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Anti-metastatic and anti-angiogenic activities of a new matrix metalloproteinase inhibitor, TN-6b

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Abstract

We investigated the anti-metastatic and anti-angiogenic effects of TN-6b, a new broad-spectrum inhibitor of matrix metalloproteinases (MMPs), against Lewis lung carcinoma (LLC) and hepatic sinusoidal endothelial (HSE) cells. TN-6b potently inhibited the activities of MMP-2 and -9 secreted by LLC and HSE cells in a zymogram assay. TN-6b, at non-cytotoxic concentrations, caused a marked inhibition of invasion and migration of LLC, and tube-like formation of HSE cells. In contrast, TN-6d, an inactive enantiomer of TN-6b, did not inhibit the invasion and tube-like formation. Daily subcutaneous (s.c.) administration of TN-6b at doses of 30 and 60 mg/kg in mice resulted in a potent inhibition of tumour-induced angiogenesis of B16 melanomas and lymph node metastasis of LLC cells. In conclusion, TN-6b effectively inhibited lymph node metastasis of LLC cells through its anti-invasive and anti-angiogenic properties. These findings suggest that the MMP inhibition correlates well with its anti-angiogenic and anti-metastatic efficacy and TN-6b has the therapeutic potential to inhibit angiogenesis and metastasis *in vivo* and *in vitro*.

Keywords: MMP inhibitor; Angiogenesis; Lymph node metastasis

1. Introduction

The essential characteristics of cancer are the ability to invade surrounding tissues and metastasise to regional and remote sites. Metastasis is the major cause of mortality in cancer patients. The extent of lymphatic metastasis is the most important influencing factor with regard to the prognosis of non-small cell lung cancer (NSCLC) patients. The 5-year survival rate after resection for patients with pN_2 disease was only 23% [1]. Therefore, suppression of lymphatic metastasis results in the improved survival of lung cancer patients [2].

The growth of a tumour and its ability to metastasise is dependent on angiogenesis [3]. The generation of blood vessels is an essential step in the transition of a tumour from a small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body [4]. The principal barriers to tumour growth and spread are the extracellular matrix (ECM) compartments. During the metastatic process, tumour cells need to attach to other cells and ECM proteins. Translocation of neoplastic cells across ECM barriers is also a part of the metastatic process and lysis of matrix proteins by specific proteinases is required for invasion [5].

Matrix metalloproteinases (MMPs) are classified as zinc-dependent proteinases with a proteolytic activity for components of the basement membrane (BM) and ECM. MMPs can mediate cell death, cell proliferation, cell differentiation, tumour-associated angiogenesis and malignant conversion. Changes in MMP levels can markedly affect the invasive behaviour of tumour cells and their ability to metastasise in experimental animal models [6]. During normal physiological processes, endogenous inhibitors regulate the proteolytic activities of these proteinases. However, if this balance is disrupted and the MMP levels increase, their enzyme

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activities directly contribute to the pathology of cancer and other disease states. In several cases, the stage of tumour progression is positively correlated with the expression of the MMP family members [7]. An elevated level of MMPs has been shown in many histological types of cancer, including breast, lung, prostate, colorectal, ovarian and gastric cancers [8].

Synthetic MMP inhibitors have been shown to successfully limit the growth and metastasis of a variety of tumours in mice and are currently undergoing clinical assessment in phase I and II trials in various human cancers [9–12]. TN-6b is a synthetic, low molecular weight (MW = 483.61) MMP inhibitor with a broad spectrum of MMP substrates (MMP-1, -2, -3, -7, -8, -9, -12, -13 and MT1-MMP). In the present study, we investigated the effects of TN-6b on the *in vitro* metastatic properties of LLC cells and angiogenic properties of heptatic sinusoidal endothelial (HSE) cells and assessed the *in vivo* ability of TN-6b to inhibit lymph node metastasis and tumour-induced angiogenesis.

2. Materials and methods

2.1. Reagents

TN-6b (MW = 483.61) and TN-6d, the inactive enantiomer of TN-6b, were synthesised at the Daiichi Fine Chemical Co., Ltd. (Fig. 1a). For the *in vitro* experiment, TN-6b and TN-6d were dissolved in dimethylsulphoxide (DMSO) at a concentration of 100 mg/ml for the stock solution and stored at −20 °C until use. For the *in vivo* experiment, TN-6b was dissolved in saline and adjusted to pH 7.0.

2.2. Animals

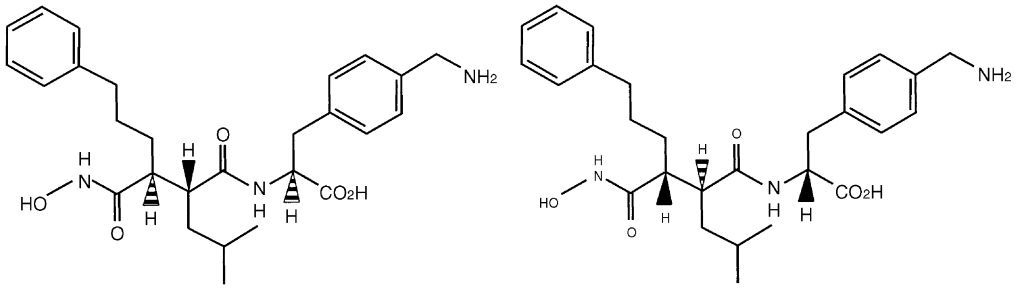
C57BL/6 mice (6-week-old females) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were provided with food and water *ad libitum* and maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical Pharmaceutical University, under laminar air flow conditions with a 12-h light/dark cycle at 22–25 °C.

