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Cytochalasin E, an Epoxide Containing *Aspergillus*-Derived Fungal Metabolite, Inhibits Angiogenesis and Tumor Growth[1]

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ABSTRACT

Several previously identified inhibitors of angiogenesis have been epoxide-containing fungus-derived metabolites. We therefore hypothesized that novel epoxide-containing low molecular weight compounds structurally resembling known antiangiogenic agents may also exhibit antiangiogenic activity. Cytochalasin E was found to be a potent and selective inhibitor of bovine capillary endothelial (BCE) cell proliferation. Cytochalasin E differed from other cytochalasins by the presence of an epoxide. The epoxide was required for activity, because acid-catalyzed hydrolysis of the epoxide abrogated the specificity and potency of cytochalasin E. Phalloidin staining indicated that disruption of actin stress fibers by cytochalasin E occurred only at relatively high concentrations. Lower concentrations of cytochalasin E preferentially inhibited BCE cell proliferation without disrupting actin stress fibers. In vivo, cytochalasin E inhibited angiogenesis induced by basic fibroblast growth factor by 40% to 50% in the mouse cornea assay and inhibited the growth of Lewis lung tumors by approximately 72%. Cytochalasin E is a potent antiangiogenic agent that may hold promise for the treatment of cancer and other types of pathologic angiogenesis.

During angiogenesis, a gradient of growth factors induce sprouting from vessels by stimulating proliferation and migration of endothelial cells (Folkman and Klagsbrun, 1987). Agents that inhibit the migration or the proliferation of endothelial cells may potentially be used as treatments for angiogenesis-dependent diseases such as cancer, diabetic retinopathy, and arthritis (Folkman, 1995a,b). In addition, therapy that specifically targets endothelial cells should have fewer side effects than cytotoxic chemotherapy, which targets tumor cells but also affects normal cells due to a lack of selectivity (Folkman, 1995b).

For these reasons, there has been significant interest in the discovery and the identification of low molecular weight inhibitors of angiogenesis. A number of reported low molecular weight inhibitors of angiogenesis are fungus- or microbe-derived metabolites containing epoxides (Ingber et al., 1990; Oikawa et al., 1991, 1995; Onozawa et al., 1997). The cytochalasins are a family of compounds with diverse activities on cellular function, including inhibition of actin polymerization and glucose transport (Carter, 1967; Buchi et al., 1973; Brenner and Korn, 1980; Mookerjee et al., 1981). We found that cytochalasin E, an epoxide-containing metabolite of *Aspergillus clavatus*, contains a substructure spanning an epoxide group found in TNP-470 (AGM-1470) (Ingber et al., 1990). TNP-470, an *Aspergillus*-derived angiogenesis inhibitor, is currently in phase III trials for the treatment of cancer.

Due to structural similarities between cytochalasin E and TNP-470, we hypothesized that cytochalasin E may exhibit antiangiogenic activity. We found that cytochalasin E was a particularly potent and selective inhibitor of endothelial cells in vitro and that it inhibited angiogenesis and tumor growth in vivo. Unlike TNP-470, however, cytochalasin E did not inactivate methionine aminopeptidase-2 (Griffith et al., 1997; Sin et al., 1997). Thus, cytochalasin E is a novel inhibitor of angiogenesis and tumor growth, which may be useful in the treatment of cancer and other types of angiogenesis-dependent diseases.

Materials and Methods

Reagents and Cell Culture. Cytochalasins E, D, H, and A were purchased from Aldrich Chemical Co. (Milwaukee, WI). The cytocha-

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ABBREVIATIONS: BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; DMSO, dimethyl sulfoxide; TRITC, tetramethylrhodamine isothiocyanate; MetAP-2, methionyl aminopeptidase-2; T/C, tumor volume of treated animals/tumor volume of control animals.

lasin E derivative JHS-2-35 was formed by bubbling HCl gas in a solution of cytochalasin E dissolved in chloroform for 1 h. The product was confirmed by silica gel HPLC as well as by NMR (Kajimoto et al., 1989). The ozonolysis product of cytochalasin E was synthesized as described (Aldridge et al., 1973). Primary bovine capillary endothelial (BCE) cell cultures (Folkman et al., 1979) were plated in plastic tissue culture wells pretreated with 1.5% gelatin/PBS for 30 min at room temperature. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine calf serum, 1 ng/ml basic fibroblast growth factor (bFGF), 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin-streptomycin in an atmosphere of 10% CO_2. NIH-3T3 [American Type Culture Collection (ATCC), Manassas, VA], the ST7 human gastric carcinoma (Yadav et al., 1996), A375 (ATCC), and MMAN (obtained from Dr. J. Arbiser, Emory University, Atlanta, GA) melanomas, retinal pigment epithelial cells (obtained from Dr. A. Adamis, Children's Hospital, Boston, MA), bovine smooth muscle cells (Dr. P. D'Amore, Children's Hospital), and C6 glioblastama (ATCC), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin-streptomycin. The low metastatic variant of the Lewis lung carcinoma line (obtained from M. S. O'Reilly, Children's Hospital) was maintained in C57BL/6 mice as described (O'Reilly et al., 1994).

