Enhanced Antitumor Efficacy of Clinical-Grade Vasculature-Targeted Liposomal Doxorubicin

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Abstract


Experimental Design: Mice were implanted with lung, ovarian, or neuroblastoma tumor cells via the pulmonary, peritoneal, or orthotopic (adrenal gland) routes, respectively, and treated, at different days post inoculation, with multiple doses of doxorubicin, administered either free or encapsulated in untargeted liposomes (Caelyx) or in TVT-DOX. The effect of TVT-DOX treatment on tumor cell proliferation, viability, apoptosis, and angiogenesis was studied by immunohistochemical analyses of neoplastic tissues and using the chick embryo chorioallantoic membrane assay.

Results: Compared with the three control groups (no doxorubicin, free doxorubicin, or Caelyx), statistically significant improvements in survival was seen in all three animal models following treatment with 5 mg/kg (maximum tolerated dose) of TVT-DOX, with long-term survivors occurring in the neuroblastoma group; increased survival was also seen at a dose of 1.7 mg/kg in mice bearing neuroblastoma or ovarian cancer. Minimal residual disease after surgical removal of neuroblastoma primary mass, and the enhanced response to TVT-DOX, was visualized and quantified by bioluminescence imaging and with magnetic resonance imaging. When treated with TVT-DOX, compared with Caelyx, all three tumor models, as assayed by immunohistochemistry and chorioallantoic membrane, showed statistically significant reductions in cell proliferation, blood vessel density, and microvessel area, showing increased cell apoptosis.

Conclusion: TVT-DOX should be evaluated as a novel angiostatic strategy for adjuvant therapy of solid tumors.

Targeting the tumor's blood supply is not a new concept. The development of a functional blood supply is essential to meet the oxygen and nutrient demands of growing solid tumors (1). The neovasculature that arises from the normal host vessels by the process of angiogenesis also is the principal vehicle for metastatic spread (2). Thus, tumor neovasculature is a potential therapeutic target (3).

There are two major approaches to controlling tumor vasculature. One strategy is to prevent the development of tumor blood vessels by inhibiting the angiogenesis process (angiogenesis-inhibiting agents); the other strategy acts by compromising the function of established tumor blood vessels [vascular-targeting agents (VTA)]. Recently, the use of VTAs, such as ligand-targeted liposomes and drug conjugates, has started to fulfill its promise (4). The VTA strategy builds on the clinical success of nanomedicines such as Caelyx, which are used to improved therapeutic outcome and/or minimized damage to normal tissues such as heart or bone marrow, thereby increasing the selective toxicity of chemotherapeutics in cancer (5). Further increases in therapeutic activity can be...
Translational Relevance

Conventional anticancer therapy with chemotherapeutic drugs has been a mainstay of cancer therapy for several decades, but the poor selective toxicity of chemotherapeutics has led to high levels of dose-limiting side effect, compromising their clinical utility. Liposomal anticancer drugs like Stealth liposomal doxorubicin (DoxiI in the United States and Caelyx in the rest of the world) has reduced the side effects of the entrapped drugs and has resulted in improved therapeutic efficiencies. The therapeutic indices for anticancer drugs are increased by liposome encapsulation. The mechanism for this effect stems from increased localization of the liposomal drugs to tumors through increased permeability of the tumor vasculature, the so-called passive targeting or enhanced permeability and retention effect. Direct cell kill of the tumor cells results from increased localization of the liposomal drug to the tumor interstitial space, sustained release of the drug, and uptake of the released drug into the tumor cells. We think that this article will be of widespread interest to scientists and members the public who are interested in cancer medicine, because a single formulation of our clinical-grade TVT-DOX resulted in anticancer activity via two different mechanisms that resulted in both direct and indirect tumor cell kill. This led to enhanced antitumor efficacy in clinically relevant animal models of neuroblastoma, ovarian, and lung cancers. The clinical relevance of our novel approach relies on a combination of the two strategies; indeed, aspargine-glycine-arginine-targeted liposomal doxorubicin (TVT-DOX) binds to and kills angiogenic blood vessels and, indirectly, the tumor cells that these vessels support. We believe that this combination approach is a promising one in the search for more effective and less toxic cancer treatments.

achieved by using ligand-targeted nanomedicines that have surface-conjugated, tumor-selective antibodies or peptides (6), particularly when targeting is via internalizing ligands that facilitate the delivery of the therapeutic contents to intracellular sites of activity via the endosome/lysosome pathway (6, 7).

