Frequent Administration of Angiogenesis Inhibitor TNP-470 (AGM-1470) at an Optimal Biological Dose Inhibits Tumor Growth and Metastasis of Metastatic Human Transitional Cell Carcinoma in the Urinary Bladder

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ABSTRACT

Purpose: **The angiogenic inhibitor TNP-470 (AGM-1470, *O*-chloracetyl-carbamoyl fumagillol) has been reported to inhibit the growth of human transitional cell carcinoma (TCC) in the urinary bladder. However, it is still unknown whether TNP-470 inhibits metastasis of TCC. Here, we identify an efficient protocol using TNP-470, and optimize its antitumor and antimetastatic effects on human TCC in the urinary bladder.**

Experimental Design: In vitro, **the human metastatic TCC cell line 253J B-V and human umbilical vascular endothelial cells were treated with TNP-470, and examined for cell growth and protein production of angiogenic factors. To study *in vivo* effects of TNP-470, 253J B-V cells were implanted orthotopically into athymic nude mice. TNP-470 was administered in several dosing and scheduling regimens, and its effects on tumor growth, metastasis, intratumor neovascularization, and mRNA expression of angiogenic factors were determined in both nonestablished and established tumors.**

Results: In vitro **treatment with TNP-470 inhibited cell growth and production of basic fibroblast growth factor protein in 253J B-V and human umbilical vascular endothelial cells in a dose-dependent manner. *In vivo* daily administration of TNP-470 significantly inhibited tumor growth ($P < 0.001$), metastasis ($P < 0.05$), intratumor neovascularization ($P < 0.005$), and mRNA expression of basic fibroblast growth factor and MMP-9 ($P < 0.005$), in both nonestablished and established tumors. Increasing the daily dose did not increase the effect on tumor growth, metastasis, and angiogenesis; however, the drug-induced toxicity did increase in a dose-dependent manner.**

Conclusions: **Frequent administration of TNP-470 at an optimal biological dose provided maximal antitumor and antimetastatic effects of human TCC of the urinary bladder. It may function by down-regulating angiogenic factors.**

INTRODUCTION

TCC[2] of the urinary bladder is the fourth most common malignancy in the United States. This malignancy is diagnosed in ~54,300 patients and results in 12,400 deaths annually (1). The standard treatment for operable invasive bladder cancer is surgery, whereas chemotherapy offers the only viable therapeutic and preventive option for distant metastasis and local recurrence of advanced bladder cancer. A combination of methotrexate, vinblastine, doxorubicin, and cisplatin chemotherapy induces complete pathological responses in primary bladder (2), nodal (3), or metastatic (4) TCC, and results in long-term survival (5). The overall response rate in clinical trials using cisplatin-based combination neoadjuvant chemotherapy was reported previously to be 40–70%, with a high percentage of complete response (4, 6). In our institution, patients with superficial disease have an uniformly good prognosis after radical cystectomy, whereas the prognosis for patients with more invasive disease is variable. Most deaths from bladder cancer are caused by invasion and subsequent metastases, which are resistant to conventional therapy (7). Up to 70% of patients with advanced TCC will achieve an initial response to chemotherapy, only to progress rapidly with chemoresistant disease (7). Therefore, it is important to develop a novel, more effective therapy strategy for tumor invasion and subsequent metastasis.

It is well established that tumor growth and metastasis depend on the induction of new blood supply (8). Angiogenesis is mediated in part by the secretion of angiogenic factors such as bFGF (9, 10), VEGF (11, 12), and IL-8 (13, 14) by tumors. MVD has been shown to predict the early progression of muscle-invasive disease (15–17). Overexpression of bFGF (9, 10) and VEGF (11, 12) has been found in the tissue, serum, and urine of patients with bladder cancer, and has also been associated with disease progression. IL-8 is a putative angiogenic factor, and we have found that IL-8 expression regulates tumorigenicity, angiogenesis, and metastasis in an orthotopic murine

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[2] The abbreviations used are: TCC, transitional cell carcinoma; MVD, microvessel density; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial cell growth factor; IL, interleukin; MMP, matrix metalloproteinase; GC, guanosine cytosine; ISH, *in situ* hybridization; FBS, fetal bovine serum; IHC, immunohistochemical staining; TNP-470, AGM-1470, *O*-chloracetyl-carbamoyl fumagillol; HUVEC, human umbilical vascular endothelial cell.

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model for human TCC (18). Moreover, we demonstrated recently that MVD and the expression of angiogenic factors could identify the patients with advanced TCC who will fail chemotherapy and cystectomy (16). Therefore, angiogenesis inhibitors are promising agents for tumor dormancy therapy.