Proliferation Assay. To determine proliferation, 4000 cells per well were plated in 200 μl of the appropriate media in 96-well tissue culture plates together with drugs in varying concentrations. The final dimethyl sulfoxide (DMSO) vehicle concentration did not exceed 0.1%. The cells were placed in a 37°C, humidified incubator containing 10% CO_2. After 2 days, the cells were stained with methylene blue according to the method of Goldman and Bar-Shavit (1979). Briefly, the plates were inverted to remove media, the wells were washed once with 100 μl of 1× PBS, and then the cells were fixed to the plates with 50 μl/well of 100% ethanol for 5 min at room temperature. The wells were washed with 100 μl/well of 0.1 M sodium borate, pH 8.9, and the cells were then stained with 50 μl/well of 1% methylene blue dissolved in the sodium borate buffer. After 10 min at room temperature, the excess stain was removed by inversion, and the plates were rinsed in a bucket of tap water with several changes. The dye was solubilized with 100 μl/well of 0.1 N HCl, and the absorbance was read at 630 nm using an enzyme-linked immunosorbent assay plate reader (Dynatech MR 5000; Dynex, Chantilly, VA). The absorbance values at 630 nm were linear with respect to the number of cells used in the assays.

Phalloidin Staining. BCE cells (20,000 cells/well) were plated on gelatinized circular coverslips in 24-well tissue culture dishes and allowed to attach overnight. After treatment with cytochalasin E, the cells were washed once with PBS and then fixed with 4% paraformaldehyde in PBS containing Ca^{2+} (1.71 mM final) and Mg^{2+} (0.93 mM final) for 30 min at room temperature. The fixed cells were washed once with PBS and then permeabilized for 20 min at room temperature in PBS containing 0.5% Triton X-100, 1.71 mM Ca^{2+}, 0.93 mM Mg^{2+}, and 0.5% bovine serum albumin. The permeabilized cells were incubated with 1 μM tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Sigma) dissolved in permeabilization buffer (diluted from a 100 μM stock in DMSO) for 60 min at 37°C. The labeled cells were washed twice with PBS containing Ca^{2+} and Mg^{2+} and then mounted onto slides with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL).

Mouse Corneal Neovascularization. The mouse cornea model for neovascularization was performed as previously described (Kenyon et al., 1996). Briefly, a corneal pocket was created in the eyes of 7- to 9-week-old C57BL/6 mice, and a 0.4- × 0.4-mm, sucrose octasulfate (Sigma) pellet containing 80 to 100 ng of bFGF coated with hydropolymer was implanted in the micropocket. The pellet was positioned 0.7 to 1.0 mm from the temporal limbus, and erythromycin was applied once to the operated eye. A 10 mg/ml stock solution of drugs dissolved in DMSO was stored in aliquots at −20°C. On the day of injection, the drugs were thawed, diluted to 0.5 mg/ml in olive oil, vortexed for 5 min at room temperature, and then administered s.c. starting on the day of implantation. For each treatment group, at least seven eyes were quantitated and repeated at least one time. Five days after pellet implantation, the maximal vessel length and number of clock hours of neovascularization was measured using a slit lamp biomicroscope as described previously (Kenyon et al., 1996). The area of corneal neovascularization was calculated according to a modified formula for a half-ellipse: Area (mm^2) = [π × clock hours × length (mm) × 0.2 mm].

In Vivo Tumor Growth. The Lewis Lung tumor line was maintained by in vivo passage as described (O'Reilly et al., 1994). In brief, C57BL/6 mice bearing Lewis lung tumors of 600- to 1200-mm^3 volume were sacrificed, and the tumors were resected under aseptic conditions. A suspension of the resected tumor cells was made by passage through a sieve in 0.9% normal saline and then sequentially through 22- to 30-gauge needles. Approximately 1 million tumor cells in 0.1 ml of saline were injected s.c. in the dorsa of mice (weighing approximately 25 g) in the proximal midline. Starting 5 days after tumor implantation, the mice were injected with drugs s.c. away from the tumor near the flank using at least a 26-gauge needle. Each treatment group consisted of at least four mice, and each experiment was repeated. Tumor measurements were made using a caliper, and the volumes were calculated according to the formula: tumor volume = $(\text{width})^2 \times \text{length} \times 0.52$.

Methionyl Aminopeptidase Assay. Varying amounts of the inhibitors were incubated with 10 nM purified human methionyl aminopeptidase-2 (MetAP-2) in buffer H [containing 10 mM HEPES, pH 7.35, 100 mM KCl, 10% glycerol, and 0.1 mM Co(II)] and incubated at 37°C for 15 min. To start the enzyme reaction, 1 mM Met-Gly-Met-Met was added to the reaction mixture. Released methionine was quantified at 0, 3, and 5 min using the method of Zuo et al. (1994).

Results

Cytochalasin E Shows a Unique Inhibition of BCE Cells. We investigated the effect of cytochalasin E and related molecules (Fig. 1, compounds 1, 4–8) on the proliferation of capillary endothelial cells as well as other nonendothelial cell lines. In Table 1, the sensitivity of BCE cells were compared with several different cell lines, including primary smooth muscle and retinal pigment epithelial cells, the NIH-3T3 fibroblast line, and the tumor lines ST7, A375, and C6. Among these cell lines, the BCE cells were the most sensitive to inhibition by cytochalasin E, particularly at lower concentrations. We then compared the inhibition of BCE proliferation by cytochalasins E, A, and H and found that, among these compounds, cytochalasin E was the most potent (Fig. 2). Cytochalasins A and H inhibited BCE cell proliferation at nanomolar concentrations, whereas cytochalasin E inhibited BCE proliferation at low picomolar concentrations.

