RESEARCH COMMUNICATION

# Inhibition of Rat Urinary Bladder Carcinogenesis by the Antiangiogenic Drug TNP-470

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## Abstract

**Potential inhibitory effects of the antiangiogenic drug TNP-470 on rat urinary bladder carcinogenesis were investigated in F344 male rats initiated with 0.05% BBN in the drinking water for 8 weeks. Group 1 was then continuously treated with TNP-470 by subcutaneous injection using osmotic minipump until the end of the experiment; group 2 served as the control with only initiation. The incidences and multiplicities of papillomas and carcinomas in the TNP-470-treated group were significantly decreased compared to the control group values along with the tumor vascular density. In conclusion, TNP-470 can inhibit rat urinary bladder carcinogenesis, presumably through its effects on angiogenesis.**

**Key Words**: Angiogenesis - inhibition - urinary bladder carcinogenesis - TNP-470 - anti-angiogenic drug

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## Introduction

Angiogenesis, the process of new blood vessel formation, is essential for the progression and particularly for the growth of solid tumors and metastasis. Tumor growth will be impaired and the size of the tumor nodule will be restricted to a smaller size when capillaries are inhibited from growth due to angiogenesis (Hanahan et al., 1996). An angiogenic switch is usually activated during the early stages of neoplasia preceding the appearance of solid tumors, with extensive vascularization and ongoing angiogenesis is readily apparent in the end-stage tumors in transgenic mouse models with cancer (Hanahan et al., 1996). Therefore, angiogenesis could be considered as one of the most important enhancing factors for most types of cancers.

In rat urinary bladder carcinogenesis induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), formation of bladder tumors is related to tumor angiogenesis (Tatematsu et al., 1978; Cohen et al., 1980; Wanibuchi et al., 1996). At an early stage of carcinogenesis, from 7-8 weeks after the start of BBN treatment, the bladder epithelium forms putative preneoplastic lesions, papillary or nodular hyperplasia (PN hyperplasia), which is epithelial cell hyperplasia accompanied by a site-specific vascular proliferation appearing in the underlying submucosa (Fukushima et al., 1982; Wanibuchi et al., 1996). With the growth of tumors, vascular proliferation is conspicuous and the tumor forms an extensive papillary architecture. Thus, inhibition of angiogenesis could be an effective means for suppressing rat urinary bladder carcinogenesis.

TNP-470, a semisynthetic analogue of fumagillin derived from Aspergillus fumigatus, possesses potent anti-angiogenic activity in vitro and in vivo (Ingber et al., 1990). TNP-470 exerts its anti-angiogenic effects via inhibition of proliferation of endothelial cells (Kusaka et al., 1994)and has been shown to inhibit tumor growth and metastasis by the suppression of tumor angiogenesis in various in vitro (Farinelle et al., 2000; Fernandez et al., 2001) and in vivo experimental models (Gervaz et al., 2000; Hotz et al., 2001; Dabrowska-Iwanicka et al., 2002), including various xenografts of human carcinomas in nude or SCID mice, such as uterine carcinomas (Emoto et al., 2003), pancreatic cancer (Prox et al., 2003), metastatic transitional cell carcinomas (Inoue et al., 2003), neuroblastomas (Lindskog et al., 2003), and colon cancer (Huang et al., 2003). However, potential inhibitory effects of TNP-470 and related underlying molecular mechanisms have hitherto not been investigated in a rat urinary bladder chemical carcinogenesis model .

An angiogenic switch is speculated during the early stage of carcinogenesis preceding the appearance of solid tumors based on observations from transgenic mouse models (Hanahan et al., 1996). In such models, however, oncogene

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expression occurs in targeted cells independently of vascularity and inflammation, which are both observed in the early stage of carcinogenesis. Although an angiogenic switch has been demonstrated in transgenic models, the contributions of inflammatory cells, stromal cells and angiogenesis to promotion of the earliest stage of carcinogenesis may be underestimated.

In the present study, the potential inhibitory effects of the anti-angiogenic agent TNP-470 on rat urinary bladder carcinogenesis induced by BBN and its underlying molecular mechanisms were investigated using subcutaneous implantation of mini-osmotic pumps to the rats for constant systemic administration of the drug.