TNP-470 is an analogue of fumagillin, which is derived from Aspergillus fumigatus, and strongly inhibits vascular endothelial cell growth and migration (17). It is also less toxic than fumagillin (19). It has been reported previously that TNP-470 has an inhibitory effect on the growth and metastasis of human cancers, such as melanoma (20), and gastric (21), colon (22), pancreatic (23), hepatocellular (24), renal (25), ovarian (26), uterine endometrial (26), breast (20), and prostate (20) cancers. In human TCC of the urinary bladder, TNP-470 has also been reported to inhibit tumor growth through inhibition of the growth of vascular endothelial cells (27, 28), but an efficient protocol for the administration of TNP-470 has not been well defined. Moreover, although it was reported previously that *in vivo* therapy with TNP-470 inhibits liver metastasis of gastric (21) and colon (22) cancer, lung metastasis of hepatocellular carcinoma (24) and choriocarcinoma (26), and lung and liver metastasis of renal cell carcinoma (25), it is still unclear whether TNP-470 inhibits the metastasis of TCC in the urinary bladder. Therefore, the purpose of the present study is to identify an efficient protocol using TNP-470, and to optimize its antitumor and antimetastatic effects on human TCC in the urinary bladder. Moreover, we hypothesize that selective down-regulation of angiogenic factors by the tumor cells after TNP-470 therapy leads to involution of tumor vessels, contributing to the growth inhibition of the primary tumors and the reduction of spontaneous metastasis from these highly metastatic tumors.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The highly metastatic human bladder carcinoma cell line 253J B-V and HUVECs were grown as a monolayer in modified Eagle's MEM supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (CMEM; Ref. 29).

Reagents. TNP-470 (AGM-1470; molecular weight: 401.89) was a kind gift of Takeda Chemical Industries, Ltd., Osaka, Japan. Stock solutions of TNP-470 were prepared in absolute ethanol and suspended in 5% gum arabic and normal saline.

***In Vitro* Cell Growth Inhibition.** The *in vitro* dose-dependent antiproliferative effect of TNP-470 was evaluated after incubating 5×10^3 253J B-V cells and HUVECs for 48 h in serum-free medium, then exchanging the medium for 10% FBS-supplemented MEM containing increasing concentrations of TNP-470 (0–100 μg/ml). Growth inhibition was determined after 48 h by microscopic cell count and expressed as the ratio of the number of viable cells in each group treated with TNP-470 to the number in a control group treated with ethanol containing 5% gum arabic and normal saline or MEM.

***In Vitro* Assay for bFGF, VEGF, and IL-8.** Viable 253J B-V cells (5×10^3) or HUVECs (5×10^3) were seeded in a 96-well plate. Conditioned medium was removed after 24 h, and then the medium was exchanged for 10% FBS-supplemented MEM containing increasing concentrations of TNP-470 (0–25 μg/ml). The cells were then washed with 200 μl of HBSS, and 200 μl of fresh MEM supplemented with 10% bovine serum was added. Forty-eight h later, the amounts of VEGF and IL-8 in cell-free culture supernatants and cell-associated bFGF in freeze-thaw cell lysates were determined using the commercial Quantine ELISA kit (R&D System, Minneapolis, MN). The protein concentration for each factor was then determined by comparing the absorbance with that of a standard. Results were expressed in terms of cell numbers (18).

Animals. Male athymic BALB/cA Jc1-nu nude mice were obtained from Clea Japan Inc., Osaka, Japan. The mice were maintained in a laminar-airflow cabinet in pathogen-free conditions and used at 8–12 weeks of age.

Orthotopic Implantation of Tumor Cells. Cultured 253J B-V cells (60–70% confluent) were prepared for injection as described previously (29). Mice were anesthetized with Nembutal. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (1×10^6/0.05 ml) in HBSS were implanted into the bladder wall. The formation of a bulla was a sign of a satisfactory injection. The bladder was returned to the abdominal cavity and the abdominal wall closed with a single layer of metal clips.

***In Vivo* Therapy of Human TCC Growing in the Bladders of Athymic Nude Mice.** To study nonestablished tumors, treatment commenced 3 days after tumor implantation. Mice were randomly separated into six groups and treated for 3 weeks with s.c. injections of TNP-470 (105 mg/kg/week) at different dosing schedules (1, 2, 7 times/week; Fig. 3*A*). Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied ~4 weeks later. To study established tumors, treatment commenced 21 days after tumor implantation. Mice were randomly separated into seven groups and treated for 3 weeks with s.c. injections of TNP-470 (105 mg/kg/week) at different dosing schedules (1, 2, 7 times/week; Fig. 3*B*).

Tissue Processing. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied ~4 weeks later. The primary tumors were removed and weighed, and the presence of metastases in the lymph nodes and lungs was determined grossly and microscopically. The bladders were then either quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or mechanically dissociated and put into tissue culture. The lungs and lymph nodes were fixed in 10% buffered formalin, or mechanically dissociated and put into tissue culture.

ISH Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequences: bFGF (CGG′GAA′GGC′GCC′GCT′GCC′GCC′), 85.7% GC content (30); VEGF/vascular permeability factor (TGG'-TGA'TGT'TGG'ACT'CTT'CAG'TGG'GCU), 57.7% GC content (31); IL-8 (CTC′CAC′ACC′CCT′CTG′CAC′CC), 66.0% GC content (13); MMP-9 (CCG′GTC′CAC′CTC′-GCT′GGC′GCT′CCG′GU), 80.0% GC content (32); MMP-2 (GGC′-CAC′ATC′TGG′GTT′GCG′GC), 70.0% GC content (32); E-cadherin (mixture; TGG′AGC′-GGG′CTG′GAG′-TCT′GAA′CTG), 62.5% GC content and (GAC′GCC′G-GC′GGC′CCC′-TTC′ACA′GTC), 75.0% GC content (33).