The inhibition of BCE cell proliferation by cytochalasin E was biphasic and occurred over a broad concentration range (Fig. 2). The first phase of inhibition occurred in the femtomolar to picomolar concentrations. At these dose levels, there was no visible cytotoxic effect (not shown). The cells were able to proliferate, because cell numbers increased after 2 days, but proliferation was significantly reduced. The second phase occurred in the nanomolar to micromolar range, which corresponded roughly to the range of inhibition seen using other cytochalasins. In this range, there were gross changes in cell morphology and loss of adhesion to the substratum. At these higher concentrations, the observed inhibition was due in part to cell death (as determined by trypan blue staining) and to loss of adhesion to substratum. The biphasic nature of

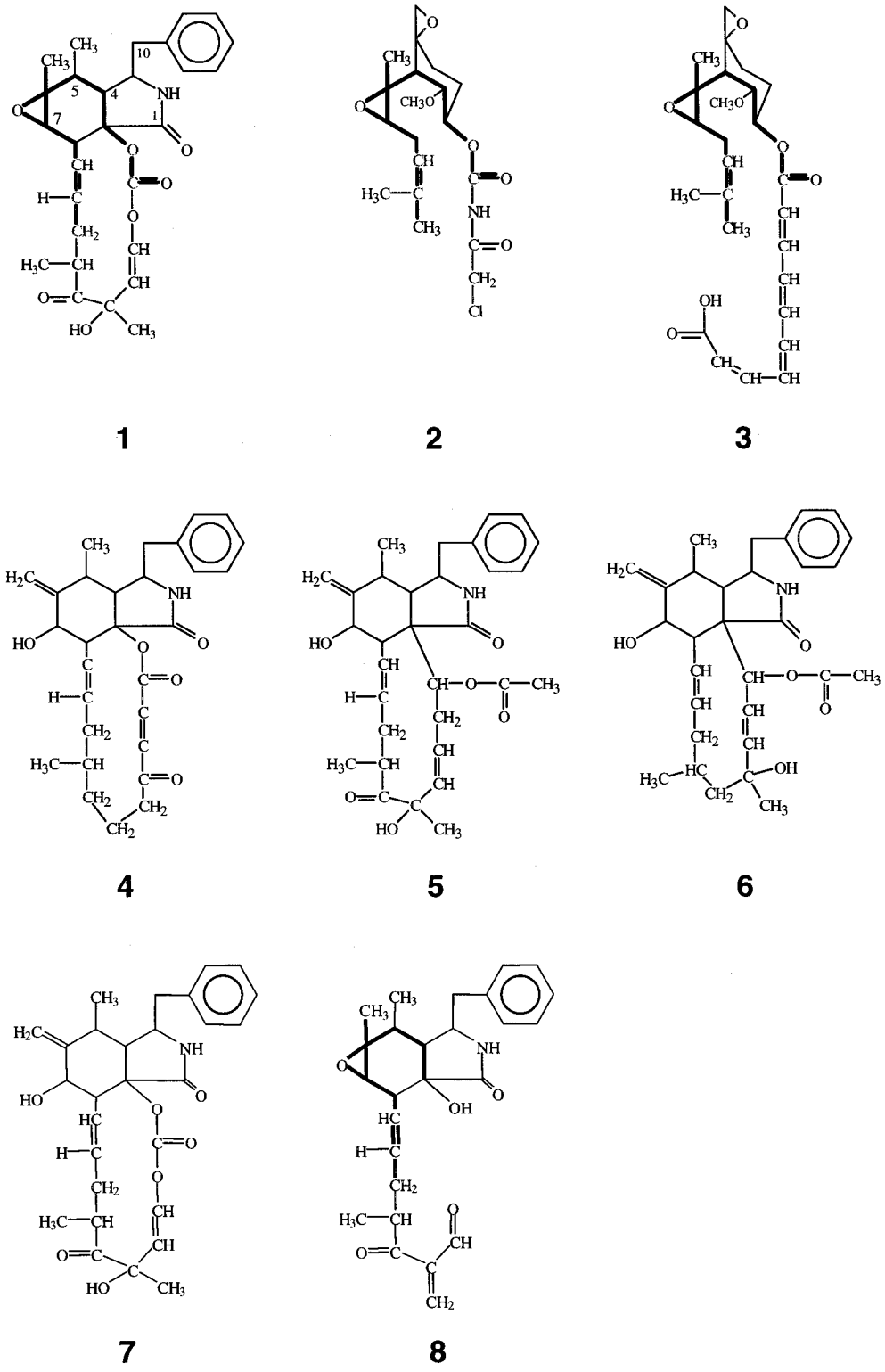

Fig. 1. Structure of fungus-derived metabolites and angiogenesis inhibitors. Cytochalasin E (1); TNP-470 (2); fumagillin (3); and cytochalasins A (4), D (5), and H (6). Rearrangement product JHS-2-35 (7) and ozonolysis product JHS-1-99 (8) of cytochalasin E. Regions of similarity containing epoxides are indicated by the bold lines.

the inhibition and the associated differences in morphology suggested that cytochalasin E inhibited proliferation through more than one site of action. At high concentrations (10^{-9} to 10^{-6} M), cytochalasin E, as well as cytochalasins A and H, inhibited proliferation predominantly through its well known antiactin effect (Brenner and Korn, 1980; Flanagan and Lin, 1980). Inhibition at lower concentrations (10^{-14} to 10^{-9} M), however, was mediated by an interaction with a different target.

In contrast to BCE cells, cytochalasin E inhibited NIH-3T3 fibroblasts only in the nanomolar to micromolar range, whereas inhibition at lower concentrations of cytochalasin E was not observed. In fact, the fibroblast line was equally sensitive to inhibition by cytochalasins E, A, and H as shown

TABLE 1
Inhibition of proliferation by cytochalasin E
Cells were cultured in the presence of indicated concentrations of cytochalasin E, and proliferation was determined by methylene blue staining after 48 h as described in under *Materials and Methods*. Values indicate mean percentage proliferation relative to vehicle control ± SE.