Compared with normal blood vessels, tumor blood vessels have an abnormal wall structure and a branching pattern with uneven diameters (8). Endothelial cells in angiogenic vessels express several proteins that are absent or barely detectable in established blood vessels, including αv integrins (9), receptors for angiogenic growth factors (10), and other types of membrane-spanning molecules, such as the aminopeptidases N (CD13) and A (APA; refs. 11, 12). In vivo panning of phage libraries in tumor-bearing mice have proven useful for selecting peptides that bind to receptors that are either overexpressed or selectively expressed on tumor-associated vessels and that home to neoplastic tissues (13). Thus, it may be possible to develop ligand-targeted chemotherapy strategies based on peptides that are selective for tumor vasculature. Among the various tumor-targeting ligands identified to date, peptides containing the aspargine-glycine-arginine motif, which binds to CD13, have proven useful for delivering various antitumor compounds to tumor vasculature (14, 15). Although there are several subpopulations of CD13 (16), relatively widely distributed in the body, only one isoform is believed to be the receptor for the aspargine-glycine-arginine-containing peptides. This isoform has been shown to be expressed exclusively in angiogenic vessels, such as the neovasculature found in tumor tissues (17).

Because the CD13 isoform that is recognized by aspargine-glycine-arginine-containing peptides is expressed on endothelial cells within most, if not all, solid tumors, in this work, we validated the potential of the VTA strategy by evaluating aspargine-glycine-arginine-targeted liposomal doxorubicin (TVT-DOX) in several murine xenografts of doxorubicin-resistant human cancer, including lung, ovarian, and neuroblastoma. Contrary to previous experiments (14, 18), the TVT-DOX used in this study was manufactured as large-scale good manufacturing practice (GMP) preparation (19) suited to human clinical trials. Specifically, the antitumor potential of several concentrations of TVT-DOX have been validated, using different treatment schedules, designed to act specifically against more or less mature neovasculature within the tumor mass. Compared with an untargeted formulation of doxorubicin (Caelyx), in clinical use for the treatment of ovarian cancer and other solid tumors (20, 21), the good manufacturing practice preparation of TVT-DOX was able to more effectively kill angiogenic tumor blood vessels and, indirectly, the tumor cells that these vessels support. In conclusion, the antitumor activity of TVT-DOX was higher than that of Caelyx, in all three murine tumor xenograft models, particularly when administered at the higher dose treatment. Our results suggest that TVT-DOX should be evaluated as a novel VTA strategy for adjuvant therapy of solid tumors.

Materials and Methods

Drugs. TVT-DOX and Caelyx were purchased from Northern Lipids and Schering Canada, respectively. Doxorubicin was purchased from Sigma.

Tumor cell lines, cotransfection, and selection of cell line. The aggressive neuroblastoma cell line GI-LI-N, the lung cancer cell line Colo-699N, and the ovarian cancer cell line OVCAR-3 were grown in complete DMEM or RPMI 1640 as reported previously (18). In some experiments, GI-LI-N cells were infected with retrovirus expressing the firefly luciferase gene. The retroviral plasmid was obtained by cloning the firefly luciferase gene, derived from the pGL3-control vector (Promega), into the retroviral pLXIN bicistronic vector (Clontech), to obtain pl-Luc-IN. Retroviruses were prepared by calcium phosphate transient transfection of HEK293 T cells by mixing 5 μg pkat2ampac and 5 μg pl-Luc-IN (22). The retrovirus-containing supernatant was collected at 48 h post-transfection, filtered, supplemented with 8 μg/mL Polybrene (Sigma), and used to infect cells. After 48 h, the transduced cells were selected with 1 mg/mL G418 (MP Biomedicals). Luciferase activity of retrovirally transduced cells was confirmed by IVIS imaging (Caliper Life Sciences) after a 10 min incubation with 150 μg/mL ą-luciferin (Caliper Life Sciences) and diluted in tissue culture medium as described previously (7).

