Docetaxel Enhances the Therapeutic Effect of the Angiogenesis Inhibitor TNP-470 (AGM-1470) in Metastatic Human Transitional Cell Carcinoma

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ABSTRACT

We demonstrated recently that chronic frequent administration of an adequate biological dose of the angiogenesis inhibitor TNP-470 (AGM-1470, *O*-chloracetyl-carbamoyl fumagillol) completely inhibits spontaneous lymph node metastasis but does not have a complete response on tumor growth of nonestablished or established human metastatic transitional cell carcinoma (TCC) 253J B-V growing orthotopically into athymic nude mice. Therefore, in this study, we evaluated whether docetaxel (Taxotere) enhances the therapeutic effect of TNP-470, especially on tumor growth.

Docetaxel enhanced *in vitro* antiproliferation but not basic fibroblast growth factor down-regulation by TNP-470 in 253J B-V and human umbilical vascular endothelial cells. Docetaxel significantly enhanced *in vitro* apoptosis by TNP-470 in human umbilical vascular endothelial cells but not in 253J B-V. *In vivo* combination was most effective when docetaxel was administered before TNP-470, and increased significantly the complete response on tumor growth of nonestablished and established TCCs growing orthotopically into athymic nude mice compared with either therapy alone ($P < 0.05$). The incidence of spontaneous lymph node metastasis was inhibited completely by the combination therapy ($P < 0.05$). Drug-induced body weight loss was not significantly different in any treatment groups. The combination of TNP-470 and docetaxel inhibited intratumor neovascularization, the expression of bFGF and matrix metalloproteinases type-9 compared with controls ($P < 0.005$), and enhanced apoptosis in tumors compared with each therapy alone ($P < 0.005$).

These studies indicate that docetaxel markedly enhances the ability of TNP-470 to inhibit tumorigenicity and metastasis in both nonestablished and established TCCs. These effects are mediated, in part, by the complementary cytotoxicities of angiogenesis inhibition, down-regulation of bFGF and matrix metalloproteinases type-9, and induction of apoptosis.

INTRODUCTION

Radical cystectomy is the standard treatment for operable invasive TCC[2] of the urinary bladder, whereas the combination of methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) chemotherapy offers the only viable therapeutic and preventive option for distant metastasis and local recurrence. Although TCC of the urinary bladder is a chemosensitive tumor, most deaths from bladder cancer are caused by invasion and subsequent metastases that are resistant to conventional chemotherapy (1, 2). Therefore, the development of a novel, more effective therapeutic strategy for invasion and subsequent metastasis are mandatory if we are to improve the outcome for patients with advanced bladder cancer.

It is well established that tumor growth and metastasis depend on the establishment of a new blood supply (3). This process of angiogenesis is mediated in part by the secretion of angiogenic factors such as bFGF (4, 5), VEGF (6, 7), IL-8 (8, 9), and metastasis-related factors such as MMP-9 (10, 11) and MMP-2 (10, 11), and E-cadherin (12, 13), which are produced by tumors growing in their relevant microenvironment. Therefore, angiogenesis inhibitors are promising agents for tumor dormancy therapy. TNP-470 (AGM-1470, *O*-chloracetyl-carbamoyl fumagillol), C225 (14, 15) and DC101 (16), are promising antiangiogenic agents in clinical trials. TNP-470, a less toxic analogue of fumagillin (17), is derived from *Aspergillus fumigatus,* and inhibits vascular endothelial cell growth and migration (18). It has been reported previously that TNP-470 has an inhibitory effect on the growth and metastasis of human cancers, including breast (19), gastric (20), colon (21), hepatocellular (22), renal (23), ovarian (24), and prostate (25) cancers. TNP-470 has also been reported to inhibit tumor growth through inhibition of the growth of vascular endothelial cells in human TCC of the urinary bladder (26, 27). *In vivo* therapy with TNP-470 reportedly inhibits liver metastasis of gastric (20) and colon (21) cancer, lung metastasis of hepatocellular carcinoma (22) and ovarian cancer (24), and lung and liver metastasis of renal cell carcinoma (23). However, it is still

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[2] The abbreviations used are: TCC, transitional cell carcinoma; MVD, microvessel density; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; IL, interleukin; MMP, matrix metalloproteinase; ISH, *in situ* hybridization; IHC, immunohistochemical; HUVEC, human umbilical vascular endothelial cell; Mab, monoclonal antibody; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase (Tdt) -mediated nick end labeling; PCNA, proliferating cell nuclear antigen.

unclear whether TNP-470 inhibits metastasis of TCC. We demonstrated recently that the chronic frequent administration of an adequate biological dose of the angiogenesis inhibitor TNP-470 significantly inhibits angiogenesis, tumor growth, and metastasis of human TCC in the urinary bladder of athymic nude mice. Therapy with the single-agent TNP-470 completely inhibited the development of spontaneous lymph node metastasis, but did not give a complete response on tumor growth of either nonestablished or established TCCs (28). Therefore, in this study, we evaluated whether the chemotherapeutic agent docetaxel (Taxotere) enhances the therapeutic effect of TNP-470, especially on tumor growth.

The taxanes, paclitaxel (Taxol) and docetaxel, are effective chemotherapeutic agents used in the treatment of a number of major solid tumors including lung (29), colon (29), pancreatic (29), prostate (30), and bladder (31, 32) cancers. Taxanes bind to the β-subunit of tubulin and interfere with microtubular polymerization by promoting abnormal assembly of microtubules and inhibiting their subsequent disassembly in the mitotic spindle, resulting in arrest of the G_2-M phase of the cell cycle, leading to programmed cell death (33, 34). It has been reported recently that docetaxel causes apoptosis by inducing phosphorylation of an apoptosis suppressing oncogene, *bcl-2,* in prostate (30) and bladder (31, 32) cancers. Moreover, docetaxel was described as the most potent inducer of *bcl-2* phosphorylation; docetaxel was >100 times more potent in this effect than paclitaxel (32). We demonstrated previously that paclitaxel enhanced the effects of the antiangiogenic agent MAb C225 (which blocks EGFR function; Ref. 15) and MAb DC101 (which blocks VEGF receptor-2 function; Ref. 16) to inhibit tumorigenicity and metastasis of human TCC.

Therefore, we hypothesized from these data that taxane cytotoxicity would compliment antiangiogenic agents and provide additive or synergic therapeutic effects on tumorigenicity and metastasis. In the present study described herein, the combination therapy of docetaxel followed by TNP-470 markedly inhibited tumorigenicity and metastasis in nonestablished and established human TCC tumors compared with therapy with each agent alone. This effect is mediated, at least in part, by the complementary cytotoxicities of angiogenesis inhibition, downregulation of bFGF and MMP-9, and the induction of apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The highly metastatic human bladder carcinoma cell line 253J B-V was grown as a monolayer in modified Eagle's MEM supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (35). HUVECs were grown as a monolayer in sterile endothelial growth medium (EGM-2, Clonetics, San Diego, CA; Ref. 36). These cells were maintained at 37°C in a 5% CO_2 environment.

