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Original Paper

Effect of Novel CAAX Peptidomimetic Farnesyltransferase Inhibitor on Angiogenesis *In Vitro* and *In Vivo*

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Ras oncogenes can contribute to tumour development by stimulating vascular endothelial growth factor (VEGF)-dependent angiogenesis. The effect of Ras on angiogenesis may be affected by farnesyltransferase inhibitors (FTI) since farnesylation of Ras is required for its biological activity. In this paper we evaluated the effect of A-170634, a novel and potent CAAX FTI on angiogenesis. Human umbilical vein endothelial cell (HUVEC) tube formation and VEGF secretion were used to assess the effect of A-170634 on angiogenesis *in vitro*. *In vivo*, nude mice were injected with the K-*ras* mutant colon carcinoma cell line HCT116 and treated subcutaneously with A-170634 using osmotic minipump infusion for 10 days. The effect of A-170634 on corneal angiogenesis *in vivo* was assessed using pellets containing hydron, VEGF, A-170634 or vehicle. *In vitro*, A-170634 selectively inhibited farnesyltransferase activity over the closely related geranylgeranyltransferase I, inhibited Ras processing, blocked anchorage-dependent and -independent growth of HCT116 K-*ras* mutated cells, decreased HUVEC capillary structure formation, decreased VEGF secretion from tumour cells and HUVEC growth stimulating activity in a dose-dependent manner. *In vivo*, tumour growth was decreased by 30% and vascularisation in and around the tumours was reduced by 41% following drug-treatment with no apparent toxicity to the animals. VEGF-induced corneal neovascularisation was reduced by 80% following A-170634 treatment for 7 days. The data presented here demonstrated that A-170634 was a potent and selective peptidomimetic CAAX FTI with anti-angiogenic properties. These results implied that A-170634 may affect tumour growth *in vivo* by one or more antitumour pathways. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

ONCOGENIC K-ras MUTATION is a frequent genetic event in certain human cancers including pancreatic, colorectal, lung and bladder carcinomas [1–3]. Ras protein p21, a GTP binding protein, is involved in the regulation of cell growth through its various pathways in signal transduction [4–6]. Ras participates in the signalling cascade initiated by platelet derived growth factor [1, 7, 8] through receptor protein tyrosine kinase and various adaptor molecules to downstream signal transducers [6, 9]. However, when oncogenic *ras* loses its intrinsic GTPase activity and is constitutively bound to GTP, it leads to continuous propagation of growth signals within the cell [1]. To acquire biological activity, Ras must associate with the inner surface of the plasma membrane, both in normal and transformed cells [10–12]. In order to be active, Ras requires a series of post-translational modifications. The first and obligatory step, the addition of a farnesyl group to its carboxy-terminal 'CAAX' motif, is catalysed by farnesyl transferase (FTase) [4, 13]. Inhibition of Ras farnesylation is a promising approach for developing a new gen-

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eration of mechanism-based anticancer drugs [13–15] since farnesyl transferase inhibitors (FTI) disrupt mutant Ras protein farnesylation *in vitro* and suppress tumour growth *in vivo*.

There is evidence to suggest that *ras* oncogenes can contribute to tumour development directly by promoting tumour cell proliferation and indirectly by stimulating vascular endothelial growth factor (VEGF)-dependent angiogenesis. Mutant *ras* expression is not only required to maintain the transformed phenotype of tumour cells but also critical for tumour progression and metastasis, which is partly mediated by constitutively upregulated expression of tumour angiogenesis factors [16, 17]. There is considerable interest in the role of angiogenesis factors such as VEGF/vascular permeability factor (VEGF/VPF) on tumorigenesis since it is expressed by many human and animal tumours [17, 18] and yet it is a growth factor specific for vascular endothelial cells. The expression of VEGF in a variety of tumours suggests that the growth of solid tumours is dependent on VEGF angiogenesis [9, 19–21]. This has been demonstrated in several recent studies supporting the idea that inhibition of VEGF-induced angiogenesis suppresses tumour growth *in vivo* [22, 23].

We have recently developed and investigated the effect of A-170634, a novel CAAX peptidomimetic FTI, on tumour angiogenesis *in vitro* and *in vivo*. In this report the effect of A-170634 on angiogenesis was assessed by human umbilical vein endothelial cell (HUVEC) tube formation assay as well as tumour cell VEGF secretion *in vitro*. *In vivo* effects of A-170634 were investigated in a xenograft mouse model and in a rat corneal angiogenesis model.

MATERIALS AND METHODS

Chemistry of A-170634

A-170634 was designed as a non-cysteine containing peptidomimetic of the CAAX motif of the ras protein. The compound was derived from FTI-277 as the template [24] by replacing the N-terminal cysteine with a 3-pyridylaminomethyl group through combinatorial chemistry efforts. The structure of A-170634 is N-[4-(3-pyr-NH-CH_2)-2-phenylbenzoyl]methionine methyl ester·HCl (Figure 1a). Details on the structure activity relationship will be reported elsewhere.