2.3. Cell cultures

Lewis lung carcinoma (LLC) and B6-derived melanoma (B16-BL6) cell lines were maintained as monolayer cultures in Eagle's Minimal Essential Medium (EMEM; GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS; Cansera International Inc., Canada). LLC and B16-BL6 cells were collected by brief treatment with ethylene diamine tetraacetic acid (EDTA) or trypsin-EDTA, respectively, and then used for the experiments.

Murine hepatic sinusoidal endothelial (HSE) cells were kindly provided by Dr. G.L. Nicolson (The Uni-

(a) Chemical structures (MW = 483.61)



TN-6b (original drug, S-R-S) TN-6d (enantiomer, R-S-R)

(b) Profile of MMP inhibitory activity (IC_{50}, nM)

	Collgenases			Gelatinase		Stromelysins			MT-MMPs
	MMP-1	MMP-8	MMP-13	MMP-2	MMP-9	MMP-3	MMP-7	MMP-12	MMP-14
TN-6b	78	6.0	3.0	60	19	16	6.7	0.053	470
TN-6d	16%[a]	72 000	46 000	48%[a]	39%[a]	16%[a]	23%[a]	8000	−1.0[a]

[a] Inhibition % at o.1 mM

Fig. 1. Chemical structures and matrix metalloproteinase (MMP) inhibitory activities of TN-6b and its enantiomer, TN-6d: (a) chemical structures of TN-6b and TN-6d (molecular weight (MW) = 483.61); (b) profile of inhibitory activity of TN-6b and TN-6d against the MMPs.

versity of Texas, MD Anderson Cancer Center, TX, USA) [13]. HSE cells were maintained in Attachment Factor (Cell Systems, Kirkland, WA, USA)-coated culture flasks in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (GIBCO BRL, Life Technologies Inc., NY, USA) supplemented with 5% FBS and 100 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA). All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2 air.

2.4. MMP inhibition assay

MMPs inhibitory activity of a synthetic compound was determined by modifying the method of Yamamoto and colleagues described in Ref. [14] using a fluorogenic substrate, (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitropheny]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (Peptide Institute, Inc., Japan) [15].

ProMMP-1 and proMMP-3 were purified from the media of human skin fibroblast NB1RGB cells (RIKEN Cell Bank, RCB0222) stimulated with interleukin (IL)-1α (Genzyme Corp. MPLS, MN, USA) [16], and recombinant human proMMP-2, -8, -9 and -13 were purchased from Genzyme Corp. (MPLS, MN, USA). ProMMP-1, -2, -3, -8, -9 and -13 were activated with 1 mM 4-aminophenylmercuric acetate (APMA, Nacalai Tesque, Inc., Japan) at 37 °C for 2, 18, 1, 1, 24 and 2 h, respectively. Recombinant human MMP-7 was purchased from the Oriental Yeast Co., Ltd. (Japan) and the catalytic domain of recombinant human MMP-12 was purchased from R&D systems (MPLS, MN, USA). Recombinant human MT1-MMP lacking the transmembrane domain (Ala^{536}-Val^{582}) was expressed and purified from *Escherichia coli* [16].

One unit of MMP was defined as the amount of enzyme required to degrade 1 pmol of the substrate in 1 min at 37 °C. A mixture of enzyme solution (one unit/40 μl) and inhibitor solution (10 μl) was incubated with 50 μl of substrate solution in 50 mM Tris–HCl (pH 7.5) buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and 0.02% NaN_3. Each substrate concentration in MMP-1, -2, -3, -7, -8, -9, -12, -13 and the MT1-MMP assay was adjusted to 10, 5, 10, 10, 3, 5, 10, 3 and 3 μM, respectively. The enzyme reaction was performed at 37 °C, for 1 h, and quenched with 100 μl of 3% aqueous acetic acid. The emergence of fluorescence intensity (excitation 325 nm, emission 405 nm) was measured using a Biolumin 960 (Molecular Dynamics Pth. Ltd., Key East, VIC, Australia).

2.5. Gelatin zymography

To prepare conditioned medium, LLC and HSE cells were grown to subconfluence in T-25 culture flasks in EMEM. After several washes with phosphate-buffered solution (PBS), LLC and HSE cells were recultured in EMEM containing 0.1% bovine serum albumin (BSA) or DMEM/F-12 containing 0.1% FBS for 24 h, respectively. The culture medium was then collected and centrifuged to remove debris and the supernatants were subjected to gelatin zymography as previously reported in Ref. [17] with some modifications [18]. Briefly, aliquots of the supernatants were applied directly to a 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.1% (w/v) gelatin. After electrophoresis, the gels were soaked in rinsing buffer containing 50 mM Tris–HCl, 2.5% (w/v) Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% (w/v) NaN_3 at room temperature for 60 min to remove the SDS. The renatured gelatinases were incubated in reaction buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% (w/v) NaN_3) for 24 hr at 37 °C. To access the MMP inhibitory activity of TN-6b, the gels were incubated in reaction buffer containing TN-6b, stained in 10% (w/v) acetic acid/10% (w/v) isopropanol containing 0.1% (w/v) Coomassie Brilliant Blue for 30 min and destained in the same solution in the absence of dye. Gelatinolytic activity was detected as a clear band in the background of uniform staining and MMP activity was measured by scoring the intensity of bands by computerised image analysis using a Scion Image System (Scion Corporation, MD, USA).

