Bastadin 6, a spongean brominated tyrosine derivative, inhibits tumor angiogenesis by inducing selective apoptosis to endothelial cells

Shunji Aoki[a], Seok-hwan Cho[a], Mayumi Ono[b], Takashi Kuwano[b], Shintaro Nakao[b], Michihiko Kuwano[c], Shinsaku Nakagawa[a], Jian-Qing Gao[a], Tadanori Mayumi[a], Masabumi Shibuya[d] and Motomasa Kobayashi[a]

Bastadin 6, a macrocyclic tetramer of a brominated tyrosine derivative, was isolated from a marine sponge and its anti-angiogenic activity was evaluated. Bastadin 6 was found to inhibit vascular endothelial growth factor (VEGF)- or basic fibroblast growth factor (bFGF)-dependent proliferation (IC_{50} = 0.052 μmol/l) of human umbilical vein endothelial cells (HUVECs) 20- to 100-fold selectively in comparison with normal fibroblast (3Y1) or several tumor cells (KB3-1, K562 and Neuro2A). Bastadin 6 also inhibited VEGF- or bFGF-induced tubular formation (0.1 μmol/l, 6 h treatment) and VEGF-induced migration (1 μmol/l, 4 h treatment) of HUVECs. Moreover, bastadin 6 almost completely blocked VEGF- or bFGF-induced *in vivo* neovascularization in the mice corneal assay and suppressed growth of s.c. inoculated A431 solid tumor in nude mice (100 mg/kg, i.p.). Bastadin 6 induced cell death of HUVECs with an apoptotic phenotype, whereas it showed no effect on the VEGF-induced auto-phosphorylation of VEGF receptors Flt-1 and KDR/Flk-1. These results suggest that the anti-angiogenic effect of bastadin 6 is closely related to selective induction activity of apoptosis against endothelial cells. *Anti-Cancer Drugs* 17:269–278

Anti-Cancer Drugs 2006, **17**:269–278

Keywords: apoptosis, anti-angiogenesis, anti-tumor activity, bastadin 6

[a]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan, [b]Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [c]Research Center for Innovative Cancer Therapy of the 21st Century COE Program for Medical Science, Kurume University, Fukuoka, Japan and [d]Department of Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Correspondence to M. Kobayashi, Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka, 565-0871, Japan.
Tel: +81 66879 8215; fax: +81 66879 8219;
e-mail: kobayasi@phs.osaka-u.ac.jp.

Sponsorship: This study was supported by grants from Takeda Science Foundation and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Received 23 August 2005 **Accepted** 18 November 2005

Introduction

Tumor angiogenesis involves several processes such as proliferation, migration, invasion and tubular formation of endothelial cells. These processes are regulated by multiple factors such as specific angiogenic growth factors [i.e. vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)] or cytokines produced by tumor cells and the surrounding stroma [1]. Several anti-angiogenic agents have been tested in preclinical studies and clinical trials. A number of agents such as neutralizing antibodies to angiogenic proteins [2,3], natural products [4] and synthetic angiogenic inhibitors [5] have been studied for their anti-angiogenic ability and anti-tumor effect.

Angiogenesis inhibitors have been classified into two categories, i.e. 'indirect' and 'direct' [6]. Indirect inhibitors interfere with pro-angiogenic communication between the tumor and endothelial cell compartments. Most anti-angiogenic agents such as blockers of the angiogenic growth factor [7,8], inhibitors of matrix metalloproteinase [9] and integrin antagonists [10] have been developed on the basis of this concept. On the other hand, direct inhibitors disrupt directly endothelial cells of new blood capillaries in the tumor and arrest blood flow. For example, endostatin [11], angiostatin [12,13] and thrombospondin [14,15] target microvascular endothelial cells and induce apoptosis of the endothelial cells selectively. ZD6126, a vascular-targeting agent, selectively disrupts the cytoskeleton of endothelial cells in tumors [16].

In the course of our study of bioactive substances from marine organisms, we focused on a search for anti-angiogenic substances and isolated a novel sulfated sterol named lembehsterol as a thymidine phosphorylase (TP) inhibitor from a marine sponge [17]. TP has been shown to be an angiogenic factor [18]. We further isolated bastadin 6 [19] as a selective inhibitor of proliferation of human umbilical vein endothelial cells (HUVECs) (Fig. 1). In-vitro studies have demonstrated that bastadin 6 inhibits VEGF- or bFGF-dependent proliferation of

0959-4973

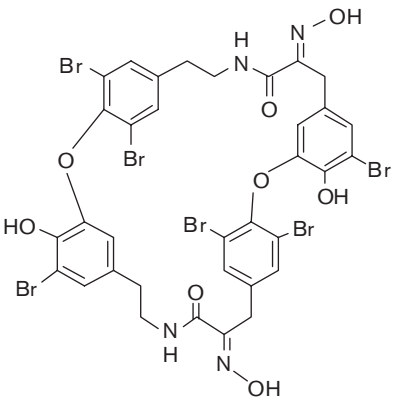
HUVECs at concentrations 20- to 100-fold less than those required to inhibit proliferation of normal fibroblasts or several tumor cells. Anti-angiogenic effects of bastadin 6 were shown in a VEGF-induced migration assay and VEGF- or bFGF-induced Matrigel tubular formation assays. Moreover, bastadin 6 inhibited in-vivo neovascularization in the mice corneal micropocket assay and suppressed tumor growth in the mice xenograft model. Bastadin 6 induced apoptosis against HUVECs, whereas it showed no effect on VEGF-induced autophosphorylation of VEGF receptors Flt-1 and KDR/Flk-1. This paper presents details of the anti-angiogenic effect of bastadin 6.

Materials and methods

Materials

DMEM and RPMI 1640 were purchased from Nissui Pharmaceutical (Tokyo, Japan). WST-8 colorimetric reagent was from Nakalai Tesque (Kyoto, Japan). The chemotaxicell chamber (8 μm) was obtained from Kurabo (Osaka, Japan). Human recombinant $VEGF_{165}$, mouse recombinant $VEGF_{165}$ and human recombinant bFGF were from R & D Systems (Minneapolis, Minnesota, USA). The monoclonal phosphotyrosine antibody (PY-20) was from ICN Biochemicals (Costa Mesa, California, USA). Anti-Flt-1 antibody and anti-KDR antibody were from Santa Cruz Biotechnology (Santa Cruz, California, USA). The fluorometric TUNEL system was from Promega (Madison, WI). Hydron pellets were from IFN Science (New Brunswick, NJ). Carboxymethyl cellulose sodium salt (CMC) was from Nakalai Tesque. Mice (6 weeks old, BALB/c) and nude mice (6 weeks old, BALB/c, *nu/nu*) were from SLC (Hamamatsu, Japan). Doxorubicin, etoposide and other reagents were purchased from Sigma (St Louis, Missouri, USA) or Nakalai Tesque.

