Diaminotriazoles Inhibit Angiogenesis Efficiently by Suppressing Akt Phosphorylation[‡]

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Abbreviations: ¹VEGF - Vascular Endothelial Growth Factor, HUVEC - Human Umbilical Vein Endothelial Cell, MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LY 294002 - 2-(4-morpholinyl)-8-phenyl-chromone, CAM - Chorioallantoic membrane, DAT1 - 4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole

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Abstract

The prevention of neo vessel formation or angiogenesis is a recent popular strategy for the limiting and cure of cancer. Diaminotiazoles are a class of compounds that have been reported to show promise in the treatment of cancer by inhibiting cancer cell proliferation and inducing apoptosis due to their effect on microtubules and as inhibitors of cdks. Many microtubule targeting agents are being studied for their antiangiogenic activity and a few have shown promising activity in the treatment of cancer. Here we report that diaminotiazoles can be highly effective as antiangiogenic agents as observed in chick membrane assay. The lead compound DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole] inhibits the endothelial cell processes like invasion, migration and tubule formation which require a functional cytoskeleton. DAT1 also decreases the expression of cell-adhesion markers. The antiangiogenic activities of DAT1 occur at concentrations that are not cytotoxic to the normal endothelium. Analysis of the intracellular signalling pathways shows that DAT1 inhibits the Akt phosphorylation, which is actively involved in the angiogenic process. The antiangiogenic properties of diaminotiazoles in addition to their promising antimitotic and cytotoxic properties in cancer cell lines, give them an extra advantage in the treatment of cancer.

Introduction

Angiogenesis, a crucial requirement for tumour progression and metastasis, is a potential target in cancer chemotherapy. As tumours require the development of a vascular supply in order to grow beyond 1-2 mm in diameter, constant vascular growth and remodelling is necessary. Compromising this vascular supply leads to the indirect killing of the tumour. Due to the genetic stability of the endothelial cells which line the blood vessels and the accessibility of the tumour vasculature, this approach promises less resistance (Hinnen and Eskens, 2007).

The activation of the angiogenic switch in tumours results in the degradation of the basement membrane, invasion of the extracellular matrix followed by endothelial cell migration, proliferation and capillary network formation (Denekamp, 1982). Thus, each stage can be a target for therapeutic strategies. A number of cytotoxic chemotherapeutic agents exhibit antiangiogenic activity. In addition, novel antiangiogenic compounds have been developed and many are undergoing clinical trials which work at different stages of angiogenesis (Kerbel and Folkman, 2002; Eichhorn et al., 2004). However, agents that inhibited only endothelial cell proliferation in vitro did not have any effect on angiogenesis in vivo (Belotti et al., 1996; Klauber et al., 1997), indicating that cytotoxicity was not sufficient to inhibit angiogenesis. Colchicine was among the first agents that were shown to possess antiangiogenic activity. Subsequently, a number of microtubule targeting agents have been shown to inhibit angiogenesis efficiently (Belotti et al., 1996; Klauber et al., 1997; Vacca et al., 1999; Hotchkiss et al., 2002). At low concentrations, tubulin binding agents disrupt the dynamic instability of microtubules thereby causing changes in the endothelial cytoskeleton (Vacca et al., 1999; Bocci et al., 2002; Grant et al., 2003; Wang et al., 2003). Tubulin depolymerising agents have also been shown to increase vascular permeability due to their role in the maintenance of the endothelial barrier (Watts et al., 1997). Combretastatin A4

phosphate (CA4P) is a highly effective antiangiogenic agent that causes vascular shutdown leading to tumour death (Iyer et al., 1998). CA4P and a few other tubulin binding agents that show antiangiogenic activity are undergoing clinical trials (Mauer et al., 2008; Mita et al., 2008).

Diaminothiazole analogues were found to be cytotoxic in different types of cancer cells, a few of the series being effective even at nanomolar concentrations [(Romagnoli et al., 2009), unpublished results from our laboratory]. The lead compound DAT1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole] was shown to bind tubulin at the colchicine binding site causing mitotic arrest and cell death (Sengupta et al., 2005). The promising activity of CA4P and a few other tubulin binding agents in the clinical trials prompted us to study the antiangiogenic activity of diaminothiazoles. Though there are reports of the probable antiangiogenic activity of diaminothiazoles due to their inhibition of the protein tyrosine kinase Axl and cyclin dependent kinases (Chu et al., 2000; Goff et al., 2011), our studies in angiogenic models prove their antiangiogenic activity. We have also studied the probable signalling pathway underlying the antiangiogenic activity of the lead compound DAT1.