Animal models. Mice were purchased from Harlan Laboratories, Harlan Italy, S. Pietro al Natisone Italy and housed under specific pathogen-free conditions. All experiments were reviewed and approved by the licensing and ethical committee of the National Cancer Institute (Genoa, Italy) and by the Italian Ministry of Health. All in vivo experiments were done using 5-week-old female athymic (nude-nu), 10 to 12 mice per group, and were repeated at least twice.

For the neuroblastoma animal model, mice were anesthetized with a mixture of xylazine (10 mg/kg) and ketamine (90 mg/kg; Imalgene 1000; Merial Italia), subjected to laparotomy, and orthotopically

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injected with the neuroblastoma cell line, GI-LI-N (1 x 10^6 cells), in the capsule of the left adrenal gland, as reported previously (7, 14).

In another set of experiments, mice were orthotopically injected with 1 x 10^6 luciferase-transfected GI-LI-N cells on day 0 and half of the mice had their tumors surgically resected on day 20 as reported previously (7). The resected neuroblastoma animal model, referred to in the future as "neuroblastoma-resected," was used to visualize, by bioluminescence imaging (BLI) and magnetic resonance imaging (MRI), the response of minimal residual disease (MRD) to therapy, after surgical removal of the primary mass, as well as to monitor orthotopic expansion over time and organ-specific homing of tumor cells.

For the lung tumor animal model, mice received intrapulmonary injections of Colo-699N cells (1.5 x 10^6) as reported previously (18).

For the ovarian cancer animal model, mice were injected intra-peritoneally with OVCAR-3 cells (2.5 x 10^5) as described previously (18).

**In vivo therapeutic experiments.** For the neuroblastoma animal models, tumors were allowed to grow from the injected cells for 20 days, and animals were treated with a single intravenous injection per week x 5 weeks with 5 mg/kg [maximum tolerated dose (MTD)], 3.33 mg/kg (2/3 MTD), or 1.7 mg/kg (1/3 MTD) of free doxorubicin or encapsulated doxorubicin, either Caelyx or TVT-DOX.

In the neuroblastoma-resected animal model, at 48 h after the surgical removal of the primary mass (day 22), mice were treated with TVT-DOX once a week x 5 weeks at a dose 5 mg/kg/injection doxorubicin.

In the first set of experiments, at 5 days after cells implantation, mice bearing lung or ovarian tumors were injected intravenously with 5 mg/kg doxorubicin (the MTD), 3.3 mg/kg doxorubicin (2/3 MTD), or 1.7 mg/kg doxorubicin (1/3 MTD) of free doxorubicin, Caelyx, or TVT-DOX. In a second set of experiments, to allow the additional development of tumor vasculature, mice were treated at 7 days after cell inoculation, using the same doxorubicin formulations as above, but only at the MTD or 1/3 MTD.

In every experiment, an untreated control group of mice received HEPES-buffered saline (HBS). Body weight and general physical status of the animals were recorded daily until they were judged to be in comfort by animal caretakers. Specifically, once showing signs of poor health [abdominal dilatation, dehydration, paraplegia, and severe weight loss], the veterinarian (M.C.) from the Animal Research Facility and his collaborator (F.P.) euthanized the mice, following anesthesia with xylazine (Xilor 2%; Bio98 Srl), and the day of euthanasia was recorded as the day of death. The method of Kaplan and Meier was used to estimate survival distribution and survival time. Survival time is defined as the time, in days, between the day of resection, and response to TVT-DOX therapy were all readily visualized and quantified via a 1.5 T magnetic resonance scanner (Philips Gyroscan NT-Intera) before and after gadolinium injection. Briefly, mice were anesthetized by an intraperitoneally injection of a mixture of xylazine and ketamine and were placed on a surface coil in prone position; body temperature was maintained at 37°C with a water-filled support. Images were obtained of the coronal and axial planes, perpendicular to the vertebral column of the animal. MRI was done before and after intravenous injections of 0.5 mmol/kg gadolinium (Magnevist, gadopentetate dimeglumine; Bayer, HealthCare). The temperature during the experiment was 28°C. The mean acquisition time was 15 min for each experiment.

We performed the following sequences: T1-weighted turbo spin echo sequences (repetition time in ms/echo time in ms/flip angle in degrees), T2-weighted turbo spin echo sequences (repetition time in ms/echo time in ms/flip angle in degrees), and T2-weighted turbo spin echo fat saturation (repetition time in ms/echo time in ms/flip angle in degrees), with a field of view of 100 x 100 mm, a matrix of 256 x 256 pixels, and a slice thickness of 2 mm.

