (VLB), vincristine (VCR) and vindesine (VDS). Since TZT-1027 at 10 μM almost completely inhibited the assembly of porcine brain microtubules, its mechanism of antitumor action seems to be, at least in part, ascribable to the inhibition of microtubule assembly (19). Natsume and coworkers have compared the activity of TZT-1027 with that of dolastatin 10 as well as these vinca alkaloids (23). In their study, Scatchard analysis of the binding of 3H-VLB suggested one binding site, while that for 3H-TZT-1027 suggested two binding sites, one of high affinity and one of low affinity. 3H-TZT-1027 was completely displaced by dolastatin 10 but only incompletely by VLB. TZT-1027 affected the binding of VLB to tubulin, but its binding site was not completely identical to that of VLB. These results suggested that TZT-1027 had a potent inhibitory effect on tubulin polymerization and differed from vinca alkaloids in its mode of action against tubulin polymerization. Watanabe and colleagues (23) have used three cancer cell lines, human leukemia HL-60, human breast cancer MCF-7 and human prostate cancer DU145, and reported that TZT-1027 induced DNA fragmentation. The flow-cytometric analysis revealed that the cells treated with TZT-1027 were arrested at the G2/M-phase and subsequently showed fragmented DNA smaller than that of G1-phase cells. From these results, they suggested an association of the growth-inhibitory effect of TZT-1027 with the induction of apoptosis, which was followed by G2/M arrest. Natsume and colleagues (24) have investigated the sensitivity of surgical specimens obtained from patients with non-small cell lung carcinoma (NSCLC) and renal cell carcinoma (RCC) to TZT-
1027 and other DNA-damaging agents, comparing the status of the p53 gene in those specimens. When 29 NSCLC specimens and 22 RCC specimens were analyzed for their sensitivity by flow cytometry and their p53 status was compared, TZT-1027 was less influenced by the p53 status of specimens than the DNA-damaging agents.

In our present study, R-27 was sensitive to TZT-1027 in vivo with a T/C ratio of 28.6%, similar to that of MCF-7 at 30.0%, although R-27 was highly resistant to TZT-1027 when detected by CD-DST using the primary cultured R-27 that was sensitive to TZT-1027 in vivo. Since the control cells grew until they reached a density 2.3 times that of the initial seeding, this CD-DST was successfully conducted and actually docetaxel was evaluated to be effective against R-27 tumor cells in this assay. On the other hand, MCF-7 was also sensitive to TZT-1027 in CD-DST, which reproduced the cytotoxicity of this drug on MCF-7 in nude mice. This discrepancy between the in vitro and in vivo sensitivity of Hhashiguchi et al: TZT-1027 Against MCF-7 and R-27
R-27 was considered to be due to the indirect cytotoxicity of TZT-1027, which is mediated through an anti-vascular effect, the cytotoxicity on tumor vascular endothelial cells as reported by Otani and colleagues (17). They evaluated TZT-1027-induced tumor vascular collapse and tumor cell death in an advanced tumor model, murine colon 26 adenocarcinoma. Tolerable doses of TZT-1027 induced tumor-selective hemorrhage within 1 hour, which occurred mainly in the peripheral area of the tumor mass. They measured tumor hemoglobin content and dye permeation detected by Evans blue. When 2% Evans blue is injected, Evans blue molecules will bind to plasma albumin and behave like albumin (18). In their study, it was revealed that the hemorrhage occurred first and tumor blood flow stopped secondarily. They also studied the effects of TZT-1027 on cultured human umbilical vascular endothelial cells, where TZT-1027 induced marked cell contraction with membrane blebbing in 30 minutes, which was completely inhibited by a broad-spectrum inhibitor of protein kinases, K252a. As a result, they concluded that TZT-1027 quickly attacked the well-developed vascular system of advanced tumors, resulting in the blockade of tumor blood flow. These results may explain the indirect cytotoxicity of TZT-1027 against R-27 through vascular blockade detected by Evans blue assay. It was interesting that docetaxel did not exert this anti-vascular effect, while it was effective against R-27 as detected by CD-DST.

In conclusion, TZT-1027 exerts its antitumor activity through direct cytotoxicity for MCF-7, as well as via selective blockade...
Figure 7. A. Sensitivity pattern of MCF-7 in vivo. MCF-7 was sensitive to mitomycin C (MMC), doxorubicin (DXR) and UCN-01 and the effect of TZT-1027 followed from that of MMC and UCN-01, while its efficacy was more potent than that of DXR, 5-FU, cyclophosphamide (CPA) and tamoxifen (TAM). B. Sensitivity pattern of R-27 in vivo. R-27 was more sensitive to MMC, CPA and TZT-1027 than to paclitaxel (PAC), DXR, hexamethylmelamine (HMM) and UFT (a mixed compound of tegafur and uracil at a molar ratio of 1:4).
of tumor blood flow for R-27, resulting in its remarkable antitumor activity against both strains in vivo. TZT-1027 has potent antitumor activity against human breast carcinoma in vivo compared with conventionally available agents for breast cancer, including DXR, CPA, 5-FU and PAC. Further investigation of TZT-1027 is warranted, in particular its unique mode of action. Because of its promising preclinical activity, TZT-1027 has been entered into phase I clinical trials and the recommended dose for phase II study was reported to be 1.8 mg/m² in a weekly regimen for 3 weeks with the dose-limiting toxicities being neutropenia and constipation (25).

References


Received October 20, 2003
Revised February 11, 2004
Accepted April 5, 2004