Cell cultures

NIH3T3 transformed H- and K-*ras* cell lines were kindly provided by Channing Der (University of North Carolina, U.S.A.). HCT116 was purchased from American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.). Both NIH3T3 and HCT116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic (Gibco, Grand Island, New York, U.S.A.). NIH3T3 *ras* transformed cells were cultured at 37°C in a humidified chamber containing 90% air and 10% CO_2, whilst HCT116 was cultured in a humidified 95% air and 5% CO_2 atmosphere. Normal HUVEC were purchased from Clonetics (San Diego, California, U.S.A.) and cultured with endothelial growth medium (EGM Bulletkit) supplemented with 3% FBS, bovine brain extract (12 μg/ml), human epidermal growth factor (10 ng/ml), hydrocortisone acetate (1 μg/ml) and gentamycin-1000 (50 μg/ml). Matrigel was obtained from Collaborative Research (Bedford, Massachusetts, U.S.A.).

Ras processing assay

Cells for Ras processing were harvested from subconfluent cultures of NIH3T3 H-*ras* transformed cells and HCT116 cell lines. Cell lysates were prepared by removing culture medium, washing twice with PBS, and the addition of Laemmli sample electrophoresis buffer (Sigma, St Louis, Missouri, U.S.A.). Lysates were boiled for 5 min and then separated on a 15% Tris-Glycine gel (Bio-Rad, Richmond, California, U.S.A.). Cellular proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Optitran BA-S 83) and incubated in blocking buffer (2% nonfat dry milk, 3% bovine serum albumin) overnight. The HCT116 blot was probed with a pan anti-Ras antibody from Transduction Labs (Lexington, Kentucky, U.S.A.) whereas the H-Ras specific antibody Y13-238 was used for the NIH3T3 H-*ras* blot. The blots were subsequently probed with horseradish conjugated antimouse Ig (Amersham, Arlington Heights, Illinois, U.S.A.). Ras bands were visualised by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, Illinois, U.S.A.) and quantified by densitometry using an image analysis program Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland, U.S.A.).

Anchorage-dependent proliferation assay

HCT116 cells were harvested from subconfluent culture in 75 cm^2 culture flasks (Costar, Cambridge, Massachusetts, U.S.A.). Approximately 1×10^4 HCT116 cells were seeded in each well of 24-well culture plates (Costar) and allowed to grow for 24 h. Cells were treated with varying concentrations of A-170634 in medium containing 5% FBS and re-fed every 2 days with medium containing compound or vehicle. After 4 days of incubation, cell proliferation was quantified using non-radioactive alamarBlue (Alamar Biosciences, Sacramento, California, U.S.A.). The plates were read by a CytoFluor

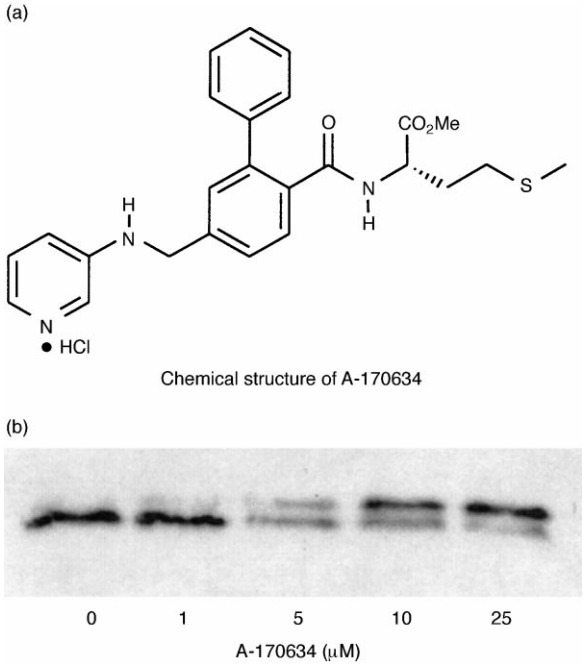


Figure 1. (a) Chemical structure of A-170634. (b) Western blot analysis of *ras* processing. A-170634 inhibited ras processing in HCT116 cells. The growing cells were treated with varying concentrations of A-170634 for 48 h. Upper bands are unprocessed ras and lower bands are processed ras.

2300 fluorescence measurement system (Millipore, Bedford, Massachusetts, U.S.A.) and cell growth was measured by fluorescence intensity and expressed by percentage of inhibition relative to controls.

Soft agar colony formation assay

For anchorage-independent growth, 15 000 HCT116 cells were seeded on to each well of 24-well culture plates on a 0.35% top agar layer overlaid with 0.7% agar with the same medium supplemented with 10% FBS. Both agar layers contained the test compound A-170634. Cultures were re-fed with compound or vehicle twice a week. After 10–14 days of incubation, colonies from duplicate wells were photographed using a Sony CCD camera and quantified by Image-Pro Plus.