Methods

DAT1 and its analogues were synthesized by a green chemistry route that involved some process simplification of an earlier report (Sreejalekshmi et al., 2006). The details of the synthesis and characterization of the analogues would be published subsequently.

MCDB131 medium was from Invitrogen and FBS was from PAN Biotech. Matrigel and the invasion plates were from BD Bioscience. E-selectin, CD31, Akt and phospho Akt (Thr 308) antibodies were from Cell Signaling Technology, USA. β -actin antibody and 1VEGF were from Santacruz Biotechnology. PI3-K inhibitor, Antimouse HRP and antirabbit HRP were from Sigma, St Louis, USA. ECL Plus western blotting detection system was from Amersham, UK. All other fine chemicals were from Sigma, St Louis, USA.

Isolation and culture of HUVECs. Endothelial cells were isolated by controlled collagenase perfusion of the umbilical vein (Jaffe et al., 1973). The endothelial cells were selected with two markers, presence of the factor von-WF and uptake of acetylated LDL. For characterization, the cells were grown on 12 mm coverglass in 24 well plates for 48h. The cells were then washed with PBS and fixed with 4% para-formaldehyde. After blocking the monolayer with PBS containing 3% BSA, the cells were permeabilised with 0.2 % TritonX 100 in PBS. They were subsequently incubated with the primary antibody against von WF (Sigma, USA) at concentration of 5 μ g / ml for 4h at 37°C followed by incubation with Alexa 546 conjugated secondary antibody for 45 minutes. The monolayer was extensively washed and mounted with glycerol and imaged under fluorescent microscope respective filter sets (Nikon TE2000E). Images were captured with Retiga Exi camera (Photometrics, Canada) and analysed with NIS element software (Nikon, Japan). For analyzing of Ac-LDL uptake, the cells grown on glass coverslips were incubated with 5 μ g/ml of acetylated low density lipoprotein, labelled with 1,1'-dioctadecyl-3,3',3',3'-tetramethyl-indocarbocyanine perchlorate, (Dil-Ac-LDL, Biomedical Technologies Inc., USA) for 4h at 37°C and 5% CO₂.

The cells were washed and imaged with rhodamine filter sets of fluorescent microscope using 40X objective. Culture containing 100% endothelial cells were only used for experiments. For experiments, the cells were resuspended in 5 ml of fresh MCDB131 medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated at 37°C under 5% CO₂ and used within the 3rd passage.

Proliferation Assay HUVECs were seeded onto 96-well microtiter plates at a density of 3×10^3 cells/well and grown in MCDB131 medium supplemented with 10% fetal bovine serum for 24 hours. The effect of DAT1 on the growth of HUVECs was measured after 48 hours using the MTT dye reduction assay (Mossman, 1983).

HUVEC invasion assay Invasion of HUVECs was determined in an invasion chamber (BD Biosciences) consisting of a multiwell insert plate that contains 8.0 µm pores occluded with matrigel. The cells were seeded in the upper compartment of the chamber in 0.2 ml MCDB131 without serum. The lower compartment contained MCDB131 supplemented with 5% FBS. Following incubation for 2 hours at 37°C, MCDB131 containing different concentrations of the drug was added to the upper compartments and incubated for a further 22 hours. The cells that had migrated (cells on the lower surface of the filter) were stained with propidium iodide and quantitated using a bottom reading spectrofluorimeter (Tecan Infinite M200) with the excitation and emission wavelengths of 529 nm and 550 nm, respectively. The inhibition of migration was calculated as $[1 - (\text{migrated cells treated} / \text{migrated cells control})] \times 100\%$.

Migration assay (scratch assay) Endothelial cells were seeded in MCDB medium containing 10% FBS in 35 mm graduated dishes at a density of 2,00,000 cells per plate and incubated at 37°C. HCT116 cells were seeded in DMEM medium containing 10% FBS in 60 mm graduated dishes at a density of 500,000 cells per plate and incubated at 37°C. When the cells were 80-90% confluent, wounds were made through the cell layer using a sterile 200 µl

pipette tip. The cells were then rinsed carefully with PBS without disturbing the monolayer. The cells were then treated with fresh medium or DAT1 containing medium and imaged at 0, 6, 12, 24 and 48 hours in a phase contrast microscope with the 4X objective.

