

A comparative study on the anti-angiogenic effects of DNA-damaging and cytoskeletal-disrupting agents

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Abstract The discovery of molecules with anti-angiogenic properties has led to promising new strategies for the treatment of diseases characterized by excessive new vessel growth, such as cancer and haemangioma. We have assessed the effects of DNA-damaging and cytoskeletal-disrupting agents *in vitro* on several endothelial cell functions. We report that bleomycin, mitomycin C and cytoskeletal-disrupting drugs (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D) exhibit anti-angiogenic activities of varying potency. Bleomycin and the various cytoskeletal-disrupting drugs inhibited endothelial cell migration, while mitomycin C had a marginal effect. Both DNA-damaging and cytoskeletal-disrupting drugs decreased endothelial cell growth in a dose-dependent manner, and this was accompanied by the induction of apoptosis. The growth inhibitory and apoptotic effects of cytoskeletal-disrupting drugs were the most pronounced. We also show that both classes of drugs inhibited capillary-like tube formation in an assay of

in vitro angiogenesis, with cytoskeletal-disrupting agents inhibiting *in vitro* angiogenesis with greater potency. A targeted approach incorporating several compounds with different mechanisms of action may be useful for the treatment of angiogenesis-dependent diseases such as hemangiomas of infancy.

Keywords Angiogenesis · Hemangioma · Bleomycin · Mitomycin C · Cytoskeletal-disrupting agents · Angiogenesis · Endothelial cell · Cell growth · Cell migration · Tube formation

Introduction

Hemangiomas are benign vascular tumors frequently encountered in pediatrics, and although benign, may present serious complications [1–3]. Although several approaches have been tried, there is to date no definitive treatment for hemangiomas. Recently however, the effectiveness of intralesional bleomycin was evaluated by the Pretoria Vascular Malformation Study Group in 37 hemangioma patients. Complete resolution or significant improvement was seen in 87% of patients [2]. Hemangioma development is associated with an imbalance between negative and positive regulators of angiogenesis, and light microscopic examination of haemangioma tissue has demonstrated that the hallmark of a growing hemangioma is proliferating endothelial cells [4, 5]. Furthermore, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have been implicated in the pathogenesis of these lesions [6].

The process of angiogenesis is characterized by alterations in at least three endothelial cell functions, each of which is a potential target for anti-angiogenic strategies: (1) modulation of interactions with the extracellular matrix, (2)

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an initial increase and subsequent decrease in migration, and (3) an increase in proliferation, which provides new cells for the growing and elongating vessel [7]. Angiogenesis is also dependent on the suppression of endothelial cell apoptosis [8–10]. The recognition that excessive angiogenesis underlies hemangioma development, offers an opportunity for the development of therapeutic strategies based on the inhibition of angiogenesis.

Therapeutic strategies that target angiogenesis have relied on an understanding of the mechanisms of action of anti-angiogenic molecules and a re-evaluation of conventional chemotherapeutic drugs [11]. With respect to the re-examination of ‘classical’ anticancer agents, mitomycin C has previously been shown to inhibit human microvascular endothelial cell growth [12]. However, the anti-angiogenic effects of both mitomycin C and bleomycin have not been well documented.

Microtubule-disrupting agents (including 2-methoxyestradiol, taxol, vincristine, vinblastine, and colchicine) and the microfilament-disrupting drug, cytochalasin D, have been reported to affect certain elements of the angiogenic process, such as endothelial cell migration and *in vitro* capillary-like tube formation [13–15].

Previously, drugs with anti-angiogenic activity were developed and tested individually. However, because of the complexity of the angiogenesis process and the number of regulatory molecules involved in blood vessel growth, a multi-drug approach may be required to optimize the anti-angiogenic effect of therapy aimed at treating vascular tumors of infancy.

In the present study we evaluated and compared the effects of bleomycin, mitomycin C and cytoskeletal-disrupting drugs (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D), on two critical endothelial cell functions namely, endothelial cell migration and growth, which characterize angiogenesis. The effect of these drugs on endothelial cell apoptosis and *in vitro* angiogenesis were also assessed. To our knowledge, this is the first comparative study undertaken to investigate the anti-angiogenic effects of these drugs under similar conditions.

Materials and methods

Cell culture

Adrenal cortex-derived bovine microvascular endothelial (BME) cells were maintained in a 37°C incubator in a humidified atmosphere containing 5% CO₂. The cells were grown in α -modified minimum essential medium (α -MEM) supplemented with 15% donor calf serum (DCS), penicillin (500 U/ml), and streptomycin (100 μ g/ml).

Cell migration

Confluent monolayers of BME cells in 35 mm tissue culture dishes were wounded with a blade to mark the initial wound edge. Wounded cultures were washed twice with serum-free α -MEM, then serum-free α -MEM/0.1% gelatin and test drugs (bleomycin, mitomycin C, 2-methoxyestradiol (2-ME), taxol, colchicine, nocodazole, and, cytochalasin D) were added at the indicated concentrations (Figs. 1, 2). After 15 h, monolayers were stained with 0.1% crystal violet in 20% methanol for 30 min. Fields measuring 1.0 \times 1.4 mm were selected and further subdivided into smaller fields measuring 100 μ m each. The fields were then photographed under phase-contrast using a Nikon Diaphot TMD inverted photomicroscope (Nikon, Tokyo, Japan). The total number of cells which had crossed the original wound edge was determined. At least six wells were analyzed from each treatment per experiment. Results were from at least two independent experiments.