2.6. Invasion assay

The invasion assay was carried out using Transwell cell culture chambers (Corning Costar No. 3422, MA, USA) as previously described in [19] with some modifications [20]. Briefly, polyvinylpyrrolidone-free polycarbonate filters (8.0 μm pore size, Nuclepore, CA, USA) were precoated with 1 μg of fibronectin (Asahi Technoglass Co., Japan) on the lower surface, and 5 μg of Matrigel (BD Biosciences, MA, USA) on the upper surface. These cells were harvested with 1 mM EDTA and then LLC cells were resuspended in EMEM supplemented with 0.1% BSA and HSE cells were resuspended in DMEM/F12 supplemented with 0.1% FBS. Cell suspensions (1×10^5 cells) were added to the upper compartment of the chamber with various concentrations of TN-6b and TN-6d. After a 24-h incubation, the filters were fixed with 30% methanol, stained with 0.5% crystal violet in 20% methanol and the cells that remained on the upper side of the filters were removed. The filters containing the stained cells that had invaded to their lower sides were removed from the Transwell chambers and individually transferred to separate wells in a 96-well culture plate. The crystal violet dye retained on the filters was extracted with 30% acetic acid and colorimetrically assessed by measuring its absorbance at 590 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon InterMed K.K., Japan).

2.7. Haptotactic migration assay

Haptotatic cell migration was assayed in a Transwell cell-culture chamber according to the methods previously reported in Ref. [19]. The lower surface of the filters was precoated with 1 μg of fibronectin. LLC or HSE cells (1×10^5 cells) and various concentrations of TN-6b were added to the upper compartment and incubated for 6 h at 37 °C in a 5% CO_2/95% O_2 atmosphere. The subsequent procedures were the same as those for the invasion assay.

2.8. Tube formation assay

Assessment of *in vitro* capillary formation used growth factor-reduced Matrigel (BD Biosciences, MA, USA). Matrigel (100 μl/well of 10 mg/ml) was plated in 48-well culture plates after thawing on ice, and allowed to polymerise at 37 °C for 30 min. HSE cells (3×10^4 cells/500 μl in DMEM/F-12 medium containing 0.1% FBS) were added to each well, in the absence or presence of TN-6b and TN-6d. The plates were then incubated for 6 h at 37 °C in a 5% CO_2/95% O_2 atmosphere. After the incubation, cells were photographed (five fields per well at 40× magnification) and the length of tube formation was measured using digital curvimeter (Uchida Yoko Co., Ltd., Japan). Briefly, a connecting branch between two discrete endothelial cells was measured as a tube-like structure. The extent of inhibition of the tube-like formation induced by TN-6b and TN-6d was estimated by comparing the length of the tube-like structures formed in the control treatments.

2.9. Tumour-induced angiogenesis assay

Tumour-induced angiogenesis was assessed according to the previously described methods in Ref. [21], with some modifications. Mice were inoculated intradermally with B16-BL6 cells (5×10^5 cells/50 μl) on the back. TN-6b was administered by subcutaneous (s.c.) injections of 30 and 60 mg/kg per day starting on the day of tumour-cell inoculation. Six days after the tumour inoculation, the mice were sacrificed and the tumour-inoculated skin was separated from the underlying tissues. Angiogenesis was quantified by counting only the vessels directly supplying the tumour under a dissecting microscope. Tumour growth was assessed by measuring with a caliper square along the longer axes (a) and the shorter axes (b). Tumour volumes (mm^3) were calculated by the following formula. Tumour volume (mm^3) = $ab^2/2$.

2.10. Intrapulmonary implantation of LLC cells and evaluation of antitumour activity

Orthotopic implantation of LLC cells into the lung was performed as previously described in Ref. [22], with some modification. The left chest of the anaesthetised mouse was incised (approximately a 5-mm incision), and 20-μl aliquots of LLC cell suspension (3×10^3 cells) admixed with 20 μg of Matrigel were injected into the left lung parenchyma through the intercostal space. The skin incision was closed with a surgical skin clip. Mice were sacrificed on day 21 after tumour implantation, and the weights of the tumour at the implantation site in the lung and the metastasised tumour in the mediastinal lymph node were measured. TN-6b was administered by s.c. injections of 30 or 60 mg/kg for 14 days starting on day 1 after the intrapulmonary implantation of LLC cells.

2.11. Statistical analysis

Representative data from each experiment are presented as mean values ± standard error of the means (SEM), as described in the figure legends. The statistical differences between the groups were determined by applying the Student's two-tailed t-test. The Dunnett's test was performed to decrease the multiplicity in comparisons of drug-treated groups with the control group during the *in vivo* experiments. Statistical significance was defined as a P value of <0.05.

3. Results

3.1. MMP inhibitory activity of TN-6b and TN-6d

The inhibitory activity of TN-6b and TN-6d against various human MMPs was examined (Fig. 1b). TN-6b inhibited all of the MMP activities examined with IC_{50} values $\leqslant$ 470 nM. However, TN-6d, an enantiomer of TN-6b, showed very low inhibitory activities (IC_{50} values >8000 nM).