Tube formation assay

The effect of A-170634 on the capacity of HUVEC to form a capillary-like network on matrix substratum was studied using a modified method described previously by Schnaper [25]. Briefly, HUVEC passages two to eight grown subconfluently on 75 cm^2 culture flask were used for the assays. Matrigel was thawed at 4°C overnight. 80 µl of matrigel/well was coated on to each well in two-chamber slides (Nunc, Naperville, Illinois, U.S.A.) and allowed to polymerize at 37°C for 30 min. Approximately 60 000 cells in 1 ml of medium were seeded into each well. After 1 h of incubation, another 1 ml of medium with different doses of test compounds or vehicle was added to each well. A two-dimensional capillary structure was formed after a 12 h incubation. The cell cultures were fixed and stained with Diff-Quik differential staining set (Baxter, Waukegan, Illinois, U.S.A.) and photographed with a Nikon Diaphot 300 microscope and Nikon N6006 camera. Tube formation was scored manually and expressed relative to controls.

VEGF secretion

VEGF secretion was measured by quantitative sandwich enzyme immunoassay technique with a quantikine kit (R&D, Minneapolis, Minnesota, U.S.A.). The assay was performed according to manufacturer instructions [26]. Briefly, a monoclonal antibody (MAb) specific for VEGF165 was prepared from mice immunised with purified protein derived from VEGF antigen of insect SS21 cells and precoated on to a 96-well microtitre plate. 200 µl of control, compound treated culture supernatant or serially diluted VEGF standards were added to the plates and incubated for 2 h at room temperature. After washing the wells three times, 200 µl of polyclonal antibody against VEGF conjugated to horseradish peroxidase was added to each well and incubated for 2 h. Wells were washed three times and then the enzyme reaction was carried out at 37°C for 20 min with stabilised hydrogen peroxide and tetramethylbenzidine as substrates The plates were read by a plate reader (SLT Labinstruments, Austria) and VEGF content of the samples was estimated from the standard curve determined from serially diluted VEGF standards. The VEGF secretion was normalised to cell number by dividing it by the fluorescence intensity obtained from previous HCT116 cell proliferation assays.

HUVEC growth stimulating activity

We tested whether endothelial cell proliferation was stimulated by VEGF from HCT116 conditioned medium. Briefly, 5000 HUVEC were seeded in each well of a 96-well microtitre plate and allowed to grow for 24 h. The conditioned medium from either control or compound treated HCT116 cells from the previously described proliferation assay was added to each well of the HUVEC culture. After 2 days of incubation, endothelial cell growth was quantified by alamarBlue assay as described above.

Human tumour xenograft in nude mice

Male BR nu/nu mice aged 8 weeks (Charles River, Maine, U.S.A.) were housed in barrier facilities with food and water *ad libitum*. 5 000 000 HCT116 cells were inoculated intradermally into mouse ventral skin on day 0. Mice with tumours of equal size were randomly divided into control ($n = 8$) and treatment ($n = 8$) groups, and minipumps (Alzet, Palo Alto, California, U.S.A.) containing 50 mg/kg/day A-170634 or vehicle (0.1% DMSO in saline) were implanted subcutaneously (s.c.) into the back of each mouse on day 3. Tumour volume was estimated by measuring length and width of the tumour mass with digital calipers and by applying the formula $(L \times W^2)/2$ every 3 days. After 10 days of treatment, tumour skin was dissected from each mouse and analysed for vessel density. To quantify macroscopic blood vessels, we removed the tumours with the surrounding skin. The tumours and skin were laid out flat, imaged using a Sony CCD camera attached to a dissection microscope. Image-Pro Plus was then used to segment the blood vessels based on contrast differences and quantified by determining the total pixels (area) of the blood vessels. The area of blood vessels from drug treated animals was expressed as a per cent of untreated controls.

Rat corneal angiogenesis model

The effect of A-170634 was assayed *in vivo* using the corneal angiogenesis model in female Sprague–Dawley rats (Charles River, Maine, U.S.A.), as previously described [27]. Rat eyes were divided into control, low-dose (0.5 µg/kg) and high-dose (1 µg/kg) treatment groups ($n = 4$ for each group). Briefly, 10 µl aliquots made of hydron (12% in ethanol), a slow-release polymer, polyhydroxyethylmethacrylate (polyHEMA, Sigma, St Louis, Missouri, U.S.A.), human recombinant VEGF (Collaborative Research, Bedford, Massachusetts, U.S.A.), A-170634 or vehicle were pipetted on to the flat surface of an inverted sterile polypropylene column, and allowed to polymerise for 2 h in a laminar flow hood. Using a dissection microscope, a 2 mm lamellar corneal incision was cut approximately 1 mm from the centre of the cornea into the stroma and 1–1.5 mm from the temporal limbus with a no. 11 surgical blade (Bard-Parker, Becton Dickson Acute-Care, Franklin Lakes, New Jersey, U.S.A.). The pocket was made with a curved iris spatula (Fine Science Tools, Belmont, California, U.S.A.) and the pellets were implanted into corneas of anesthetised rats. Postsurgery, antibiotic ointment was applied to the operated eyes to prevent infection and to decrease irritation of the ocular surface. After 7 days the anesthetised rats were perfused with 250 ml of saline via the left ventricle followed by 20 ml of India ink (1:50 dilution). Corneas were carefully removed and fixed with formalin. The corneas were laid flat by making three counterlateral cuts and imaged using a Sony CCD camera attached to a dissection microscope. Neovascularisation was determined by measuring blood vessel density as described above. Neovascularisation from animals receiving drug was expressed as a per cent of untreated controls ± standard deviation (S.D.).