HUVEC tube formation assay Matrigel (0.05 ml/well) was coated on a 96 well plate and allowed to solidify for 1 hour at 37°C. HUVECs were then seeded at a density of 2×10^5 cells/well in MCDB131 containing 5 % FBS, VEGF (5 ng/ml) and different concentrations of DAT1. Following incubation for 24 hours, images of the cords formed were taken using an inverted microscope (Nikon Eclipse TE300). The effect of DAT1 on the preformed cords was also studied by adding it to the cords after formation and monitoring their disruption with time.

Chicken chorioallantoic membrane (CAM) assay Fertilized chicken eggs (white leghorn) were incubated at 37°C and 50% humidity and allowed to grow for 4 days. A 1 cm² window was made perpendicular to the broad end of the egg. The eggs were incubated for 4 more days and different concentrations of the drug and VEGF (5 ng/ml) was added within sterile plastic O-rings between preexisting vessels. PBS was used as the vehicle for delivery of the drug and VEGF. Following incubation for 24 hours, the eggs were viewed and photographed with a stereomicroscope. Angiogenesis was quantified by counting the number of blood vessel branch points in the images using image proplus software. The percentage inhibition of neovessel formation was obtained by comparison with the untreated control.

Western blotting The cells to be analyzed were harvested and lysed with phospholysis buffer (1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-Cl pH 7.4, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton X 100, 5 mM phenyl methyl sulphonyl fluoride, 1 µg/ml each of aprotinin and leupeptin). The whole cell lysates (40-50 µg) were immunoblotted with appropriate dilutions of primary antibody followed by incubation in Horse Radish Peroxidase or Alkaline Phosphatase conjugated secondary

antibody. The proteins were visualized using enhanced chemiluminescence detection system or nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate reagent. β -actin was used as a loading control.

Analysis of intracellular signaling pathways HUVECs in culture were treated with the inhibitor of PI3K-Akt (LY294002) at a concentration of 20 μ M for 48 hours and the expression of the angiogenic markers E-selectin and CD31 were analysed by immunoblotting of equivalent amounts of cell lysates with specific antibodies as described in the materials and methods section. The effect of DAT1 on the activation of Akt by phosphorylation was also studied by western blotting.

Results

To establish diaminothiazoles as highly promising anticancer agents, we have studied their effect on tumour angiogenesis. Blocking of tumour angiogenesis is an essential strategy to prevent cancer progression and metastasis. Some proangiogenic growth factors are actively involved in tumour growth. VEGF is one of the most potent proangiogenic factors required for the growth of tumour. In order to simulate the diseased condition in cancer where there is excess blood vessel formation, VEGF was used in all the assays.

DAT1 and its analogues show antiangiogenic activity

The chick CAM is used as a favourable model to study the antiangiogenic activity of compounds as it provides a natural medium of growing blood vessels with all the components of the complex host interaction (Auerbach et al., 2003). We analysed the antiangiogenic activity of DAT1 and its analogues (Supplemental Figure 1) in this model after 24 hours or 48 hours. The effects of five of the compounds tested are shown in Table 1. The concentrations of the compounds that caused 50% inhibition of neovessel formation and the number of vessel branches within the ring area are indicated. The percentage inhibition was calculated as the percentage decrease compared to the untreated control. Representative images of the effect of the drugs are shown in Figure 1. The compounds showed similar effects after 24 hours or 48 hours.

Isolation and characterization of HUVECs

Endothelial cells isolated by the collagenase perfusion method of the umbilical vein were characterized by the expression of two markers. The von Willebrand factor (VWF) glycoprotein is a prominent marker for endothelial cells. It participates in the platelet-vessel wall interactions (Zanetta et al., 2000). Another characteristic of endothelial cells is the uptake of acetylated LDL. We have stained the cells by an antibody against VWF and viewed them using a secondary antibody conjugated with Alexa 546. Endothelial cells also take up

acetylated LDL via the scavenger cell pathway of LDL mechanism (Voyta et al., 1984). We have followed the uptake of Ac-LDL by the cells by using a fluorescent labelled analogue Dil Ac-LDL. Preparations that contained more than 99% endothelial cells (Supplemental Figure 2) were only used for the experiments. In addition, the cells also expressed CD31 (Figure 5).