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. Docetaxel (taxotere) was a kind gift of Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

In Vitro **Cell Growth Inhibition.** The *in vitro* dose-dependent antiproliferative effect of TNP-470 and/or docetaxel was evaluated after incubating 5×10^3 253J B-V and HUVEC cells 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) and/or docetaxel (0–50 μg/ml). Attached cells were trypsinized for microscopic cell count. Growth inhibition was determined after 48 h by cell count in a hemocytometer and expressed as the ratio of the number of viable cells in each group treated with TNP-470 and/or docetaxel to the number in the 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 (5×10^3) or HUVEC (5×10^3) cells were seeded in a 96-well plate. Conditioned medium was removed after 24 h. The medium was exchanged for 10% FBS-supplemented MEM containing increasing concentrations of TNP-470 (0–25 μg/ml) and/or docetaxel (0–25 μg/ml). The cells were then washed with 200 μl of HBSS and 200 μl of 10× bovine serum supplemented with fresh MEM. Forty-eight h later, the amount 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 (9).

In Vitro **Apoptosis.** The *in vitro* dose-dependent apoptotic effect of TNP-470 and/or docetaxel was evaluated by incubating 1×10^6 253J B-V cells for 24 h in serum-free medium, then exchanging the medium for 10% FBS-supplemented MEM containing increasing concentrations of TNP-470 (0–25 μg/ml) and/or docetaxel (0–25 μg/ml). Cells were harvested by centrifugation and incubated at 4°C for 24 h in 10× bovine serum supplemented with fresh MEM. Quantification of DNA fragmentation was accomplished using the Apoptosis *in Situ* Detection kit.

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 (35). 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 separated randomly into 6 groups and treated for 4 weeks with s.c. injections of TNP-470 (105 mg/kg/week) and/or i.p. injections of docetaxel (20 mg/kg/week) according to the schedule shown in Fig. 4*A*. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied ~5 weeks later. To study established tumors, treatment commenced 21 days after tumor implantation.
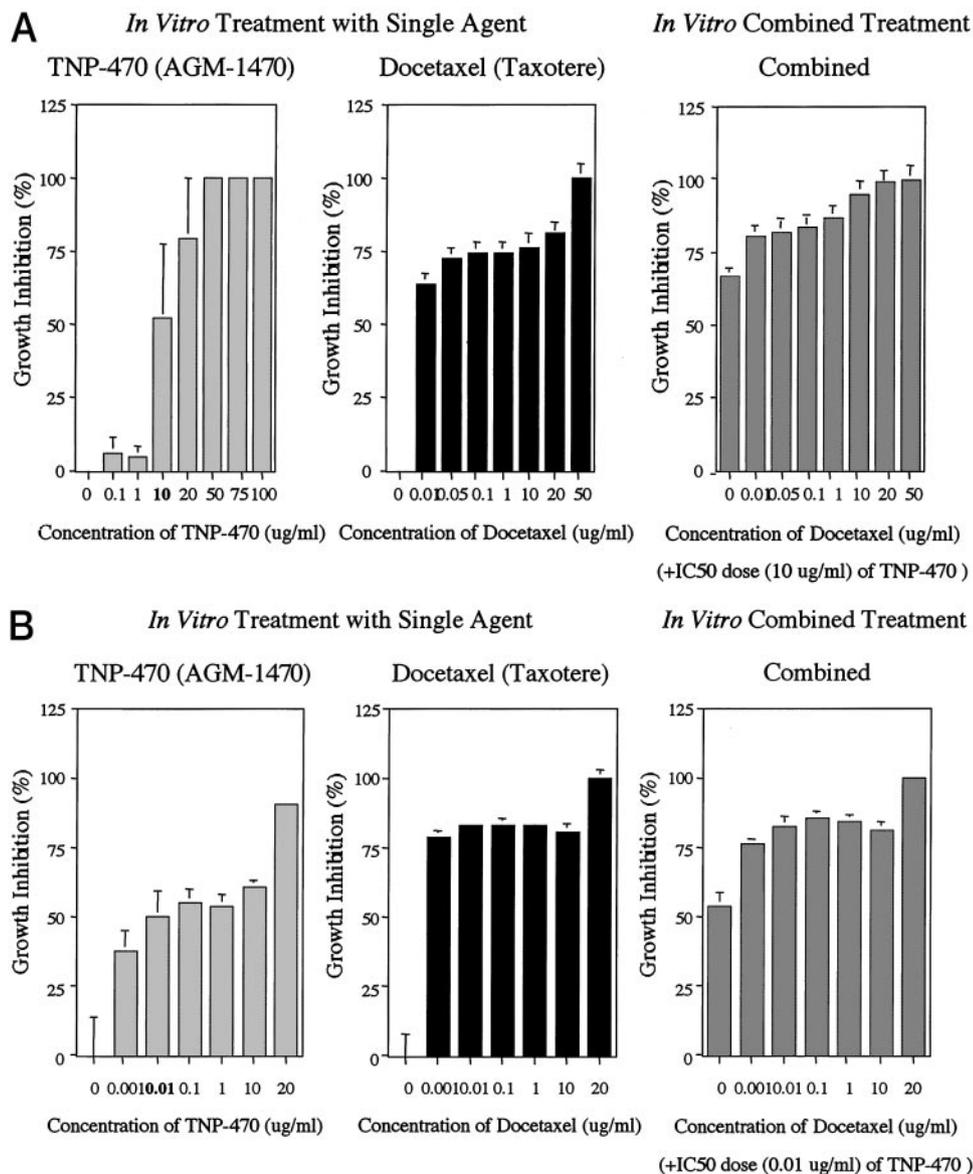
Fig. 1 In vitro inhibition of cell growth by the combined treatment with the angiogenesis inhibitor TNP-470 and docetaxel, in human TCC 253J B-V cells (*A*) and HUVECs (*B*). TNP-470 or docetaxel inhibited antiproliferation of 253J B-V and HUVEC cells in a dose-dependent manner. Docetaxel enhanced the antiproliferative effects of TNP-470 in a dose-dependent manner (*A* and *B*); *bars,* ±SD.

Mice were randomly separated into 7 groups and treated for 4 weeks at the same dose and schedule as that for nonestablished tumors (Fig. 4*B*).

Tissue Processing. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied ~5 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.

mRNA ISH Analysis. Specific antisense oligonucleotide DNA probes were designed to complement the mRNA transcripts based on published reports of the cDNA sequences:

bFGF (CGG′GAA′GGC′GCC′GCT′GCC′GCC′), 85.7% guanosine cytosine (GC) content (4); VEGF (TGG′TGA′TGT-′TGG′ACT′CTT′CAG′TGG′GCU), 57.7% GC content (6); IL-8 (CTC′CAC′ACC′CCT′CTG′CAC′CC), 66.0% GC content (8); MMP-9 (CCG′GTC′CAC′CTC′GCT′GGC′GCT′C-CG′GU), 80.0% GC content (11); MMP-2 (GGC′-CA-C′ATC′TGG′GTT′GCG′GC), 70.0% GC content (11); and E-cadherin (mixture; TGG′AGC′-GGG′CTG′GAG′TCT′GAA′-CTG), 62.5% GC content and (GAC′GCC′GGC′GGC′CCC′-TTC′ACA′GTC), 75.0% GC content (13). 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 se-
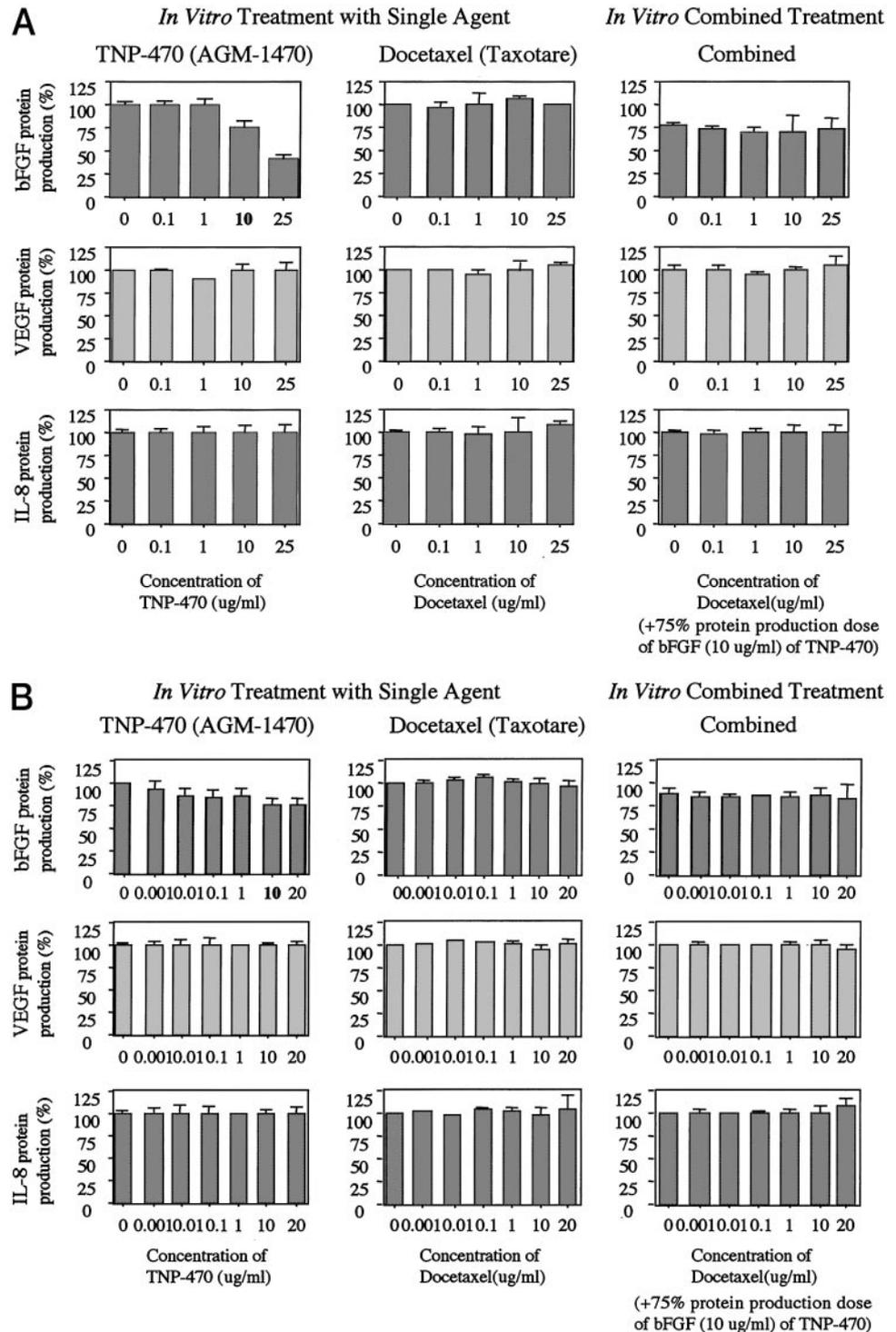
Fig. 2 In vitro protein production after combined treatment with the angiogenesis inhibitor TNP-470 and docetaxel in human TCC 253J B-V cells (*A*) and HUVECs (*B*). Although TNP-470 did not affect protein expression of VEGF or IL-8, protein production of bFGF in 253J B-V and HUVEC cells was inhibited by TNP-470 in a dose-dependent manner. Docetaxel did not influence protein expression or the effects of TNP-470 (*A* and *B*); *bars,* ±SD.

quences. The specificity of each of the sequences was also confirmed by Northern blot analysis (37). 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).

mRNA ISH was performed as described previously, with

Table 1 *In vitro* induction of apoptosis by the treatment with angiogenesis inhibitor TNP-470 (AGM-1470) and/or Docetaxel (Taxotere) for human transitional cell carcinoma 253J B-V cells and HUVECs

	Apoptosis index[a]	
Treatment	In 253J B-V Mean $\pm$ SD (Range) (percentage)	In HUVEC Mean $\pm$ SD (Range) (percentage)
CTRL (EtOH in arabic gum/saline)	7 $\pm$ 1 (5–10)	11 $\pm$ 4 (5–19)
TNP-470 [10 μg/ml(253J B-V), 0.01 μg/ml(HUVEC)]	9 $\pm$ 3 (5–15)	42 $\pm$ 8 (23–53)[b]
Docetaxel [0.01 μg/m (253J B-V), 0.001 μg/ml(HUVEC)]	69 $\pm$ 14 (36–95)[c]	36 $\pm$ 10 (20–55)[b]
TNP-470 + Docetaxel	61 $\pm$ 17 (41–81)[c]	73 $\pm$ 17 (51–93)[d]

[a] The density of apoptosis by TUNEL assay was expressed as an average percentage of five highest area identified within a single 200 $\times$/field.
[b] $P < 0.005$ against CTRL.
[c] $P < 0.005$ against CTRL and TNP-470.
[d] $P < 0.001$ against CTRL, $P < 0.05$ against both single therapy groups with either Docetaxel or TNP-470 (Mann-Whitney statistical comparison).
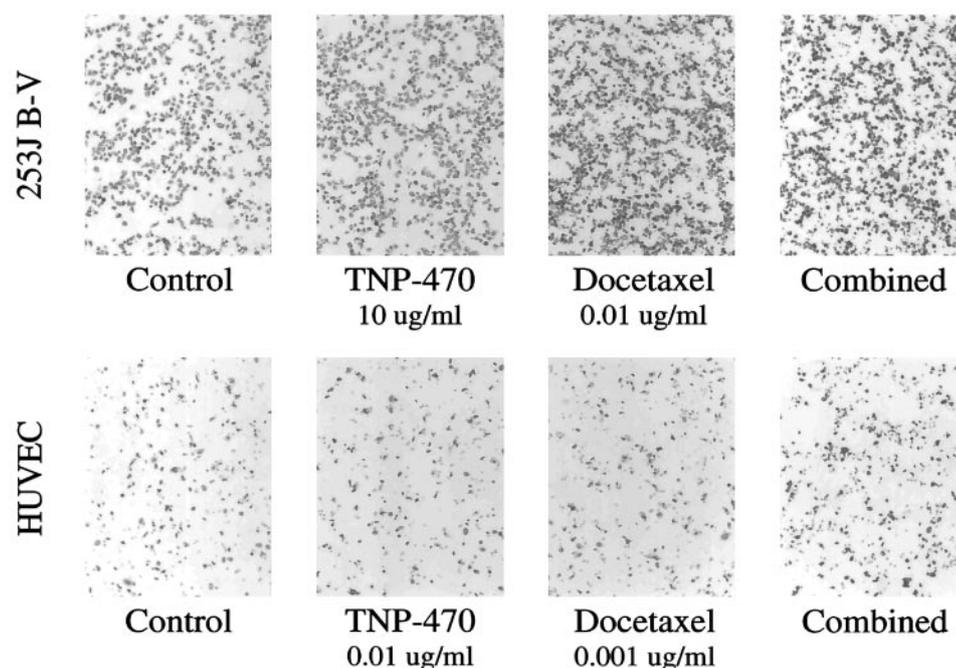