**Histologic analysis of murine neuroblastoma and lung tumor models.** Histologic evaluation of primary tumors was done at 29 and 42 days after lung and neuroblastoma cancer cell inoculations, respectively. Briefly, orthotopically implanted athymic mice were treated with either Caelyx or TVT-DOX at 7 and 20 days after cell inoculation for lung and neuroblastoma cancers, respectively. Mice received intravenous injections weekly x 5 weeks with the MTD of doxorubicin (5 mg/kg). One day after the fourth treatment, mice were anesthetized with xylazine and killed by cervical dislocation. Tumors were collected, divided in two, and then embedded in either paraffin or OCT (Miles Chemical) compound. Tissue sections (5 µm thick) were examined after staining with Mayer's H&E (Sigma).

Tumor frozen sections from treated neuroblastoma-bearing mice were analyzed for CD31 expression and microvessel area assessed as reported (see Supplementary Data; refs. 18, 23, 24).

Paraffin-embedded tissue sections derived from the lung carcinoma model were stained with primary antibodies against the proliferation antigen Ki-67, the endothelial cell marker CD34, and the mouse anti-human desmin and for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis as reported previously (see Supplementary Data; refs. 18, 23, 24).

**Chorioallantoic membrane assay.** Chorioallantoic membrane (CAM) experiments were done according to the method of Ribatti et al. (25) on biopsy fragments taken from murine xenografts of neuroblastoma (GI-LI-N). Lung (Colo-699N), or ovarian (OVCAR-3) cancers and microvessel area was assessed as described previously (see Supplementary Data; refs. 23, 24, 26).

**Statistical methods.** All the in vivo experiments were done at least twice, with similar results. Results are expressed as mean ± 95% confidence intervals. The statistical significance of differential findings between experimental groups and controls was determined by ANOVA, with the Tukey’s multiple comparison test, in GraphPad Prism 3.0 software (GraphPad Software). The significance of the differences between experimental groups (n = 8-12 mice per group) in the survival experiments was determined by Kaplan-Meier curves by the use of the x^2 log-rank test (GraphPad Prism 3.0). These findings were considered significant if P values < 0.05.

**Results**

In vivo antitumor activity of TVT-DOX against neuroblastoma xenograft models. To determine whether tumor vasculature-targeted doxorubicin (TVT-DOX) could be used to improve the therapeutic outcome in neuroblastoma xenografts, compared with free doxorubicin or untargeted liposomal doxorubicin (Caelyx), we injected GI-LI-N cells into the left adrenal gland of...
nude mice and allowed them to grow until they reached a size of \( \sim 200 \text{ mm}^3 \) (20 days). Neuroblastoma tumor-bearing mice were then treated at weekly intervals with 5 intravenous injections of free doxorubicin, Caelyx, or TVT-DOX, at a doxorubicin dose of 5 mg/kg/injection (MTD), at 2/3 of the MTD (1.66 mg/kg), or at 1/3 of the MTD (1.7 mg/kg). As shown in Fig. 1A to C, neuroblastoma-bearing mice treated with Caelyx outlived control mice (HBS) and mice treated with free doxorubicin (5 mg/kg doxorubicin in Caelyx versus HBS or free doxorubicin, \( P < 0.001 \)). Mice were not treated at lower free doxorubicin doses, because, even at the MTD for free doxorubicin, they all died from widespread disease. Mice treated at each of the three TVT-DOX doses had statistically significant increases in lifespan, compared with the HBS and the free doxorubicin treatment groups, and, of note, also compared with those treated with Caelyx (5 mg/kg doxorubicin in TVT-DOX, \( P < 0.0001 \) versus HBS or free doxorubicin and \( P < 0.001 \) versus Caelyx; 3.3 mg/kg doxorubicin in TVT-DOX, \( P < 0.001 \) versus PBS and \( P < 0.001 \) versus Caelyx; and 1.7 mg/kg doxorubicin in TVT-DOX, \( P < 0.0001 \) versus HBS and \( P < 0.001 \) versus Caelyx). Long-term survivors were obtained only for mice treated at the MTD of TVT-DOX.