DAT1 induces cytotoxicity in HUVECs in a relatively high concentration

Diaminotiazoles have been shown to inhibit cell viability of various cancer cells due to their action on microtubules. To determine whether the observed antiangiogenic effect of DAT1 and its analogues was due to a similar action on endothelial cells, the cytotoxic activity of the lead compound DAT1 against HUVECs was evaluated by MTT assay. A dose response curve of the effect of DAT1 on the endothelial cell viability is indicated in Figure 2. DAT1 reduced the cell viability by 50% following treatment for 48 hours at a concentration of $4.47 \pm 0.99 \mu\text{M}$. This is about 14 times higher than the cytotoxicity of DAT1 in a variety of human tumor cell lines (average IC_{50} $0.35 \mu\text{M}$ - (Sengupta et al., 2005)).

DAT1 inhibits the invasion, migration and cord formation of HUVECs

The effect of DAT1 on HUVEC invasion and migration was studied using the BD Biocoat angiogenesis system. This system consists of a multiwell insert plate that contains $3.0 \mu\text{m}$ pores occluded with matrigel. It replicates the process of new blood vessel formation, where endothelial cells escape from the vessels they line by secreting matrix metalloproteases and migrate toward the angiogenic stimulus.

A concentration of $0.5 \mu\text{M}$ DAT1 inhibited the invasion of HUVECs to the bottom compartment by $81.45 \pm 26.23\%$, while $0.05 \mu\text{M}$ DAT1 inhibited invasion by $27.8 \pm 3.75\%$.

Migration of endothelial cells was also monitored by scratch assay in the presence of DAT1. In the case of the untreated control, cells started migrating after 6 hours and in 24 hours the entire area of the scratch was closed with cells. Migration of HUVECs were inhibited in the presence of DAT1 from a concentration as low as $0.04 \mu\text{M}$ even up to 24 hours (Figure 3a).

Since in case of tumour metastasis, cancer cells migrate, the effect of DAT1 on the migration of colon cancer cells HCT116 was also monitored by scratch assay. In the case of the untreated control, cells started migrating after 6 hours and in 48 hours the entire area of the scratch was closed with cells. In the presence of 0.04 μM DAT1, though the migration started at 24 hours at a lower extent, there seemed to be no further migration at 48 hours. In the cells treated with 0.2 μM DAT1, migration started only at 48 hours (Figure 3b). These results indicated that DAT1 inhibited the migration of cells in a dose dependent manner.

Following the new outgrowth of endothelial cells, there is the need for their reorganization into a tubular structure. This can be replicated in vitro as endothelial cells form cords spontaneously on extracellular matrix components.

Visible inhibition of cord formation (observed as sparse and disrupted cords) was observed at concentrations as low as 16 nM DAT1 following treatment for 24 hours (Figure 4a). The inhibition was quantitated by counting the number of 3 point branching in different fields and in 3 repeated experiments (Figure 4b). When DAT1 was added to preformed cords, it resulted in their disruption as early as 2 hours at a concentration of 40 nM (Figure 4c). This proves that DAT1 is able to inhibit and disrupt the tube formation of endothelial cells.

DAT1 decreases the expression of cell-adhesion markers

E-selectin is a cell-adhesion molecule that is involved in cell-cell contact. Cell-cell interactions between endothelial cells are required for the formation of capillary tubes suggesting a functional role for E-selectin in angiogenesis (Brooks, 1996; Yasuda et al., 2002). CD31, essential for the migration and tubular structure formation, increases prior to the endothelial cell network formation (Yang et al., 1999; Cao et al., 2009).

We observed a decrease in the cell-associated levels of both of these markers in the immunoblot of HUVEC samples following treatment with DAT1 at both the concentrations. (Figure 5a)

DAT1 targets the Akt pathway

Akt is one of the most important proteins for the activation of the angiogenic process in endothelial cells (Jiang et al., 2000). In order to study the signalling pathways inhibited by DAT1, we analyzed the expression of E-selectin following the addition of LY 294002 (2-(4-morpholinyl)-8-phenyl-chromone), a potent inhibitor of PI3-kinase activity, which has been shown to exhibit antitumoral effect both in vitro and in vivo (Brognard et al., 2001).