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The specificity of the oligonucleotide sequences was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis (34). A poly$(dT)_{20}$ oligonucleotide was used to verify the integrity of the mRNA in each sample. All of the DNA probes were synthesized with six biotin molecules (hyperbiotinylated) added to the 3′ end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 g/liter in 10 mmol/liter Tris (pH 7.6) and 1 mmol/liter EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

In situ mRNA hybridization was performed as described previously, with minor modifications (35, 36). ISH was carried out using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 37). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 35, 36). The slides were placed in a Microprobe slide holder, dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was carried out for 45 min at 45°C, and the samples were then washed with alkaline phosphatase-labeled avidin for 30 min at 45 °C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with fresh chromogen substrate if necessary to enhance a weak reaction. A positive reaction in this assay appears as a red stain. The control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone.

Quantification of Color Reaction. Stained sections were examined with a Zeiss photomicroscope (Carl Zeiss, Thomwood, NY) equipped with a three-chip, charge-coupled device color camera (DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed using Optimas image analysis software (version 4.10; Bothell, WA). The slides were prescreened by one of the investigators to determine the range in staining intensities. This range was captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All of the subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the absorbance was attributable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity was determined by comparison with the integrated absorbance of poly$(dT)_{20}$. The results for each cell line are presented relative to the control, which was set to 100 (18).

IHC. For immunohistochemical analysis, frozen tissue sections (8-μm thick) were fixed with cold acetone. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution, containing 5% normal horse serum and 1% normal goat serum in PBS (pH 7.5). Excess blocking solution was drained, and the samples were incubated for 18 h at 4 °C with the appropriate dilution (1: 100) of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 38). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody, peroxidase-conjugated anti-rat IgG (H+L; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories, San Ramon, CA), and again washed three times with PBS. The slides were mounted with Universal Mount (Research Genetics).

Quantification of MVD. MVD was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies according to the procedure of Weidner *et al.* (39). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue images were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation, Tokyo, Japan). The MVD was expressed as the average of the five highest areas identified within a single 200×/field (18).

Statistical Analysis. The statistical differences in the number of vessels; staining intensity for mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin; and the amount of cell proliferation and apoptosis within the bladder tumors were analyzed with the Mann-Whitney test. The incidence of tumors and metastases were statistically analyzed with the χ^2 test. A value of $P < 0.05$ was considered significant.

RESULTS

***In Vitro* Cell Growth Inhibition by TNP-470.** *In vitro* treatment of 253J B-V and HUVEC cells with TNP-470 for 48 h resulted in a dose-dependent antiproliferative effect, as measured by microscopic cell count and expressed as the ratio of the number of viable cells in the group treated with TNP-470 to the number of viable cells in a control group treated with ethanol containing 5% gum arabic and normal saline.

The IC50 of 253J B-V and HUVEC treated with TNP-470 was 10 μg/ml and 0.1 μg/ml, respectively. TNP-470 inhibited the proliferation of 253J B-V and HUVEC in a dose-dependent manner (Fig. 1*A* and *B*).

***In Vitro* Inhibition of Production of Angiogenic Factor Proteins by TNP-470.** *In vitro* treatment with TNP-470 for 48 h resulted in a dose-dependent inhibitory effect on 253J B-V and HUVEC cells as measured by ELISA.

The protein production of bFGF in 253J B-V and HUVEC cells was inhibited by TNP-470 in a dose-dependent manner. However, TNP-470 did not influence the protein production of VEGF and IL-8 (Fig. 2, *A* and *B*).

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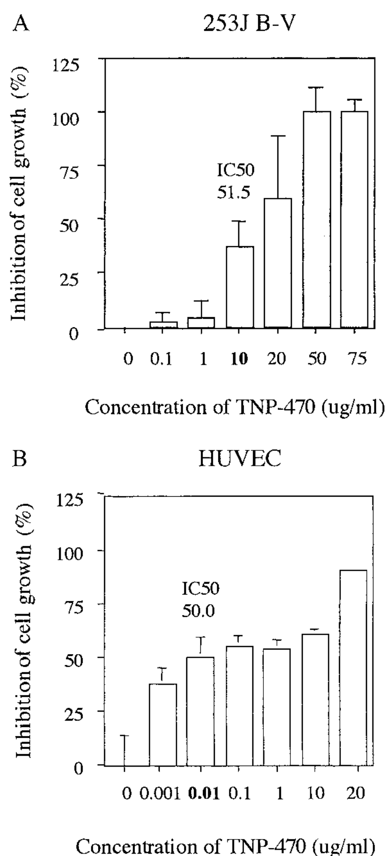

Fig. 1 *In vitro* inhibition of cell growth by treatment with the angiogenesis inhibitor TNP-470, in human TCC 253J B-V cells (*A*) and HUVECs (*B*). The IC_{50} of 253J B-V and HUVEC by TNP-470 was 10 μg/ml and 0.1 μg/ml, respectively. TNP-470 inhibited proliferation of 253J B-V and HUVEC in a dose-dependent manner; *bars,* ±SD.