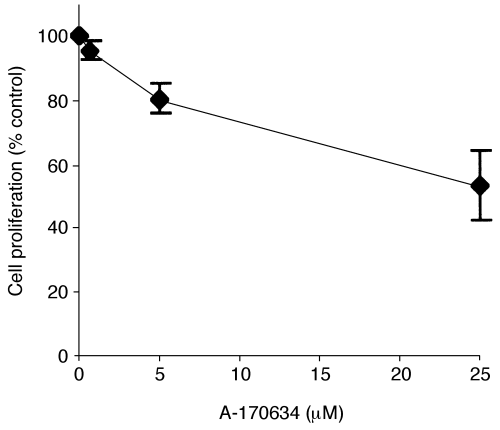


Figure 2. A-170634 blocked HCT116 anchorage-dependent growth. HCT116 cells were plated in 24-well culture plates, allowed to attach for 24 h and then treated with different concentrations of A-170634 for 4 days. Cell proliferation was determined by alamarBlue assay. Data represents the average (± standard deviation, S.D.) of three experiments.

RESULTS

A-170634 *inhibits anchorage-dependent and -independent tumour cell growth*

For anchorage-dependent growth, HCT116 cell proliferation was inhibited 5, 20 and 47% by 0.5, 5 and 25 μM A-170634, respectively (Figure 2). We further tested whether anchorage-independent growth of NIH3T3 H- and K-*ras* as well as HCT116 cell lines was inhibited by A-170634. We observed that colony formation was inhibited in a dose-dependent manner following treatment with A-170634 in all three cell lines. HCT116 anchorage-independent growth was inhibited by 2, 35 and 48% following 0.1, 0.5 and 1 μM A-170634 treatment, respectively. The ED_{50} was 0.5, 3.1 and 1.1 μM for NIH3T3 H-, K-*ras* and HCT116 cells, respectively. We also observed that a reduction in soft agar colony number was accompanied by a reduction in colony size (data not shown).

A-170634 was a potent and selective FTI on FTase over geranylgeranyltransferase I (GGTase I). The concentration of A-170634 required to inhibit ras farnesylation by 50% (IC_{50}) was 120 nM whilst for geranylgeranylation it was as high as 18 000 nM. The IC_{50} of the free acid form of A-170634 was 6.8 nM. In whole cells A-170634 inhibited post-translational Ras processing in a dose-dependent manner in both NIH3T3 H-*ras* and HCT116 cells (Figure 1b). The ED_{50} was 3.3 and 5 μM, respectively.

A-170634 *inhibits HUVEC tube formation*

We tested the effect of A-170634 on HUVEC tube formation *in vitro*. Tubulogenesis was induced in vascular endothelial cells by plating them on to the surface of matrigel for several hours. Figure 3 shows the branching vessel-like structures formed by HUVEC. When A-170634 was added to the culture, there was a decrease in both the number and thickness of vessel formation in a dose-dependent manner. There was approximately a 60% reduction in tubular structure following 25 μM A-170634 treatment for 12 h. The ED_{50} of A-170634 was 16 μM.

A-170634 *reduces VEGF secretion*

At the end of the HCT116 proliferation experiment, the culture supernatant was harvested and stored at $-80°C$ to evaluate VEGF levels by ELISA assay. The test cell number was approximately 1×10^6 per well in 24-well culture plates. The VEGF level released from 1×10^6 cells after 24 h of incubation was ~800 pg/ml. VEGF levels declined following A-170634 treatment in a time (data not shown) and dose-dependent manner. VEGF levels were reduced by 16, 31 and 45% relative to the control following treatment with 0.5, 5 and 25 μM A-170634 for 2 days, respectively (Figure 4a). The data of VEGF secretion was normalised to HCT116 cell number.

A-170634 *inhibits HUVEC growth stimulating activity*

To test the effect of VEGF on endothelial cell growth stimulating activity, HUVEC were cultured with conditioned medium from HCT116 cell cultures. Endothelial cell growth increased 80% when cultured with conditioned medium for 2 days as compared with controls. There was less growth stimulating activity (56, 38 and 5%) in HUVEC when they were incubated with the conditioned medium from HCT116 cells previously treated with 0.5, 5 and 25 μM A-170634, respectively (Figure 4b). The decrease in HUVEC growth stimulating activity of the conditioned medium from HCT116 cells treated with A-170634 was probably not due to the presence of residual compound in the medium since A-170634 had little direct effect on HUVEC cell proliferation (data not shown).