Fig. 1



Chemical structure of Bastadin 6.

Isolation of bastadin 6

Bastadin 6 was isolated from the marine sponge, *Ianthella basta*, which was collected in Indonesia in 1998. The dried sponge (50 g) was repeatedly extracted with methanol and the methanol-soluble portion was concentrated *in vacuo* to obtain a methanol extract. The methanol extract was partitioned into an EtOAc:H_2O mixture (1 : 1) and the H_2O-soluble portion was further partitioned with *n*-butanol. The EtOAc-soluble portion (2.2 g), which showed selective anti-proliferative effect against HUVECs, was separated by SiO_2 column chromatography (eluent, hexane:EtOAc and $CHCl_3$:MeOH:H_2O) and ODS column chromatography (eluent, MeOH:H_2O). The active fraction (460 mg) was further purified by ODS HPLC (eluent, MeOH:H_2O) to afford bastadin 6 (78 mg). The isolated bastadin 6 was identified against an authentic sample by mass and NMR analysis. Bastadin 6 is characterized as a macrocyclic tetramer of a brominated tyrosine derivative (Fig. 1).

Cell culture and cDNA transfection

HUVECs (5×10^5 cells/vial) were obtained from Kurabo and grown in HuMedia-EG2 medium with growth supplements (Kurabo). Human KB epidermoid carcinoma cells (KB3-1) were cultured in RPMI 1640 medium, and neuroblastoma cells (Neuro2A), human chronic myelogenous leukemia cells (K562) and rat fibroblasts (3Y1) were cultured in DMEM supplemented with heat-inactivated 10% FBS and kanamycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. A mouse NIH 3T3 cell line overexpressing human KDR/Flk-1 or Flt-1 receptor was established as described previously [20,21]. KDR/Flk-1 or Flt-1 expression vector was transfected into NIH 3T3 cells followed by selection of G418-resistant clones to establish NIH 3T3-KDR or NIH 3T3-Flt-1. Each cell line was maintained in DMEM medium supplemented with heat-inactivated 10% calf serum, kanamycin (40 μg/ml) and G418 sulfate (200 μg/ml; Nakalai Tesque)

Growth inhibition assay

A suspension of HUVECs in proliferation medium (HuMedia-EG2) with growth supplements was plated into each well of 96-well plates (2×10^3 cells/well/100 μl). After 24 h, the culture medium was removed and replaced with fresh essential minimal medium (HuMedia-EB2) with growth factor [bFGF (30 ng/ml) or VEGF (30 ng/ml)] of endothelial cells and various concentrations of bastadin 6. Plates were incubated for an additional 72 h in a humidified atmosphere of 5% CO_2 at 37°C and cell proliferation was detected by WST-8 colorimetric reagent. Cell proliferations of other cell lines were measured by the MTT colorimetric assay. The IC_{50} value was determined by linear interpolation from the growth inhibition curve. We selectivity assessed anti-proliferative activity [selective index (SI)] from the differences of IC_{50} values against HUVECs and other cell lines.

VEGF- or bFGF-induced Matrigel tubular formation assay

Ninety-six-well plates were coated with 50 μl of Matrigel (11.55 mg/ml) and incubated at 37°C for 1 h to promote gelation. HUVECs were trypsinized, washed with HEPES buffer, counted and resuspended in HuMedia-EB2 medium supplemented with bFGF (30 ng/ml) or VEGF (30 ng/ml) and 0.2% FBS in the presence or absence of different concentrations of bastadin 6. HUVECs (1×10^4 cells/well) were seeded onto the solidified Matrigel in 96-well plates. Plates were incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 4–6 h, tubular network patterns were captured through an inverted phase contrast microscope and photographed.

VEGF-induced migration assay

A polycarbonate filter of the inner chamber of a Chemotaxicell chamber (8 μm) was soaked in fibronectin solution (1.33 μg/ml) for 1 h at 37°C and dried *in vacuo*. HUVECs (3×10^5 cells) were suspended in HuMedia-EB2 medium containing 0.2% FBS and seeded in the inner chamber. The inner chamber was put into the outer chamber (24-well plate), which was filled with the same medium containing VEGF (20 ng/ml) and various concentrations of bastadin 6. After 4 h incubation at 37°C, non-migrated cells on the upper surface of the filter were removed by wiping with cotton swabs, and the filter was fixed with 70% EtOH and stained with Giemsa. The cells that migrated through the filter to the reverse side were counted manually at six different areas under a microscope (× 200).

Immunoblotting assay

Confluent NIH 3T3-Flt-1 or NIH 3T3-KDR cells were cultured in six-well plates supplemented with serum-depleted DMEM medium for 48 h. The cells were then pre-incubated with several concentrations of bastadin 6 for 2 h and stimulated with VEGF (20 ng/ml) for 5 min at 37°C. The cells were rinsed with ice-cold PBS and lysed with HNTG buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 1% Triton X-100, and 10% glycerol containing 1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mmol/l sodium vanadate). The cell lysate was subjected to SDS–PAGE and transferred onto Immobilon membranes (Millipore, Bedford, Massachusetts, USA). The membrane was incubated with the blocking solution and probed with the primary antibodies [anti-phosphotyrosine (PY-20) antibody, anti-Flt-1 receptor antibody or anti-KDR antibody]. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham, New Jersey, USA).

TUNEL assay for detection of apoptosis

A fluorescein apoptosis detection system (Promega, Madison, Wisconsin, USA) was used. Cells were inoculated on a chamber slide and incubated 24 h before treatment with bastadin 6. After exposure to bastadin 6, the cells were fixed in 4% paraformaldehyde (pH 7.4) for 25 min on ice. The cells were immersed in PBS with 0.2% Triton X-100 solution for 5 min, equilibrated in the manufacturer's buffer and then incubated with fluorescein-12 dUTP in the presence or absence of TdT for 60 min at 37°C in the dark. The reaction was terminated by immersion in the stop solution for 15 min. The cells were rinsed 3 times in PBS and immersed in PBS containing 1 μg/ml propidium iodide. The fluorescein-labeled DNA strand breaks (TUNEL-positive cells) were then visualized using a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) and pictures were taken with a digital camera. The TUNEL-positive nuclei were compared with the stained nuclei by propidium iodide to determine the percentage of apoptosis induction by the various concentrations of bastadin 6.