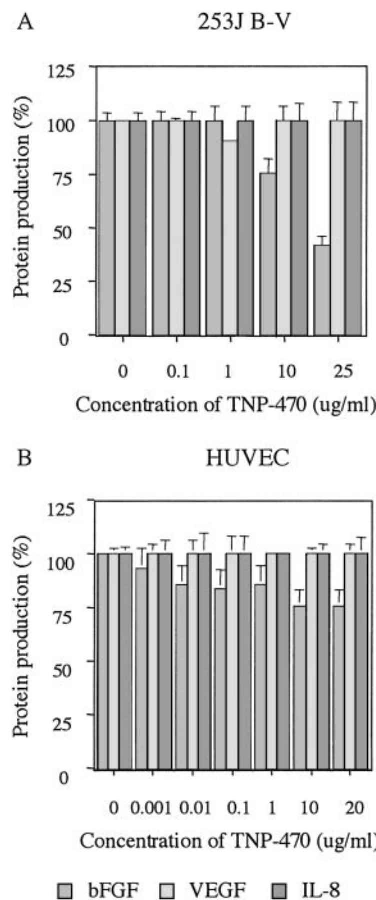

Fig. 2 *In vitro* inhibition of protein production by treatment with the angiogenesis inhibitor TNP-470, in human TCC 253J B-V cells (*A*) and HUVECs (*B*). The protein production of bFGF in 253J B-V and HUVECs was inhibited by TNP-470 in a dose-dependent manner; *bars,* ±SD.

Inhibition of Growth and Metastasis of Nonestablished Human TCC. To determine whether TNP-470 therapy of nonestablished human TCC growing within the bladder of athymic nude mice would be effective, therapy was commenced 3 days after tumor implantation (Fig. 3*A*). Treated mice were closely monitored for any signs of progressive disease and were sacrificed if they became moribund. The results of the therapy are summarized in Table 1. The therapy with TNP-470 resulted in significant regression of bladder tumors after daily administration at a dose of 15 mg/kg (median tumor weight 37 mg; $P < 0.001$), 35 mg/kg (38 mg; $P < 0.001$), and 105 mg/kg (35 mg; $P < 0.001$), compared with administration of ethanol with 5% gum arabic and normal saline as a control (147 mg). The therapy with TNP-740 given 3 times/week at a dose of 35 mg (median tumor weight 68 mg; $P < 0.001$) and weekly at a dose of 105 mg (80 mg; $P < 0.005$) also inhibited the tumor growth significantly. However, complete inhibition of tumor growth in 1 of 9 mice (11.1%) and 2 of 6 mice (33.3%) bearing nonestablished TCCs was demonstrated by daily therapy with TNP-470 at a dose of 15 mg/kg and 105 mg/kg, respectively. Lymph node metastasis was also inhibited completely by TNP-470 therapy given daily or 3 times/week ($P < 0.05$ compared with control). With daily TNP-470 therapy, changing the dose had no significant effect on tumor growth or metastasis, but body weight loss increased as the dose increased (Fig. 4).

Inhibition of Growth and Metastasis of Established Human TCC. To determine whether TNP-470 therapy would also be effective in established tumors, we commenced treatment 21 days after tumor implantation (Fig. 3*B*). At the time the therapy commenced, the tumors had a median weight of 241 mg (range, 100–340 mg). Treated mice were closely monitored for any signs of progressive disease and were sacrificed if they became moribund. The results of the therapy are summarized in Table 2. The therapy with TNP-470 resulted in significant regression of bladder tumors after daily administration at a dose of 15 mg/kg (median tumor weight 49 mg; $P < 0.001$), 35 mg/kg (45 mg; $P < 0.001$), and 105 mg/kg (44 mg; $P < 0.001$), compared with administration of ethanol with 5% gum arabic and normal saline as a control (440 mg). The therapy with TNP-470 given 3 times/week at a dose of 35 mg (median tumor weight 156 mg; $P < 0.005$) also inhibited the tumor growth significantly. However, complete inhibition of tumor growth in 1 of 6 mice (16.7%) bearing established TCCs was demonstrated by daily therapy with TNP-470 at a dose of 105 mg/kg. Although daily therapy with TNP-470 completely inhibited

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Fig. 3 *A,* the schedule of single-agent therapy with the angiogenesis inhibitor TNP-470 for nonestablished human TCC 253J B-V cells growing orthotopically in athymic nude mice. Treatment commenced 3 days after tumor implantation. Mice were randomly separated into six groups and treated for 3 weeks according to the schedule. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied 4 weeks after the initial therapy. *B,* the schedule of single-agent therapy with the angiogenesis inhibitor TNP-470 for established human TCC 253J B-V cells growing orthotopically in athymic nude mice. Treatment commenced 21 days after tumor implantation. Mice were randomly separated into seven groups and treated for 3 weeks according to the schedule. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied 4 weeks after the initial therapy.

lymph node metastasis as compared with the control ($P < 0.05$) lymph node metastasis of established tumors developed in 3 of 7 mice (42.9%) treated 3 times/week and in 5 of 7 mice (71.4%) treated weekly. With daily TNP-470 therapy, changing the dose had no significant effect on tumor growth or metastasis, but body weight loss increased as the dose increased (data not shown).