Treatment of HUVECs with DAT1 in the presence of the PI3K/Akt inhibitor LY294002 resulted in a reduction in the expression of E-selectin compared to those cells treated with inhibitor alone (Figure 5b).

DAT1 also inhibited the phosphorylation of Akt at Thr-308 in concentrations as low as 100 nM as observed by the decreased levels of pAkt following probing with antibody that detected phosphorylation at the Threonine 308 site (Figure 5c).

Discussion

With the emergence of anti-angiogenic strategies, the scope of cancer treatment has expanded widely. The development of antiangiogenic compound based therapy may improve the outcome of cancer treatment, with the promise of reduced side effects and increased treatment efficacy. DAT1 inhibited the proliferation of HUVECs at a higher concentration (IC_{50} - 4.47 μ M) compared to its IC_{50} in cancer cells (0.35 μ M) indicating that it would be less toxic to normal endothelial cells. Some microtubule targeting agents show maximum anti-angiogenic effects when administered at low doses over a longer time span - metronomic therapy (Belotti et al., 1996). Metronomic therapy has been shown to be effective for the treatment of human cancers with low side effects in several pre-clinical and clinical studies (Kerbel and Kamen, 2004). The antiangiogenic activity that DAT1 showed at a much lower effective concentration compared to the concentration required for its antiproliferative activity and apoptosis induction in cancer cells (unpublished result) could indicate its promising activity in this mode of treatment. This, in turn, could be due to its effect on the microtubule dynamics of the endothelial cells. Earlier studies showed that low concentrations of microtubule targeting agents induced an unexpected increase in MT dynamic instability in human endothelial cells, associated with an inhibition of cell motility (Pasquier et al., 2005; Pourroy, 2006). This could be due to impairment of cell polarization or of microtubule-induced focal adhesion turnover (Hotchkiss et al., 2002).

In all the assays, the effect of DAT1 was observed at concentrations well below the concentration at which cytotoxicity was observed. In the cord formation assay, the disruption of preformed cords was observed at a very low concentration and following a short treatment time, which rules out the cytotoxic effect of DAT1. This disruption may be attributed to the effect of DAT1 on the endothelial cytoskeleton and the adhesion molecules.

Increased levels of phosphorylated Akt have been observed in various cancers that are resistant to radiation (Gupta et al., 2002; Choe et al., 2003; Schmitz et al., 2004). On activation, Akt has been reported to activate NF- κ B and upregulate the antiapoptotic protein Bcl2, thus promoting cell survival (Beraud et al., 1999; Kane et al., 1999; Pugazhenthil et al., 2000). The PI3K-Akt pathway is involved in the regulation of the angiogenic processes of survival, migration and tube formation (Gupta et al., 1999; Yu and Sato, 1999). Many reports have also shown that the expression of adhesion molecules in endothelial cells are regulated by the Akt/PI3-kinase pathway. It has been found that the expression of the vascular Adhesion molecule-1 expression in human intestinal microvascular endothelial cells is regulated by PI3-K/Akt/MAPK/NF- κ B pathway (Binion et al., 2009). Blockade of the PI3-Akt pathway abrogated the suppression of the TNF- α induced cell surface expression of E-selectin in HUVECs (Gong et al., 2006). Therefore endothelial cells require the activation of this pathway during angiogenesis (Zhu et al., 2002; Bullard et al., 2003). We observed downregulation of phosphorylated Akt following treatment with DAT1 indicating that this is one of the signalling pathways through which DAT1 reduces the expression of E-selectin.

Inhibition of angiogenesis is desirable for the treatment of a number of conditions like rheumatoid arthritis, muscular degeneration and cancer. Our experiment with colon cancer cells indicates the antiangiogenic activity that diaminothiazoles might possess in a cancer scenario. The antiangiogenic property of diaminothiazoles observed both in the *in vitro* and *in vivo* models at concentrations well below the cytotoxic dose, along with their promising cytotoxicity against cancer cells, makes them more promising in the clinic.

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Author contributions

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Footnotes:

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Parts of the work have been submitted to the University of Kerala in the Ph.D. thesis of Sannu Ann Thomas entitled "Study of the Mechanism of Action of a Diaminotiazole, a Potential Anticancer Agent".