Assay for caspase-3/7 activity

HUVECs and KB3-1 cells (1×10^4 cells/well) were inoculated into white-walled 96-well plates. After 24 h, the culture medium of HUVECs was exchanged with HuMedia-EB2 medium containing VEGF (30 ng/ml) and various concentrations of bastadin 6. KB3-1 cells were also treated with various concentrations of bastadin 6 and incubated for 11 h in a humidified atmosphere of 5% CO_2 at 37°C. A caspase luminescent assay (Promega) was used to determine the enzymatic activity of caspase-3/7 according to the manufacturer's instructions. Cleavage of the pro-luminescent substrate (z-DEVD-aminoluciferin) containing the DEVD sequence by caspase-3/7 was monitored by a luminometer (Berthold Technologies, Bad Wildbad, Germany). Quantification of caspase-3/7 was calculated as fold increase over the control.

Corneal micropocket assay in mice

The corneal micropocket assay was performed as described previously [22]. Hydron pellets containing human bFGF (40 ng) or murine VEGF (200 ng) were prepared and implanted in the corneas of male BALB/c mice (SLC). Bastadin 6 was administered by adding directly to the hydron pellet (1.0 or 0.1 μg/pellet) or by i.p. injection (100 or 50 mg/kg/day) on days 1–3. On day 5, the corneal vessels of anesthetized mice were photographed.

In-vivo experiment using tumor xenografts

All procedures were conducted in accordance with the institute's animal care committee guidelines. Human epidermoid carcinoma cells, A431, were cultured in DMEM medium supplemented with heat-inactivated 10% FBS and kanamycin (50 μg/ml). A431 cells (1×10^5) were implanted s.c. into the right ventral flank of 6-week-old female athymic mice (BALB/c, *nu/nu*; SLC). Bastadin 6 was administered by two protocols starting at the early or late stage of xenograft development. In the former, the administration (100 or 50 mg/kg/day) of bastadin 6 was

started at the day of A431 cell implantation (every other day for 2 weeks; 7 times in total). Bastadin 6 was administered by i.p. injection as a suspension of 0.1% CMC. Control groups were treated with 0.1% CMC. In the latter, the treatment with bastadin 6 was started 1 week after cell implantation, when the tumor volume had grown to 50–100 mm^3. Tumor growth was measured twice a week by calipers. The tumor length data were converted to tumor volume (V) using the formula $V = W^2 \times L/2$, where W and L are defined as the smaller and larger diameters, respectively.

Results

Selective anti-proliferative effect of bastadin 6 against endothelial cells

The anti-proliferative effect of bastadin 6 against endothelial cells (HUVECs) and several tumor cells derived from different organs was evaluated. HUVECs were treated with various concentrations of bastadin 6 for 72 h and cell viability was determined by the WST-8 colorimetric assay. The anti-proliferative activity was represented by the IC_{50} value and SI between HUVECs and other cell lines. Bastadin 6 showed a selective anti-proliferative effect against HUVECs in comparison with other cell lines (KB3-1, Neuro2A, K562 and 3Y1). In particular, when HUVECs were cultured under VEGF- or bFGF-dependent conditions, the growth-inhibitory activity ($IC_{50} = 0.052$ μmol/l) of bastadin 6 was 41-, 35-, 17- and 106-fold stronger than that of KB3-1, Neuro2A, K562 and 3Y1, respectively (Table 1). No significant difference in the effects of bastadin 6 was observed between the bFGF- and VEGF-dependent conditions. Clinical chemotherapeutic agents such as doxorubicin and etoposide showed no selective anti-proliferative activities against HUVECs (Table 1).

Inhibitory activity of bastadin 6 on bFGF- or VEGF-induced tubular formation of endothelial cells

The anti-angiogenic property of bastadin 6 was examined by the Matrigel tubular formation assay. HUVECs were plated on Matrigel (a gel of mouse basement membrane protein) in the presence of bFGF (30 ng/ml); the cells aligned with high motility and cell–cell communication, and formed a tight tubular network within 6 h. When HUVECs were plated on Matrigel without addition of exogenous growth factor, the cells showed only a few spontaneous tubular formations. Treatment with bastadin 6 (0.1 μmol/l) inhibited bFGF-induced tubular formation of HUVECs. A dose-dependent inhibition of the formation of a tubular structure was observed in the range of 0.01–1 μmol/l bastadin 6 (Fig. 2). Bastadin 6 also inhibited tubular formation induced by VEGF (30 ng/ml) in the same manner as bFGF-induced tubular formation (data not shown).

Inhibitory effect of bastadin 6 on VEGF-induced migration of HUVECs

Cell proliferation, migration and tubular formation of vascular endothelial cells are essential for the development of the neovasculature. We performed a migration assay using a chemotactic chamber to evaluate the inhibitory activity of bastadin 6 on VEGF-induced migration of HUVECs. In this assay, HUVECs in the inner chamber migrate to the reverse side of the membrane filter coated with fibronectin by stimulation with VEGF in the outer chamber. A concentration of 1 μmol/l of bastadin 6 in the outer chamber inhibited 50% migration of HUVECs in comparison with the control (Fig. 3).

Effect of bastadin 6 on VEGF-induced auto-phosphorylations of Flt-1 and KDR/Flk-1

To elucidate the inhibitory mechanisms of bastadin 6 for proliferation, tubular network formation and migration of HUVECs, we examined whether VEGF-induced tyrosine auto-phosphorylation of Flt-1 and KDR/Flk-1 would be blocked by bastadin 6 or not. The treatment of NIH 3T3/Flt-1 or NIH 3T3/KDR cells with VEGF (20 ng/ml) enhanced the auto-phosphorylation of Flt-1 or KDR/Flk-1. Concentrations of 0.1–5 μmol/l of bastadin 6 showed no inhibitory activity against VEGF-induced auto-phosphorylations of Flt-1 and KDR/Flk-1 (Fig. 4).