We next investigated the inhibitory activity of TN-6b against murine MMPs (MMP-2 and-9) using the gelatin zymography. Conditioned serum-free media of LLC and HSE cells were used in the gelatin-zymogram assays. Gelatinolytic activity was analysed *in vitro* by an overnight gel incubation in buffer containing increasing concentrations of TN-6b (1–50 μg/ml). The densitometric analysis of zymography revealed that the degradation of gelatin substrate by MMP-2 and MMP-9 was inhibited by TN-6b in a concentration-dependent manner (Fig. 2). In contrast, the synthesis and secretion of MMP-2 and-9 were not affected in the TN-6b-treated LLC and HSE cells (data not shown).

3.2. Effect of TN-6b on the growth, migration and invasion of LLC cells and HSE cells in vitro

Next, we examined the *in vitro* effect of TN-6b and its inactive enantiomer, TN-6d, on the growth, invasion and migration by tumour and endothelial cells. *In vitro*

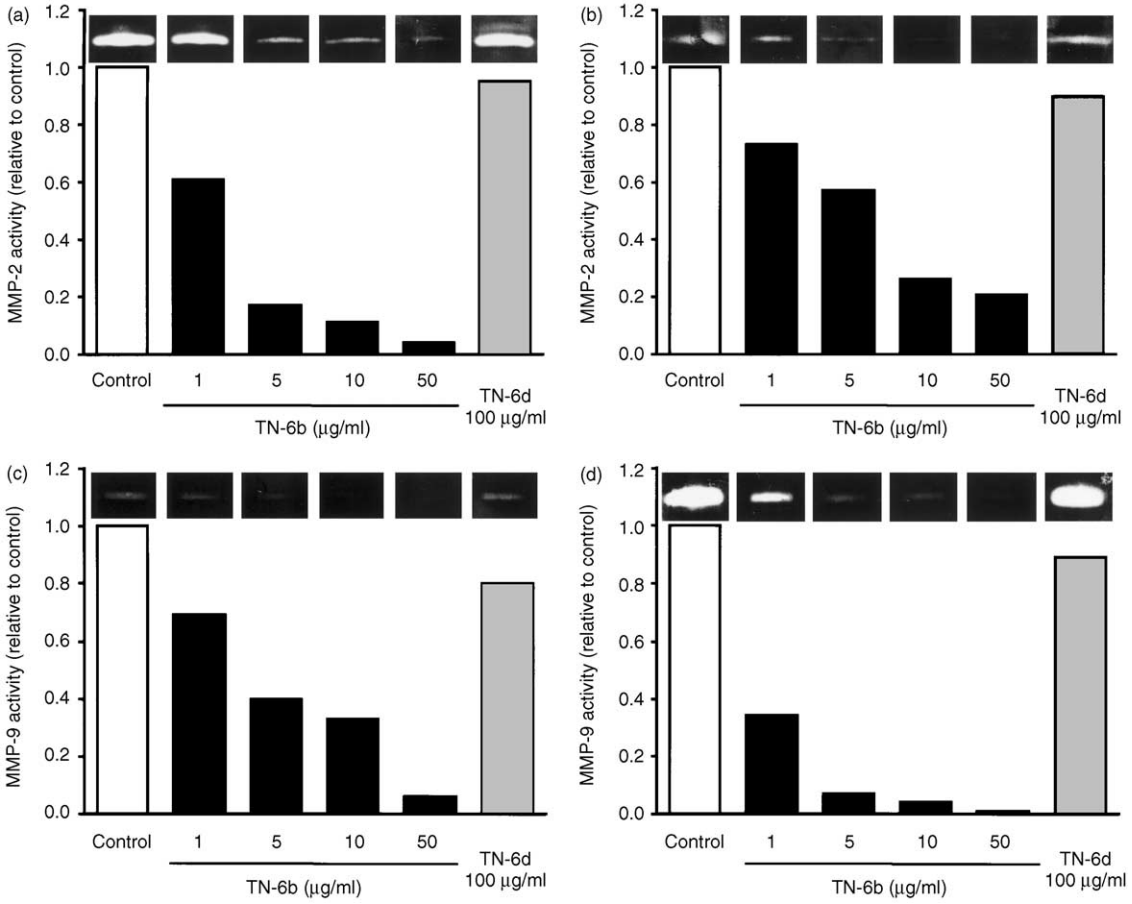


Fig. 2. Zymographic analysis of gelatinolytic activity from conditioned media of Lewis lung carcinoma (LLC) and hepatic sinusoidal endothelical (HSE) cells treated with TN-6b. Samples from conditioned serum-free media of LLC and HSE cells were run on gelatin-embedded gels. Gelatinolytic activity was analysed *in vitro* by overnight gel incubation in buffer containing increasing concentrations of TN-6b (1–50 μg/ml). (a) and (c) show matrix metalloproteinase (MMP)-2 activity by LLC and HSE cells, respectively. (b) and (d) show MMP-9 activity by LLC and HSE cells, respectively. Similar results were obtained in three independent experiments.

proliferation of LLC and HSE cells was not affected by TN-6b at concentrations less than 1000 μg/ml (data not shown). MMPs are implicated in a wide variety of roles that can assist tumour initiation, growth, migration, angiogenesis, invasion and metastasis. Therefore, we also examined the effect of TN-6b on the invasion and migration of tumour and endothelial cells. The invasion and migration of LLC and HSE cells through Matrigel/fibronectin-coated and fibronectin-coated filters were significantly inhibited by TN-6b in a concentration-dependent manner (Figs. 3 and 4). In contrast, the inactive enantiomer TN-6d, at a concentration of 100 μg/ml, did not inhibit the invasion of LLC and HSE cells (Fig. 3b and c), suggesting that the MMP inhibitory activity is essential for the inhibition of invasion and migration by TN-6b.