Cell growth

Cell growth studies were performed to assess the effects of test drugs on BME cell growth using a FACScan Analyzer. Briefly, BME cells were seeded into gelatine-coated 24 well-plates at a density of 10,000 cells/well. Cells were grown in α -MEM supplemented with 5% DCS. After 24 h, fresh medium was added together with test drugs (bleomycin, colchicine, 2-methoxyestradiol, and taxol). Control cells were exposed to drug vehicles. Two days later, medium and test drugs were renewed, and further after 2 days, cells were trypsinised and counted in a FACScan Analyzer (Becton–Dickinson, San José, CA). Results represent the mean \pm SEM. Three wells were analyzed for each concentration. Data shown are representative of least two independent experiments.

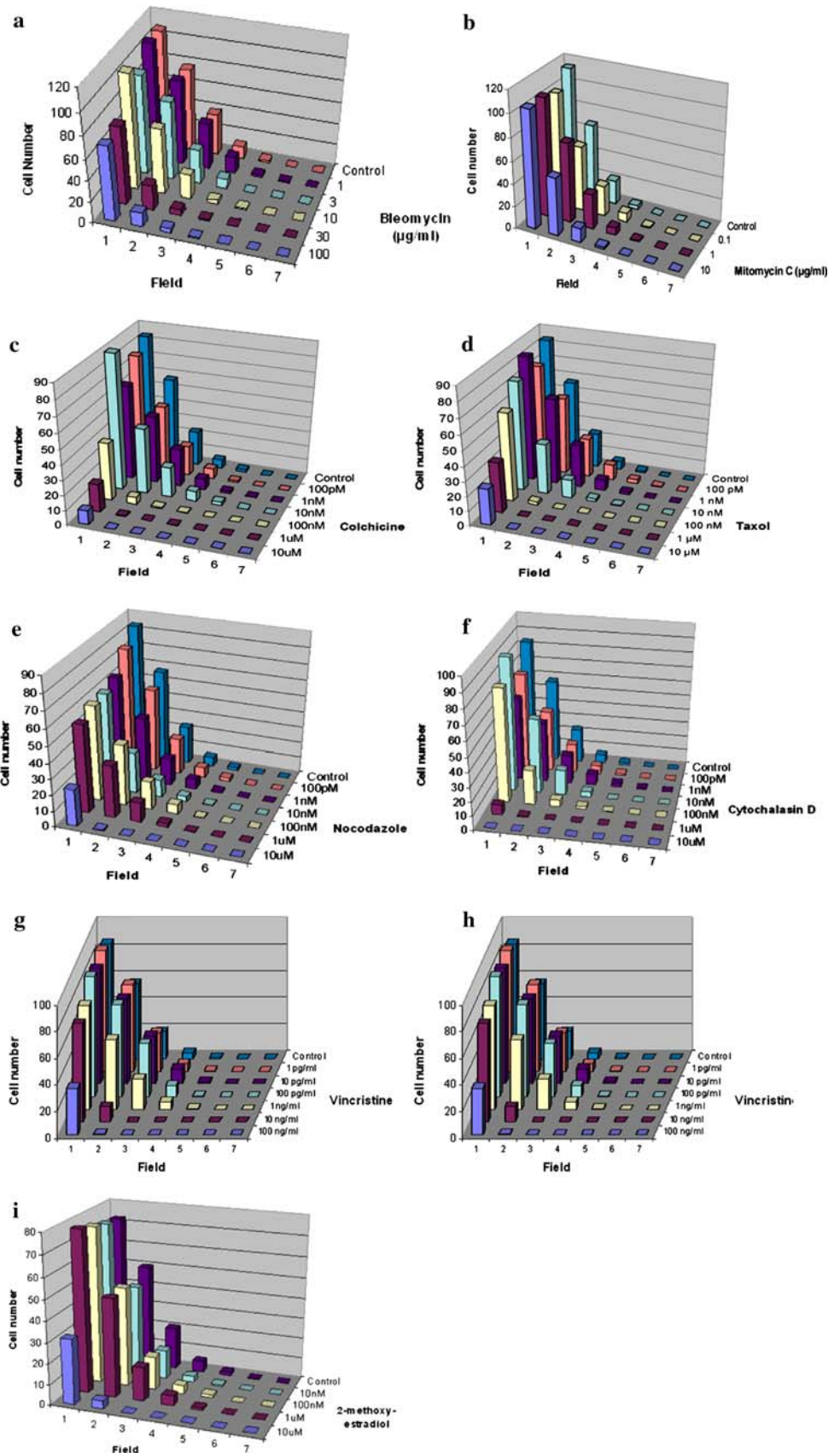
Analysis of DNA fragmentation

Confluent BME cell monolayers in 60 mm tissue culture dishes (1 \times 10⁶ cells/plate) were incubated with test drugs/vehicles for 48 h. At the end of the incubation, floating cells were recovered from culture media by centrifugation, and adherent cells were washed twice with PBS.