Induction of apoptosis of endothelial cells by bastadin 6

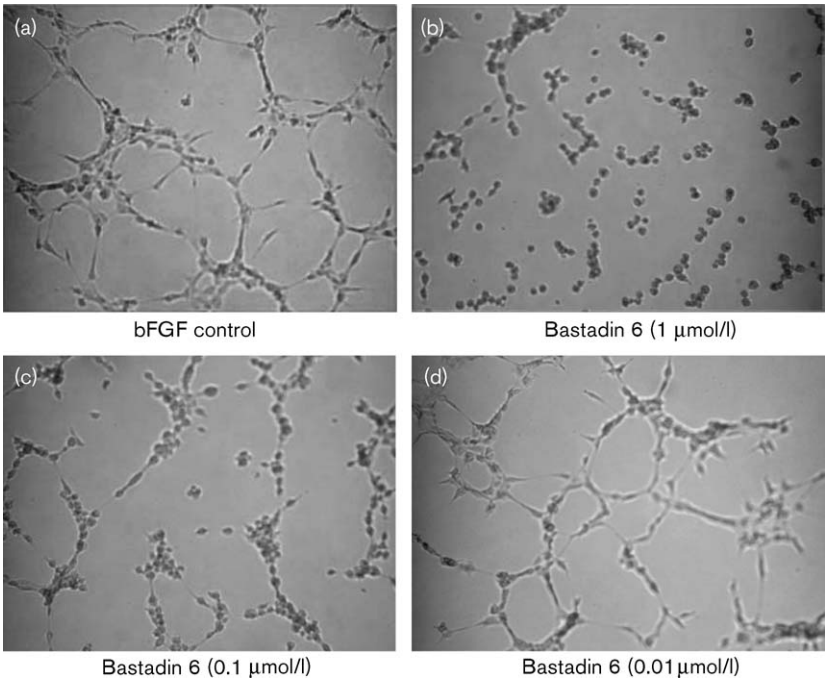
We found that bastadin 6 selectively induced cell death of HUVECs. Several compounds that induce selective cell

Table 1 Growth inhibition of bastadin 6 and known anti-cancer agents against several cell lines

	Bastadin 6		Doxorubicin		Etoposide	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
HUVECs (VEGF)[a]	0.052	1	0.22	1	3.2	1
HUVECs (bFGF)[b]	0.052	1	0.18	0.8	3.3	1
HUVECs (normal)[c]	0.18	3.5	0.13	0.6	1.6	0.5
KB3-1	2.1	41	0.36	1.6	4.3	1.3
N2A	1.8	35	0.40	1.8	1.1	0.3
K562	0.85	17	0.095	0.4	0.91	0.3
3Y1	5.5	106	0.52	2.4	8.0	2.5

[a,b]Cultured in medium containing only VEGF or bFGF as a growth factor.
[c]Cultured in commercially available proliferating medium.
SI = IC_{50} against tested cells/IC_{50} against HUVECs (VEGF-dependent condition).

Fig. 2



Effect of bastadin 6 on tubule formation of HUVECs induced by bFGF. HUVECs (1×10^4 cells) were suspended in essential minimal medium supplemented with bFGF (30 ng/ml) in the absence (a) or presence (b–d) of the indicated concentrations of bastadin 6 and inoculated onto solidified Matrigel in 96-well plates. After 6 h, images were captured under a microscope ($\times$ 200).

death of endothelial cells are reported to be anti-angiogenic agents [11,12]. To clarify whether bastadin 6 induces apoptosis against endothelial cells or not, we performed the TUNEL assay for analyzing DNA fragmentation. As shown in Fig. 5, HUVECs treated with bastadin 6 (0.01–1 μmol/l, 12 h) showed a dose-dependent increase of TUNEL-positive cells (Fig. 5A).

The pathways of apoptotic cell death involve activation of caspases, which lead to degradation and inactivation of key cellular proteins concerned with DNA repair, signaling and cell structure. We examined whether bastadin 6 (0.01–1 μmol/l, 11 h) activates caspase-3/7 in HUVECs or not. The activity of caspase-3/7 in HUVECs increased 3.5- and 2.5-fold by bastadin 6 treatment at 1 and 0.1 μmol/l, respectively, while the activity of caspase-3/7 in KB3-1 cells did not increase at the same concentrations, and a 2.4-fold increase was observed at 10 μmol/l bastadin 6 (Fig. 5B).

Inhibitory effect of bastadin 6 on bFGF- or VEGF-induced neovascularization in mice cornea

We examined the in-vivo effect of bastadin 6 on angiogenesis in newly formed mice corneas by VEGF or bFGF treatment. VEGF (200 ng/pellet) as well as bFGF (40 ng/pellet) induced marked angiogenesis in the non-vascular area of mice corneas as shown in Fig. 6. When bastadin 6 was administered by adding directly in a pellet (0.1 μg/pellet) or by injecting (i.p., 100 mg/kg/day) on days 1–3, bFGF- or VEGF-induced neovascularization in mice corneas was completely inhibited (Fig. 6).

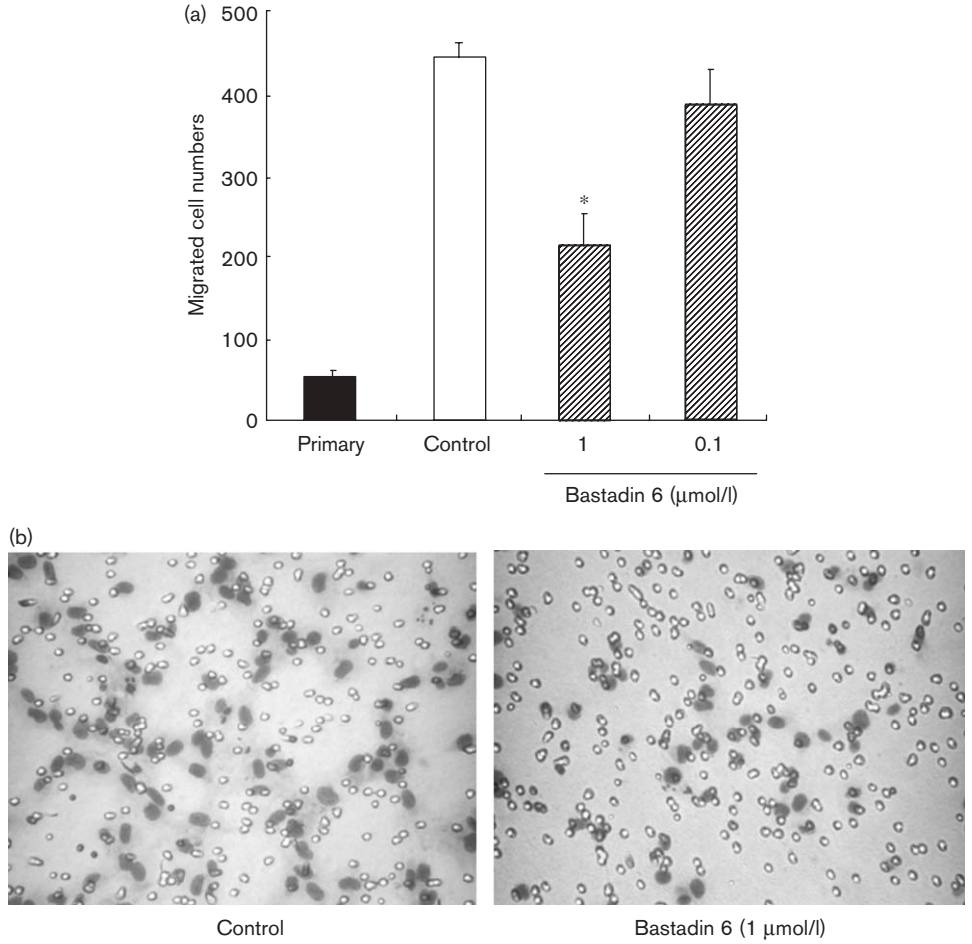
Inhibitory effect of bastadin 6 on the growth of xenograft tumor