Inhibition of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-Cadherin Expression, and Reduction of MVD by TNP-470 in a Nonestablished Tumor Model. The mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin were analyzed by ISH, and MVD was analyzed by IHC (Fig. 5; Table 3). In the nonestablished-tumor model, the mRNA expression of bFGF and MMP-9 within the tumors of mice treated with daily TNP-470 was significantly reduced 56–59% ($P < 0.005$) and 54–64% ($P < 0.005$), respectively, compared with control. However, the expression was unaltered by TNP-470 administered either 3 times/week or weekly. Using any protocol, TNP-470 did not significantly alter the expression of VEGF, IL-8, MMP-2, and E-cadherin (Fig. 5; Table 3).

MVD was significantly lower in tumors treated with daily TNP-470 (55 ± 11 at a dose of 15 mg/kg, 53 ± 10 at a dose of 35 mg/kg, 46 ± 9 at a dose of 105 mg/kg) than in control tumors (108 ± 24; $P < 0.005$). Either 3 times/week or weekly TNP-470 did not reduce MVD (Fig. 5; Table 3).

Inhibition of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-Cadherin Expression and Reduction of MVD by TNP-470 in an Established Tumor Model. The mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin were analyzed by ISH, and MVD was analyzed by IHC (Fig. 6; Table 4). In this model, the mRNA expression of bFGF and MMP-9 within the tumors of mice treated with daily TNP-470 was also significantly reduced, by 68–72% ($P < 0.005$) and 60–69%

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Table 1 The single agent therapy of angiogenesis inhibitor TNP-470 for nonestablished human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Tumorigenicity[a]		LN metastasis[b]
	Incidence	Median bladder weight (Range) (mg)	Incidence
CTRL (10% ethanol in 5% arabic gum/saline (sci)) daily ($n = 9$)	9/9	147 (120–263)	4/9
TNP-470 (15 mg/kg (sci)) daily ($n = 9$)	8/9	37 (20–53)[c]	0/9[d]
TNP-470 (35 mg/kg (sci)) daily ($n = 9$)	9/9	38 (24–59)[c]	0/9[d]
TNP-470 (105 mg/kg (sci)) daily ($n = 6$)	4/6	35 (22–42)[c]	0/6
TNP-470 (35 mg/kg (sci)) 3 times/week ($n = 8$)	8/8	68 (29–111)[e]	0/8[d]
TNP-470 (105 mg/kg (sci)) weekly ($n = 9$)	9/9	80 (38–210)[f]	1/9

[a] Tumorigenicity (P, Mann-Whitney statistical comparison).
[b] LN metastasis (P, χ^2 test).
[c] $P < 0.001$ against CTRL, $P < 0.005$ against TNP-470 (35 mg/kg (sci)) 3 times/week and TNP-470 (105 mg/kg (sci)) weekly.
[d] $P < 0.05$ against CTRL.
[e] $P < 0.001$ against CTRL.
[f] $P < 0.005$ against CTRL.

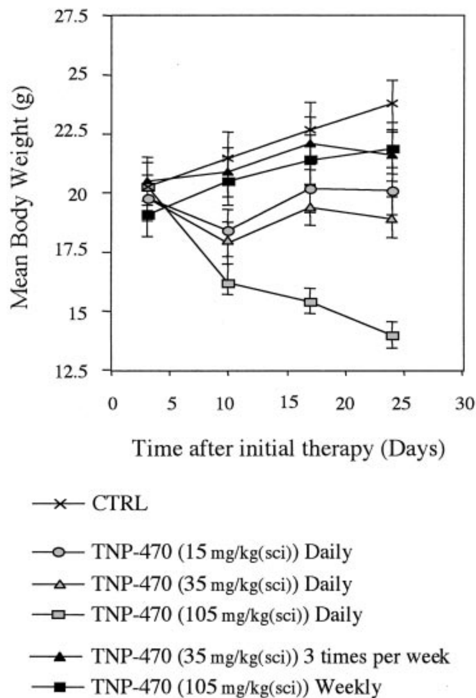

Fig. 4 Mean body weight of athymic nude mice after TNP-470 therapy of nonestablished 253J B-V cells growing orthotopically. With daily TNP-470 therapy, the antitumor and antimetastatic effects did not change with increases in dose, but the drug-induced body weight loss increased in a dose-dependent manner; *bars,* ±SD.

($P < 0.005$), respectively, compared with control; however, the expression was unaltered by TNP-470 administered either 3 times/week or weekly. TNP-470 did not alter significantly the expression of VEGF, IL-8, MMP-2, and E-cadherin with any protocol (Fig. 6; Table 4).

MVD was significantly lower in tumors treated daily with TNP-470 (90 ± 18 at a dose of 15 mg/kg, 88 ± 12 at a dose of 35 mg/kg, and 78 ± 8 at a dose of 105 mg/kg) than in control tumors (133 ± 26; $P < 0.005$). TNP-470 administered 3 times/week or weekly did not reduce MVD (Fig. 6; Table 4).