Floating and adherent cells were lysed together in 250 μ l/tissue culture dish of 20 mM Tris–HCl, pH 7.4, 5 mM EDTA, pH 7.4, 0.4% Triton X-100. Cell lysates were centrifuged at 13,000 rpm for 30 min at 4°C to pellet nuclei.

Supernatants were extracted with equal volumes of phenol–chloroform, precipitated with 0.5 volume of 7.5 M ammonium acetate, pH 7.5, and 3 volumes of ethanol, and then centrifuged at 13,000 rpm for 30 min at 4°C. Samples were left overnight at –20°C. Pellets were resuspended in

Fig. 1 Effects of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on BME cell migration. Confluent BME cell monolayers were wounded with a blade to mark the initial wound edge and washed. Cells were then treated with the indicated test substances (a–i). After 15 h, control and treated cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven sequential 100 μm -deep fields parallel to the original wound edge measuring 1.0×1.4 mm were delineated from the original normal edge. The total number of cells in each of the field was determined. At least six wells were analyzed from each treatment per experiment. Data shown is from at least two separate experiments per drug and concentration

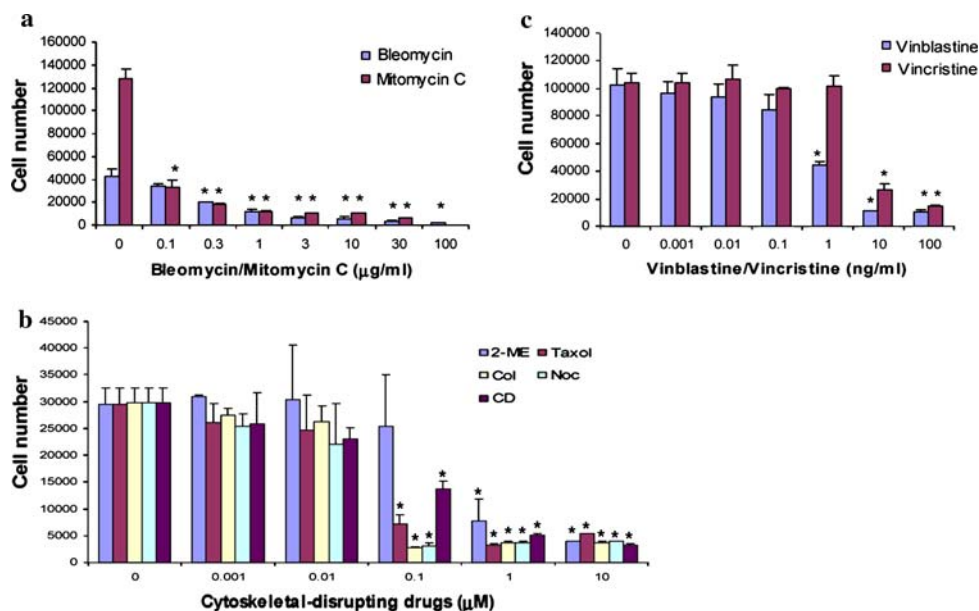


10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, containing DNase-free pancreatic ribonuclease (20 µg/ml), incubated at 37°C for 30 min and loaded onto 1.8% agarose gels containing ethidium bromide (1 µg/ml). Samples were electrophoresed at 10 V/cm for 2 h. DNA was visualized by UV fluorescence. Results were from at least two separate experiments.

Acridine-orange staining

Cells were seeded in 35 mm diameter wells at a density of 20,000 cells per well. After 24 h, BME cells were grown in the presence of the various drug preparations/vehicles for 48 h. Cells were harvested as follows: medium and floating cells were transferred to 15 ml tubes. The rest of the adherent cells were detached with 0.25% trypsin-1 mM EDTA. The floating and detached cells from the same sample were pooled together in 15 ml tubes, pelleted by centrifugation at 1,000 rpm for 5 min using a Beckman Model centrifuge, and cell pellets were washed with 1 ml PBS, and subsequently re-suspended in 25 µl PBS. Cells were then incubated with 10 µg/ml acridine orange for 5 min. Stained cell suspensions (10 µl) were placed on microscope slides and covered with coverslips. Cells were viewed and counted using a Zeiss Axiovert microscope (Carl Zeiss, Jena, Germany) at 400× magnification. Pictures were taken with a Nikon digital camera. Apoptosis was identified as nuclear chromatin condensation. Randomly selected microscopic fields were examined, and the percentage of cells undergoing apoptosis in each field was determined. Results were expressed as the mean percentage (±SD) of cells with evidence of apoptosis. Tests were done in triplicate.

Fig. 2 Effect of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on BME cell growth. Cells were seeded at a density of 10,000 cells per well in 24-well culture plates and exposed to test drugs indicated in **a–c** for 4 days. At termination, drug-treated and control cells were counted following trypsinization using a FACScan analyser. Three wells were analyzed for each concentration. Results represent the mean ± SEM. Data shown is from least two independent experiments per drug and concentration. * Significant difference between the number of control and treated cells: $P < 0.05$



Preparation of collagen gels

The in vitro angiogenesis assay was performed using BME cells as previously described by Montesano and Orci (1987) [16]. Type 1 collagen was solubilized by stirring adult rat tail tendons for 48 h at 4°C in sterile 1:1000 (vol/vol) acetic acid solution (300 ml for 1 mg of collagen). The resulting solution was filtered through sterile triple gauze and centrifuged at 16,000×g for 1 h at 4°C.