We further evaluated the anti-tumor property of bastadin 6. Bastadin 6 (100 or 50 mg/kg/day) was administered every other day for 2 weeks (7 times in total) via i.p. injection. The administration of bastadin 6 was started on the day of A431 inoculation (early treatment) or after 1 week (late treatment). Significant inhibition of xenograft growth was observed on the early treatment. At the third week after inoculation of tumor cells, the early and late administrations of bastadin 6 reduced tumor growth up to 30 and 60% of that to the control, respectively (Fig. 7). No weight loss of the inoculated mice treated with bastadin 6 was observed during 2 weeks, and also no toxicities such as diarrhea, infection and weakening were observed.

Discussion

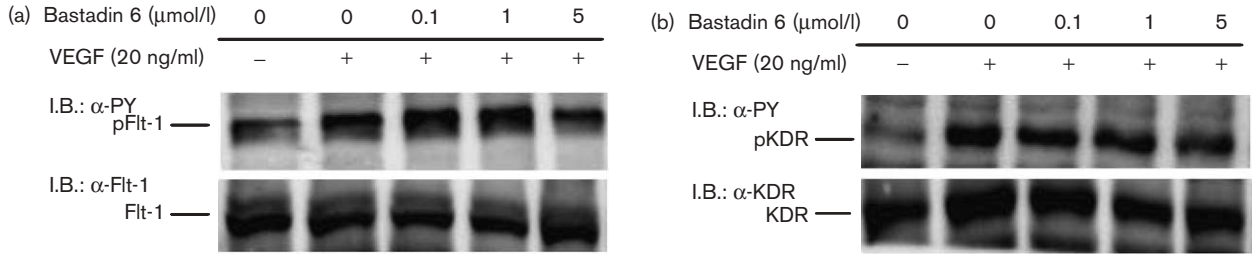
Angiogenesis requires several processes that must be tightly regulated by multiple angiogenic factors in a spatial and temporal manner. Each of these processes presents possible targets for the development of therapeutic agents against tumor angiogenesis. Based on

Fig. 3



Effect of Bastadin 6 on migration of HUVECs induced by VEGF. HUVECs (2×10^5 cells/well) were placed in the inner chamber. The indicated concentrations of bastadin 6 were added in the outer chamber and then stimulated with VEGF (20 ng/ml). After 4 h, the migrated cells were counted in six different microscopic fields ($\times 200$). The data are presented as the means ± SD (a). Representative figures of migrated HUVECs are shown in (b). *Statistically significant difference ($P < 0.05$) from the value for VEGF alone.

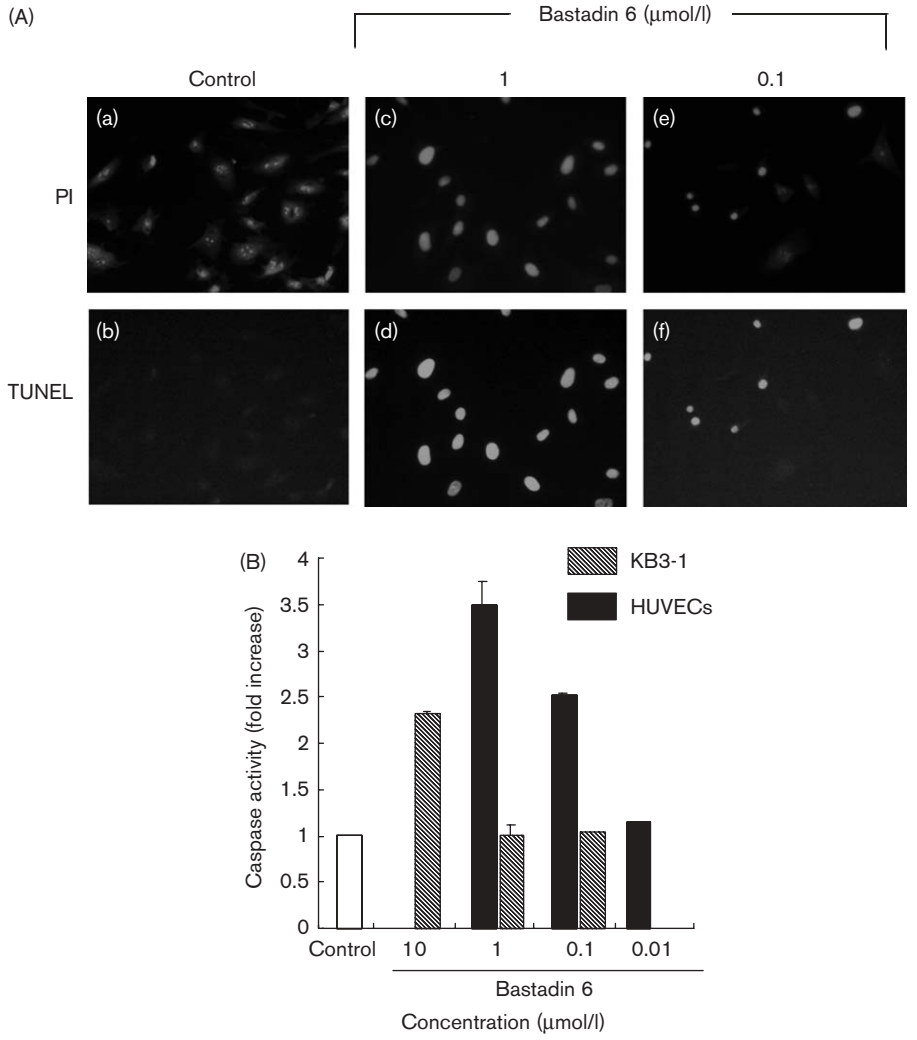
Fig. 4



Effect of bastadin 6 on auto-phosphorylation of VEGF-induced Flt-1 or KDR/Flk-1 receptor. The cells were pre-incubated with the indicated concentrations of Bastadin 6 for 2 h and 20 ng/ml of VEGF was added for 5 min at 37°C. Protein extracts were resolved by 7.5% SDS–PAGE, and detected by anti-phosphotyrosine (PY-20) antibody and anti-Flt-1 receptor antibody (a) or anti-KDR receptor antibody (b).