We then asked if TVT-DOX affected neuroblastoma tumor growth and CD31 expression specifically via its inhibitory effects on angiogenesis. Histopathologic analysis of excised tumors from control mice (PBS), or mice treated with doxorubicin at a doxorubicin dose of 5 mg/kg as Caelyx or as TVT-DOX, revealed a significant (80-90%) suppression of blood vessel density in mice treated with TVT-DOX compared with PBS and a lesser, but still significant, suppression compared with Caelyx (Fig. 1B; Supplementary Fig. S1).

**BLI and MRI visualization of primary tumor regrowth and development of metastatic tumors after neuroblastoma surgical resection in response to TVT-DOX therapy.** To assess the effect of TVT-DOX on controlling MRD and helping to prevent tumor relapse, “neuroblastoma-resected” mice were treated with TVT-DOX (5 mg/kg doxorubicin) weekly \( \times \) 5 injections at 48 h after surgery. Figure 2A presents lateral images (from the side where cells were implanted) from five neuroblastoma-bearing mice on day 20 after surgical resection. Images were evaluated for BLI intensity over time (T21 and T44). The results show that in vivo imaging is a noninvasive tool, with applications in cancer xenografts for the measurement of primary tumor regrowth and the development of metastatic tumors. Treatment of neuroblastoma-resected mice with TVT-DOX induced a partial arrest in primary tumor regrowth and possibly an inhibition of MRD in 4 of 5 treated mice, whereas images from PBS-treated neuroblastoma-resected mice show tumor mass relapse and expansion in 4 of 5 mice (Fig. 2A and B). In neuroblastoma-resected mice receiving TVT-DOX treatment, there was a decreased lifespan compared with PBS controls, with 2 of 5 animals still alive at 130 days after tumor challenge (Fig. 2C).

MRD was visualized, and metastatic tumor growth was evaluated, in neuroblastoma-resected mice in response to TVT-DOX therapy using a 1.5 T MRI scanner. In a scan of a typical neuroblastoma-resected mouse, on the T2-weighted sequences, we observed a hypotense solid lesion of 1 cm in diameter on the back of the mice (Fig. 2D, arrow), which appeared hyperintense on T2-weighted fat saturation sequences (Fig. 2D, 3). Compared with sequences before gadolinium injection (Fig. 2D, 4), T1-weighted sequences after gadolinium injection showed a considerable increase in the signal intensity of this lesion (Fig. 2D, 5), related to the hypervascularization of the neuroblastoma tumor. Importantly, whereas BLI evaluation only allowed the visualization of a solid lesion on the back of the mouse (Fig. 2D, 1), on the T1-weighted sequence from a neuroblastoma-resected, PBS-treated mouse, we observed an enlargement of the bilateral axillary nodes (Fig. 2D, 6 and 7).
before and after gadolinium injection (arrows). On the contrary, in a neuroblastoma-resected mouse treated with TVT-DOX, the MRI did not show any lesions both before and after gadolinium injection (Fig. 2D, 6 and 7) as also observed by BLI evaluation (Fig. 2D, 1).

**In vivo antitumor activity of TVT-DOX against human lung and ovarian carcinoma xenografts.** We next evaluated the efficacy of TVT-DOX in two aggressive xenograft models of adult cancers. Lung and ovarian carcinoma are two of the most commonly diagnosed cancers and are also one of the most common causes of cancer deaths. Thus, two recently developed lung and ovarian cancer xenograft models (18) were used to ascertain if TVT-DOX had wider potential to inhibit tumor growth in vivo. In an initial set of experiments, lung or ovarian tumor-bearing mice were treated, at 5 days after cell implantation, with doxorubicin at its MTD, 2/3 of MTD, or 1/3 of MTD, using free doxorubicin, Caelyx, or TVT-DOX. In a second set of experiments, treatment started at 7 days after cell inoculation using the same doxorubicin formulations as above but only at 5 and 1.7 mg/kg doxorubicin.