Eight volumes of the supernatant were mixed with 1 volume of 10× minimum essential medium and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile flask placed on ice, to prevent immediate gelation. The cold mixture was then dispensed into 18 mm tissue culture wells and allowed to gel for 10 min at 37°C.

In vitro angiogenesis assay

BME cells were seeded onto collagen gels at 1.0×10^5 cells/well in 500 µl α -MEM supplemented with 5% DCS, and were grown to confluence (3–4 days), at which point treatment with growth factors and/or drugs was begun. Medium and treatments were renewed every second day. After 6 days, cultures were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4). Fixed cultures were photographed for quantification of invasion.

Quantification of invasion

Three randomly selected fields measuring 1.0×1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy.

Table 1 Effects of test drugs on endothelial cell growth

Drug	Cell type	IC ₅₀
Bleomycin	BME cells	0.105 μM
Bleomycin + bFGF		0.053 μM
Mitomycin C	BME cells	0.149 μM
Mitomycin C + bFGF		0.180 μM
2-Methoxyestradiol	BME cells	0.398 μM
2-Methoxyestradiol + bFGF		0.018 μM
Taxol	BME cells	0.040 μM
Taxol + bFGF		0.063 μM
Vincristine	BME cells	0.004 μM
Vincristine + bFGF		0.004 μM
Vinblastine	BME cells	0.690 nM
Vinblastine + bFGF		0.900 nM
Colchicine	BME cells	0.032 μM
Colchicine + bFGF		0.017 μM
Nocodazole	BME cells	0.031 μM
Nocodazole + bFGF		0.022 μM
Cytochalasin D	BME cells	0.063 μM
Cytochalasin D + bFGF		ND

Results are expressed as IC₅₀ values or the concentration of the respective substance, which resulted in half the numbers of cells being present at the end of the experiment that were obtained in the control. Data is representative of at least two independent experiments per drug and concentration

ND Not determined

bFGF basic fibroblast growth factor

Invasion was quantitated by determining the total additive sprout length in μm ± SEM, and the results were from at least two separate experiments.

Statistical analysis

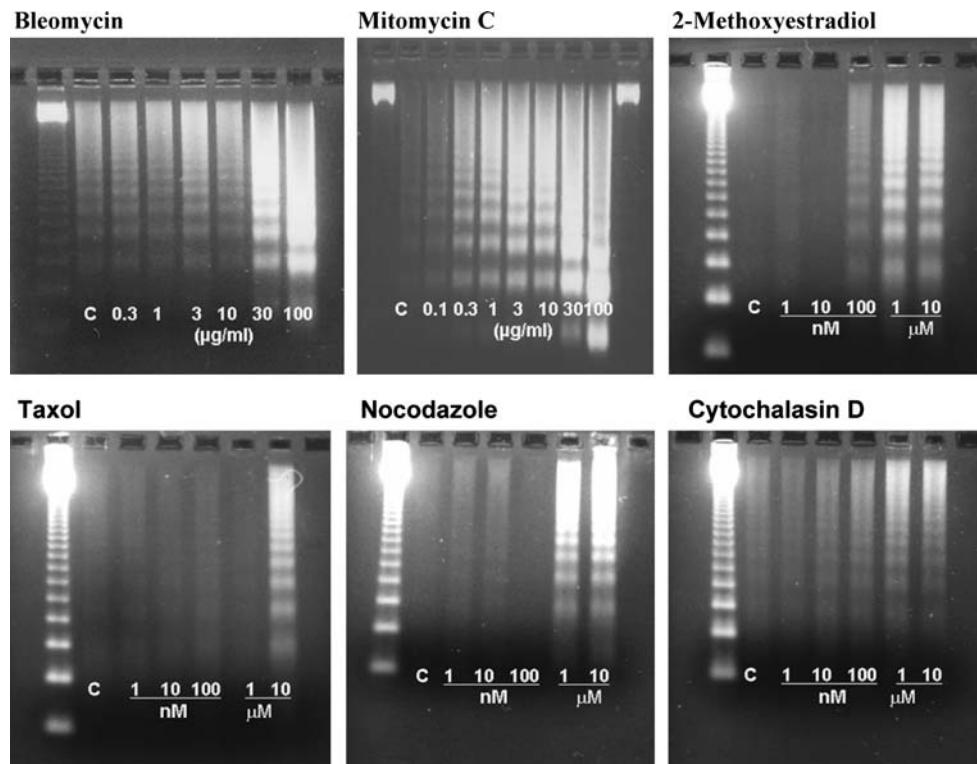
Statistics were performed using 1-way ANOVA, and where statistical significance was reached, pair-wise multiple comparisons were made using an ad hoc test (Bonferroni’s method). When data were not normally distributed, ANOVA on ranks was performed, followed by pair-wise comparisons, again employing the Bonferroni approach. Significance was designated as *P* < 0.05.