Cell Line	Cyochalasin E			
	Control	10 pg/ml	1 ng/ml	100 ng/ml
	% control			
BCE	100 ± 4	66 ± 2	36 ± 1	34 ± 2
Smooth muscle	100 ± 3	89 ± 2	86 ± 2	63 ± 4
RPE	100 ± 6	94 ± 6	97 ± 7	39 ± 5
NIH-3T3	100 ± 6	96 ± 12	100 ± 2	58 ± 2
ST7	100 ± 7	94 ± 7	87 ± 5	38 ± 1
A375	100 ± 4	100 ± 8	100 ± 4	53 ± 2
C6	100 ± 6	100 ± 5	88 ± 1	68 ± 3
MMAN	100 ± 1	98 ± 2	96 ± 6	65 ± 1

by roughly overlapping dose responses (Fig. 2). Other cell types, including primary smooth muscle cells, tumor lines, and retinal pigment epithelial cells, similar to NIH-3T3 fibroblasts, were much less sensitive to cytochalasin E (Table 1). The inhibitory concentrations for NIH-3T3 fibroblasts by cytochalasins approximately corresponded to the second phase (10^{-9} to 10^{-6} M) of BCE cell inhibition. Therefore, at the lower concentrations, cytochalasin E inhibited BCE cell proliferation through a unique interaction, which was not observed in other cell types. At the higher concentrations (10^{-9} to 10^{-6} M), cytochalasin E exhibited a cytochalasin-like effect characterized by disruption of actin leading to growth inhibition and cytotoxicity of various cells types.

BCE cells were treated with varying concentrations of cytochalasin E for 16 h and then stained with phalloiden to visualize the effect of cytochalasin E on actin (Wulf et al., 1979). At 2 pM, dissolution of actin filament was not evident compared with controls (Fig. 3), even though the proliferation of BCE cells was inhibited at this concentration (Fig. 2). Dissolution of actin stress fibers was not seen until much higher concentrations of cytochalasin E were used. At 20 nM and higher, an antiactin effect was revealed by the absence of stress fiber staining, retraction of the cytoplasm, and cellular detachment from the substratum.

Because the epoxide group was one of the distinguishing structural elements of cytochalasin E, the contribution of the epoxide was examined by acid-catalyzed rearrangement of the epoxide group under nonaqueous conditions. This resulted in a single rearrangement product, JHS-2-35 (Fig. 1, compound 7), whose perisohydroindole core ring was now identical with other cytochalasins. JHS-2-35 exhibited a sharper inhibitory dose response, which was evidence for a more restricted mechanism of action. The rearrangement product was no longer inhibitory for BCE proliferation at picomolar concentrations but was still active at the higher doses (Fig. 4). The inhibition at the higher doses was associated with cytoplasmic contraction and cell rounding (not shown), which demonstrated that JHS-2-35 was still able to inhibit actin polymerization. The similar dose-response profile of JHS-2-35 and cytochalasin A revealed that the two compounds were active at similar concentrations. Therefore, the epoxide of cytochalasin E participated in the unique inhibition of BCE cell proliferation but, as might be expected, was not required for inhibition of cellular proliferation caused by dissolution of actin. Cleavage of the macrocyclic ring by ozonolysis resulted in compound JHS-1-99 (Fig. 1, compound 8), which was inactive in the proliferation assays (Fig. 4). This suggests that elements of the macrocyclic ring participate in both the selective inhibition of BCE proliferation as well as nonselective inhibition of actin polymerization.

Inhibition of Angiogenesis by Cytochalasin E in an Experimental Eye Model. To determine whether cytochalasin E exhibited antiangiogenic activity, cytochalasin E was tested in vivo in a mouse cornea angiogenesis model (Kenyon et al., 1996). In Fig. 5, neovascularization was induced by bFGF released slowly from a polymer implanted in the mouse cornea. Starting on the day of surgery, the animals were treated with doses of cytochalasin E based on previous animal studies (Trirawatanapong et al., 1980). Cytochalasin E was mixed and diluted in olive oil to retard its release and then administered s.c. Delivery by oral or i.p. routes resulted in reduced effectiveness. At a maximum tolerated dose of 2.5 mg/kg every other day, cytochalasin E inhibited bFGF-induced angiogenesis by approximately 50% (Fig. 5) without evidence of toxicity. Vascular endothelial growth factor (VEGF)-induced angiogenesis was also equally inhibited (not shown).

Table 2 shows a comparison of cytochalasin E with other cytochalasins. At 2.5 mg/kg/day, cytochalasin E inhibited bFGF-induced angiogenesis by 50%, but at this dose, cytochalasins A and H were both toxic and resulted in significant weight loss (>5% of body weight). At lower doses, cytochalasins A and H also exhibited some antiangiogenic activity. At comparable doses, however, cytochalasin E was the most effective (Table 2). At 2.5 mg/kg every other day, cytochalasin E inhibited 39 ± 3%, whereas cytochalasin A and cytochalasin H inhibited 26 ± 4% ($P < .02$) and 22 ± 3% ($P < .001$), respectively. At 2.0 mg/kg every other day, cytochalasin E inhibited angiogenesis by 36 ± 2%, whereas cytochalasin A and cytochalasin H inhibited 22 ± 2% ($P < .002$) and 27 ± 4% ($P < .04$), respectively. Thus, cytochalasin E was less toxic and significantly more potent than either cytochalasin A or H.

Inhibition of Tumor Growth in Vivo by Cytochalasin E. Because cytochalasin E inhibited angiogenesis induced by bFGF and VEGF in mice, cytochalasin E was also tested for inhibiting the growth of the Lewis lung tumor in mice. Mice were treated over a 2-week period at the maximum tolerated dose of 2.5 mg/kg every other day starting 5 days after inoculation of tumor cells. Tumor volumes and animal weights were monitored throughout the course of treatment.