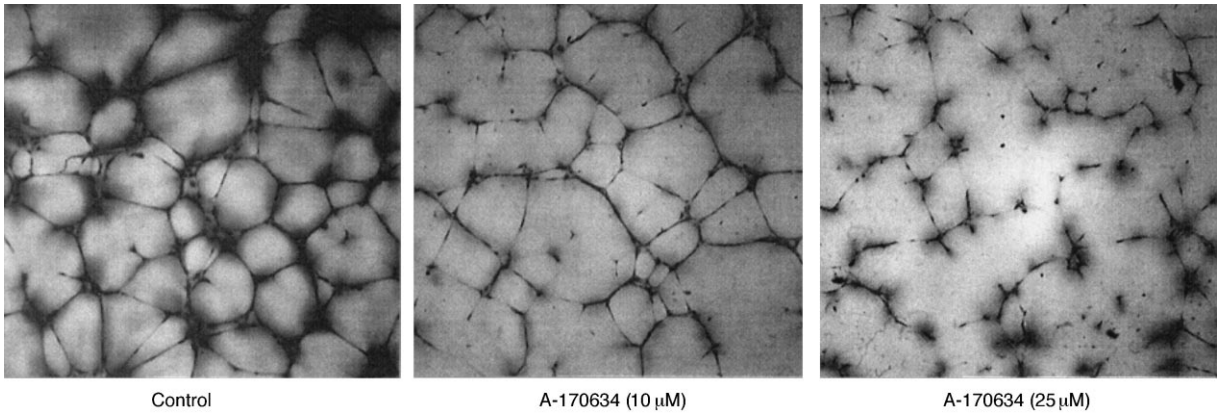


Figure 3. A-170634 disrupted HUVEC tube formation. HUVEC tube formation was reduced in a dose-dependent manner following treatment with A-170634 (EC_{50} = 16 μM). The cells were fixed with methanol, stained by Diff-Quik and imaged with a colour CCD camera attached to a Nikon inverted microscope.

A-170634 *reduces growth and angiogenesis of human xenograft in nude mice*

All nude mice developed tumours after intradermal inoculation of HCT116 cells. Tumours in mouse skin became palpable after 2 days of inoculation. HCT116 tumour volumes between 200–300 mm^3 were obtained after 7–10 days of growth. In the treatment group, 50 mg/kg/day of A-170634 was administered via minipumps for 10 days and tumour growth was reduced by approximately one-third as compared with controls. The reduction of HCT116 tumour growth in nude mice was accompanied by a 41% reduction in vascularisation in and around the tumour following A-170634 treatment as compared with controls (Figure 5).

A-170634 *inhibits corneal angiogenesis in rats*

Pellets containing slow release hydroxyethyl methacrylate (hydron) and VEGF with vehicle or A-170634 were implanted into rat intracorneal micropockets. Limbal capillaries started to sprout into the avascular corneas towards the pellet