DISCUSSION

Radical cystectomy is the standard treatment for operable invasive bladder cancer, whereas methotrexate, vinblastine, doxorubicin, and cisplatin combination chemotherapy offers the only viable therapeutic and preventive option for distant metastasis and recurrence. Although TCC of the urinary bladder is a chemosensitive tumor, most deaths from bladder cancer are caused by metastases that are resistant to conventional chemotherapy (7). Therefore, the development of novel therapeutic strategies is mandatory if we are to improve the outcome for patients with advanced bladder cancer.

In the present study, we demonstrate that the systemic administration of a novel therapeutic strategy, TNP-470, significantly inhibits tumor growth, metastasis, and angiogenesis of human TCC growing in the urinary bladder of athymic nude mice. Altering the dose and schedule of administration profoundly influenced therapeutic outcome. Daily administration of TNP-470 provided maximal antitumor, antimetastatic, and antiangiogenic effects regardless of dose, whereas the drug-induced toxicity increased with dose. Therefore, daily therapy at a dose of 15 mg/kg of TNP-470 is the safest and most effective dose for inhibiting tumor growth, metastasis, and angiogenesis in both nonestablished and established TCCs.

Recently, TNP-470 has been reported to suppress the development of bladder cancer only when the treatment is started at an early stage of tumor development (27). In our study, TNP-470 significantly inhibited tumor growth of both nonestablished and established TCCs. A complete inhibition of tumor growth in 1 of 9 mice (11.1%) and 2 of 6 mice (33.3%) bearing nonestablished TCCs was demonstrated by daily therapy with TNP-470 at a dose of 15 mg/kg and 105 mg/kg, respectively. This complete inhibition of growth was seen in 1 of 6 mice (16.7%) bearing established TCCs treated with daily TNP-470 at a dose of 105 mg/kg, which may support the conclusions of the previous report. Moreover, TNP-470 has been reported to be equally effective for reducing tumor growth in superficial and invasive bladder cancer models in

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Table 2 The single agent therapy of angiogenesis inhibitor TNP-470 for established human TCC 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Tumorigenicity[a]		LN metastasis[b]
	Incidence	Median bladder weight (Range) (mg)	Incidence
Nontreated CTRL ($n = 7$)	7/7	241 (100–340)	3/7
CTRL (10% ethanol in 5% arabic gum/saline (sci)) daily ($n = 8$)	8/8	440 (154–553)	7/8
TNP-470 (15 mg/kg (sci)) daily ($n = 8$)	8/8	49 (27–101)[c]	0/8[d]
TNP-470 (35 mg/kg (sci)) daily ($n = 8$)	8/8	45 (20–83)[c]	0/8[d]
TNP-470 (105 mg/kg (sci)) daily ($n = 6$)	5/6	44 (21–55)[c]	0/6[e]
TNP-470 (35 mg/kg (sci)) 3 times per week ($n = 7$)	7/7	156 (36–222)[f]	3/7
TNP-470 (105 mg/kg (sci)) weekly ($n = 7$)	7/7	263 (214–428)	5/7

[a] Tumorigenicity (*P*, Mann-Whitney statistical comparison).
[b] LN metastasis (*P*, χ^2 test).
[c] $P < 0.001$ against nontreated CTRL and CTRL, $P < 0.005$ against TNP-470 (35 mg/kg (sci)) 3 times per week, and $P < 0.001$ against TNP-470 (105 mg/kg (sci)) weekly.
[d] $P < 0.05$ against nontreated CTRL, $P < 0.001$ against CTRL, $P < 0.05$ against TNP-470 (35 mg/kg (sci)) 3 times per week, and $P < 0.005$ against TNP-470 (105 mg/kg (sci)) weekly.
[e] $P < 0.005$ against CTRL and $P < 0.01$ against TNP-470 (105 mg/kg (sci)) weekly.
[f] $P < 0.005$ against CTRL.