Both Caelyx and TVT-DOX significantly enhanced the antitumor activity against in vivo doxorubicin-resistant lung and ovarian cancers relative to control mice (HBS) or those treated with free doxorubicin. In the lung tumor model, when mice were treated at 5 days after cell implantation, the antitumor efficacy of TVT-DOX was marginally significant at the MTD ($P < 0.05$), when compared with Caelyx, and not at lower doses (Fig. 3A; Supplementary Fig. S2). Of note, the increase in lifespan in the lung cancer-bearing mice, in response to TVT-DOX treatment, was greater when the tumors may have been more angiogenic (at 7 days after cell inoculation) compared with 5 days (Fig. 3B). The antitumor effects, relative to HBS-treated control mice or those treated with Caelyx, were significantly greater both at the MTD of doxorubicin and at 1/3 of its MTD (5 mg/kg doxorubicin in TVT-DOX, $P < 0.0001$ versus HBS and $P < 0.05$ versus Caelyx; 1.7 mg/kg doxorubicin in TVT-DOX, $P < 0.0001$ versus HBS and $P < 0.05$ versus Caelyx).

Tumor growth after Caelyx treatment was not significantly different to that of a HBS control group, when animals were treated at 1.7 mg/kg doxorubicin, at either 5 or 7 days after cell implantation (1.7 mg/kg doxorubicin of Caelyx versus HBS, $P > 0.05$ and $P > 0.1$ at 5 and 7 days, respectively; Fig. 3A and B). In contrast, TVT-DOX maintained its antitumor activity...
versus HBS (1.7 mg/kg doxorubicin of TVT-DOX versus HBS, \( P < 0.01 \) and \( P < 0.0001 \) at 5 and 7 days, respectively). These findings clearly indicate the importance of the vascular targeting mechanism of TVT-DOX in inhibiting tumor progression.

Figure 4 shows the antitumor activity of TVT-DOX against human ovarian carcinoma xenografts. When treatment was commenced at 5 days post-implantation, the difference in treatment efficacy between TVT-DOX and Caelyx was either not significant or marginally significant at 1/3 of the MTD (1.7 mg/kg doxorubicin in TVT-DOX versus Caelyx, \( P < 0.05 \); Fig. 4A; Supplementary Fig. S3). However, when treatment with TVT-DOX was commenced at 7 days post-inoculation, similar to the neuroblastoma model, greater tumor growth control relative to Caelyx was observed both at the MTD and at 1/3 of the MTD (5 mg/kg doxorubicin in TVT-DOX versus Caelyx, \( P < 0.001 \); 1.7 mg/kg doxorubicin in TVT-DOX versus Caelyx, \( P < 0.01 \); Fig. 4B).

**Effect of treatment on tumor cell proliferation, apoptosis, and angiogenesis in vivo.** To assess the effect of TVT-DOX on tumor cell proliferation, viability, and apoptosis, we stained cryosections taken from lung cancer xenografts at day 29 and examined them at low and medium magnification for changes in blood vessels and in the surrounding tumor parenchyma (Fig. 5). Histopathologic analysis of tumors excised from untreated animals or animals treated at the MTD with Caelyx or TVT-DOX showed that both types of treatments inhibited tumor cell proliferation as assessed by the drastic decrease in Ki-67-positive cells. However, TVT-DOX resulted in a statistically significant increased antiproliferative effect compared with Caelyx (\( P < 0.001 \)). Moreover, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay showed an increased level of apoptosis in tumor and endothelial cells in mice treated with TVT-DOX compared with those treated with the nontargeted formulation (\( P < 0.01 \) versus Caelyx). No observable apoptosis was observed in normal tissue such as heart, lung, kidneys, liver, and spleen in all TVT-DOX-treated animals (data not shown).

Finally, angiogenesis-specific studies, done in parallel sections, clearly showed a pronounced destruction of the tumor vasculature with a marked decrease in CD34+ and desmin-positive endothelial cells following treatment of the mice with TVT-DOX. A statistically significant improvement in the angiostatic effects were observed in mice treated with TVT-DOX compared with those treated with Caelyx at the same doxorubicin concentration (CD34+ and desmin-positive cells: \( P < 0.01 \) and \( P < 0.05 \) versus Caelyx, respectively).