successful preclinical data, several agents are now in clinical trials. These agents are categorized as (i) interfering with angiogenic ligands, their receptors or downstream signaling (e.g. SU5416 [23,24], SU6668 [25], bevacizumab [26]), (ii) upregulating or delivering endogenous inhibitors (e.g. IL-12 [27]), or (iii) directly

Fig. 5

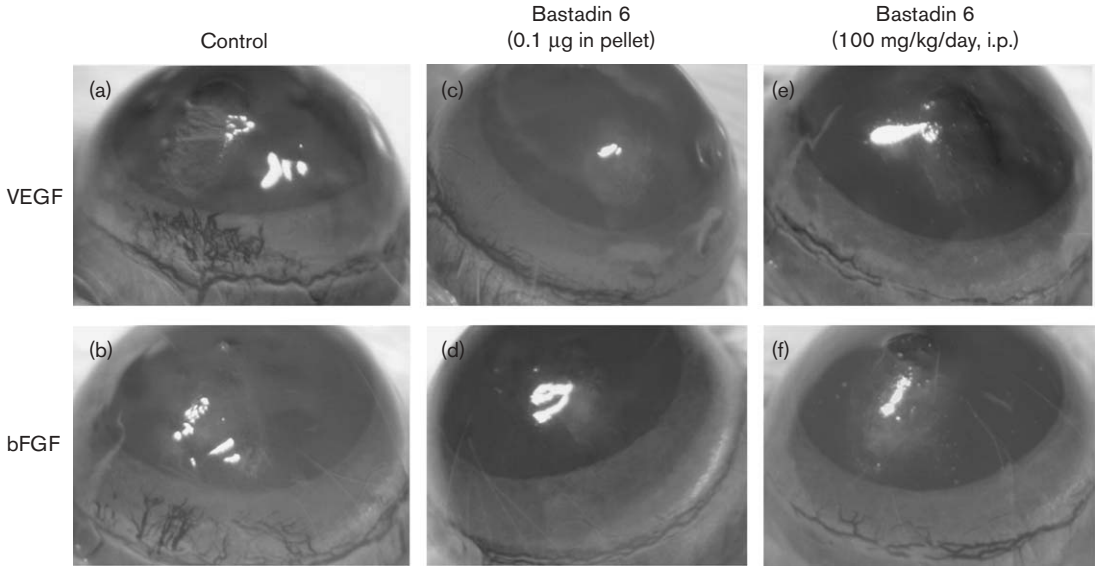


Detection of Bastadin 6-induced apoptosis in HUVECs by the TUNEL assay and caspase-3/7 activation. Apoptotic bodies of bastadin 6-treated HUVECs were detected after 12 h by fluorescent microscopy. Representative figures of the TUNEL assay are shown in (A). Panels (a), (c) and (e) are fields of cells stained with propidium iodide (1 μg/ml). Panels (b), (d) and (f) are the same fields under green fluorescent light (TUNEL-positive cells). Activation of caspase-3/7 in HUVECs and KB3-1 treated with bastadin 6 (B). HUVECs and KB3-1 cells (1×10^4 cells/well) were treated with the indicated concentrations of bastadin 6 for 11 h, and caspase-3/7 activity was detected by the caspase luminescence assay. The result for the control cells was taken as 1.0. Values are means ± SD for three independent experiments.

disrupting the tumor vasculature (e.g. endostatin [11], TNP470 [28,29]). Recently, the US Food and Drug Administration approved bevacizumab (Avastin), a recombinant humanized antibody to VEGF, as a first-line treatment for patients with metastasized colorectal cancer [26]. This indicates the possibility of an angiogenic inhibitor as a cancer chemotherapeutic agent.

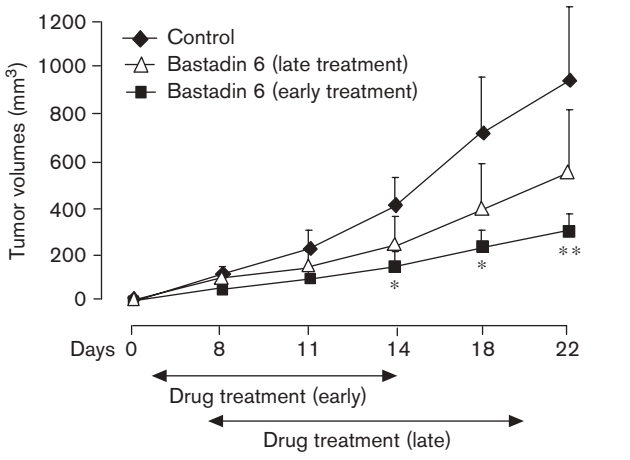
Bastadin 6, a cyclic tetramer of a brominated tyrosine derivative, was isolated as a selective growth inhibitor of HUVECs from a marine sponge. Bastadin 6 selectively inhibited VEGF- or bFGF-dependent proliferation of HUVECs at concentrations 20- to 100-fold less than those required to inhibit proliferation of normal fibroblasts or several tumor cells. This selective anti-proliferation against endothelial cells was expected to guarantee low toxicity in *in vivo* experiments. Bastadin 6 also inhibited tubular formation and migration of HUVECs. Moreover, i.p. administration of bastadin 6 prevented in-vivo neovascularization in the mice corneal assay. Therefore, we evaluated the anti-tumor effect of bastadin 6 on an in-vivo xenograft model. Bastadin 6 (100 mg/kg/day, 7 times during 2 weeks) showed inhibitory activity on tumor growth in an A431 xenograft model without any acute toxicity. These results suggested that bastadin 6 showed in vivo anti-tumor activity based on its anti-angiogenic property.

Fig. 6



Inhibitory effect of bastadin 6 on VEGF- or bFGF-induced corneal neovascularization. Hydron pellets containing 200 ng of VEGF (a) or 40 ng of bFGF (b) were implanted into corneal micropockets of mice. At the same time as pellet implantation, mice were treated with bastadin 6 [1 or 0.1 μg in a pellet (c and d), by i.p. injection, 100 or 50 mg/kg/day (e and f) on days 1–3]. After 5 days, new vessels developing in the region around the implanted pellet were photographed. Representative figures of mouse corneas are shown.