Results

Effects on endothelial cell migration

Bleomycin inhibited BME cell migration at a dose of 100 μg/ml, while no significant inhibition of endothelial cell migration occurred following exposure of cells to mitomycin C (Fig. 1a, b). Inhibition of endothelial cell migration following exposure to colchicine occurred at a dose of 1 μM (Fig. 1c) while taxol inhibited cell migration at 0.1 μM (Fig. 1d). Nocodazole, cytochalasin D, vinblastine, vincristine and 2-ME inhibited BME cell migration in a dose-dependent manner (Fig. 1e–i). Both vinblastine and

Fig. 3 Analysis of internucleosomal fragmentation in DNA preparations following exposure to bleomycin, mitomycin C and cytoskeletal-disrupting drugs for 48 h. Representative experiments are shown



vincristine had a more profound effect by inhibiting cell migration at doses of 0.011 and 0.001 μM , respectively. The doses at which test drugs induced significant inhibition of endothelial cell migration are summarized in Table 4.

Effects on endothelial cell “growth”

Cell “growth”, as determined in the assays performed, measures the absolute number of cells after a given time period under defined conditions. It does not distinguish between cell proliferation and cell death, but is the net result of the two. It is therefore a crude test aimed at determining whether the test substance affects the cells in question, and the cause for any variation needs to be determined in greater detail.

Bleomycin and mitomycin C inhibited BME cell growth (Fig. 2a), with IC_{50} values of 0.16 $\mu\text{g/ml}$ (0.11 μM) and 0.05 $\mu\text{g/ml}$ (0.15 μM), respectively, (Table 1). Bleomycin also inhibited bFGF-induced BME cell growth, with the inhibitory effect being more pronounced at 100 $\mu\text{g/ml}$. The level of 2-ME-induced cell growth inhibition was less than that seen with taxol (Fig. 2b). A maximum inhibitory effect following exposure to 2-ME occurred at a dose of 10 μM , while taxol exerted a maximum inhibitory effect on endothelial cell growth at a dose of 1 μM . Colchicine induced a dose-dependent inhibition of BME cell growth with an IC_{50} of 0.032 μM (Table 1). The effects of colchicine on BME cell growth were mimicked by nocodazole (IC_{50} 0.031 μM). Cytochalasin D induced a significant decrease in cell numbers at doses of 1 μM and above (Fig. 2b).

Vinblastine and vincristine inhibited growth of endothelial cells in a dose-dependent manner (Fig. 2c); however, vinblastine was more potent as shown by the IC_{50} values of 0.004 μM and 0.690 nM, respectively, (Table 1).

Effects on endothelial cell apoptosis

DNA isolated from untreated BME cells was compared with DNA from cells exposed to varying concentrations of the different drugs. Bleomycin induced DNA strand breaks in a dose-dependent manner (Fig. 3). The intensity of the ladders increased with increasing drug concentrations, with the most intense ladders observed following exposure to 30 and 100 $\mu\text{g/ml}$ bleomycin. 2-ME, taxol, Colchicine, nocodazole and cytochalasin D also induced DNA strand breaks (Fig. 3). In 2-ME treated cells, internucleosomal DNA fragmentation was detected only after exposure to the highest dose (10 μM), while colchicine and taxol induced DNA strand breaks at doses of only 100 nM.

The percentage of acridine orange labeled-BME cells was increased in cultures exposed to the various test drugs (Table 2), indicating that they induced apoptosis.

Table 2 Induction of apoptosis in BME cells

	% Acridine orange-labeled cells
Control	5.92 \pm 4.28
Bleomycin	
0.1 $\mu\text{g/ml}$	11.26 \pm 9.62
10 $\mu\text{g/ml}$	21.03 \pm 11.61*
100 $\mu\text{g/ml}$	41.72 \pm 16.35*
Mitomycin	
0.1 $\mu\text{g/ml}$	22.55 \pm 18.60*
1 $\mu\text{g/ml}$	33.76 \pm 18.21*
10 $\mu\text{g/ml}$	58.10 \pm 20.42*
Colchicine	
0.01 μM	6.21 \pm 4.02
1 μM	33.52 \pm 11.26*
10 μM	31.05 \pm 9.65*
Nocodazole	
0.01 μM	12.53 \pm 9.36
1 μM	43.87 \pm 9.75*
10 μM	40.25 \pm 8.15*
Taxol	
0.01 μM	17.82 \pm 6.22*
1 μM	45.37 \pm 10.80*
10 μM	44.00 \pm 8.01*
2-ME	
0.01 μM	18.57 \pm 8.05*
1 μM	32.81 \pm 12.50*
10 μM	52.21 \pm 10.02*
Vincristine	
0.001 ng/ml	5.92 \pm 6.10
0.1 ng/ml	5.87 \pm 4.25
10 ng/ml	27.26 \pm 11.20*
Vinblastine	
0.001 ng/ml	6.04 \pm 8.22
0.1 ng/ml	12.01 \pm 8.75
10 ng/ml	37.20 \pm 11.26*

Confluent monolayers of BME cells were exposed to the agents indicated above, and apoptosis (acridine orange labeling) was determined after 48 h. Values are the mean \pm SD. Results are pooled from at least three separate experiments per drug and concentration. An average of 12 fields was analyzed per treatment dose in each experiment. * Significant difference between control and drug-treated cells: $P < 0.05$

Effects on in vitro angiogenesis

Control untreated BME cells formed a monolayer on the surface of 3-dimensional collagen gels (Fig. 4a) with a cobblestone appearance, while BME cells treated with a combination of 10 ng/ml bFGF and 30 ng/ml VEGF invaded the underlying gel and organized into branching cords inside the collagen matrix (Fig. 4b); few such cords were observed in cultures treated with bleomycin alone or

with a combination of bFGF/VEGF and bleomycin (Fig. 4c–d).