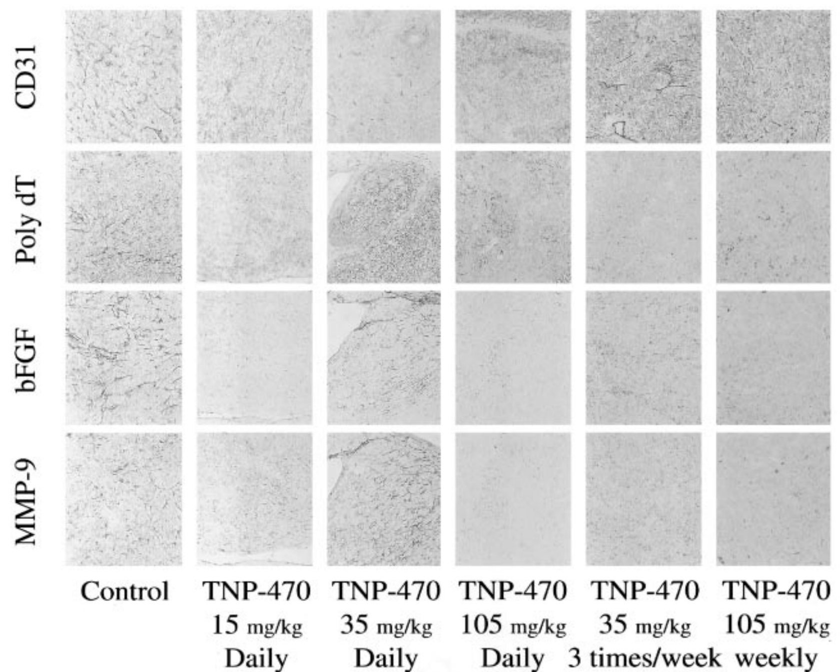

Fig. 5 *In vivo* mRNA expression level and MVD after TNP-470 therapy of nonestablished 253J B-V cells growing orthotopically in athymic nude mice. The specific mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin was analyzed by ISH. MVD was determined by immunostaining with anti-CD31 antibodies. The expression levels of bFGF and MMP-9, and the MVD were reduced 40–50% in treated tumors, especially those treated daily administration with a dose of 15 mg/kg of TNP-470 ($P < 0.005$) compared with control tumors.

mice (28), whereas it is still unknown whether TNP-470 inhibits metastasis of TCC. In the present study, we demonstrated that therapy with TNP-470 significantly inhibits not only tumor growth but also spontaneous lymph node metastasis in an invasive bladder cancer model. In particular, daily therapy with TNP-470 at any dose completely inhibits the development of spontaneous lymph node metastasis of both nonestablished and established TCCs. These results indicate that chronic frequent administration of TNP-470 at an adequate biological dose is the optimal protocol for human TCCs. This exact mechanism why daily administration of TNP-470 is more effective than 3 times a week or weekly administration is unclear. However, Slaton *et al.* (40) showed that chronic frequent administration of IFN-α at biologically optimal doses inhibited tumorigenicity and mediated down-regulation of angiogenesis-related genes in TCC of the urinary bladder. They suggested that the half-life of IFN-α is measured in hours; consistent exposure of the tumor and host to IFN could, thus, be presumed to require frequent administration. Moreover, Boehm *et al.* (41) suggested that antiangiogenic therapy including TNP-470 of experimental cancer does not induce acquired drug resistance. These previous reports supported our results.

The present data confirm the finding that the effect of TNP-

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Table 3 *In vivo* mRNA expression level and microvessel density after the therapy with angiogenesis inhibitor TNP-470 for nonestablished human TCC 253J B-V cells growing orthotopically in athymic nude mice

Therapy	mRNA expression index[a]						Microvessel density[b]
	bFGF	VEGF	IL-8	MMP-9	MMP-2	E-cadherin	(per 200 ×/field)
CTRL (ethanol in arabic gum/saline) daily	100	100	100	100	100	100	108 ± 24
TNP-470 (15 mg/kg) daily	59[c]	96	99	64[c]	96	106	55 ± 11[c]
TNP-470 (35 mg/kg) daily	56[c]	96	98	62[c]	95	108	53 ± 10[c]
TNP-470 (105 mg/kg) daily	56[c]	94	99	54[c]	94	106	46 ± 9[c]
TNP-470 (35 mg/kg) 3 times per week	85	100	101	82	103	103	70 ± 15
TNP-470 (105 mg/kg) weekly	96	101	100	99	106	105	93 ± 19

[a] The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of poly(dT) color reaction in each sample. The results were presented as a number of each cell line compared with CTRL defined as 100.

[b] Microvessel density was expressed as an average number of five highest areas identified within a single 200 ×/field.

[c] $P < 0.005$ against CTRL (Mann-Whitney statistical comparison).