## Materials and Methods

*Chemicals*

BBN was purchased from Tokyo Kasei Co., Tokyo, Japan. TNP-470 was kindly provided by Takeda Chemical Industries, Ltd., Osaka, Japan.

*Animals*

A total of 130, five-week-old, male F344/DuCrj rats (Charles River Japan, Inc., Hino, Japan) were housed 5 per cage in an animal facility with a 12-hour light, 12-hour dark cycle at a targeted temperature of 22 ± 2∞C and 44 ± 5% relative humidity and were given free access to tap water and food (Oriental MF; Oriental Yeast Co., Tokyo, Japan). The animals were observed daily and body weights were measured weekly throughout the experiment.

*Treatments and Processing*

Experiment 1: Fifty male F344 rats, 20 weeks old (purchased at five weeks old, and maintained for one week before experimentation without any chemical treatment), were administered 0.05% BBN in their drinking water for 8 weeks, and then divided into controls and a TNP-470 treatment group continuously receiving subcutaneous infusion of TNP-470 at the dose of 2.5 mg/kg/week using subcutaneously implanted osmotic minipumps (Model 2002, ALZA Co., Palo Alto, CA, USA.). Minipumps were changed every two weeks, and the total treatment period of TNP-470 was 20 weeks. Rats of each group were killed under ether anesthesia at week 12, 20 and 28 after the commencement of the experiment, and their urinary bladders were removed and processed for histopathological and immunohistochemical analysis.

For pathological analysis, each urinary bladder was inflated by intraluminal injection of 10% phosphate-buffered formalin solution. After fixation, the urinary bladders were divided sagittally and weighed. Macroscopically, the number of tumors in each urinary bladder was counted. For histological analysis the urinary bladders were then cut into eight longitudinal strips, routinely processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin (H.E.). Areas of tumors in the H&E-stained specimens were measured by image analyzer (IPAP, Sumika Technos, Osaka, Japan).

For proliferating cell nuclear antigen (PCNA) and cyclin D1 immunohistochemistry, sections were blocked with goat serum at 37°C for 30 minutes and then incubated with mouse monoclonal anti-PCNA antibody (PC-10,IgG2a; Dako, Japan) at 1:250 dilution or with mouse monoclonal anti-cyclin D1 antibody (A-12, IgG2a ; Santa Cruz Biotechnology, California, USA) at 1: 1000 dilution overnight at 4°C. Staining was achieved with Vector Stain elite (Vector Lab. Inc., Burlinghame, CA) and developed with DAB. The results of PCNA immunohistochemistry were quantitated on an IPAP image analyzer (Sumitomo Chemical Technology, Osaka, Japan). For papillary or nodular (PN) hyperplasia, all cells within the lesions were evaluated; for the tumors(papilloma and carcinoma), three areas totaling over 500 nuclei were evaluated. The results of cyclin D1 immunohistochemistry were analyzed semiquantitatively(-: negative, +: < 50 % positive cells in the lesions, ++: > 50 % positive cells in the lesions).

For the detection of blood vessels, endothelial cells were stained immunohistochemically using polyclonal antibody against human FVIII(1:3000, Dako, Kyoto) and the reaction product was visualized by the avidin-biotin complex method using Dako ABC kit (Dako). For the analysis of the vascular density, blood vessels were counted on an IPAP image analyzer (Sumika Technos, Osaka, Japan).

Experiment 2: Twenty male F344 rats, 6 weeks old were divided into two groups, an experimental group (5 rats) and a control group (15 rats). Rats in the experimental group were administered 0.05% BBN in their drinking water for 8 weeks followed by tap water. The control group received no chemical treatment. Rats of each group were killed under ether anesthesia at week 28. Three bladder tumors from the experimental group and the bladder mucosa from the control group were removed and quickly frozen in liquid nitrogen and stored at -80∞C until analysis for mRNA levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor b (TGFb). Two RNA samples for the control were prepared by pooling RNA from 7 and 8 control rats, respectively.

