Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly

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Many conventional chemotherapeutics, such as the microtubule-stabilizing anticancer drug paclitaxel (Taxol), have been shown to have anti-angiogenic activity and clinical application of a continuous low dose of these agents has been suggested for cancer therapy. In this study, we show that paclitaxel selectively inhibits the proliferation of human endothelial cells (ECs) at ultra low concentrations (0.1–100 pM), with an $IC_{50} = 0.1$ pM, while it inhibits non-endothelial type human cells at 10^4- to 10^5-fold higher concentrations, with $IC_{50} = 1–10$ nM. The selectivity of paclitaxel inhibition of cell proliferation is also species specific, as mouse ECs are not sensitive to paclitaxel at ultra low concentrations. They are inhibited by higher concentrations of paclitaxel with $IC_{50} = 1–10$ nM. Inhibition of human ECs by paclitaxel at ultra low concentrations does not affect the cellular microtubule structure, and the treated cells do not show G_2/M cell cycle arrest and apoptosis, suggesting a novel but as yet unidentified mechanism of action. In an *in vitro* angiogenesis assay, paclitaxel at ultra low concentrations blocks human ECs from forming sprouts and tubes in the three-dimensional fibrin matrix. In summary, paclitaxel selectively inhibits human EC proliferation and *in vitro* angiogenesis at low picomolar concentrations. The data support a clinical application of continuous ultra-low-dose paclitaxel to treat cancer. *Anti-Cancer Drugs* 14:13–19 © 2003 Lippincott Williams & Wilkins.

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Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, is an essential component of a variety of pathological states including tumor growth, diabetic retinopathy, macular degeneration, arthritis and inflammation [1,2]. Proliferation of normally quiescent endothelial cells (ECs) is one of the critical steps required for angiogenesis. Inhibition of endothelial proliferation has been shown to be effective in blocking angiogenesis and tumor growth in animal models [3,4]. Anticancer chemotherapeutic agents, while targeting cancer cells directly, have inhibitory effects on vascular ECs, which may have contributed to the anti-cancer efficacy of these agents [5]. In fact, anti-endothelial effects have been demonstrated for vinblastine, cyclophosphamide, 5-flurouracil (5-FU), paclitaxel, doxorubicin and others [6,7]. Furthermore, Browder *et al.* has reported that anti-angiogenic scheduling of chemotherapy improved efficacy against drug-resistant tumors in animal models [8]. The anti-angiogenic-dosing schedule was designed to administer chemotherapeutics at shorter intervals without interruption, to prevent recovery of ECs and, therefore, to achieve more effective suppression of the proliferating ECs in the tumor bed. Klement *et al.* showed that continuous low-dose vinblastine administration caused a direct anti-angiogenic effect *in vivo*, and that combination treatment of low-dose vinblastine with VEGF receptor-2 antibody resulted in full and sustained regressions of large established tumors without an ensuing increase in host toxicity or drug resistance [9]. These studies have demonstrated the potential usefulness of EC growth inhibitors in cancer treatment.

In an effort to identify more potent EC inhibitors, we tested many conventional chemotherapeutics in a proliferation assay of human microvascular ECs. Paclitaxel showed selective inhibitory activity in human ECs at ultra low concentrations (0.1–100 pM). We further show that human ECs are more sensitive than mouse ECs and that the mechanism of this activity of ultra low concentrations of paclitaxel is not through disrupting cellular microtubules. The data support the use of ultra low paclitaxel in treating cancers in the clinic.

Materials and methods

Paclitaxel was purchased from Sigma (St Louis, MO), and was dissolved in DMSO at 10 mM and stored frozen at −20°C in aliquots. Other reagents were commercially obtained in the highest quality available.

Cell culture and proliferation assay

All normal human primary cells and their recommended culture media were purchased from Clonetics (San Diego, CA) as described earlier [10]. These cells included

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human neonatal dermal microvascular ECs (HMVECs), human umbilical vein ECs (HUVECs), human umbilical artery ECs (HUAVECs), normal human astrocytes (NHAs), normal human dermal fibroblasts (NHDFs), normal human epidermal keratinocytes (NHEKs), human mammary epithelial cells (HMEpCs), human prostate epithelial cells (PrEpCs) and human umbilical artery smooth muscle cells (UASMCs). Cells were grown according to instructions from Clonetics, and cell proliferations were performed in 96-well plates using cells between passages 6 and 12. Cells were seeded at 3000–5000 cells/well and allowed to attach for 4 h. Paclitaxel and other agents, diluted in culture medium, were added in quadruplicate wells and the cells were incubated for 3 days before MTS reagents (Promega, Madison, WI) were added to quantitate the live cells in each well.

Transformed HUVECs were prepared in order to extend the *in vitro* proliferative lifespan of primary HUVECs (Clonetics); the cells were transformed using SV40 Tag [11]. Briefly, the HUVECs were infected (m.o.i. = 1) with a recombinant, defective retrovirus transducing the SV40 TAg and *hygB* genes. Clonal cell lines were obtained from the hygromycin-resistant cell populations. Flow cytometric analysis indicated that tumor necrosis factor-α-induced expression of E-selectin, ICAM-1 and VCAM was similar in both the primary and Tag-transformed HUVECs.