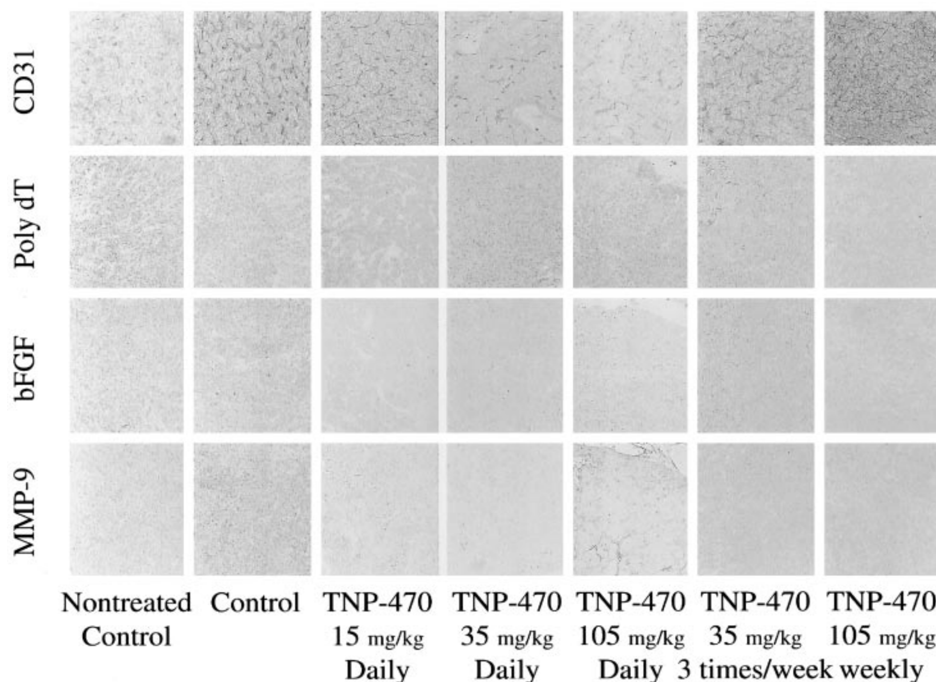

Fig. 6 *In vivo* mRNA expression level and MVD after TNP-470 therapy of established 253J B-V cells growing orthotopically in athymic nude mice. The specific mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin was analyzed by ISH. MVD was determined by immunostaining with anti-CD31 antibodies. The expression levels of bFGF and MMP-9, and the MVD were reduced 30–40% in treated tumors, especially those treated with daily administration at a dose of 15 mg/kg of TNP-470 ($P < 0.005$) compared with control tumors.

470 is mediated, in part, by the inhibition of angiogenesis. It was demonstrated previously that vascular endothelial cells produce various growth factors, including bFGF, that stimulate their proliferation and migration (42, 43). In TCC, bFGF regulates growth and metastasis, in part by regulating the process of angiogenesis (44, 45). TNP-470 reduces the recognition of bFGF by low-affinity growth factor binding sites (46). The reduction of bFGF signaling by TNP-470 results in the inhibition of vascular endothelial cell growth and migration, and, hence, bFGF-induced angiogenesis (17, 47). Recently, we reported that therapy with IFN-α (40), the anti-epidermal growth factor receptor monoclonal antibody C225 (48), or the adenovirus-mediated antisense bFGF gene (49) inhibits tumor growth and metastasis of human TCC secondary to the down-regulation of bFGF and MMP-9 expression, with a subsequent regression of tumor-induced neovascularization. Our own *in vitro* data demonstrate that adenovirus-mediated antisense bFGF gene therapy directly inhibits proliferation and enhanced apoptosis in HUVEC cells (49). The down-regulation of bFGF with either neutralizing antibodies or antisense oligonucleotides also inhibits the growth of human malignant glioblastoma (50) and melanoma (51), and liposome-mediated gene transfer with vectors containing antisense-oriented bFGF or fibroblast growth factor receptor-1 inhibits angiogenesis and growth of human melanoma (52). These effects are mediated by a reduction of bFGF expression by the tumor cells, which results in the down-regulation of MMP-9 and an inhibition of tumor-induced neovascularization. MMP-9 facilitates angiogenesis and invasion by altering the extracellular matrix or by initiating signaling pathways that promote angiogenesis by facilitating the migration of endothelial cells toward the source of the angiogenic stimulus. MMP-9 is regulated by various factors, including tumor necrosis factor-α (53), IL-1 (54), transforming growth factor-β1 (54), epidermal growth factor (54, 55), hepatocyte growth factor (55), IL-8 (18), and bFGF (30). Therefore, bFGF, which up-regulates MMP-9 and induces neovascularization,

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Table 4 *In vivo* mRNA expression level and microvessel density after the therapy with angiogenesis inhibitor TNP-470 for established human TCC 253J B-V cells growing orthotopically in athymic nude mice

Therapy	mRNA expression index[a]						Microvessel density[b]
	bFGF	VEGF	IL-8	MMP-9	MMP-2	E-cadherin	(per 200 ×/field)
Nontreated CTRL	144	86	88	119	100	96	84 ± 12
CTRL (ethanol in arabic gum/saline) daily	100	100	100	100	100	100	133 ± 26
TNP-470 (15 mg/kg) daily	72[c]	100	100	69[c]	100	101	90 ± 18[c]
TNP-470 (35 mg/kg) daily	70[c]	99	99	66[c]	99	103	88 ± 12[c]
TNP-470 (105 mg/kg) daily	68[c]	99	96	60[c]	99	103	78 ± 8[c]
TNP-470 (35 mg/kg) 3 times per week	96	103	101	93	99	101	101 ± 11
TNP-470 (105 mg/kg) weekly	99	105	101	100	101	104	115 ± 13

[a] The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of poly(dT) color reaction in each sample. The results were presented as a number of each cell line compared with CTRL defined as 100.

[b] Microvessel density was expressed as an average number of five highest areas identified within a single 200 ×/field.

[c] $P < 0.005$ against CTRL (Mann-Whitney statistical comparison).

is a good target for the inhibition of angiogenesis, tumor growth, and metastasis.

In summary, the present study provides evidence that TNP-470 therapy can inhibit tumor growth, metastasis, and tumor-induced neovascularization of human TCC growing in athymic nude mice. The antiangiogenic activity of TNP-470 is dependent on frequent administration of an optimal biological dose rather than the maximal tolerated dose. Moreover, we demonstrated that this therapy blocks the release of both bFGF and MMP-9 by the tumor cells and host vasculature, and represents a novel and effective antiangiogenic therapy for treating TCC. It may also be useful for prevention of human TCC of the urinary bladder.

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