As shown in Fig. 6, cytochalasin E inhibited the growth of Lewis lung tumors with a final T/C (tumor volume of treated animals/tumor volume of control animals) of 0.28 (72% inhibition). Table 3 summarizes the effect of cytochalasins at varying doses. At 1.0 mg/kg/day, cytochalasin E inhibited tumor growth with a final T/C value of approximately 0.77 (23% inhibition). A dose of 2.0 mg/kg every 3 days gave approximately the same degree of inhibition (T/C = 0.7). Increasing the dose to 2.0 mg/kg administered every other day resulted in a final T/C value of 0.28 (72% inhibition). Higher doses resulted in weight loss. At the maximum tolerated dose, cytochalasin A and cytochalasin H were much less effective with final T/C values of 0.59 (41% inhibition), and 0.65 (35% inhibition), respectively. These results showed

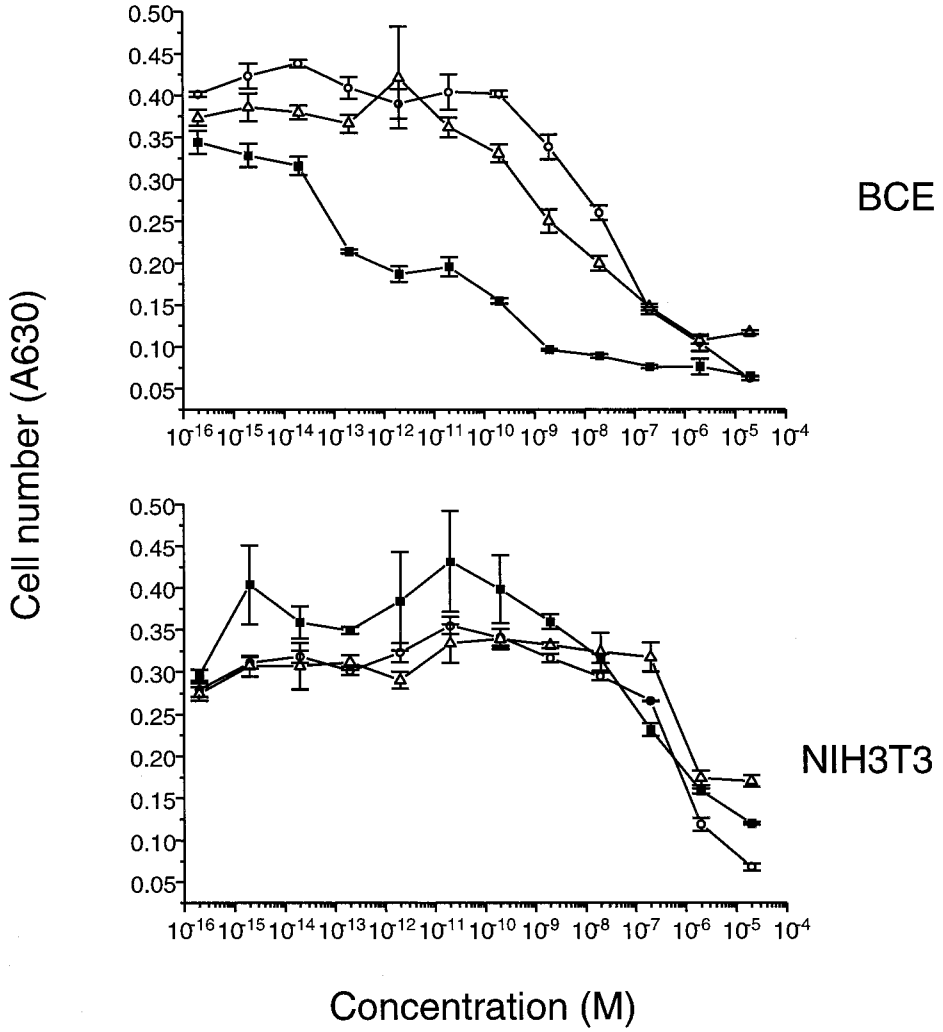

Fig. 2. Biphasic inhibition of BCE cell proliferation by cytochalasin E. BCE (top) and NIH-3T3 cells (bottom) were plated in 96-well plates, allowed to attach for 3 to 6 h, and then treated with the indicated concentrations of cytochalasins E (■), A (○), or H (△). After 2 days, the cells were then harvested and quantitated by methylene blue staining as described under *Materials and Methods*.

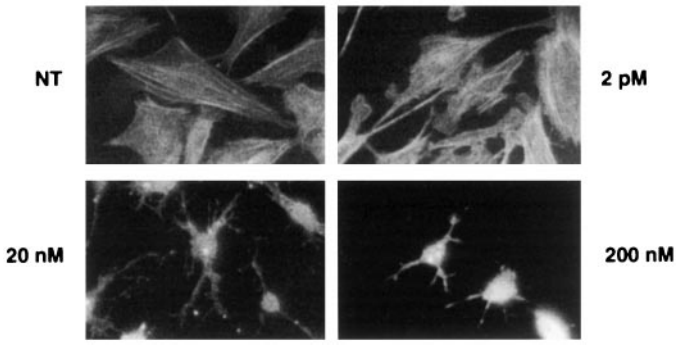

Fig. 3. Effect of cytochalasin E on actin polymerization by phalloidin staining. BCE cells (40,000 cells/well) were plated onto gelatinized circular cover slips, placed in 24-well tissue culture dishes, and allowed to attach overnight. The cells were then treated with the indicated concentrations of cytochalasin or left untreated (NT) for 16 h. The cells were fixed and stained with TRITC-phalloidin as described. Immunofluorescence photographs were taken at 400× magnification.