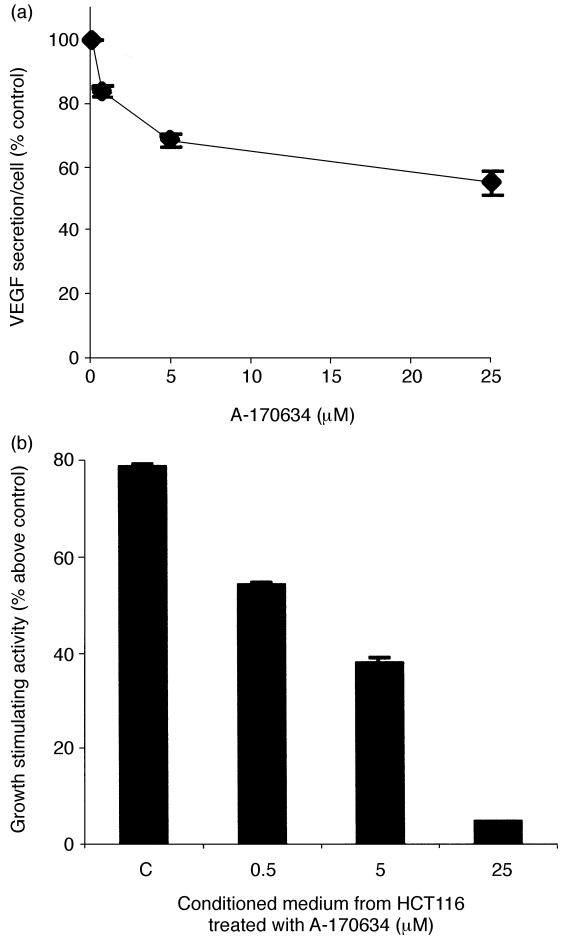


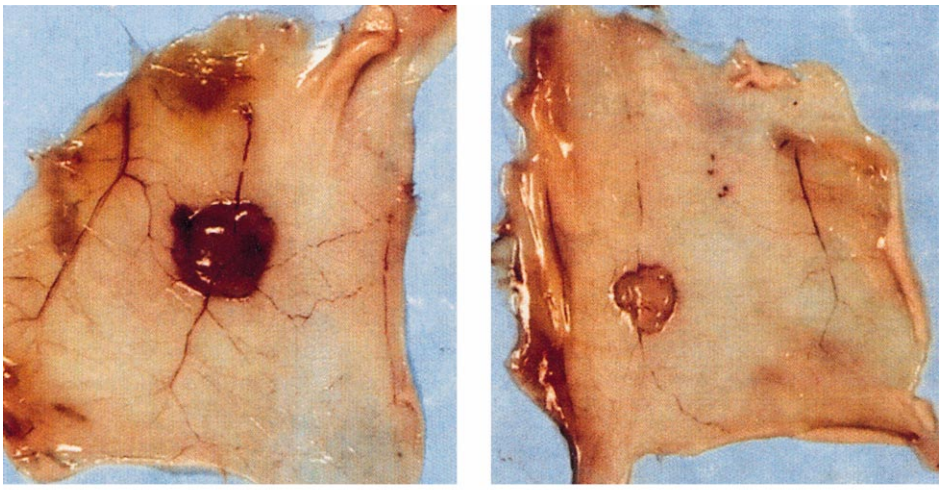
Figure 4. (a) VEGF secretion by HCT116 cells decreased in a dose-dependent manner following treatment with different concentrations of A-170634. VEGF was measured in culture medium with ELISA assay (R&D) and normalised for cell number using alamarBlue assay. Data represents the average (± standard deviation, S.D.) of three experiments. (b) The effect of A-170634 on HUVEC growth stimulating activity *in vitro*. HUVEC were seeded in 96-well culture plates and grown for 24 h. Cells were treated with conditioned medium harvested from A-170634 treated HCT116 cells. HUVEC growth was determined by alamarBlue assay. Data represents the average (± standard deviation, S.D.) of three experiments.

2–3 days after surgery. In control eyes the number of vessels increased and hair-like vessels approached or reached the pellet at day 7. In contrast to controls, 0.5 and 1 μg/kg A-170634 treatment significantly inhibited sprouting neovessel growth (Figure 6a). Vessel density from controls and treatment rat corneas were quantified by image analysis at day 7. Neovascular density in A-170634 (0.5 and 1 μg/kg) treated corneas was reduced by 42 and 80% as compared with controls ($P<0.05$ and $P<0.00001$, respectively) (Figure 6b).

DISCUSSION

The data presented here demonstrated that A-170634 is a potent and selective peptidomimetic CAAX FTI that may exert its effects on tumour growth *in vivo* by suppressing tumour angiogenesis. We selected HCT116 cells for *in vitro* and *in vivo* studies because it is a representative K-*ras* mutated human colon carcinoma cell line and it is also known to express high levels of VEGF [28]. The reduction of HCT116 tumour growth following A-170634 treatment *in vivo* may be due to a combination of direct and indirect effects. A-170634 may directly affect tumour cell proliferation through the inhibition of Ras processing. From our *in vitro* results, it was clear that A-170634 suppressed HCT-116 K-*ras* proliferation as well as Ras processing. In addition, we do not exclude the possible direct effect of FTI on the induction of apoptosis in HCT116 tumour *in vivo*. The idea that FTI could suppress tumour cell proliferation directly through apoptosis was first suggested by Rak and colleagues [29], and later demonstrated in both cell culture [30] and in transgenic tumour models [31]. In MMTV-V-H-*ras* model, L-744,832 treated salivary tumour exhibited more than a 10-fold increase in apoptosis as measured by TUNEL analysis, and this apoptotic pathway was largely p53 independent. However, in a mammary *ras*/*myc* transgenic tumour model, the increase in apoptosis was much more modest, even though tumour regression occurred in both tumour models after FTI treatment [31]. These studies showed that both apoptotic and non-apoptotic pathways could be involved in tumour regression, and the relative contribution of such pathways may vary with different tumour types.

The direct effects of A-170634 on tumour growth *in vivo* may not adequately account for the fact that the effect of some FTIs *in vivo* was greater than what would be predicted *in vitro* [9]. The observation that A-170634 suppressed VEGF release by HCT116 cells *in vitro* also suggested that it may exert its effect on tumour growth *in vivo* by suppressing tumour angiogenesis. A decrease in the vascularisation in and around HCT116 tumour xenografts in nude mice and a reduction in neovascularisation in the rat cornea *in vivo* helped to demonstrate further the anti-angiogenic effects of A-170634. The cornea model is a reliable and reproducible system to evaluate angiogenic and anti-angiogenic compounds *in vivo* since it induced a persistent and aggressive neovascular response by directly stimulating blood vessel growth rather than indirectly by stimulating inflammation [27, 32]. One could argue that local concentrations of A-170634 released by the hydron pellet in the cornea are higher than that in the circulation and acting in a nonspecific, cytotoxic manner. This did not appear to be the case since we did not observe any necropathy within the cornea following treatment. We chose to use recombinant VEGF rather than HCT116 conditioned medium to demonstrate the effect of A-170634 on neovascularisation due to the technical diffi-