There were no endothelial cell cords in cultures exposed to colchicines or 2-ME or only (Fig. 4e, g); however, colchicine- and bleomycin-treated cells appeared similar in morphology to control cells, while 2-ME-treated cells appeared polygonal in morphology (e). Quantitative analysis of in vitro tube formation showed that bleomycin, mitomycin C, colchicine, 2-ME, nocodazole, cytochalasin D and taxol inhibited bFGF-induced BME cell cord formation (Fig. 5a–d; Tables 3, 4).

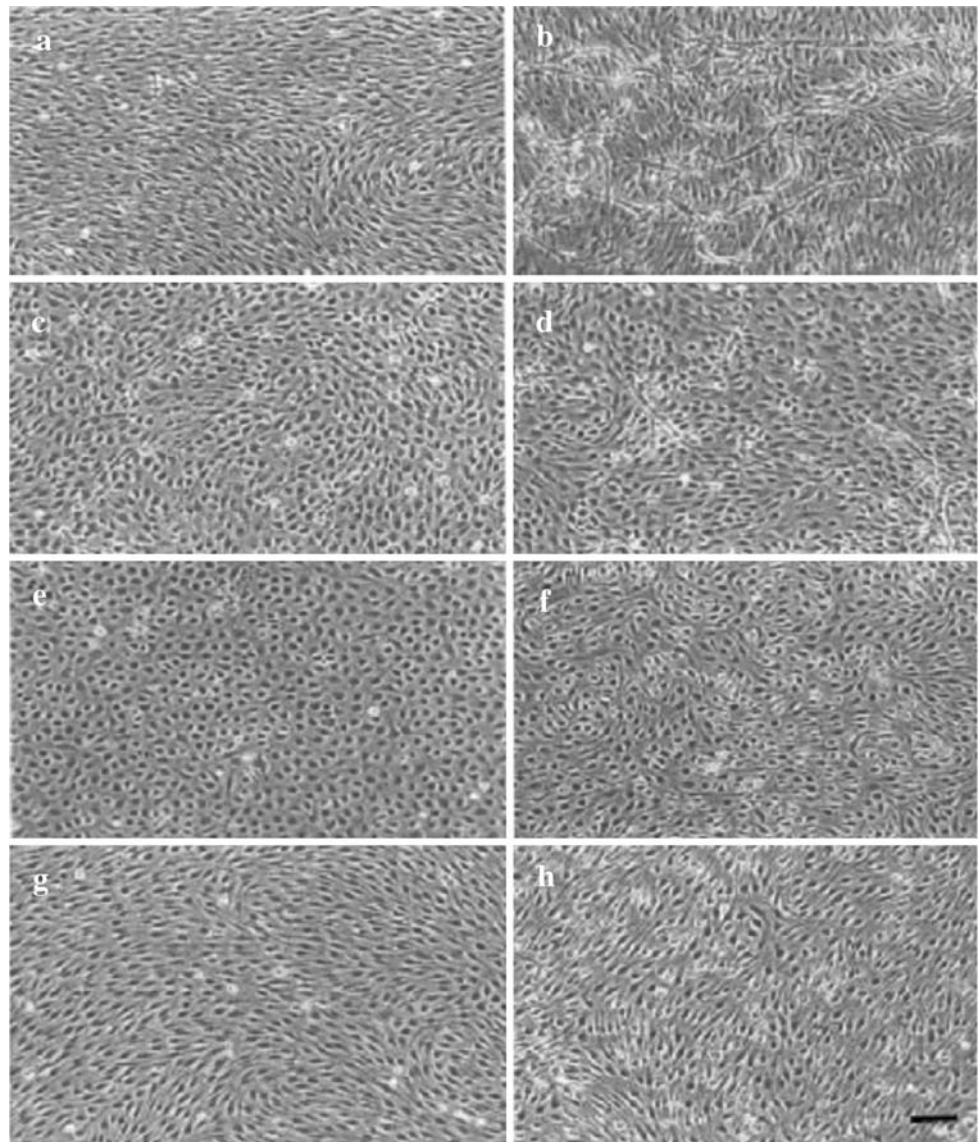
Discussion

Herein we describe studies undertaken to compare the anti-angiogenic effects of DNA-damaging drugs and agents that

affect cytoskeletal dynamics. Our observations revealed that bleomycin, a DNA-damaging chemotherapeutic drug, as well as cytoskeletal-disrupting agents (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D) inhibited BME cell migration. However, the inhibitory effects of cytoskeletal-disrupting drugs on endothelial cell migration were more pronounced. In addition to endothelial cell migration, angiogenesis is characterized by endothelial cell growth, sometimes uncontrolled, as well as capillary-like tube formation. Therefore further studies on the anti-angiogenic effects of test drugs were evaluated using assays of cell growth and in vitro tube formation.