3.3. Inhibitory effect of TN-6b on angiogenesis

Angiogenesis requires invasion by endothelial cells and localised proteolytic modification of the ECM. MMPs are expressed by and around forming blood vessels and modulate endothelial cell proliferation and tube formation [23]. Since invasion and migration of endothelial cells are crucial steps in angiogenesis, we next investigated the effect of TN-6b against *in vitro* tube-like formation and *in vivo* tumour-induced angiogenesis. The incubation of HSE cells in Matrigel-coated wells caused tube-like formations within 6 h. Correlating with the results of the invasion and migration assay, treatment with various concentrations of TN-6b, but not with the inactive enantiomer TN-6d, inhibited the tube-like formation of HSE cells in a concentration-dependent manner (Fig. 5).

To confirm the anti-angiogenic activity of TN-6b *in vivo*, we employed an angiogenesis model by orthotropic implantation of melanomas. B16-BL6 melanomas were inoculated on the back skin intradermally and TN-6b (30 and 60 mg/kg, s.c.) was administered for 6 days from the day of tumour cell inoculation. As shown in Fig. 6b, treatment with TN-6b reduced the number of vessels oriented to the tumour mass, indicating an inhibition of angiogenesis. In addition, TN-6b inhibited the primary tumour growth in a dose-dependent manner (Fig. 6a). TN-6b did not show any side-effects during

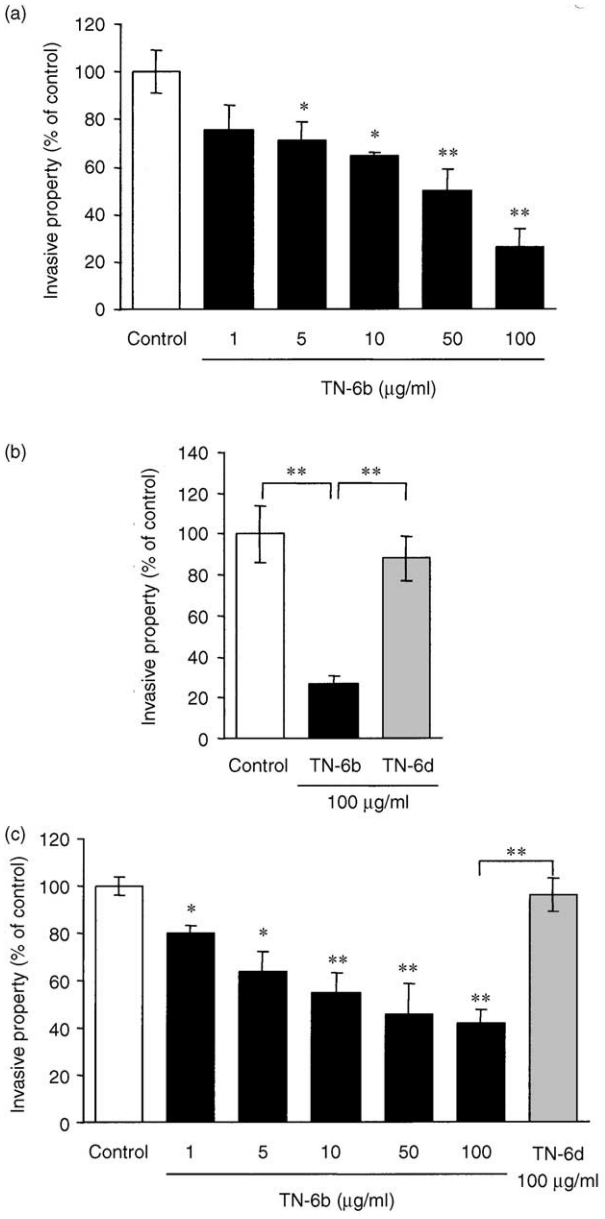


Fig. 3. Effect of TN-6b and TN-6d on tumour and endothelial cell invasion through Matrigel/fibronectin-coated filters. Lewis lung carcinoma (LLC) cells and hepatic sinusoidal endothelial (HSE) cells were seeded into Transwell chambers in quadruplicate and incubated with or without TN-6b and TN-6d. After 24 h, the invading cells were detected by the crystal violet staining method. (a) and (b), invasive properties of LLC cells with or without TN-6b and TN-6d; (c) invasive properties of HSE cells with or without TN-6b and TN-6d. All data are represented as the mean±SEM. * $P<0.05$; ** $P<0.01$.

the *in vivo* experiments (data not shown). These results demonstrated that TN-6b had a potent inhibitory activity against tumour-induced angiogenesis *in vivo*.

3.4. Effects of TN-6b on tumour growth and mediastinal lymph node metastasis of LLC

Finally, the *in vivo* efficacy of TN-6b was examined in a lymph node metastasis model by orthotopic implantation of LLC cells. Mice were treated with TN-6b (30 and 60 mg/kg, s.c.) for 14 days from 1 day after tumour inoculation. The tumour growth and mediastinal lymph node metastasis were assessed 21 days after the tumour cell inoculation. Administration of TN-6b failed to inhibit the growth of LLC tumours at the implantation site (Fig. 7a). However, metastasis of LLC cells to the mediastinal lymph nodes was significantly inhibited by TN-6b in a dose-dependent manner (Fig. 7b). TN-6b did not cause any adverse effects in the mice during the *in vivo* experiments (data not shown).