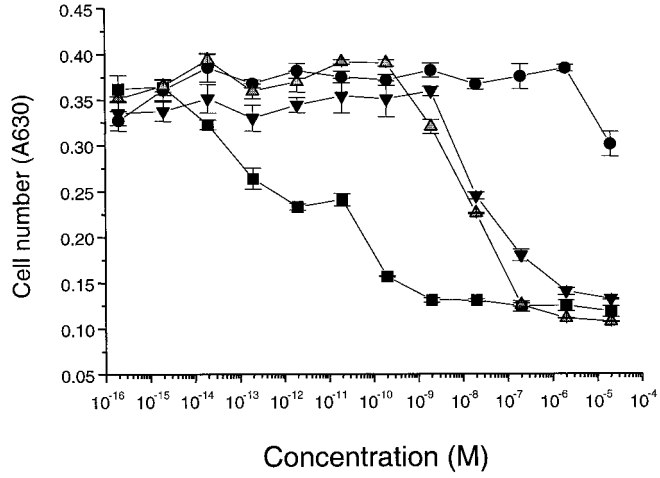

Fig. 4. Comparison between cytochalasin E and related compounds on inhibition of BCE cell proliferation. Cytochalasin E (■), rearrangement product JHS-2-35 (▲), JHS-1-99 (●), and Cytochalasin A (▼) were compared in a BCE assay. The drugs were added to BCE cells at the indicated final concentrations, and proliferation was determined by methylene blue staining.

that, among the cytochalasins, cytochalasin E was the most effective inhibitor of tumor growth as well as angiogenesis.

Cytochalasin E Does Not Inhibit MetAP-2. Based on structural resemblance of cytochalasin E to TNP-470 (Fig. 1), a previously identified inhibitor of endothelial proliferation and angiogenesis (Ingber et al., 1990), we investigated the possibility that cytochalasin E and TNP-470 might interact with a common target. The angiogenesis inhibitors fumagillin and TNP-470 are known to bind and to inactivate the enzyme MetAP-2 (Griffith et al., 1997; Sin et al., 1997). To

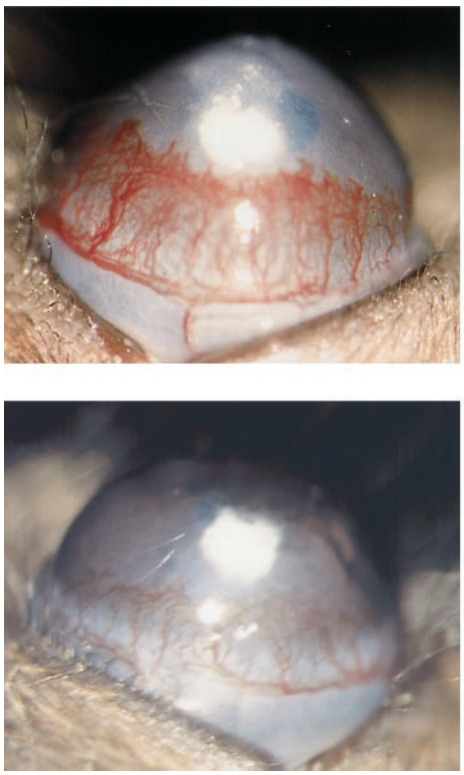

Fig. 5. Inhibition of bFGF-induced angiogenesis in the mouse cornea by cytochalasin E. Mice with corneal implants of polymers containing bFGF were treated starting on the day of implantation with cytochalasin E injected s.c. at a dose of 0 (control) or 2.5 mg/kg every other day. 5 days after pellet implantation, angiogenesis in the cornea was visualized by slit lamp biomicroscopy.

TABLE 2

Inhibition of angiogenesis in the mouse cornea by cytochalasins

Drugs were dissolved in DMSO as a stock solution, and then administered S.C. in oil. Treatment began on the day of surgery. Angiogenesis in the mouse cornea induced by bFGF was determined on day 5. Neovascularization was quantitated, and the percentage inhibition was determined by the formula described under *Material and Methods*.

Dose	% Inhibition (±S.E.)		
	Cytochalasin E	Cytochalasin A	Cytochalasin H
mg/kg			
2.5 qd[a]	50 ± 3	ND[b]	ND
2.0 qd	49 ± 4	ND	ND
2.5 qod	39 ± 3	26 ± 4 ($P < .02$)	22 ± 3 ($P < .001$)
2.0 qod	36 ± 2	22 ± 2 ($P < .002$)	27 ± 4 ($P < .04$)

[a] qd, everyday; qod, every other day.
[b] ND, not determined due to toxicity.

determine whether inhibition of MetAP-2 could also be involved in nonactin-mediated inhibition of BCE cells, the peptidase activity of recombinant human MetAP-2 on a synthetic peptide substrate was measured in the presence of increasing concentrations of cytochalasin E and TNP-470. Approximately 20 nM TNP-470 completely inhibited the activity of 10 nM MetAP-2 as measured by release of methionine using a synthetic peptide substrate at 1 mM. In contrast, cytochalasin E was unable to inhibit MetAP-2 activity even when tested at a 100-fold higher concentration of TNP-470 used to completely suppress MetAP-2 (not shown). Therefore, despite the similarity in structure, the mechanism of cytochalasin