Fig. 3 In vitro induction of apoptosis after treatment with the angiogenesis inhibitor TNP-470 and/or docetaxel in human TCC 253J B-V cells and HUVECs. TNP-470 did not significantly induce apoptosis in 253J B-V cells *in vitro*. Docetaxel induced apoptosis in 253J B-V and HUVEC cells, and significantly enhanced *in vitro* apoptosis induced by TNP-470 in HUVEC cells but not in 253J B-V.

minor modifications (38, 39). ISH was carried out using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 40). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 38, 39). The slides were placed in the 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, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corporation, 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

Fig. 4 The schedule of the combination therapy with the angiogenesis inhibitor TNP-470 and docetaxel for human TCC 253J B-V cells growing orthotopically in athymic nude mice. Treatment commenced 3 days for nonestablished tumor (*A*) or 21 days for established tumor (*B*) after tumor implantation. Mice were randomly separated into 7 groups and treated for 4 weeks according to the schedule. Tumors were harvested from a group of controls at the time therapy commenced, whereas treated mice were necropsied 5 weeks after the initial therapy.
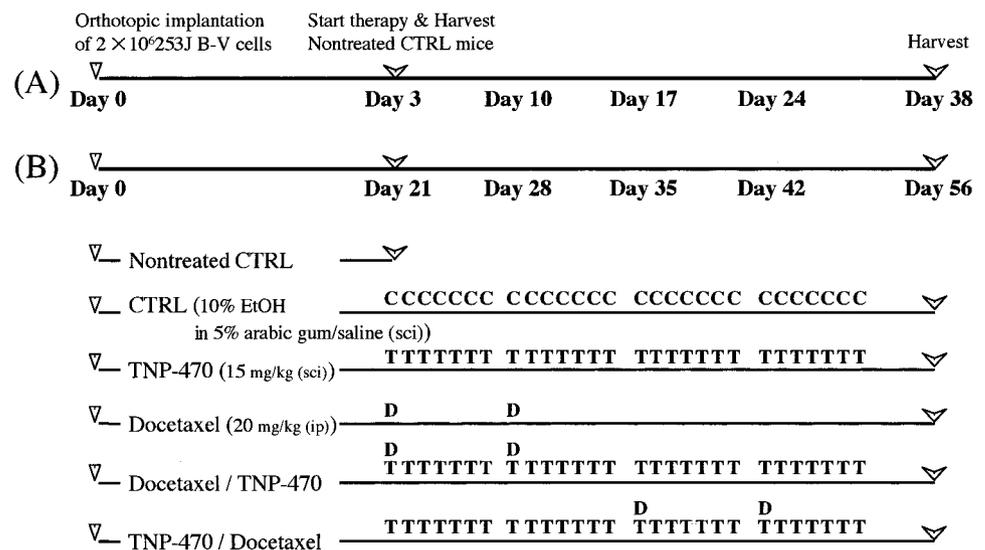
The schedule of the combination therapy of TNP-470 and Docetaxel for non-established (A) and established (B) human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice.

Table 2 The combination therapy of angiogenesis inhibitor TNP-470 and Docetaxel for nonestablished human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Tumorigenicity (Mann-Whitney statistical comparison)		LN metastasis (χ^2 test)
	Incidence	Median bladder weight (Range) (mg)	Incidence
CTRL [10% EtOH in 5% arabic gum/saline (sci)] Daily ($n = 7$)	7/7	204 (118–302)	4/7
TNP-470 [15 mg/kg (sci)] Daily ($n = 9$)	8/9	48 (21–170)[a]	1/9[b]
Docetaxel [20 mg/kg (i.p.)] weekly ($n = 10$)	10/10	87 (54–150)[c]	3/10
Docetaxel—TNP-470 ($n = 9$)	5/9[d]	28 (22–44)[e]	0/9[f]
TNP-470—docetaxel ($n = 10$)	9/10	45 (27–55)[a]	0/10[f]

[a] $P < 0.005$ against CTRL.
[b] $P < 0.05$ against CTRL.
[c] $P < 0.05$ against CTRL.
[d] $P < 0.05$ against CTRL and docetaxel [20 mg/kg (i.p.)] weekly.
[e] $P < 0.001$ against CTRL, $P < 0.05$ against TNP-470 [15 mg/kg (sci)] daily and $P < 0.001$ against docetaxel [20 mg/kg (i.p.)] weekly.
[f] $P < 0.01$ against CTRL.

was determined by comparison with the integrated absorbance of poly $(T)_{20}$. The results for each cell line are presented relative to the control, which was set to 100 (9).

IHC. For IHC 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. 41). 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 (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.* (42). 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
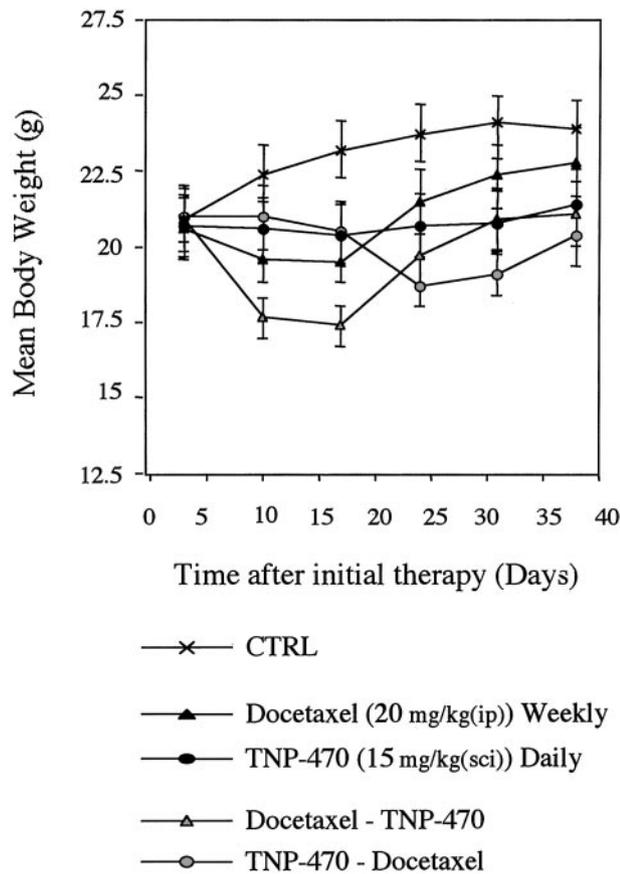
Fig. 5 Mean body weight of athymic nude mice with nonestablished human TCC 253J B-V cells growing orthotopically after treatment with the combination therapy of the angiogenesis inhibitor TNP-470 and docetaxel. Drug-induced body weight loss was not significantly different in any therapeutic groups.

(Sony Corporation). The MVD was expressed as the average of the 5 highest areas identified within a single $200\times$ field (9).

TUNEL Assay. For the TUNEL assay, tissue sections (5-μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with distilled water with BRIJ (DW/BRIJ), and treated with a 1:500 protein-ase K solution (20 μg/ml) for 15 min, and endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with DW/BRIJ and incubated for 10 min at room temperature with TDT buffer. Excess TDT buffer was drained, and the samples were incubated for 18 h at 4°C with terminal transferase and biotin-16-dUTP. The samples were then rinsed four times with TB buffer and incubated for 30 min at 37°C with a 1:400 dilution of peroxi-dase-conjugated streptavidin. 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), and again washed three times with PBS. The slides were mounted with a Universal Mount (Research Genetics).