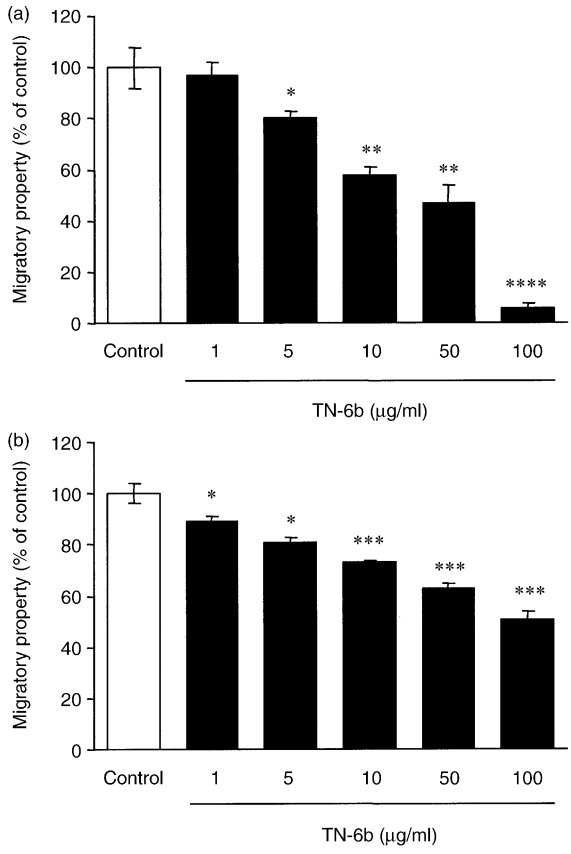


Fig. 4. Effect of TN-6b on tumour and endothelial cell migration through fibronectin-coated filters. Lewis lung carcinoma (LLC) cells (a) and hepatic sinusoidal endothelial (HSE) cells (b) were seeded into Transwell chambers in quadruplicate and incubated with or without TN-6b. After 6 h, the migrated cells were detected by the crystal violet staining method. All data are represented as the mean±SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

4. Discussion

The MMPs are a family of zinc-dependent proteinases involved in degrading and remodelling the ECM. MMP activity is tightly regulated in normal physiological states, but the loss of this regulation, in diseases such as cancer, results in the destruction of the ECM and subsequent tumour progression [6,8]. The degradation of the ECM surrounding tumour cells is considered to be a

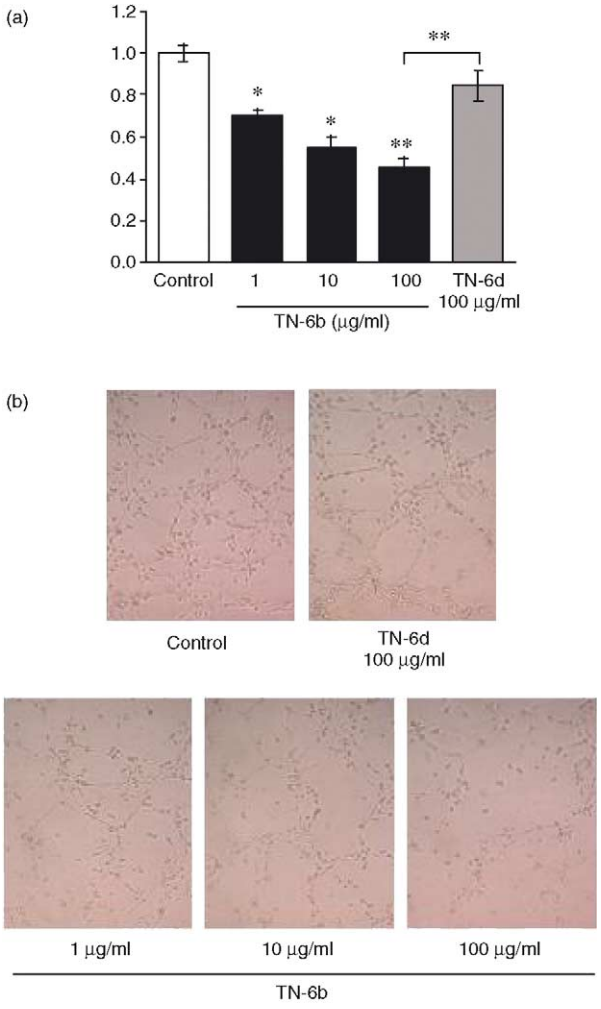


Fig. 5. Effect of TN-6b and TN-6d on the tube-like formation of endothelial cells. Hepatic sinusoidal endothelial (HSE) cells were seeded in Matrigel-coated wells and incubated in the absence or presence of TN-6b and TN-6d. The tube-like formation was monitored every 1 h, over 6 h. (a) The extent of inhibition of tube-like formations induced by TN-6b and TN-6d was estimated by comparing the length of tube-like structures formed in the control treatments. Data are represented as the mean±SEM. * $P<0.001$; ** $P<0.0001$; (b) The microscopic observation of tube-like formations in TN-6b- and TN-6d-treated wells (×40).