Experiment 3: Sixty male F344 rats, 6 weeks old, were divided into three groups (20 rats each): group 1, the BBN and TNP-470 treated group; group 2, BBN alone group; and group 3, control group. Rats of groups 1 and 2 were administered 0.05% BBN in the drinking water for 4 weeks and those in group 3 were given tap water. Then rats in group 1 continuously received subcutaneous infusion of TNP-470 for 8 weeks at a dose of 2.5 mg/kg/week using a minipump (model 2002, for 2 weeks) and groups 2 and 3 were kept without TNP-470 treatment . All rats were killed under ether anesthesia at week 12. Five from each group received a single intraperitoneal injection of BrdU (Sigma Chemical Co., St Louis, MO) at a dose of 100 mg/kg body weight 1 hour before sacrifice. The urinary bladders were inflated with 10% phosphate-buffered formalin solution, excised and divided

into eight strips and then embedded in paraffin for histological analysis. Epithelial cells incorporating BrdU were immunohistochemically demonstrated in the sections by the avidin-biotin-peroxidase complex procedure using anti-BrdU monoclonal antibody as detailed elsewhere (Hsu et al., 1981). The number of labeled cells per 1000 urothelial cells were counted under the light microscope, and labeling indices were expressed as percentage values. For detection of blood vessels, endothelial cells were stained immunohistochemically and the vascular density counted with the aid of an IPAP image analyzer. Urinary bladder mucosae of fifteen rats from each group were collected and pooled together and frozen quickly under liquid nitrogen and stored at –80°C until analysis.

*Extraction of total RNA*

Total RNA was extracted from the urinary bladder epithelia or bladder tumors. Briefly, sample tissues were homogenized with a polytron homogenizer (PCU-11 Kinematica AG) for 60s at a dial speed of 10 in denaturing solution (4 mol/L guanidium thiocyanate, 25 mmol/L sodium citrate [pH 7.0], 0.1 mol/L 2-mercaptoethanol, and 0.5% N-auroylsarcosine). The homogenate was added to 1/10 vol of water-saturated phenol, and 1/2 vol of chloroform and centrifuged at 10,000g at 4°C for 20 minutes. The resulting upper aqueous phase was transferred to a fresh tube and precipitated by addition of 1 vol of isopropanol followed by centrifugation. The precipitate was dissolved in the above solution, incubated with 3.5 mol/L lithium chloride at 4°C for 18 h and then centrifuged. The RNA pellet was washed with 3 mol/L lithium chloride and then with 70% ethanol. Finally, it was dissolved in 0.1% diethyl pyrocarbonate-treated water and stored at -80°C until applied for Northern blot analysis. RNA concentrations were spectrophotometrically determined at 260 nm.

*Northern blot hybridization*

For RNA hybridization, 10μg samples of total RNA were denatured by incubation with 1 mol/L deionized glyoxal and 50% dimethyl sulfoxide at 50°C for 1 hr and electrophoresed on 1% agarose gels at 50 V. The 28S and 18S ribosomal RNAs in gels were stained with ethidium bromide to demonstrate the integrity of applied RNA and to verify that the same amounts of RNA were applied to each lane. RNAs in the gels were then transferred to nylon membranes (GeneScreen Plus, EI du Pont de Nemours&Co. France). cDNA probes were labeled with [a-32P] dCTP (specific activity, 3000 Ci per millimoles per liter, Du Pont) using a Random Primer DNA labeling Kit (Takara). The membranes were prehybridized in a solution containing 50% formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate, and 200 mg/mL denatured salmon sperm DNA by heating for 4 h at 42°C, and hybridized with probes for VEGF, bFGF, TGFb, as well as 32P-labeled (1 to 2 x $10^6$ disintegrations per min per mL) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the method of Chomczynski and Sacchi (1987). For all RNA samples, the density of individual mRNA bands was divided by that of the GAPDH mRNA band to correct for difference in RNA loading and/or transfer.

*Statistical analysis*

All results are reported as mean ± SD values. Significances of differences between group mean values were analyzed using the unpaired Student's t-test. Data for bladder lesions were compared using the Chi-squared or Fisher's exact probability tests (StatView, ver 5.0, SAS Institute Inc., NC). P values less than 0.05 were considered significant.