Quantification of Cell Proliferation and Apoptosis. Cell proliferation and apoptosis were determined by IHC staining of tissue sections with anti-PCNA antibodies and the TUNEL assay. The tissue was recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering) linked to a computer and digital printer (Sony Corporation). The density of proliferative cells and apoptotic cells was expressed as an average number of the 5 highest areas identified within a single $200\times$ field (9).

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 and/or Docetaxel.** *In vitro* treatment of 253J B-V and HUVEC cells with TNP-470 and/or docetaxel 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 treated group (TNP-470 and docetaxel) to the number of viable cells in the control group (ethanol containing 5% gum arabic and normal saline).

The IC_{50} of 253J B-V and HUVEC cells treated with TNP-470 was 10 μg/ml and 0.01 μg/ml, respectively. The IC_{50} of 253J B-V and HUVEC cells treated with docetaxel was <0.01 μg/ml and 0.001 μg/ml, respectively. TNP-470 and docetaxel inhibited the proliferation of 253J B-V and HUVEC cells in a dose-dependent manner. *In vitro* antiproliferation of 253J B-V and HUVEC cells was increased by the combination treatment of TNP-470 and docetaxel compared with either agent alone (Fig. 1, *A* and *B*).

In Vitro **Inhibition of Protein Production of Angiogenic Factors by TNP-470 and/or Docetaxel.** *In vitro* treatment with TNP-470 and/or docetaxel for 48 h resulted in a dose-dependent inhibitory effect on 253J B-V and HUVEC cells as measured by ELISA. Although TNP-470 did not affect protein expression of VEGF or IL-8, bFGF production in 253J B-V and HUVEC cells was inhibited by TNP-470 dose-dependently. Docetaxel did not influence protein expression or the effects of TNP-470 on protein expression (Fig. 2, *A* and *B*).

In Vitro **Induction of Apoptosis by TNP-470 and/or Docetaxel.** The dose-dependent apoptotic effect of TNP-470 and/or docetaxel on 253J B-V and HUVEC cells was determined using the Apoptosis *In Situ* Detection kit. The results were expressed as the ratio of apoptotic to total cells. TNP-470 induced apoptosis in $9 \pm 3\%$ (range, 5–15%) and $42 \pm 8\%$ (range, 23–53%) of 253J B-V and HUVEC cells, respectively. Docetaxel induced apoptosis in $69 \pm 14\%$ (range, 36–95%) and $36 \pm 10\%$ (range, 20–55%) of 253J B-V and HUVEC cells, respectively. Docetaxel significantly enhanced apoptosis in $73 \pm 17\%$ (range, 51–93%) of HUVEC but not in 253J B-V cells (Table 1; Fig. 3).

Table 3 The combination therapy of angiogenesis inhibitor TNP-470 and docetaxel for established human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Tumorigenicity (Mann-Whitney)		LN metastasis (χ^2 test)
	Incidence	Median bladder weight (range) (mg)	Incidence
Nontreated CTRL ($n = 5$)	5/5	275 (170–290)	3/5
CTRL [10% EtOH in 5% arabic gum/saline(sci)] daily ($n = 7$)	7/7	408 (273–602)	7/7
TNP-470 [15 mg/kg (sci)] daily ($n = 8$)	8/8	69 (30–253)[a]	1/8[b]
Docetaxel [20 mg/kg (i.p.)] weekly ($n = 9$)	9/9	162 (42–237)[c]	3/9[d]
Docetaxel—TNP-470 ($n = 7$)	4/7[e]	28 (24–136)[f]	0/7[g]
TNP-470—docetaxel ($n = 5$)	5/5	75 (28–273)[a]	1/5[d]

[a] $P < 0.05$ against nontreated CTRL, $P < 0.005$ against CTRL.
[b] $P < 0.005$ against CTRL.
[c] $P < 0.005$ against CTRL.
[d] $P < 0.01$ against CTRL.
[e] $P < 0.05$ against CTRL, TNP-470 [15 mg/kg (sci)] daily and docetaxel [20 mg/kg (i.p.)] weekly.
[f] $P < 0.005$ against nontreated CTRL and CTRL and $P < 0.05$ against TNP-470 [15 mg/kg (sci)] daily and docetaxel [20 mg/kg (i.p.)] weekly.
[g] $P < 0.05$ against nontreated CTRL and $P < 0.0005$ against CTRL.

Table 4 *In vivo* mRNA expression level and microvessel density after the therapy with angiogenesis inhibitor TNP-470 and docetaxel for nonestablished human transitional cell carcinoma 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 (EtOH in arabic gum/saline) daily	100	100	100	100	100	100	119 ± 19
TNP-470 (15 mg/kg) daily	63[c]	99	99	64[c]	97	107	55 ± 15[c]
Docetaxel (20 mg/kg) weekly	96	97	99	96	95	109	77 ± 14
Docetaxel—TNP-470	55[c]	95	99	46[c]	99	105	49 ± 11[c]
TNP-470—docetaxel	82[c]	101	101	73[c]	103	105	63 ± 13[c]

[a] The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of poly d(T) 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).

Inhibition of Growth and Metastasis of Nonestablished Human TCC. To determine whether TNP-470 therapy of nonestablished human TCCs growing within the bladder of athymic nude mice would be effective, therapy was commenced 3 days after tumor implantation (Fig. 4*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 2. *In vivo* combination therapy of TNP-470 with docetaxel resulted in the significant regression of nonestablished human TCC tumors compared with either therapy alone. The combination was most effective when docetaxel was administered before TNP-470. The median bladder weights were 204 mg (range, 118–302 mg) in the controls administered ethanol with 5% gum arabic and normal saline, 48 mg (range, 21–170 mg) in mice treated with TNP-470 alone ($P < 0.005$), 87 mg (range, 54–150 mg) in mice treated with docetaxel alone ($P < 0.05$), and 28 mg (range, 22–44 mg) in mice treated with docetaxel before TNP-470 ($P < 0.001$). The incidence of spontaneous lymph node metastasis was completely inhibited by the combination of TNP-470 with docetaxel. Drug-induced body weight loss was not significantly different in any of the therapeutic groups (Fig. 5).