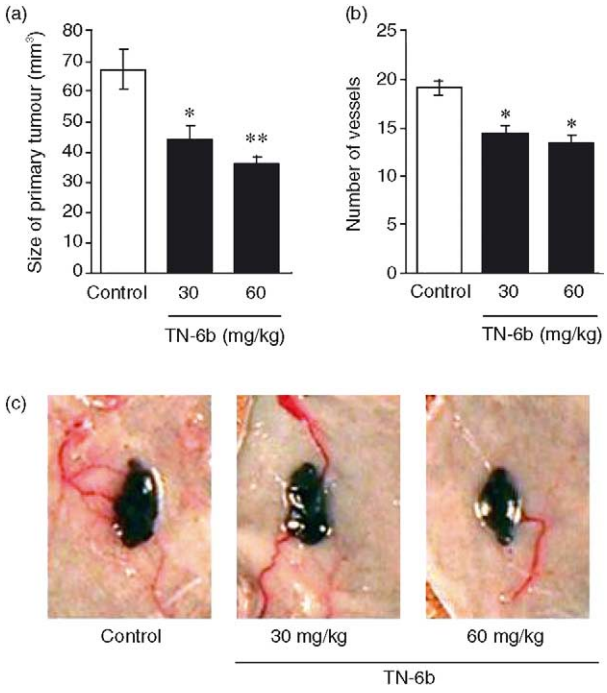


Fig. 6. Effect of TN-6b on tumour growth and angiogenesis induced by B16-BL6 melanoma cells implanted on the backs of mice. Mice were intradermally inoculated with B16-BL6 cells and subcutaneously (s.c.) administered with TN-6b or vehicle for 6 days from the day of tumour inoculation. Tumour-inoculated sites were isolated from vehicle- or TN-6b-treated mice 6 days after the tumour inoculation and the tumour-supplying vessels were counted (b) and the tumour size was measured (a). The macroscopic observation is shown in (c). Data are represented as the mean±SEM of seven mice in each group. * $P<0.05$; ** $P<0.01$.

crucial step in the processes of tumour growth, invasion and metastasis, as well as in angiogenic processes [23]. Tumours may generally be regarded as having two compartments, namely the tumour cell compartment and the endothelial compartment. Inhibitors of matrix-degrading enzymes could limit the expansion of both compartments, thereby interfering with both tumour angiogenesis and cancer spread [24]. Therefore, MMPs are attractive targets for anticancer agents and a variety of synthetic inhibitors of MMPs have been successfully developed and evaluated in animal and preclinical models of cancer [10,12].

In this study, we have investigated the effect of TN-6b, a broad-spectrum MMP inhibitor (Fig. 1b), on tumour metastasis and angiogenesis *in vitro* and *in vivo*. The primary role of MMPs in metastasis is to create a path for tumour cells to colonise host tissues by virtue of their ECM degrading ability. Invasion and metastasis require the disruption of several collagen-containing tissue barriers, and therefore most of the initial interest focused on proteinases that are capable of degrading collagen IV, the major collagen constituent of basement membranes. These include gelatinases A (MMP-2) and B (MMP-9) [6,25]. MMPs are capable of activating other members of their family. For example, MMP-1 can activate latent MMP-2. MT1- and MT-2 MMP can activate latent MMP-13, which in turn can activate latent MMP-2 and MMP-9. Latent MMP-13 can also be activated by MMP-3, -10 and -12 [25]. Therefore, TN-6b may be a potent and attractive agent to prevent cancer metastasis and angiogenesis because it is a broad-spectrum MMP inhibitor (MMP-1, -2, -3, -7, -8, -9, -12, -13 and -14).

TN-6b could directly inhibit the enzymatic activity of MMP-2 and MMP-9 produced by LLC and HSE cells (Fig. 2). In addition, TN-6b inhibited a number of *in vitro* metastatic functions of tumour cells, including invasion and migration (Figs. 3a and b and 4a). However the enantiomer TN-6d, with less MMP inhibitory

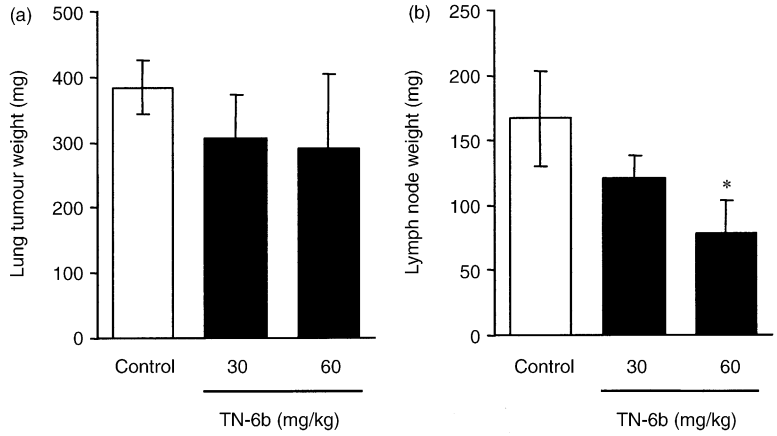


Fig. 7. Tumour growth and mediastinal lymph node metastasis after orthotopic implantation of Lewis lung carcinoma (LLC) cells. LLC cell suspension was orthotopically implanted into the left lungs of mice followed by subcutaneous (s.c.) administration of TN-6b or vehicle over 14 days from day 1 after the tumour inoculation. The weight of the tumour at the implanted site (a) and the metastasised tumour at the mediastinal lymph node (b) were measured on day 21 after the implantation. Data are represented as the mean±SEM of six mice in each group. * $P<0.05$.