## Results

*Inhibitory effects of TNP-470 on rat bladder carcinogenesis induced by BBN* (Experiment 1)

Figure 1 shows an example of the macroscopic views of bladders in a BBN alone treated rat and a rat treated with BBN followed by TNP-470 rat. Twenty-eight weeks after the start of experiment, several large, papillary tumors were observed in the BBN alone group whereas in the TNP-470-treated group, a few small tumors were present. In the BBN alone group, obvious blood vessels were seen in the bladder wall, which showed rich blood flow, however in the TNP-470 treated rats blood vessels were not obvious macroscopically. Thus by macroscopic inspection, TNP-470 strikingly inhibited urinary bladder carcinogenesis and the vascularity of the lesions.

The incidence of the bladder lesions is summarized in Table 1. At week 12, there were no obvious tumors in the urinary bladder, however, PN hyperplasia was observed microscopically. The incidence of PN hyperplasia tended to decrease in the TNP-treated group (20%) compared to the control group (40%). At week 20, the incidence of papillomas in the TNP-470-treated group tended to be reduced compared to the BBN alone group. At week 28, the incidence of papilloma (20%) and carcinoma (10%) were significantly decreased in TNP-470-treated rats compared with those (100%, 90%, respectively) in the BBN alone rats.

Figure 1 shows the multiplicity of the bladder lesions. At week 12, the number of PN hyperplasias ( 0.2 ± 0.4 ) in TNP-treated rats was decreased compared with that in the controls ( 1.2 ± 1.8 ) and at week 20, this was significant (1.9 ± 1.0 as compared to 3.8 ± 1.9) . At week 28, the numbers of papilloma (0.3 ± 0.7) and carcinoma (0.3 ± 0.9) in the TNP-470 treated rats were significantly lower compared with those of BBN alone rats (1.8 ± 1.1 and 3.2 ± 1.8, respectively).

Table 2 summarizes data for the size, PCNA-positive indices and vascular densities of bladder tumors (papillomas and carcinomas) at week 28. All three parameters were significantly reduced by the TNP-470 treatment.

To analyze the effects of TNP-470 on the early stage lesions of PN hyperplasia and on tumors, cyclin D1 immunohistochemistry was performed. The results of cyclin D1 immunohistochemistry showed no obvious change between the TNP-470-treated group and controls in PN hyperplasia or tumors (data not shown). In the adjacent

**Table 1. Incidences of Bladder Lesions in Experiment 1**

| Treatment | No of rats | PN hyperplasia[a] | Papilloma | Carcinoma |
|---|---|---|---|---|
| Week 12 | | | | |
| BBN | 5 | 2 (40) | 0 (0) | 0 (0) |
| BBN + TNP-470 | 5 | 1 (20) | 0 (0) | 0 (0) |
| Week 20 | | | | |
| BBN | 10 | 10 (100) | 6 (60) | 1 (10) |
| BBN + TNP-470 | 10 | 9 (90) | 3 (30) | 0 (0) |
| Week 28 | | | | |
| BBN | 10 | 10 (100) | 10 (100) | 9 (90) |
| BBN + TNP-470 | 10 | 8 (80) | 2 (20)* | 1 (10)* |

[a]papillary or nodular hyperplasia.
* significantly different from the BBN alone group at week 28.

tissue, there were few positive cells in both groups. In addition, no granulomatous changes were found in the subcutis at the site of osmotic mini pump implantation.

*Overexpression of VEGF mRNA in BBN induced-rat urinary bladder tumors* (Experiment 2)

BBN-induced bladder tumors (papilloma and carcinoma) expressed higher levels of VEGF mRNA compared with normal bladder mucosa (data not shown). There was no difference in expression levels of TGFb, and bFGF mRNA was not detected in any groups of rats (data not shown).

*Expression of VEGF mRNA in the rat bladder mucosa treated with BBN followed by TNP-470* (Experiment 3)

Table 3 summarizes data for the incidence, multiplicity, BrdU labeling index and vascular density of PN hyperplasia at week 12. TNP-470 suppressed the incidence and multiplicity of the PN hyperplasia. BrdU labeling indices in the PN hyperplasia were also decreased by TNP-470 treatment. The vascular density, as a marker of neovascularization, in the PN hyperplasia of TNP-470 treated rat was decreased to half of that in the BBN alone-treated rats.