Cell “growth” studies revealed a dose-dependent reduction in the percentage of viable BME cells in bleomycin- and mitomycin C-treated cultures. Cytoskeletal-disrupting drugs, with the exception of 2-ME, caused a

Fig. 4 Effects of bleomycin, colchicine, 2 methoxyestradiol (2ME) and Taxol on BME cells grown on collagen gel. Cells seeded on top of type I collagen gel were exposed to drugs in the presence or absence of bFGF for 6 days. Medium and treatments were renewed every second day. At termination, cultures were fixed with 2.5% glutaraldehyde; three randomly selected fields measuring 1.0×1.4 mm were then photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy. Results are from at least two separate experiments per drug and concentration. Bar = 100 μ m



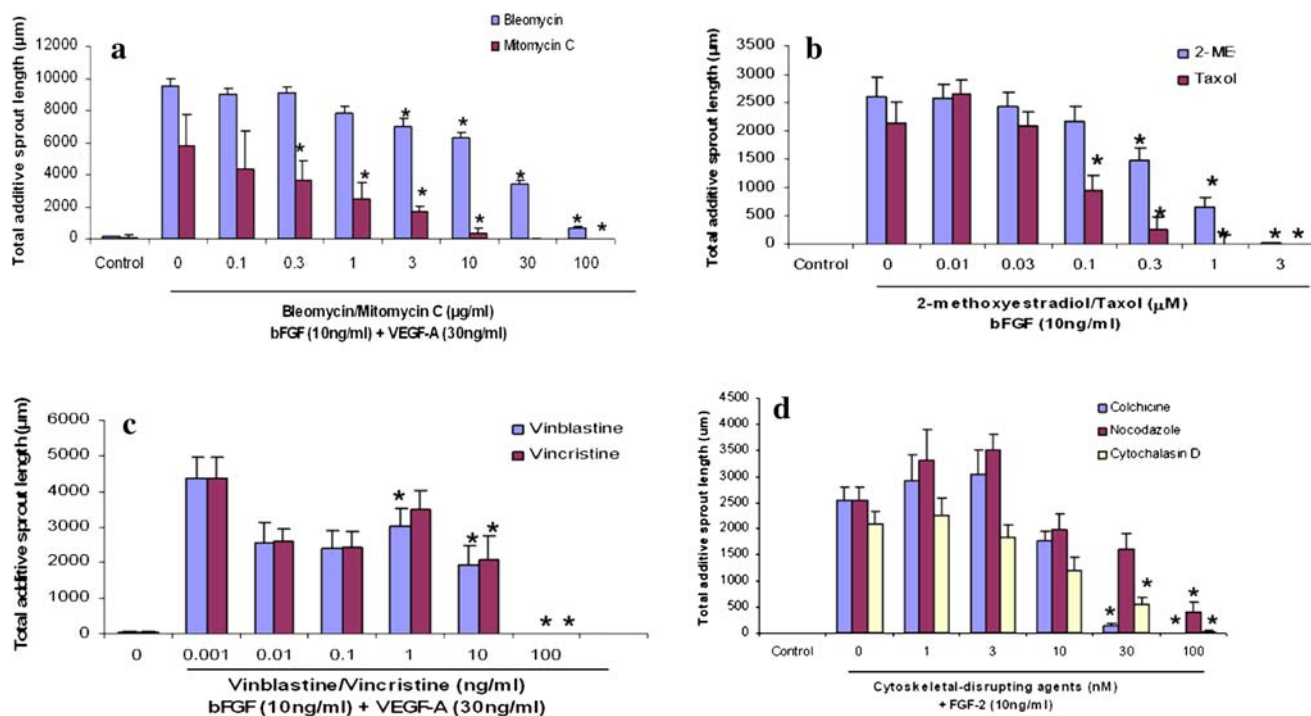


Fig. 5 Quantitative analysis of the effects of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on in vitro angiogenesis. BME cells were seeded on collagen gel in the presence of bFGF alone or bFGF and test drugs. After 6 days, invasion was quantitated by determining the total additive sprout length in $\mu\text{m} \pm \text{SEM}$, and

results are from at least two separate experiments. Two wells were analyzed for each concentration. Data shown is from at least two independent experiments per drug and concentration. *Significant difference in total additive sprout length between untreated (0 $\mu\text{g/ml}$) and drug-treated cells: $P < 0.05$

Table 3 Quantitative analysis of the effects of test drugs on in vitro angiogenesis

Drug	Cell type	IC ₅₀
Bleomycin	BME cells	11.850 μM
Mitomycin C	BME cells	2.340 μM
2-Methoxyestradiol	BME cells	0.400 μM
Taxol	BME cells	0.010 μM
Vincristine	BME cells	0.860 nM
Vinblastine	BME cells	0.870 nM
Colchicine	BME cells	0.016 μM
Nocodazole	BME cells	0.017 μM
Cytochalasin D	BME cells	0.017 μM

Results are presented as IC₅₀ values, the drug concentration at which 50% inhibition of capillary-like tube formation was observed. Data shown is from at least two independent experiments per drug and concentration

reduction in BME cell numbers in a dose-dependent manner, and with greater potency than bleomycin and mitomycin C. Bleomycin, 2-ME, colchicine and nocodazole induced a more pronounced reduction in cell numbers in bFGF-treated BME cells than when added alone. Therefore, cytokine stimulated BME cells appear to be more susceptible to the growth inhibitory effects of these

drugs. Previous reports suggest that bFGF may have a role in the development of various tumors, including hemangiomas of infancy [5].