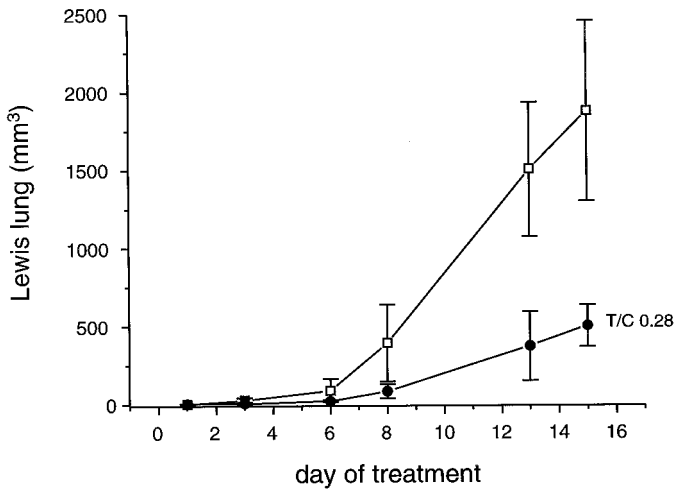

Fig. 6. Inhibition of tumor growth by cytochalasin E. Lewis lung tumors were implanted in C57BL/6 mice. On the 5th day after tumor injection, the mice were treated with either cytochalasin E administered at 2 mg/kg every other day for 15 days (●) or left untreated (□). Tumor volumes were calculated as described under *Materials and Methods*.

TABLE 3

Inhibition of tumor growth by cytochalasins

Lewis lung tumors were injected in the dorsa of C57BL/6 mice. Beginning 5 days after injecting tumors, cytochalasin E was diluted in olive oil from a stock solution of cytochalasin E at 10 mg/ml dissolved in DMSO and administered s.c. as indicated. Tumor volumes were calculated according to the formula described under *Materials and Methods*.

	Dose	T/C
Cytochalasin E	2.0 mg/kg qod	0.28
	2.0 mg/kg q3d[a]	0.70
	1.0 mg/kg qd	0.77
Cytochalasin A	2.5 mg/kg qod	0.59
Cytochalasin H	2.5 mg/kg qod	0.65

[a] q3d, every third day.

E-mediated suppression of endothelial proliferation and angiogenesis appeared distinct from that of TNP-470.

Discussion

The cytochalasins are a class of fungus-derived metabolites whose effects on cellular function include inhibition of glucose transport, inhibition of actin polymerization (Brenner and Korn, 1980; Flanagan and Lin, 1980), blockage of cytoplasmic cleavage, and inhibition of cell movement (Carter, 1967). Cytochalasin E is an epoxide-containing cytochalasin family member, which was isolated as a minor metabolite of the food storage mold *A. clavatus* (Glinsukon et al., 1973). Cytochalasin E was reported at high concentrations to exhibit unique histopathologic effects on the vasculature, including hemorrhage and injury to the vascular walls (Glinsukon et al., 1975) accompanied by alterations in vascular permeability (Aldridge et al., 1973; Lipski et al., 1987). We have found that cytochalasin E at lower doses was an inhibitor of angiogenesis and tumor growth. Among the cytochalasins tested, cytochalasin E was the most potent. In vitro, cytochalasin E exhibited a unique biphasic inhibition of BCE cell proliferation that was not observed using other nonepoxide cytochalasin analogs.

Structurally, the cytochalasins are a family of compounds (Fig. 1) characterized by a central perisohydroindole core ring and an attached large macrocyclic ring, which varies in

size and composition. A carbonyl group is present at carbon 1 of the central core ring, and a methyl group is present on carbon 5. Most cytochalasins contain a hydroxyl group on carbon 7, but cytochalasin E (Fig. 1, compound 1), instead, has a 6,7-epoxy group.

A broad, biphasic inhibitory range observed in vitro suggested the existence of at least two distinct targets. Loss of the epoxide by rearrangement resulted in a product that was no longer inhibitory for BCE cell proliferation at picomolar concentrations. At higher doses, however, the rearrangement product was still inhibitory and induced morphologic changes consistent with disruption of actin. Thus, cytochalasin E inhibited proliferation by disruption of actin at higher concentrations and also by an epoxide-dependent mechanism at lower concentrations. In vivo, inhibition of angiogenesis by several different cytochalasins suggested that disruption of actin could contribute to inhibition of angiogenesis. The greater potency of cytochalasin E, however, appeared to be attributable to an interaction between cytochalasin E and an as yet unidentified molecule involved in preferential inhibition of endothelial cells.

Interestingly, cytochalasin E contains an arrangement of atoms that span elements of the macrocyclic ring, the epoxide group, and the core ring found in the conserved regions of the angiogenesis inhibitor TNP-470 and its parent compound (Fig. 1, compounds 1 to 3). TNP-470 and fumagillin were similar over a greater portion of the molecule to cytochalasin E than to the other cytochalasins. In addition, cytochalasin E is a fungus-derived metabolite of *Aspergillus* (Aldridge et al., 1973), which exhibited potent and selective inhibition of endothelial cell proliferation similar to that of fumagillin and TNP-470 (Ingber et al., 1990). At high concentrations, fumagillin, the parent compound of TNP-470, exhibited cell-rounding activity resembling a cytochalasin-like effect (Ingber et al., 1990). Nevertheless, whereas TNP-470 in vitro completely suppressed the activity of MetAP-2 (Griffith et al., 1997; Sin et al., 1997), cytochalasin E had no effect. Thus, cytochalasin E appeared to specifically inhibit endothelial cell proliferation and angiogenesis by a mechanism distinct from that of TNP-470.

Although cytochalasin E is inhibitory for capillary cells in the picomolar range, plasma from mice injected with cytochalasin E exhibited little inhibitory effect on BCE proliferation (not shown). This suggests that cytochalasin E may be metabolized, bound to tissue, or rapidly cleared from circulation. The epoxide of cytochalasin E appears to be necessary for activity, and studies with TNP-470 have shown that the epoxide is cleaved rapidly in circulation (Figg et al., 1997). This may also explain why both cytochalasin E and TNP-470 are active at picomolar concentrations in culture but require milligram per kilogram doses in vivo.