Figure 2 shows the expression of VEGF mRNA in the bladder mucosa of rats treated with BBN. Expression of VEGF mRNA of the bladder mucosa in rats treated with

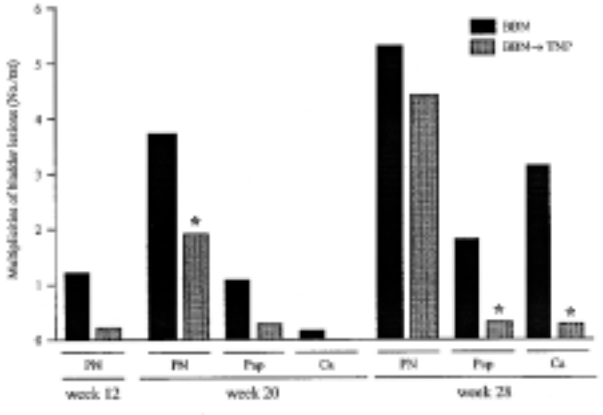


**Figure 1. Multiplicities of Urinary Bladder Lesions in BBN alone and BBN+ TNP-470 Groups (Experiment 1).** * Significantly different from the BBN alone group

**Table 2. Size, PCNA Index and Vascular Density Data for Urinary Bladder Lesions at Week 28 (Experiment 1)**

| Treatment | PCNA index (%) | Size (mm2) | Vascular density ($10^{-4}$/μm$^2$) PN hyperplasia[a] | Tumors[b] |
|---|---|---|---|---|
| BBN | 20.3±15.9 | 17.5 ± 7.4 | 28.1 ± 12.3 | 4.0 ± 0.6 |
| BBN + TNP-470 | 1.5±3.3* | 13.2 ± 2.2 | 14.8 ± 6.7* | 2.6 ± 0.4* |

[a]papillary or nodular hyperplasia [b]papillomas and carcinomas
* significantly different from the BBN alone group.

**Table 3. Incidence, Multiplicity, BrdU Labeling and Vascular Density for PN hyperplasias (Experiment 3)**

| Treatment | Incidence (%) | No /rat | BrdU index | Vascular density ($10^{-4}$/μm$^2$) |
|---|---|---|---|---|
| BBN | 3 (60) | 0.8 ± 0.8 | 9.0 ± 1.6 | 7.9 ± 3.0 |
| BBN + TNP-470 | 1 (20) | 0.2 ± 0.5 | 0.7 | 3.8 |

* significantly different from the BBN alone group.

BBN was much higher than that of the normal bladder mucosa. This indicates that VEGF is an important growth factor for neovascularization in bladder carcinogenesis from a very early stage. However, TNP-470 did not decrease the level of VEGF mRNA, although neovascularization of PN hyperplasia was decreased by TNP-470 treatment. bFGF mRNA expression was not detected in any rats (data not shown). These results implied that TNP-470 inhibited neovascularization via mechanisms other than VEGF expression, and suggests that it directly inhibits endothelial cell proliferation.

## Discussion

In the present study, we demonstrated that TNP-470 strongly inhibited rat urinary bladder carcinogenesis while significantly suppressing angiogenesis. Thus the tumor vascular density and preneoplastic lesions were both significantly reduced in TNP-470-treated rats. Of importance, TNP-470 appeared to reduce preneoplastic

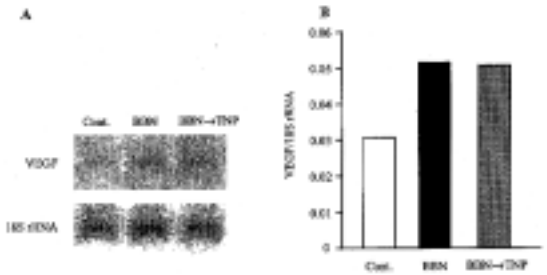


**Fig. 2. Northern blot analysis of mRNA VEGF levels in urinary bladder epithelia in BBN Æ TNP-470, BBN and control groups (Experiment 3).** A, TNP-470 had no effects on increased mRNA VEGF levels by BBN. B, Semiquantitative analysis of VEGF expression obtained in Northern blot analysis. Urinary bladders mucosae of fifteen rats from each group were collected and pooled together for Northern blot analysis.