Bleomycin has recently been employed in the treatment of juvenile hemangiomas, with promising results [17–19]. Initially, the effectiveness of bleomycin in inducing hemangioma regression was attributed to the sclerosing effect of the drug on the vascular endothelium [18]. More recently however, it was shown that bleomycin prevents human hemangioma growth in vitro by inhibiting neovessel growth [20]. Bleomycin's antiproliferative effects on growth factor-stimulated endothelial cells could be partly responsible for its ability to inhibit hemangioma growth in patients.

Also, the ability of 2-ME, colchicine and nocodazole to inhibit endothelial cell growth in the presence of this potent angiogenic growth factor may be translated in the clinical setting to enable effective growth inhibition in juvenile hemangiomas.

To determine the mode by which these test drugs decreased cell numbers, their effect on apoptosis was assessed. When DNA extracted from BME cells treated with bleomycin, mitomycin, 2-methoxyestradiol, taxol, colchicine, nocodazole, or cytochalasin D was analyzed using gel electrophoresis, characteristic internucleosomal "ladders" of DNA fragments were found. These ladders

Table 4 A summary of the effects of test drugs on the different parameters measured

Drug	Cell growth (IC ₅₀)	Cell migration ^a	Apoptosis ^b	In vitro angiogenesis (IC ₅₀)
Bleomycin	0.105 μ M	66.7 μ M	6.677 μ M	11.850 μ M
Mitomycin C	0.149 μ M	NI	2.990 μ M	2.340 μ M
2-Methoxyestradiol	0.398 μ M	10.0 μ M	0.010 μ M	0.400 μ M
Taxol	0.040 μ M	0.100 μ M	0.010 μ M	0.010 μ M
Vincristine	0.004 μ M	0.001 μ M	0.011 μ M	0.860 nM
Vinblastine	0.690 nM	0.011 μ M	0.011 μ M	0.870 nM
Colchicine	0.032 μ M	0.100 μ M	1.000 μ M	0.016 μ M
Nocodazole	0.031 μ M	10.0 μ M	1.000 μ M	0.017 μ M
Cytochalasin D	0.063 μ M	0.100 μ M	ND	0.017 μ M

^a Dose at which significant inhibition of cell migration was observed

^b Dose at which a significant number of BME cells were induced to undergo apoptosis

NI No inhibition of cell migration was observed at tested doses

ND Not determined

were not observed in control samples. Results from cultures that were exposed to vincristine and vinblastine were inconclusive. Internucleosomal DNA fragmentation is a biochemical indicator of apoptosis [21, 22].

In addition to DNA fragmentation, chromatin condensation is an early and relatively unequivocal hallmark of apoptosis [22]. Therefore, drug-induced apoptosis was further evaluated using the acridine orange stain, which assesses chromatin condensation. We found that the test drugs, including vinca alkaloids (vincristine and vinblastine), increased acridine orange staining of BME cell nuclei, indicating that the drugs induced BME cell apoptosis. Inhibition of apoptosis (and the promotion of endothelial cell survival) is important in the process of angiogenesis. Several anti-angiogenic agents such as angiostatin and endostatin have been reported to inhibit angiogenesis in part by inducing endothelial cell apoptosis [8, 9]. Results from this study further indicate that these drugs have apoptotic activity which is likely to contribute to their anti-angiogenic effect.

For in vitro angiogenesis studies, bovine microvascular endothelial (BME) cells were cultured on the surface of 3-dimensional type 1 collagen gels. Under control conditions, BME cells remain on the surface of the gels, forming a confluent monolayer. Growth factors (bFGF alone or bFGF + VEGF) induced BME cells to invade the underlying collagen matrix, within which they form networks of capillary-like tubular structures, a phenomenon that closely mimics the angiogenic process occurring in vivo. In previous studies, it was shown that bFGF and VEGF induce BME cell invasion and tube formation in a collagen gel invasion assay [23–25].

Our findings indicate that cytoskeletal-disrupting agents tested (taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D), inhibit angiogenesis in vitro with more potency than the DNA-damaging drugs, with the exception of 2-ME. The vinca alkaloids, vincristine and vinblastine, were the most potent inhibitors of angiogenesis, followed by taxol.

Taxol has been reported to exhibit anti-angiogenic activity in vitro at doses that are well below the maximum tolerated dose. In addition, vincristine, vinblastine, colchicine and nocodazole were shown in this study to inhibit angiogenesis at noncytotoxic doses.

In general, the anti-proliferative effects induced by most chemotherapeutic agents are associated with cell cycle arrest and induction of apoptosis. Interestingly, vincristine, vinblastine, colchicine and nocodazole induced an inhibition of cell growth at doses which did not result in significant apoptosis. At these cytostatic doses, the drugs inhibited angiogenesis in vitro.

A few case studies have reported on the use of vincristine to treat hemangiomas, although its mechanism of action has not been reported. It seems plausible that the positive results observed in hemangioma patients following treatment with vincristine may be due in part to its potent anti-angiogenic activity.

Furthermore, vinblastine, colchicine, nocodazole as well as taxol may have potential in the treatment of juvenile hemangiomas, as they are more potent at inhibiting angiogenesis than bleomycin. Further studies will focus on the effects of combinations of these drugs on angiogenesis in vitro and on hemangioma development in experimental models in vivo.

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