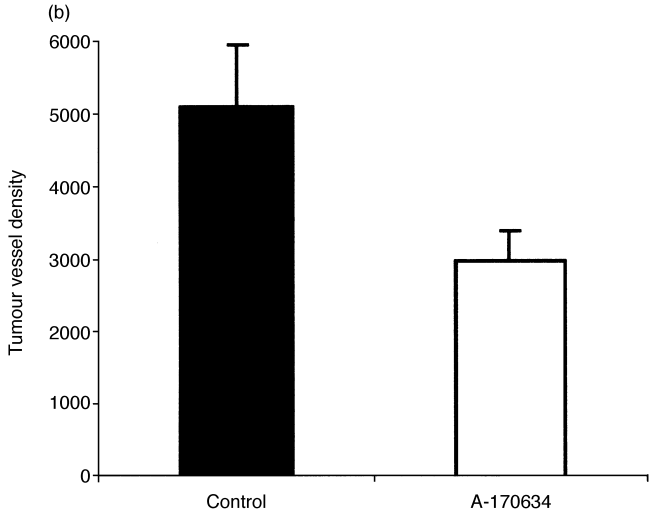


Figure 5. A-170634 reduced growth and vessel density of human tumour xenograft in nude mice. (a) mice were inoculated intradermally with HCT116 cells and treated with 50 mg/kg/day of A-170634 or vehicle for 10 days. Tumour volume decreased following A-170634 treatment as compared with controls ($P<0.05$). (b) Tumour vessel density from each mouse was measured by image analysis. Vessel density around tumours decreased following A-170634 treatment as compared with controls ($P<0.05$). Data represents the average (± standard deviation, S.D.) from each group.

culties of adding enough medium in a 10 µl pellet which also included hydron and compound. More recently, we have observed significant inhibition of angiogenesis in the mouse corneal model following systemically treatment with a A-170634 analogues (data not shown).

A-170634 may act directly as an anti-angiogenic agent *in vivo* either by inhibiting endothelial cell proliferation and differentiation or indirectly by inhibiting VEGF secretion by tumour cells. We demonstrated HUVEC proliferation was reduced following treatment with conditioned medium from HCT116 cells that were previously treated with A-170634, but it did not inhibit HUVEC cell growth directly. This suggests that A-170634 may affect endothelial growth indirectly by inhibiting tumour cell secretion of VEGF. VEGF has been shown to not only stimulate endothelial cell growth *in vivo* but it is also required for their survival [33]. This may involve induction of several survival genes by VEGF such as members of the Bcl-2 family [17], and may lead to programmed cell death when VEGF is suppressed. Thus treatment with FTI *in vivo* may result in a decrease in VEGF secretion by tumour cells which can lead to the decrease of pre-existing blood vessels by apoptosis in addition to inhibiting the formation of new ones in the tumour.

The decreased VEGF secretion *in vitro* may reflect a reduction in VEGF expression. Rak and colleagues [34] have demonstrated previously that mutant *ras* plays a role in regulating the expression of VEGF in murine and human *ras*-mutated cell lines *in vitro*. In addition to regulating VEGF expression, oncogenic *ras* is also known to regulate the transcription of other potential pro-angiogenic factors such as TGF-β and TGF-α [9]. It seems reasonable to postulate that suppression of oncogenic *ras* activity with FTIs may suppress tumour growth *in vivo* by suppressing factors important for angiogenesis. It is unclear how *ras* genes regulate the expression of VEGF and the precise mechanisms of action of A-170634 on VEGF expression are also unknown. It is conceivable that oncogenic *ras* could lead to an increase in expression of c-Jun and c-Fos, resulting in increased VEGF