lesions (PN hyperplasia) during the early stage of the urinary bladder carcinogenesis. It is reported that transitional cell hyperplasia is dependent on angiogenesis in the COX-2 transgenic mouse model (Klein et al., 2005), although a COX-2 inhibitor earlier did not inhibit development of PN hyperplasia in the BBN-induced rat urinary bladder carcinogenesis model (Grubbs et al., 2000). Our results suggest that tumor promotion could be inhibited through suppression of angiogenesis in chemical carcinogenesis. The multiplicity of urinary bladder carcinomas in rats treated with TNP-470 was significantly reduced to almost one-tenth of that in control rats at week 28 and the size of tumors was also significantly reduced, providing compelling support for our previous finding that angiogenesis is important for rat urinary bladder carcinogenesis (Wanibuchi et al., 1996).

Many kinds of cancer cells produce angiogenic factors, such as VEGF, bFGF, TGF b, PDECGF, and angiogenin. In experiment 2, we showed that expression of VEGF but not bFGF was increased in urinary bladder tumors induced by BBN in comparison with normal urinary bladder epithelium. Angiogenesis in rat urinary bladder tumors, which are of papillary, non-invasive type, may be induced by VEGF produced by the tumor cells. In human urinary bladder cancer, papillary non-invasive urothelial tumors express VEGF, whereas the non-papillary, invasive type expresses PDGF (O'Brien et al., 1995). Our data are therefore in line with the findings in humans.

The angiogenic switch during tumorigenesis could be visualized during early stages preceding the appearance of the solid tumor, suggesting that activation of angiogenesis is a discrete event in tumor development (Hanahan et al., 1996). The present study showed that VEGF expression was increased in the urinary bladder epithelium at a preneoplastic stage of rat urinary bladder carcinogenesis, and angiogenesis was induced in the preneoplastic lesion, PN hyperplasia (experiment 3). Our results support the presence of an existence of an angiogenic switch in the early stages of tumor development. TNP-470 inhibited the incidence and multiplicity of the PN hyperplasia lesions and the vascular density of PN hyperplasia in the early stage of rat urinary bladder carcinogenesis. However, TNP-470 did not inhibit VEGF expression in the bladder epithelium, so that it is direct effects on angiogenesis which are presumably responsible, consistent with results of an experiment in which addition of TNP-470 to tumor cell lines expressing VEGF did not change the VEGF expression of the tumor cells (data not shown). This is the first direct demonstration that systemic administration of the angiogenic inhibitor reduced the incidence and multiplicity of the preneoplastic lesions in a carcinogen-induced carcinogenesis model. The mechanisms by which TNP-470 inhibits angiogenesis have been studied intensively and cytostatic inhibition of capillary endothelial cell growth (Kusaka et al., 1994) and preferential effects on endothelial cell growth in tumor vasculature in vivo (Yanase et al., 1993) have both been demonstrated.

There are several reports of in vivo studies in which TNP-470 inhibited xenograft tumor growth [6] and their metastases in lung (Yanase et al., 1993), liver (Tanaka et al., 1995) and bone (Sasaki et al., 1998). However, studies in chemically induced carcinogenesis models showing inhibition by an anti-angiogenic agent are limited (Tanaka et al., 1997; Ikebe et al., 1998). Our study is the first in vivo assessment which directly revealed a relationship between angiogenesis and carcinogenesis, and inhibition by TNP-470. Tanaka et al (1995) reported that instillation of TNP-470 shortly after treatment with carcinogen reduced the tumor incidence using the heterotopically transplanted rat urinary bladder (HTB) system. They applied TNP-470 by topical administration in the HTB initiated by N-methyl-N-nitrosourea. Our results are consistent with their data, but their study showed limited effects. This might be related to the lack of continuous exposure because of application directly into the HTB rather than by continuous systemic administration (Tanaka et al., 1995). In view of the mechanism of TNP-470 action, it is reasonable to apply TNP-470 systemically rather than by instillation of the drug directly into the urinary bladder (Kusaka et al., 1994).