Fig. 7



Inhibitory effect of tumor growth in xenograft animals by bastadin 6. A431 carcinoma cells (1×10^5) were implanted s.c. into nude mice and bastadin 6 was administered using two protocols starting at the early or late stage of xenograft development. Early bastadin 6 treatment was started from day 1 of A431 cell implantation. Late treatment was started from day 7. Bastadin 6 (50 or 100 mg/kg) was administered every other day for 2 weeks by i.p. injection (7 times in total). Tumor growth is presented as the mean tumor volume (mm^3) ± SD obtained from six mice for each group. *Statistically significant difference (*$P<0.05$, **$P<0.01$) from the value for the 0.1% CMC vesicle-treated control group.

In the tumor angiogenesis process, VEGF or its receptors (KDR/Flk-1, Flt-1) have been shown to play a direct role by promoting proliferation, migration and vascular permeability of endothelial cells, which are the key processes in angiogenesis. Recently, inhibitors with a small molecular weight have been developed as anti-angiogenic agents targeting VEGF receptors. For instance, SU5416 is a potent inhibitor of KDR/Flk-1 tyrosine kinase. SU5416 inhibits tumor neovascularization and induces regression of a variety of tumors [23]. In this study, bastadin 6 showed significant and selective anti-proliferative activity against HUVECs when the culture medium was changed to the VEGF- or bFGF-dependent condition. In order to investigate the mechanism of action of bastadin 6, we examined the effect of bastadin 6 on the VEGF-induced tyrosine auto-phosphorylation of Flt-1 and KDR/Flk-1. However, bastadin 6 did not inhibit VEGF-induced auto-phosphorylation of Flt-1 and KDR/Flk-1. This means that bastadin 6 does not block the activation of Flt-1 and KDR/Flk-1 receptors induced by VEGF stimulation.

However, we demonstrated that the HUVECs treated with bastadin 6 showed a positive phenotype in TUNEL staining and bastadin 6 induced activation of caspase-3/7 in HUVECs. These results clearly indicated that the anti-proliferative effect of bastadin 6 was related to the induction of apoptosis of endothelial cells. It has been suggested that the selective induction of apoptosis of endothelial cells may represent a common feature of anti-angiogenic molecules such as angiostatin [12,13], thrombospondin [14,15], canstatin [30,31] and adiponectin [32]. Thrombospondin was reported to activate each of

four different molecules, CD36, pp59^{fyn}, caspases and p38MAPK, which were related to induction of apoptosis in microvascular cells. These molecules are essential for apoptosis of endothelial cells and may be targets for preventing tumor angiogenesis [14]. Additionally, angiostatin was reported to activate caspases such as caspase-3, -8 and -9, and induce apoptosis of endothelial cells. Angiostatin also bound to ATP synthase on the membrane surface of endothelial cells, which was thought to be a target molecule of angiostatin [15]. However, it remains unknown why these compounds can selectively induce apoptosis in endothelial cells. It is also unclear whether bastadin 6 acts on the same target molecules as thrombospondin or angiostatin or not. Fumagillin and its analogs (e.g. TNP-470, ovalicin [33], RK-805 [34], etc.) are also known to exhibit selective anti-proliferative activity against endothelial cells based on specific inhibition of methionine aminopeptidase-2 (MetAP2). They showed cytostatic growth inhibition of HUVECs and did not induce apoptosis of endothelial cells, indicating that the molecular target for bastadin 6 must be different from that for MetAP2 [28,29].

It has been clarified that bastadins modulate the skeletal muscle ryanodine receptor-1 (RyR-1) calcium channel [35–37]. RyR-1 was recognized as a major physiological pathway through which Ca^{2+} is mobilized from the sarcoplasmic reticulum during excitation/contraction coupling of the skeletal muscle. Recent studies have revealed that RyR-1 is associated with several proteins including FKBP12. FKBP12 forms a functional heteromeric complex and is essential for stabilizing the closed conformation of the Ca^{2+} release channel. Bastadins enhanced the ryanodine-binding capacity of the RyR-1 calcium channel complex by interacting with a novel modulatory domain on FKBP12 and modulated RyR-1 positively. There are three ryanodine receptor isoforms (RyR-1, -2 and -3). RyR-3 is widely expressed in a variety of cells including endothelial cells and represents an important component of Ca^{2+} signaling in endothelial cells [38]. RyR-3 may regulate various events related to angiogenesis since Ca^{2+} signaling is very important for regulating endothelial cell events such as growth or motility. It is unclear whether bastadin 6 interacts with RyR-3 to induce selective apoptosis of endothelial cells or not. Searching for a target molecule of bastadin 6 to induce selective apoptosis against endothelial cells may let us develop a new lead as a chemotherapeutic agent for cancer angiogenesis. The target molecule of bastadin 6 is now under investigation.

In conclusion, bastadin 6 was shown to inhibit several stages of angiogenesis such as proliferation, migration and tubular formation of endothelial cells, and *in vivo* neovascularization in a corneal micropocket model. These observations suggest that bastadin 6 has the potential to be an anti-cancer agent as an inhibitor of tumor angiogenesis.

Acknowledgment

The authors are grateful to Professor R. W. M. Soest, Zoologisch Museum, University of Amsterdam, for identification of the sponge.