activity, did not inhibit the invasive property of tumour cells, indicating that the MMP inhibitory activity plays an essential role in the anti-invasive effect of TN-6b (Fig. 3b). Integrins are important mediators of cell migration, and several MMPs have been shown to colocalise with various αβ integrin heterodimers at the site of cellular attachment and detachment [26]. For example, MMP-2 has been found to localise at the cell surface with MT1-MMP and αvβ3 integrin and promote cell migration [27]. In addition, MMPs play an important role in the migration process via their interaction with tumour-displayed cell-adhesion molecules such as cadherins [28] and CD44 [29]. These studies have shown that MMPs can assist a migrating tumour cell by associating with or processing cell-adhesion molecules on the tumour-cell surface and MMPs not only play a role in ECM degradation, but also can be used by stromal and tumour cells.

Solid tumours require a vascular supply and tumours are provided with nutrition through the vessels. Therefore, angiogenesis and the vascular density of tumours have been shown to be associated with tumour metastasis [30]. Many angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) and angiopoietins [30,31] and their functions, have been determined. MMP-2 and MMP-9 secretion are involved in the degradation of type IV, V, VII and X collagens, fibronectin and elastin, but not native type I collagen, proteoglycans or laminins [32], which are components of the ECM and BM. Degradation of the ECM facilitates the invasion of endothelial cells across such structures and this is an important step for neovasculature and metastatic formation. In this study, we investigated the effect of TN-6b on endothelial function, such as invasion, migration and tube formation. These processes are essential steps for neovessel sprouting [33] and angiogenesis [34,35], which is an important biological process involved in metastasis formation. The invasion, migration and tube-like formation of endothelial cells were significantly inhibited by TN-6b in a dose-dependent manner, but TN-6d, its enantiomer, did not show any inhibitory effect in the invasion and tube-like formation assays (Figs. 3–5).

During the metastatic process, the ability of tumour cells to remodel the host tissue ECM involves the secretion of a variety of proteases from all five major classes: these are serine, cysteine, threonine, aspartic and metalloproteinases [25]. TN-6b downregulated the matrix metalloproteinases (MMPs) and therefore showed antimetastatic and anti-angiogenic activities. TN-6b, at the concentration of 50 μg/ml, almost completely inhibited MMP activities in zymogram assays, but TN-6b showed approximately 50% inhibition in the invasion, migration and tube formation assays at the same doses (50 μg/ml). Similar results were obtained in the experiments using another synthetic MMP inhibitor, MMI 270 (unpublished data).

Next, we investigated the effect of TN-6b against *in vivo* tumour-induced angiogenesis. Murine B16-BL6 melanoma cells are reported to express gelatinase A (MMP-2) and MT1-MMP (MMP-14) [8], target molecules of TN-6b. TN-6b may be an effective tumour-induced angiogenic model using B16-BL6 cells because TN-6b can inhibit a variety of MMP activities. This study showed that TN-6b prevented *in vivo* angiogenesis and primary tumour growth in a dose-dependent manner (30 and 60 mg/kg, Fig. 6).

Since the metastatic spread of many tumours such as breast [35], nasopharyngeal [31] and lung cancers [1] relies on lymphangiogenesis, at least to some extent, regulation of this process is an important feature in

understanding cancer metastasis. The distribution of basement membrane collagen has been shown to correlate significantly with the presence of lymph node metastasis [36]. Therefore, we investigated the anti-metastatic activity of TN-6b in an *in vivo* mediastinal lymph node metastasis model using LLC cells expressing collagenases (MMP-1), gelatinases (MMP-2 and -9) and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 [37]. We have demonstrated that TN-6b reduced *in vivo* mediastinal lymph node metastasis in the same dose range (30 and 60 mg/kg) as was effective for reducing tumour-induced angiogenesis. Thus, this study demonstrates that inhibition of MMPs can significantly reduce angiogenesis and metastasis *in vivo* and suggests that the anti-angiogenic activity is a possible mechanism for the inhibition of lymph node metastasis by TN-6b.

In contrast to the *in vivo* angiogenic model using B16-BL6 cells, the primary tumour size in the lung was not reduced significantly in the lymph node metastasis model using LLC cells (Figs. 6a and 7a). It is possible that TN-6b is effective at an early stage of tumour progression rather than at later stages because the duration of TN-6b administration and the experimental period were different in the two models examined. Another possibility is that the tissue concentration and distribution of TN-6b at the primary tumour site, for example in the lung or the skin, might be different. Therefore, it is likely that the effect of TN-6b on the growth of the primary tumour may depend on the type of tumour, the stage of progression, duration of administration, and/or concentration of TN-6b in tissues with tumours. MMI 270, another synthetic MMP inhibitor, also did not inhibit primary tumour growth in the LLC lymph node metastasis model (unpublished data), suggesting that the primary tumour growth of LLC cells in the lung is not sensitive to the MMP inhibitor.

In conclusion, this study indicates that TN-6b, a new matrix metalloproteinase inhibitor, may have therapeutic potential for controlling tumour metastasis, based on its inhibitory effect on invasion and angiogenesis. Characterisation of the detailed biological mechanism of the inhibitory effect of TN-6b will provide new insights into the contribution of MMP activities in lymph node metastasis and the future clinical evaluation of this compound.

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