To apply TNP-470 to rats for a long period, we used an osmotic mini-pump implanted subcutaneously for a total of 20 weeks. The method avoids the formation of granulomas at the TNP-470 applied site. Granulomas frequently are produced when the drug is injected subcutaneously at effective doses. The osmotic mini-pump method reduces the applied dose of TNP-470. We applied TNP-470 at the dose of 2.5mg/kg bw/week, which was very low compared with the effective doses (30mg/kg bw/2days) of TNP-470 required when applied directly subcutaneously. In our study, TNP-470 was applied systemically with continuous subcutaneous infusion from the implanted osmotic pump. This model is useful to reduce the dose of TNP-470 and its side effect, subcutaneous granulomas.

TNP-470 and other naturally occurring or synthetic inhibitors of angiogenesis are currently being evaluated in clinical trials for the treatment of cancer. For example, preclinical studies on non-small-cell lung cancer patients suggested that synergistic treatment of TNP-470 with cytotoxic therapy of peclitaxel (paclitaxel), each at full single-agent dose, may be well tolerated with minimal pharmacokinetic interaction between the agents (Herbst et al., 2002). Moreover, TNP-470, among other anti-angiogenic agents, has produced encouraging responses in treatment of AIDS-related Kaposi`s solid sarcomas (Catellan et al., 2002) and patients with metastatic prostate cancer (Figg et al., 2002). Large clinical trials are currently in progress. In human bladder cancer therapy, angiogenesis and its role in bladder carcinogenesis suggests its usefulness as a marker of patient prognosis, and the potential anti-angiogenic therapies for future development might utilize TNP-470 and other antiangiogenesis inhibitors for further human trials (Streeter and Harris, 2002).

In conclusion, the present study clearly demonstrated that TNP-470, a angiogenesis inhibitor, can significantly inhibit rat urinary bladder carcinogenesis through blocking angiogenesis. Our results confirm the importance of

angiogenesis in rat urinary bladder carcinogenesis, a model of human popillary urothelial tumors. Further research is needed to confirm whether TNP-470 inhibits the non-papillary invasive type of bladder cancer and this will require application of a mouse model.

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## References

Cattelan AM, Trevenzoli M, Aversa SM (2002). Recent advances in the treatment of AIDS-related Kaposi's sarcoma. *Am J Clin Dermatol*, **3**, 451-62.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, **162**, 156-9.

Cohen SM, Tatematsu M, Shinohara Y, Nakanishi K, Ito N (1980). Neovascularization in rats during urinary bladder carcinogenesis induced by N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide. *J Natl Cancer Inst*, **65**, 145-8.

Dabrowska-Iwanicka A, Olszewska D, Jalili A, et al (2002). Augmented antitumour effects of combination therapy with TNP-470 and chemoimmunotherapy in mice. *J Cancer Res Clin Oncol*, **128**, 433-42.

Emoto M, Ishiguro M, Iwasaki H, Kikuchi M, Kawarabayashi T (2003). Effect of angiogenesis inhibitor TNP-470 on the growth, blood flow, and microvessel density in xenografts of human uterine carcinosarcoma in nude mice. *Gynecol Oncol*, **89**, 88-94.

Farinelle S, Malonne H, Chaboteaux C, et al (2000). Characterization of TNP-470-induced modifications to cell functions in HUVEC and cancer cells. *J Pharmacol Toxicol Methods*, **43**, 15-24.

Fernandez A, Udagawa T, Schwesinger C, et al (2001). Angiogenic potential of prostate carcinoma cells overexpressing bcl-2. *J Natl Cancer Inst*, **93**, 208-213.

Figg WD, Kruger EA, Price DK, Kim S, Dahut WD (2002). Inhibition of angiogenesis: treatment options for patients with metastatic prostate cancer. *Invest New Drugs* **20**, 183-94.

Fukushima S, Murasaki G, Hirose M, et al (1982).Histopathological analysis of preneoplastic changes during N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder carcinogenesis in rats. *Acta Pathol Jpn*, **32**, 243-50.

Gervaz P, Scholl B, Padrun V, Gillet M (2000). Growth inhibition of liver metastases by the anti-angiogenic drug TNP-470. *Liver*, **20**, 108-13.

Grubbs CJ, Lubet RA, Koki AT, et al (2000). Celecoxib inhibits N-butyl-N(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female fisher-344 rats. *Cancer Res*, **60**, 5599-602.

Hanahan D, Christofori G, Naik P, Arbeit J (1996). Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. *Eur J Cancer*, **32A**, 2386-93.