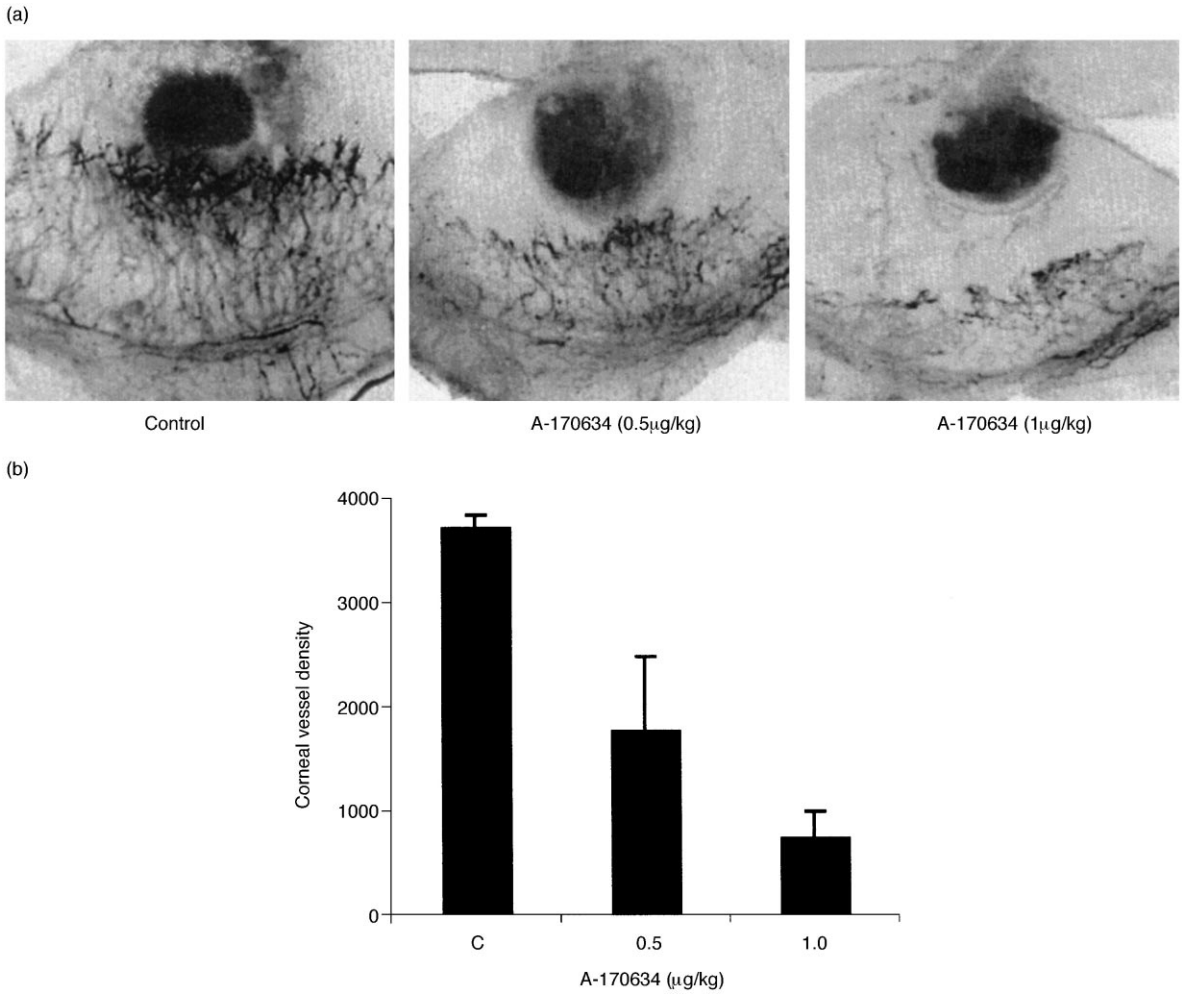


Figure 6. Reduction of corneal angiogenesis in rats by A-170634. (a) After 7 day, corneal neovascularisation was reduced following A-170634 treatments as compared with controls. (b) Corneal neovessel density from each eye was analysed using the image system. Vessel density decreased following A-170634 treatment of 0.5 and 1.0 μg/kg, respectively as compared with controls ($P<0.05$ and $P<0.00001$, respectively). Data represents the average (± standard deviation, S.D.)

transcription at the four AP1 binding sites in the VEGF promoter [18, 34]. Although we do not know the exact mechanism by which A-170634 suppresses VEGF secretion, it is important to note that VEGF expression may not be solely controlled by oncogenic *ras*, since others [9] have shown that *v-src* may also play a role in the regulation of VEGF. Furthermore, most colon carcinoma express VEGF, but only 50% have a K-*ras* mutation [35, 36]. Since HUVEC differentiation was suppressed by direct treatment with A-170634 *in vitro*, it may also have a direct effect on endothelial cell differentiation *in vivo*. However, the intracellular mechanisms of A-170634 on endothelial differentiation remains unclear.

Although the intracellular mechanisms of action of A-170634 on tumour and endothelial cells is unclear, studies have shown FTIs to inhibit tumour growth *in vivo* with little or no systemic toxicity [37]. This is remarkable despite the fact that Ras has been shown to be essential for normal cell growth and differentiation, endothelial cell migration, and embryogenesis in mice [38, 39]. Other mechanisms of FTI have been proposed recently that may account for their biological activity. For example, FTIs may affect tumour growth by *ras* independent mechanism such as Rho [37, 40], which plays an important role in the organisation of the cytoskeleton. Others have demonstrated that K-Ras can be geranylgeranylated in the presence of FTI although the biological significance of geranylgeranylated K-Ras is unclear [37, 41]. It is also clear from the literature that the potency of an FTI in cell culture and animal models does not correlate with the ras mutational status [15, 37]. Since more than 50 proteins are now known to be farnesylated [41] the true targets of FTI remains uncertain. Despite the uncertainty of whether FTI inhibit tumour growth through the inhibition of Ras function, it has been shown repeatedly that FTI can reduce tumour growth *in vivo* with little or no systemic toxicity [37]. We speculate that the mechanisms involved are likely to be multifactorial and tumour model dependent. For example, in our HCT116 xenograft model, we did not know how much contribution *ras* mutation has on the tumour growth in the context of its other genetic alterations such as overexpressed mdm2.

In conclusion, the data presented here demonstrated that A-170634 is a potent and selective peptidomimetic CAAX FTI that may exert its effects on tumour growth *in vivo* by more than one mechanism such as (a) direct and indirect suppression of tumour cell proliferation, (b) inhibition of endothelial proliferation and differentiation and (c) inhibition

of VEGF release and/or expression from tumour cells. These results open the possibility for the development of new therapeutic indications of FTIs specifically targeting angiogenesis.

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