References

1 Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992; **267**:10931–10934.
2 Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, *et al.* Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997; **57**:4593–4599.
3 Veronese ML, O'Dwyer PJ. Monoclonal antibodies in the treatment of colorectal cancer. *Eur J Cancer* 2004; **40**:1292–1301.
4 Beliveau R, Gingras D, Kruger EA, Lamy S, Sirois P, Simard B, *et al.* The antiangiogenic agent neovastat (AE-941) inhibits vascular endothelial growth factor-mediated biological effects. *Clin Cancer Res* 2002; **8**: 1242–1250.
5 Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, *et al.* ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; **62**:4645–4655.
6 Abdollahi A, Lipson KE, Sckell A, Zieher H, Klenke F, Poerschke D, *et al.* Combined therapy with direct and indirect angiogenesis inhibition results in enhanced antiangiogenesis and antitumor effects. *Cancer Res* 2003; **63**:8890–8898.
7 Beebe JS, Jani JP, Knauth E, Goodwin P, Higdon C, Rossi AM, *et al.* Pharmacological characterization of CP-547,632, a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for cancer therapy. *Cancer Res* 2003; **63**:7301–7309.
8 Dudley A, Gilbert RE, Thomas D, Cox A, Price JT, Best J, *et al.* STI-571 inhibits *in vitro* angiogenesis. *Biochem Biophys Res Commun* 2003; **310**:135–142.
9 Naglich JG, Jure-Kunkel M, Gupta E, Fargnoli J, Henderson AJ, Lewin AC, *et al.* Inhibition of angiogenesis and metastasis in two murine models by the matrix metalloproteinase inhibitor, BMS-275291. *Cancer Res* 2001; **61**:8480–8485.
10 Taga T, Suzuki A, Gonzalez-Gomez I, Gilles FH, Stins M, Shimada H, *et al.* α_v-Integrin antagonist EMD 121974 induces apoptosis in brain tumor cells growing on vitronectin and tenascin. *Int J Cancer* 2002; **98**:690–697.
11 Dhanabal M, Ramchandran R, Waterman MJF, Lu H, Knebelmann B, Segal M, *et al.* Endostatin induces endothelial cell apoptosis. *J Biol Chem* 1999; **274**:11721–11726.
12 Hanford HA, Wong CA, Kassan H, Cundiff DL, Chandel N, Underwood S, *et al.* Angiostatin$_{4.5}$-mediated apoptosis of vascular endothelial cells. *Cancer Res* 2003; **63**:4275–4280.
13 Moser TL, Kenan DJ, Ashley TA, Roy JA, Goodman MD, Misra UK, *et al.* Endothelial cell surface F_1–F_0 ATP synthase is active in ATP synthesis and is inhibited by angiostatin. *Proc Natl Acad Sci USA* 2001; **98**:6656–6661.
14 Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* 2000; **6**:41–48.
15 Guo N, Krutzsch HC, Inman JK, Robert DD. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. *Cancer Res* 1997; **57**:1735–1742.
16 Goto H, Yano S, Zhang H, Matsumori Y, Ogawa H, Blakey DC, Sone S. Activity of a new vascular targeting agent, ZD6126, in pulmonary metastases by human lung adenocarcinoma in nude mice. *Cancer Res* 2002; **62**:3711–3715.
17 Aoki S, Naka Y, Itoh T, Furukawa T, Rachmat R, Akiyama S, *et al.* Lembehsterols A and B, novel sulfated sterols inhibiting thymidine phosphorylase, from the marine sponge *Petrosia strongylata*. *Chem Pharm Bull* 2002; **49**:827–830.
18 Furukawa T, Yoshimura A, Sumizawa T, Haraguchi M, Akiyama S, Fukui K, *et al.* Angiogenic factor. *Nature* 1992; **356**:668.
19 Kazlaukas R, Lidgard RO, Murphy PT, Wells RJ, Blount JF. Brominated tyrosine-derived metabolites from the sponge *Ianthella basta*. *Aust J Chem* 1981; **34**:765–786.
20 Takahashi T, Shibuya M. The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-γ pathway and partially induces mitotic signals in NIH 3T3 fibroblasts. *Oncogene* 1997; **14**:2079–2089.
21 Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene* 1995; **10**:135–147.

22 Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M, *et al.* ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002; **62**:2554–2560.

23 Fong TAT, Shawver LK, Sun L, Tang C, App H, Powell TJ, *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999; **59**:99–106.

24 Zangari M, Anaissie E, Stopeck A, Morimoto A, Tan N, Lancet J, *et al.* Phase II study of SU5416, a small molecule vascular endothelial growth factor tyrosine kinase receptor inhibitor, in patients with refractory multiple myeloma. *Clin Cancer Res* 2004; **10**:88–95.

25 Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, *et al.* SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res* 2000; **60**: 4152–4160.

26 Willett CG, Boucher Y, Tomaso E, Duda DG, Munn LL, Tong RT, *et al.* Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004; **10**:145–147.

27 Gyorffy S, Palmer K, Podor TJ, Hitt M, Gauldie J. Combined treatment of a murine breast cancer model with type 5 adenovirus vectors expressing murine angiostatin and IL-12: a role for combined anti-angiogenesis and immunotherapy. *J Immunol* 2001; **166**:6212–6217.

28 Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, *et al.* Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). *Br J Cancer* 1994; **69**:212–216.

29 Zhang Y, Griffith EC, Sage J, Jacks T, Liu JO. Cell cycle inhibition by the anti-angiogenic agent TNP-470 is mediated by p53 and $p21^{WAF1/CIP1}$. *Proc Natl Acad Sci USA* 2000; **97**:6427–6432.

30 Kamphaus GD, Colorado PC, Panka DJ, Hopfer H, Ramchandran R, Torre A, *et al.* Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. *J Biol Chem* 2000; **275**:1209–1215.

31 Panka DJ, Mier JW. Canstatin inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells. *J Biol Chem* 2003; **278**: 37632–37636.

32 Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matusuzawa Y, Zhivotovsky B, *et al.* Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA* 2004; **101**:2476–2481.

33 Griffith EC, Su Z, Niwayama S, Ramsay C, Chang Y, Liu J. Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc Natl Acad Sci USA* 1998; **95**:15183–15188.

34 Asami Y, Kakeya H, Onose R, Chang Y, Toi M, Osada H. RK-805, an endothelial-cell-growth inhibitor produced by *Neosartorya* sp., and a docking model with methionine aminopeptidase-2. *Tetrahedron* 2004; **60**:7085–7091.

35 Mack MM, Molinski TF, Buck ED, Pessah IN. Novel modulators of skeletal muscle FKBP12/calcium channel complex from *Ianthella basta*. *J Biol Chem* 1994; **269**:23236–23249.

36 Pessah IN, Molinski TF, Meloy TD, Wong P, Buck ED, Allen PD, *et al.* Bastadins relate ryanodine-sensitive and -insensitive Ca^{2+} efflux pathways in skeletal SR and BC_3H1 cells. *Am J Physiol* 1997; **41**:C601–C614.

37 Chen L, Molinski TF, Pessah IN. Bastadin 10 stabilizes the open conformation of the ryanodine-sensitive Ca^{2+} channel in an FKBP12-dependent manner. *J Biol Chem* 1999; **46**:32603–32612.

38 Kohler R, Brakemeier S, Kuhn M, Degenhardt C, Buhr H, Pries A, *et al.* Expression of ryanodine receptor type 3 and TRP channels in endothelial cells: comparison of *in situ* and cultured human endothelial cells. *Cardiovasc Res* 2001; **51**:160–168.