Herbst RS, Madden TL, Tran HT, et al (2002). Safety and pharmacokinetic effects of TNP-470, an angiogenesis inhibitor, combined with paclitaxel in patients with solid tumors: evidence for activity in non-small-cell lung cancer. *J Clin Oncol*, **20**, 4440-7.

Hotz HG, Reber HA, Hotz B, et al (2001). Angiogenesis inhibitor TNP-470 reduces human pancreatic cancer growth. *J Gastrointest Surg*, **5**, 131-8.

Hsu SM, Raine L, Fanger H (1981). A comparative study of the peroxidase antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am J Clin Pathol*, **75**, 734-8.

Huang ZH, Fan YF, Xia H, Feng HM, Tang FX (2003). Effects of TNP-470 on proliferation and apoptosis in human colon cancer xenografts in nude mice, *World J Gastroenterol*, **9**, 281-3.

Ingber D, Fujita T, Kishimoto S, et al (1990). Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth, *Nature*, **348**, 555-7.

Ikebe T, Yamamoto T, Kubo S, et al (1998). Suppressive effect of the angiogenesis inhibitor TNP-470 on the development of carcinogen-induced hepatic nodules in rats. *Jpn J Cancer Res*, **89**, 143-9.

Inoue K, Chikazawa M, Fukata S, Yoshikawa C, Shuin T (2003). Docetaxel enhances the therapeutic effect of the angiogenesis inhibitor TNP-470 (AGM-1470) in metastatic human transitional cell carcinoma. *Clin Cancer Res*, **9**, 886-99.

Klein RD, Van Pelt CS, Sabichi AL, et al (2005). Transitional cell hyperplasia and carcinomas in urinary bladders of transgenic mice with keratin 5 promoter-driven cyclooxygenase-2 overexpression. *Cancer Res*, **65**, 1808-13.

Kusaka M, Sudo K, Matsutani E, et al (1994). Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). *Br J Cancer*, **69**, 212-16.

Lindskog M, Kogner P, Ponthan F, et al (2003). Noninvasive estimation of tumour viability in a xenograft model of human neuroblastoma with proton magnetic resonance spectroscopy (1H MRS). *Br J Cancer*, **88**, 478-85.

O'Brien T, Cranston D, Fuggle S, Bicknell R, Harris AL (1995). Different angiogenic pathways characterize superficial and invasive bladder cancer. *Cancer Res*, **55**, 510-3.

Prox D, Becker C, Pirie-Shepherd SR, et al (2003). Treatment of human pancreatic cancer in mice with angiogenic inhibitors. *World J Surg*, **27**, 405-411.

Sasaki A, Alcalde RE, Nishiyama A, et al (1998). Angiogenesis inhibitor TNP-470 inhibits human breast cancer osteolytic bone metastasis in nude mice through the reduction of bone resorption. *Cancer Res*, **58**, 462-7.

Streeter EH, Harris AL (2002). Angiogenesis in bladder cancer-prognostic marker and target for future therapy. *Surg Oncol*, **11**, 85-100.

Tanaka H, Taniguchi H, Mugitani T, et al (1995). Intra-arterial administration of the angiogenesis inhibitor TNP-470 blocks liver metastasis in a rabbit model. *Br J Cancer*, **72**, 650-3.

Tanaka Y, Kawamata H, Fujimoto K, Oyasu R (1997). Angiogenesis inhibitor TNP-470 suppresses tumorigenesis in rat urinary bladder. *J Urol*, **157**, 683-8.

Tatematsu M, Cohen SM, Fukushima S, et al (1978). Neovascularization in benign and malignant urinary bladder epithelial proliferative lesions of the rat observed in situ by scanning electron microscopy and autoradiography, *Cancer Res*, **38**, 1792-800.

Wanibuchi H, Yamamoto S, Chen H, et al (1996). Promoting effects of dimethylarsinic acid on N-butyl-N-(4-hydroxy-

butyl)nitrosamine-induced urinary bladder carcinogenesis in rats. *Carcinogenesis*, **17**, 2435-9.

Yanase T, Tamura M, Fujita K, Kodama S, Tanaka K (1993). Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines in vitro and in vivo. *Cancer Res*, **53**, 2566-70.