**Effect of treatment on angiogenic responses in the CAM assay.** The differences in the antiangiogenic activity between TVT-DOX and Caelyx in vivo using the CAM assay is shown in Fig. 6. Tumor xenografts derived from neuroblastoma, lung, and ovarian cancers were grafted onto CAMs. CAMs were then incubated with PBS (specimens alone) or with 2 \( \mu \)g doxorubicin in either Caelyx or TVT-DOX. CAMs incubated with Caelyx showed a decrease in the number of allantoic vessels radiating in a “spoked wheel” pattern toward all three of the xenografts when compared with those incubated with PBS (Fig. 6A). However, incubation of the CAMs with TVT-DOX significantly reduced the number of radiating vessels that invaded the...
implant compared with either specimens alone or CAMs incubated with Caelyx as shown by morphometric assessment of microvessel area (Fig. 6B).

Discussion

VTAs work by destroying established tumor vasculature, thereby starving the tumor cells of oxygen and nutrients (27). This strategy may overcome problems of tumor heterogeneity. Here, we report on a novel VTA, in which an anticancer therapeutic, doxorubicin, was targeted to tumor vasculature via a ligand against aminopeptidase N that is selective for tumor endothelial cells. We showed that aminopeptidase N-TVT-DOX had superior antitumor activity to Caelyx in lung, ovarian, and neuroblastoma animal tumor models. The activity was dependent on both the dose and the timing of the first TVT-DOX treatment in the lung and ovarian cancer models. In neuroblastoma-bearing mice, TVT-DOX resulted in a significant increased lifespan at all doxorubicin concentrations administered, and long-term survivors were observed at the MTD.

The formation of the vasculature by vasculogenesis and angiogenesis is essential for embryonic development (28), tissues remodeling in adults, and unrestrained growth of tumors (29). More specifically, to become clinically relevant, a tumor requires persistent neovascularization for its growth and survival (30). Neoplastic vessel density is correlated with an adverse prognosis in many types of adult and pediatric solid tumors (31, 32).

Because host endothelial cells appear to play a central role in tumor growth, progression, and metastasis through their role as primary building blocks of the tumor vasculature (29), tumor vasculature has become a compelling new target for inhibiting solid tumor growth (33). VTAs have activity against all types of solid tumors, so long as they depend on a blood supply for their growth. Hence, the targeting of therapeutics to blood vessels, using probes that bind to specific molecular addresses on the tumor vasculature, has become a major research area in cancer (33, 34).

Caelyx is hypothesized to have antitumor activity in part through its ability to elicit an antivascular effect by mimicking metronomic dosing via its sustained drug release profile (35). Here, we have shown the antivascular activity for this formulation. Nevertheless, TVT-DOX, a vascular-targeted therapeutic with some similarities to Caelyx, including similar in vivo pharmacokinetics,11 but with specificity for tumor vasculature, showed significantly greater inhibition of tumor cell proliferation and a marked decrease of blood vessels density in murine xenograft models of lung, ovarian, and neuroblastoma. Hence, the selective targeting of tumor vasculature, as opposed to its “passive” targeting, led to a greater activity against tumor endothelial cells and ultimately an improved tumor cell kill. Hence, our results support the concept that targeting tumor vasculature endothelial cells in a selective manner results in greater antitumor activity.

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11T. Allen and K. Laginha, unpublished observations.

Fig. 4. Survival curves of ovarian carcinoma-bearing mice in response to doxorubicin-containing formulations. A, mice (n = 10) were injected intraperitoneally with OVCAR-3 cells and treated at 5 d post-inoculation with 1 intravenous injection per week × 9 wk of doxorubicin, encapsulated in Caelyx or TVT-DOX, at a doxorubicin dose of 5 or 1.66 mg/kg. Free doxorubicin was only administered at the MTD. Control mice received HBS. B, in a second set of experiment, mice were treated at 7 d after cell inoculation, using the same doxorubicin formulations as above, but only at the MTD and at 1/3 of the MTD.
In this article, TVT-DOX made by good manufacturing practice (14, 19) has been used against three animal models of human cancer, and proof-of-principle for its activity has been achieved for the utilization of a large-scale good manufacturing practice formulation, which is a necessary prerequisite during the preclinical studies that precedes human clinical trials. Compared with Caelyx, which is already in clinical use, good manufacturing practice preparations of TVT-DOX were, to a greater extent, able to destroy angiogenic tumor blood vessels and, indirectly, the tumor cells that are supported by these vessels in several human solid tumors.