Inhibition of Growth and Metastasis of Established Human TCC. To determine whether the therapy would also be effective in established bladder tumors, we commenced treatment 21 days after tumor implantation (Fig. 4*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 3. *In vivo* combination therapy of TNP-470 with docetaxel resulted in significant regression of established tumors of human TCC compared with either therapy alone. The combination was most effective when docetaxel was administered before TNP-470. The median bladder weights were 275 mg (range, 170–290 mg) in control mice, 69 mg (range, 30–253 mg) in TNP-470-treated mice ($P < 0.005$), 162 mg (range, 42–237 mg) in docetaxel-treated mice ($P < 0.005$), and 28 mg (range, 24–136 mg) in mice administered docetaxel before TNP-470 ($P < 0.005$). The incidence of spontaneous lymph node metastasis was completely inhibited by the combination of docetaxel administered before TNP-470. Drug-induced body weight loss was not significantly different in any of the treatment groups (data not shown).
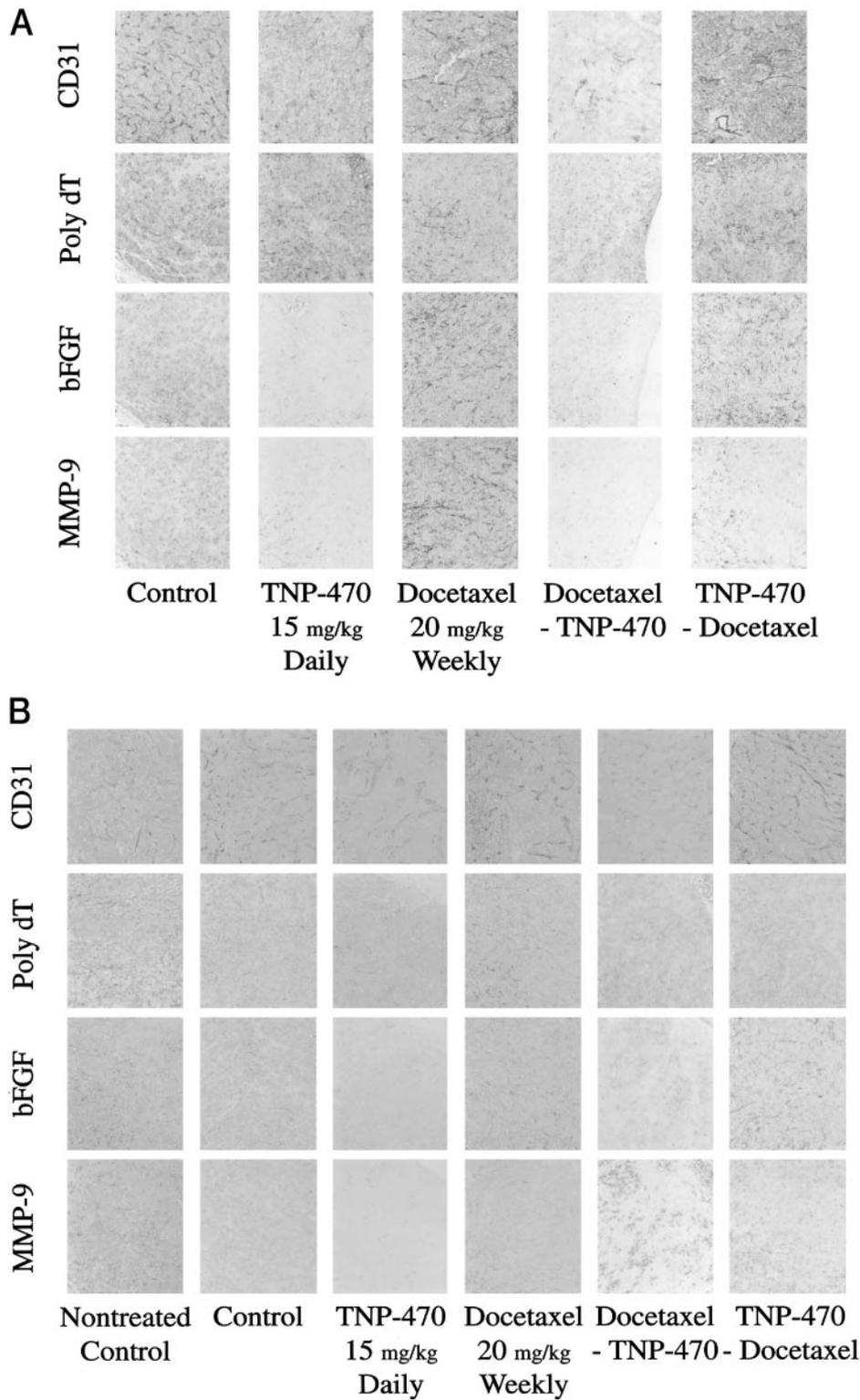
Fig. 6 A, in vivo mRNA expression level and MVD after combination therapy of TNP-470 and docetaxel for nonestablished 253J B-V cells growing orthotopically in athymic nude mice. The specific mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin were analyzed by ISH, and MVD was analyzed by IHC with anti-CD31 antibodies. The expression level of bFGF and MMP-9, and MVD were significantly reduced 40–50% in treated tumors, especially after administration of docetaxel before TNP-470, compared with control tumors; $P < 0.005$. *B, in vivo* mRNA expression level and MVD after combination therapy of TNP-470 and docetaxel for 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 were analyzed by ISH, and MVD was analyzed by IHC with anti-CD31 antibodies. The expression level of bFGF and MMP-9, and MVD were significantly reduced 20–30% in treated tumors, especially after administration of docetaxel before TNP-470, compared with control tumors; $P < 0.005$.

Inhibition of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-Cadherin Expression and MVD by TNP-470 in a Non-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 (Table 4, Fig. 6*A*). In the nonestablished-tumor model, the mRNA expression of bFGF and MMP-9 within the tumors of mice treated with docetaxel before TNP-470 was significantly reduced 55% ($P <$

Table 5 *In vivo* mRNA expression level and microvessel density after the therapy with angiogenesis inhibitor TNP-470 and docetaxel for established human transitional cell carcinoma 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	164	95	108	139	102	100	93 ± 13
CTRL (EtOH in arabic gum/saline) daily	100	100	100	100	100	100	148 ± 20
TNP-470 (15 mg/kg) daily	77[c]	101	101	70[c]	101	101	82 ± 18[c]
Docetaxel (20 mg/kg) weekly	100	99	99	99	99	103	93 ± 10
Docetaxel—TNP-470	68[c]	99	99	63[c]	99	103	69 ± 8[c]
TNP-470—docetaxel	90	105	101	88	92	100	115 ± 21

[a] The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of poly d(T) 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).

Table 6 *In vivo* induction of apoptosis and expression of proliferating cell nuclear antigen after the therapy with angiogenesis inhibitor TNP-470 and docetaxel for nonestablished human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Apoptosis index[a] Mean ± SD (Range) (per 200 ×/field)	PCNA index[a] Mean ± SD (Range) (per 200 ×/field)	Apoptosis: PCNA ratio[b]
CTRL (EtOH in arabic gum/saline) daily	5 ± 2 (2–8)	198 ± 47 (149–273)	2.5
TNP-470 (15 mg/kg) daily	6 ± 1 (3–11)[c]	81 ± 31 (45–134)[c]	7.4
Docetaxel (20 mg/kg) weekly	30 ± 14 (11–45)[c]	44 ± 10 (30–59)[c]	68.2[c]
Docetaxel—TNP-470	29 ± 17 (11–51)[c,d]	39 ± 9 (27–49)[c,e]	74.4[c,d]
TNP-470—docetaxel	15 ± 2 (9–20)[c]	51 ± 16 (33–75)[c,e]	29.4[c,e]

[a] The density of apoptosis by TUNEL assay and cell proliferation by immunohistochemistry with PCNA antibody on cancer cells was expressed as an average number of five highest area identified within a single 200 ×/field.

[b] Apoptosis:PCNA ratio: mean percentage of the number of apoptotic cells divided by the number of PCNA-positive cells.

[c] $P < 0.005$ against CTRL.

[d] $P < 0.005$ against any other groups.

[e] $P < 0.005$ against both single therapy groups with either docetaxel or TNP-470 (Mann-Whitney statistical comparison).