Current efforts are directed toward the development of cytochalasin E analogs lacking antiactin activity, which may allow higher administration of the drug. The identification of the nonactin target of cytochalasin E would facilitate the development of more specific analogs and may reveal new signaling pathways involved in angiogenesis and vascular development. Finally, we propose that cytochalasin E and analogs may be useful for the treatment of angiogenesis-dependent diseases such as cancer and age-related macular degeneration.

References

Aldridge DC, Greatbanks D and Turner WB (1973) Revised structures for cytochalasins E and F. *J Chem Soc Chem Commun* 551–552.

Brenner SL and Korn ED (1980) The effect of cytochalasins on actin polymerization and actin ATPase provide insights into the mechanism of polymerization. *J Biol Chem* **255:**841–844.

Buchi G, Kitaura Y, Yuan SS, Wright HE, Clardy J, Demain AL, Glinsukon T, Hunt N and Wogan GN (1973) The structure of cytochalasin E, a toxic metabolite of *Aspergillus clavatus*. *J Am Chem Soc* **95:**5423–5425.

Carter SB (1967) Effects of cytochalasins on mammalian cells. *Nature (Lond)* **213:** 261–264.

Figg WD, Pluda JM, Lush RM, Saville MW, Wyvill K, Reed E and Yarchoan R (1997) The pharmacokinetics of TNP-470, a new angiogenesis inhibitor. *Pharmacotherapy* **17:**91–97.

Flanagan MD and Lin S (1980) Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J Biol Chem* **255:**835–838.

Folkman J (1995a) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1:**27–31.

Folkman J (1995b) Clinical applications of research on angiogenesis. *N Engl J Med* **333:**1757–1763.

Folkman J, Haudenshild CC and Zetter RB (1979) Long-term culture of capillary endothelial cells. *Proc Natl Acad Sci USA* **76:**5217–5221.

Folkman J and Klagsbrun M (1987) Angiogenic factors. *Science (Wash DC)* **235:**442–447.

Glinsukon T, Shank RC, Wogan GN and Newberne PM (1975) Acute and subacute toxicity of cytochalasin E in the rat. *Toxicol Appl Pharmacol* **32:**135–146.

Glinsukon T, Yuan SS, Wightman R, Kitaura Y, Buchi G, Shank RC, Wogan GN and Christensen CM (1973) Isolation and purification of cytochalasin E and two tremorgens from *Aspergillus clavatus*. *Plant Foods Man* **1:**113–119.

Goldman R and Bar-Shavit Z (1979) Dual effect of normal and stimulated macrophages and their conditioned media on target cell proliferation. *J Natl Cancer Inst* **63:**1009–1016.

Griffith EC, Su Z, Turk BE, Chen S, Chang Y-H, Wu Z, Biemann K and Liu JO (1997) Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. *Chem Biol* **4:**461–471.

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H and Folkman J (1990) Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature (Lond)* **348:**555–557.

Kajimoto T, Imamura Y, Yamashita M, Takahashi K, Shibata M and Nohara T (1989) Nuclear magnetic resonance studies of cytochalasin E and its decomposition product. *Chem Pharm Bull (Tokyo)* **37:**2212–2213.

Kenyon B, Voest E, Chen C, Flynn E, Folkman J and D'Amato RJ (1996) A model of angiogenesis in the mouse cornea. *Investig Ophthalmol Vis Sci* **37:**1625–1631.

Lipski KM, McQuiggan JD, Loucy KJ and Fondy TP (1987) Cytochalasin B: Preparation, analysis in tissue extracts, and pharmacokinetics after intraperitoneal bolus administration in mice. *Anal Biochem* **161:**332–340.

Mookerjee BK, Cuppoletti J, Rampal AL and Jung CY (1981) The effects of cytochalasins on lymphocytes: Identification of distinct cytochalasin-binding sites in relation to mitogenic response and hexose transport. *J Biol Chem* **256:**1290–1300.

Oikawa T, Hasegawa M, Shimamura M, Ashino H, Murota S and Murota I (1991) Eponemycin, a novel antibiotic, is a highly powerful angiogenesis inhibitor. *Biochem Biophys Res Commun* **181:**1070–1076.

Oikawa T, Onozawa C, Inose M and Sasaki M (1995) Depudecin, a microbial metabolite containing two epoxide groups, exhibits anti-angiogenic activity *in vivo*. *Biol Pharm Bull* **18:**1305–1307.

Onozawa C, Shimamura M, Iwasaki S and Oikawa T (1997) Inhibition of angiogenesis by rhizoxin, a microbial metabolite containing two epoxide groups. *Jpn J Cancer Res* **88:**1125–1129.

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH and Folkman J (1994) Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastasis by a Lewis lung carcinoma. *Cell* **79:**315–328.

Sin N, low Meng L, Wang MQW, Wen JJ, Bornmann G and Crews CM (1997) The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc Natl Acad Sci USA* **94:**6099–6103.

Trirawatanapong T, Temcharoen P, Na Nagara B and Anukarahanonta T (1980) Alteration of vascular permeability due to cytochalasin E. *Toxicol Appl Pharmacol* **52:**209–213.

Wulf E, Deboben A, Bautz FA, Faulstich H and Wieland T (1979) Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proc Natl Acad Sci USA* **76:**4498–4502.

Yadav M, Hopwood VL, Multani AS, Mansfield PF, Takahashi Y, McIntyre BW, Udagawa T and Pathak S (1996) Non-random primary and secondary chromosomal abnormalities in human gastric cancers. *Anticancer Res* **16:**1787–1795.

Zuo S, Guo Q and Chang Y-H (1994) A protease assay via precolumn derivatization and high-performance liquid chromatography. *Anal Biochem* **222:**514–516.

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