Some novel cancer therapeutics have achieved success in the treatment of a variety of malignancies, but relapse of disease from small numbers of residual tumor cells remains a major obstacle (e.g., vascular-independent cells in the tumor periphery). Advancement of treatment regimens that effectively control MRD and prevent relapse would be greatly accelerated if sensitive, noninvasive assays could be developed that would quantitatively assess tumor burden in vivo in xenograft models of MRD that are predictive of human responses. In vivo BLI is an assay that allows the noninvasive detection of small numbers of cells enabling the repeat quantification of tumor cell growth within the internal organs (36, 37) of the same animal over a period.

The use of noninvasive diagnostic imaging methods such as BLI to establish the efficacy of angio-static therapies is becoming increasingly important as antiangiogenic agents are beginning to reach the market with the approval of Avastin by the Food and Drug Administration in 2004 for colon cancer (38), its approval in 2006 for lung cancer, and its accelerated approval for use in HER2-negative breast cancer (2008; in combination with paclitaxel). For example, MRI and BLI have been used in recent years for evaluating the antitumor (36) and antivascular effects of targeted therapies against solid tumors (39, 40). In this study, we show that BLI and MRI are viable real-time, noninvasive, quantitative, and qualitative methods for monitoring the response to VTA therapy. BLI was more sensitive than MRI for detecting early tumor responses to therapy, but MRI was able to precisely identify focal MRD and appears to be more suited to the study of tumor growth in vivo and the effects of chemotherapy in experimental animal models. Hence, we feel that MRI and BLI are complementary methods with promising roles in various types of preclinical research (41).

Fig. 5. Effect of doxorubicin-containing formulations on tumor cell proliferation, apoptosis, and endothelial tumor cell suppression. A, histologic analysis were done at day 29 on primary lung cancer removed from untreated (HBS) mice and from mice treated at 7 d after cell inoculation with 1 intravenous injection doxorubicin per week × 5 wk at a doxorubicin dose of 5 mg/kg/dose in either Caelyx or TVT-DOX. Bar = 200 μm. B, morphometric assessment of Ki-67, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, CD34, and desmin expression. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
In the last few years, nanomedicine has become a rapid growth research area (42), particularly for anticancer applications. Several nanomedicines, primarily lipid-based drug carriers such as Doxil/Caelyx (43), have received clinical approval and several more lipid-based and polymeric carriers are undergoing clinical evaluation (35). A logical extension of this success is to further improve the antitumor effects of liposomal nanomedicines, in a more selective manner, through the use of "active-targeting moieties" coupled to their external surface (6, 44). Receptor-mediated internalization of nanomedicines into tumor cells is mandatory for improved therapeutic efficacy of targeted-liposomal drugs (45, 46). It has also been established that nuclear localization of doxorubicin is required for cell killing, as it binds DNA and inhibits topoisomerase II (47). Our data indicated that liposomal doxorubicin was able to enter into the cells and localize in the nucleus when it was targeted via the aspargine-glycine-arginine-containing peptide coupled at the external surface of the nanoparticles but not in the absence of the targeting agent (19).

In this study, aminopeptidase N-targeted liposomes show an enhanced antitumor and angiostatic effect against all the tumor animal models examined and thus a candidate for progression to clinical investigation. However, it must be born in mind that destroying vascular supply with a vasculature-targeted therapy might partially limit the ability of other chemotherapeutics to reach the tumor or, at least, that survived from the vasculature-targeted therapy. Furthermore, clinical trials based on the use of single, either proangiogenic or antiangiogenic, molecules can be more challenging than anticipated, and monotherapy with single angiogenesis inhibitor might not be sufficient to control cancer and the myriad of angiogenic factors produced by cancer cells.

Phage display biopanning on viable cells is a powerful approach for identifying cell-specific peptides that mediate binding to individual tumor types (48). This technology, based on the principle that bacteriophages can present specific binding ligands on their surface, has been used for discovering peptides that can specifically bind to organs, tumors, or cell types (13, 49). Thus, in the near future, it may be possible to use phage display techniques on tumor patient specimens to develop novel ligand-targeted liposomal chemotherapeutic strategies that are based on the selective targeting of other novel molecular markers, expressed on the tumor vasculature or the tumor cell surface itself. Thus, a multiple target approach, based on a combination of antitumor and antivascular therapies, analogous to combination chemotherapy currently in widespread clinical use, could be expected to improve the therapeutic effects of nanomedicine drugs against many types of adult and pediatric solid tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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