0.005) and 46% ($P < 0.005$), respectively, compared with the expression in the control tumor model. Neither TNP-470 nor docetaxel significantly altered the expression of VEGF, IL-8, MMP-2, or E-cadherin (Table 4; Fig. 6A). MVD was significantly lower in tumors treated with docetaxel administered before TNP-470 (49 ± 11) than in control tumors (119 ± 19), $P < 0.005$ (Table 4; Fig. 6A).

Inhibition of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-Cadherin Expression and MVD by TNP-470 in an Established Tumor Model. The mRNA expression of bFGF, VEGF, IL-8, MMP-9, MMP-2, and E-cadherin was analyzed by ISH, and MVD was analyzed by IHC (Table 5; Fig. 6B). In an established-tumor model, the mRNA expression of bFGF and MMP-9 within the tumors of mice treated with docetaxel before TNP-470 was significantly reduced 68% ($P < 0.005$) and 63% ($P < 0.005$), respectively, compared with the expression in control tumors. Neither TNP-470 nor docetaxel significantly altered the expression of VEGF, IL-8, MMP-2, or E-cadherin (Table 5; Fig. 6B). MVD was significantly lower in tumors treated with docetaxel before TNP-470 (69 ± 8) than in control tumors (148 ± 20), with $P < 0.005$ (Table 5; Fig. 6B).

Enhancement of Apoptosis and Inhibition of Proliferation by Therapy with TNP-470 and Docetaxel. We evaluated the effect of therapy with TNP-470 and docetaxel on cellular proliferation by IHC for PCNA and apoptosis by

TUNEL in a nonestablished-tumor model (Table 6; Fig. 7A). The number of PCNA-positive cancer cells counted per 200× field was significantly decreased from 198 ± 47 in control tumors to 81 ± 31 and 44 ± 10 after therapy with either TNP-470 or docetaxel, $P < 0.005$, respectively. The combination of TNP-470 and docetaxel significantly inhibited proliferation compared with each agent alone, with the greatest reduction seen after therapy with initial docetaxel treatment followed by TNP-470, $P < 0.005$. The number of apoptotic cancer cells counted per 200× field was increased significantly from 5 ± 2 in controls to 6 ± 1 and 30 ± 14 after therapy with TNP-470 and docetaxel respectively, $P < 0.005$. However, TNP-470 did not significantly induce apoptosis (Table 6; Fig. 7A).

Enhancement of Apoptosis and Inhibition of Proliferation by Therapy with TNP-470. We evaluated the effect of therapy with TNP-470 on cellular proliferation by IHC for PCNA and apoptosis by TUNEL in an established-tumor model (Table 7; Fig. 7B). The number of PCNA-positive cancer cells counted per 200× field were decreased significantly from 176 ± 55 in control tumors to 102 ± 31 and 55 ± 23 after therapy with either TNP-470 or docetaxel, respectively, $P < 0.005$. The combination of TNP-470 and docetaxel significantly inhibited proliferation compared with each agent alone, with the greatest reduction seen after therapy with initial docetaxel treatment followed by TNP-470, $P < 0.005$. The number of apo-
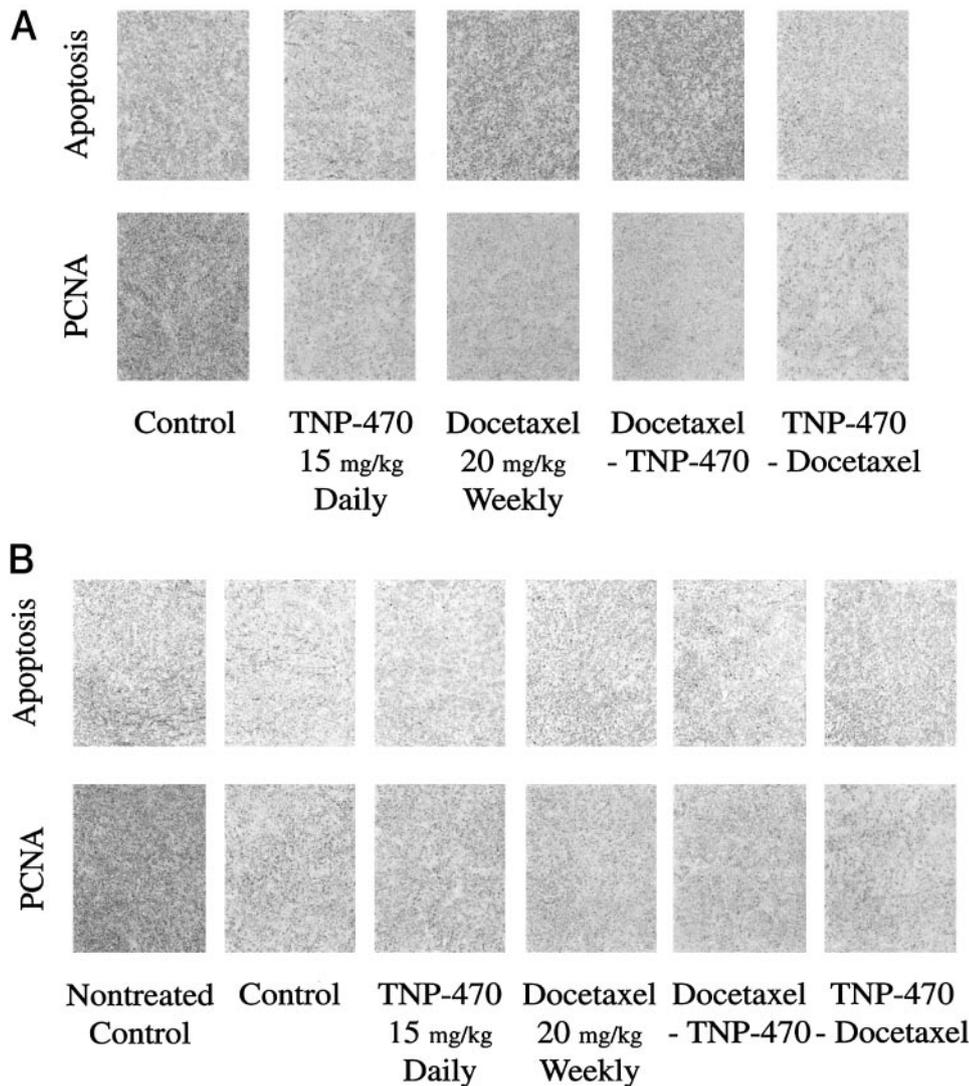
Fig. 7 *In vivo* induction of apoptosis and expression of PCNA after therapy with the angiogenesis inhibitor TNP-470 and/or docetaxel for nonestablished (*A*) and established (*B*) human TCC 253J B-V cells growing orthotopically in athymic nude mice. The combination of TNP-470 and docetaxel significantly inhibited proliferation compared with each agent alone, with the greatest reduction seen after administration of docetaxel before TNP-470; $P < 0.005$. However, TNP did not significantly increase apoptosis (*A* and *B*).

ptotic cancer cells counted per 200× field was increased significantly from 6 ± 2 in controls to 21 ± 6 after therapy with docetaxel respectively, $P < 0.005$. However, TNP-470 did not significantly induce apoptosis (Table 7; Fig. 7*B*).