An abstract covering some parts of the work has been listed in the Proceedings of the International Cancer Research Symposium, Thiruvananthapuram, India, December 20-22, 2010: Sannu Ann Thomas, Reshma Thamkachy, Bindu Ashokan, T.R.Santhoshkumar, K.N.Rajasekharan and Suparna Sengupta: "Diaminotiazoles, a new class of potential anticancer agents, inhibit angiogenesis efficiently".

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Figure Legends:

Figure 1: Inhibition of neovessel formation in the chick CAM by DAT1 and its analogues. Eight day old chick embryos were treated with different concentrations of drugs in the presence of VEGF and images were taken after 24 hours. Representative images of DAT1 and its analogues are shown. The area within the O-ring and the surrounding area are shown in the image for better comparison.

Figure 2: Effect of DAT1 on the proliferation of endothelial cells as estimated by MTT assay. A dose response curve of the endothelial cell viability following the treatment with DAT1 is shown. The error bars represent the standard deviations.

Figure 3: Effect of DAT1 on the migration of (a) endothelial cells and (b) colon cancer cells. HUVECs or HCT116 cells were seeded in 35 mm or 60 mm dishes and allowed to grow to 80 % confluency. Scratches were made in the monolayer and the cells were left untreated (top panel) or treated with DAT1 (lower panels). Migration of the cells into the vacant spaces was monitored over time. Images at different time points are shown.

Figure 4: Inhibition of cord formation and disruption of preformed cords following DAT1 treatment: (a) HUVECs were seeded on matrigel coated plates with VEGF containing medium in the absence or presence of DAT1. Cord formation was observed over time and images were taken after 24 hours. (b) The number of three point tubule branching was counted and the standard deviations from three independent experiments were calculated. (c) DAT1 was added to preformed vessels and their disruption was observed with time. Images taken at 2 and 4 hours after DAT1 addition are represented.

Figure 5: Effect of DAT1 on the level of cell adhesion markers and on the Akt signalling pathway. (a) HUVECs were treated with different concentrations of DAT1 and their cell lysates were immunoblotted and probed with the respective antibodies. β -actin was used as a loading control. (b) HUVECs were treated with DAT1 in the presence of the PI3-kinase

inhibitor LY294002 for 24 hours. Following immunoblotting of the cell lysates, they were probed with E-selectin antibody. (c) The levels of pAkt were quantitated following immunoblotting of the drug treated cell lysates using an antibody against Akt phosphorylated at Thr 308. The lysates were also probed with antibodies against Akt and β -actin as loading controls.

Table 1: Inhibition of neovessel formation by diaminothiazoles in the chick CAM model.

% Inhibition was calculated by counting the number of branching in the area of treatment and comparing with the untreated control. The drug concentrations which produced 50% inhibition in the number of branching points of the blood vessels (IC_{50}), were calculated from the semilogarithmic dose response plots using the nonlinear regression programme Origin. The standard deviations were calculated from three or more experiments for each analogue. Representative values of the number of vessel branches within the area of treatment at a concentration (shown in parenthesis) close to the IC_{50} concentration are also indicated for each analogue.

Drug	Number of branches	IC_{50} concentration (nM)
Control	40	-
DAT34	18 (0.7 nM)	0.529 ± 0.049
DAT22	14 (1 nM)	0.688 ± 0.22
DAT1	17 (8 nM)	6.45 ± 1.77
DAT7	18 (40 nM)	49 ± 11.31
DAT10	26 (8 nM)	10.8 ± 2.12

VEGF Control

DAT34 (0.1 nM)

DAT22 (0.1 nM)

DAT1 (8 nM)

DAT7 (0.2 μ M)

DAT10 (0.2 μ M)

Figure 1

Figure 2

(a)

0 HOUR

6 HOURS

12 HOURS

24 HOURS

CONTROL

DAT1 (0.04 μ M)

DAT1 (0.2 μ M)

Figure 3

(b)

0 HOUR

6 HOURS

12 HOURS

24 HOURS

CONTROL

DAT1 (0.04 μ M)

DAT1 (0.2 μ M)

Figure 3

(a)

Control

DAT1 16 nM

DAT1 80 nM

DAT1 400 nM

(b)

(c)

Control

40 nM DAT1 at 2 hours

40 nM DAT1 at 3 hours

Figure 4

Figure 5