DISCUSSION

We demonstrated recently that frequent administration of an adequate biological dose of the angiogenesis inhibitor TNP-470 significantly inhibits angiogenesis, tumor growth, and metastasis of human TCC growing in the urinary bladder of athymic nude mice. Although daily therapy with TNP-470 at the most effective single-agent dose of 15 mg/kg TNP-470 completely inhibited the development of spontaneous lymph node metastasis in nonestablished and established TCCs, a complete response on tumor growth was observed in only 1 of 9 mice (11.1%) bearing nonestablished TCCs. Therefore, in this study, we evaluated whether docetaxel enhanced the therapeutic effect of TNP-470, especially on tumor growth. Our results show a complete response on tumor growth in 5 of 9 mice (55.6%)

bearing nonestablished TCCs and 4 of 7 mice (57.1%) bearing established TCCs after combined therapy with docetaxel and TNP-470. Moreover, the combination therapy completely inhibited the development of spontaneous lymph node metastasis in nonestablished and established TCCs. These studies indicate that docetaxel enhances the ability of TNP-470 to inhibit tumorigenicity and metastasis in nonestablished and established human TCC tumors.

In the present study described herein, docetaxel treatment followed by TNP-470 markedly inhibited tumorigenicity and metastasis in nonestablished and established human TCC tumors compared with each single-agent therapy. This effect is mediated, at least in part, by the inhibition of angiogenesis, expression of bFGF and MMP-9, and the induction of apoptosis. We demonstrated recently that therapy with the single-agent TNP-470 significantly inhibited angiogenesis, and expression of bFGF and MMP-9. Although docetaxel did not influence protein expression or the effects of TNP-470 on protein expression, the expression of bFGF and MMP-9 was inhibited by TNP-470. It

Table 7 *In vivo* induction of apoptosis and expression of proliferating cell nuclear antigen after the therapy with angiogenesis inhibitor TNP-470 and docetaxel for established human transitional cell carcinoma 253J B-V cells growing orthotopically in athymic nude mice

Therapy	Apoptosis index[a] Mean ± SD (Range) (per 200 ×/field)	PCNA index[a] Mean ± SD (Range) (per 200 ×/field)	Apoptosis: PCNA ratio[b]
Nontreated CTRL	3 ± 1 (1–4)	284 ± 82 (192–399)	0.1
CTRL (EtOH in arabic gum/saline) daily	6 ± 2 (3–9)	176 ± 55 (106–283)	3.4
TNP-470 (15 mg/kg) daily	7 ± 2 (4–10)	102 ± 31 (65–142)	6.9
Docetaxel (20 mg/kg) weekly	21 ± 6 (13–34)[c]	55 ± 23 (27–89)[c]	38.2[c]
Docetaxel—TNP-470	21 ± 10 (10–43)[c,d]	48 ± 13 (30–70)[c,e]	43.8[c,d]
TNP-470—docetaxel	13 ± 3 (9–18)[c]	63 ± 26 (34–98)[c,e]	20.6[c,e]

[a] The density of apoptosis by TUNEL assay and cell proliferation by immunohistochemistry with PCNA antibody on cancer cells was expressed as an average number of five highest area identified within a single 200 ×/field.

[b] Apoptosis:PCNA ratio: mean percentage of the number of apoptotic cells divided by the number of PCNA-positive cells.

[c] $P < 0.005$ against CTRL.

[d] $P < 0.005$ against any other groups.

[e] $P < 0.005$ against both single therapy groups with either docetaxel or TNP-470 (Mann-Whitney statistical comparison).

was demonstrated previously that vascular endothelial cells produce various growth factors, including bFGF, which stimulate their own proliferation and migration (43). In TCC also, bFGF regulates growth and metastasis, in part, by regulating the process of angiogenesis (44, 45). TNP-470 reduces recognition of the bFGF low-affinity growth factor binding site (46). The reduced bFGF signaling by TNP-470 results in inhibition of vascular endothelial cell growth and migration, and, hence, bFGF-induced angiogenesis (47, 48). We also reported recently that therapy with IFN-α (49, 50), anti-EGFR MAb C225 (14, 15) or the adenoviral mediated antisense bFGF gene (51) inhibits tumor growth and metastasis of human TCC secondary to the down-regulation of bFGF and MMP-9 expression, and subsequent regression of tumor-induced neovascularization. Moreover, our own *in vitro* data demonstrate that adenoviral-mediated antisense bFGF gene therapy directly inhibits proliferation and enhances apoptosis in HUVEC cells (51). Moreover, the down-regulation of bFGF by the tumor cells results in the down-regulation of MMP-9 and 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 (52), IL-1 (53), transforming growth factor β1 (53), epidermal growth factor (53, 54), hepatocyte growth factor (54), IL-8 (9), and also bFGF (44). Therefore, bFGF, which up-regulates MMP-9 and induces neovascularization, is a prime target to inhibit angiogenesis, tumor growth, and metastasis.

In the present study, we demonstrated that TNP-470 and docetaxel inhibit the proliferation of cancer cells and vascular endothelial cells in a dose-dependent manner; the combined drugs had an additive effect on tumor growth inhibition. Previous reports have also demonstrated that TNP-470 inhibits the growth of HUVEC (55) as well as other cancer cells (56), including TCC in the urinary bladder (26, 27). Although the growth inhibition of cancer cells by TNP-470 was shown previously to be mediated, in part, by the induction of apoptosis in breast cancer (19) and prostate cancer (25), the growth of cancer cells was inhibited earlier and more extensively by the chemotherapeutic agents, Adriamycin and cisplatin, than by TNP-470.

Moreover, the growth of endothelial cells was inhibited by TNP-470, but not by chemotherapeutic agents, at the dose effective for tumor growth inhibition (57). In human TCC of the urinary bladder, taxanes induced apoptosis linked to antiproliferation through Bcl-2 phosphorylation (31, 32). Our results also show that TNP-470 induces apoptosis in vascular endothelial cells but not in cancer cells, whereas docetaxel induces more apoptosis in cancer cells than in vascular endothelial cells. Several reports show that the antitumor effect of TNP-470 was additionally or synergistically enhanced in combination with cytotoxic agents, such as mitomycin C, Adriamycin, cisplatin, and 5-fluorouracil in melanoma and Lewis lung carcinoma (58), minocycline, paclitaxel, and carboplatin in non-small-cell lung and breast cancer (59), Taxol in non-small cell lung cancer (60), cisplatin in liver metastasis of human pancreatic cancer (61), and 5-fluorouracil in liver metastasis of colorectal cancer (62). Our data, as well as these reports, indicate that docetaxel and TNP-470 have complementary cytotoxicities, providing a novel and effective biochemotherapy of TCC. We also demonstrated previously that paclitaxel enhances the antiangiogenic agent MAb C225, which blocks EGFR function (15), and MAb DC101, which blocks VEGF receptor-2 function (16), to inhibit tumorigenicity and metastasis of human TCC. These effects are mediated by the inhibition of angiogenesis and the induction of both tumor cell and endothelial cell apoptosis. We conclude from these reports, as well as from the present study, that taxanes have complementary cytotoxicities to antiangiogenic agents and can provide additive or synergical therapeutic effects on tumorigenicity and metastasis.

In summary, the present study provides evidence that docetaxel enhances the therapeutic effects of TNP-470 by increasing its inhibitory effect on tumor growth, metastasis, and tumor-induced neovascularization of human TCC cells growing in athymic nude mice. Induction of apoptosis by docetaxel and the antiangiogenic activity would appear to work synergistically together to enhance efficacy. These studies indicate that docetaxel and TNP-470 have complementary cytotoxicities, providing a clear rationale for investigation in future clinical trials.

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