Primary mouse brain microvascular ECs (MBMECs) and growth medium were purchased from Cell Applications (San Diego, CA). A mouse EC line derived from brain microvasculature (BEND3) was kindly provided by W. Risau [12]. SVEC4-10, a mouse EC line derived by SV40 transformation of ECs from auxiliary lymph node vessels, was purchased from ATCC (Rockville, MD). Both BEND3 and SVC4-10 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Proliferation assays were performed in a similar fashion as described above.

Cell cycle analysis by flow cytometry

HMVECs were grown in EGM2 media (containing growth factors VEGF, FGF, EGF and IGF, and 5% FBS) in T75 culture flasks at 50–60% confluency when paclitaxel was added for final concentration of 0 or 0.1 pM to 10 μM. The cells were incubated at 37°C and 5% CO_2 for 48 h. Cell morphology was recorded, and then cells were detached by trypsinization and stained with propidium iodide for flow cytometric analysis. Briefly, cells were washed with PBS twice and fixed with 70% ethanol. Cell pellets were then resuspended in PBS containing 0.6% NP-40, 10 mg/ml RNase and 0.1 mg/ml propidium iodide. The cells were analyzed on a FACS-Calibur instrument (Becton Dickson, San Jose, CA).

Cellular microtubule staining

HMVECs were grown on eight-well chamber slides at 20 000 cells/well in EGM2 media. The cells were incubated with 0 or 0.1 pM to 1 μM paclitaxel for 48 h. The cells were fixed with 10% formalin and stained with a rat anti-tubulin IgG followed by FITC-conjugated goat anti-rat antibody (Chemicon, Temecula, CA). Cellular microtubule structures were viewed in a confocal fluorescence microscope and digital images were taken.

EC tube formation in the fibrin matrix

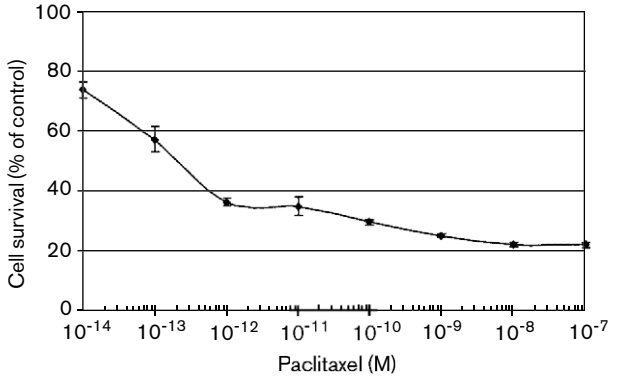
This protocol was adapted from published methods [13]. HMVECs were mixed with gelatin-coated Cytodex microcarrier beads (Sigma) at 1×10^6 cells/ml and 30% (v/v) beads in EGM2 media (containing growth factors supplements and 5% FBS). The cells/beads was incubated at 37°C/5% CO_2 for 4 h with gentle mixing every 30 min.Then, 10-fold more EGM2 was added and the mix was incubated overnight at 37°C/5% CO_2. Cells were normally at confluency on the bead surface after the incubation. The cells/beads were resuspended in fresh EGM2 media at 1% (v/v) and mixed with an equal volume of 6 mg/ml human fibrinogen (Sigma) in EBM2 basic medium. Human thrombin (Sigma) was then added to give a final concentration of 0.05 U/ml and the mixture was dispersed to a 24-well plate, 1 ml/well. The fibrin gel formed within 4 min at room temperature and 1 ml of EGM2 media with 0 or 0.1 pM to 10 μM paclitaxel was then added into the wells with HMVECs/beads embedded in the fibrin matrix. The plate was incubated at 37°C/5% CO_2 for 72 h, and tube formation was checked under phase-contrast microscopy and digital images were taken. Similar experiments were carried out with BEND3 cells.

Results

Paclitaxel inhibits EC proliferation at ultra low concentrations

Several widely used cancer chemotherapeutics were tested for their effects on human EC proliferation, among which paclitaxel showed exceptional potency against the growth of HMVECs. Paclitaxel showed an inhibitory effect on HMVEC proliferation at ultra low concentrations (0.1–100 pM) with an $IC_{50} = 0.1$ pM (Fig. 1 and Table 1). 5-FU, camptothecin, cisplatin and doxorubicin were active against HMVEC proliferation, but their effect is at higher concentrations in the nanomolar range (Table 1), not drastically different from their activity against other cells as reported [14]. The anti-proliferative activity of paclitaxel at ultra low concentrations on HMVECs prompted us to study its activity against primary cultures of a variety of cell types.

Fig. 1



Inhibition of HMVEC proliferation by paclitaxel. HMVECs grown in 96-well plates with complete growth medium EGM2 containing FBS and growth factors were exposed to paclitaxel at various concentrations as shown. The cells were allowed to grow for 3 days in the presence of the drug. Total live cells in each well at the end of incubation were quantified with MTS reagents. Data shown are from one typical experiment which were confirmed by two independent tests. Paclitaxel showed potent inhibition of HMVEC proliferation even at ultra low concentrations (0.1–100 pM).

Table 1 **Inhibition of HMVEC proliferation by chemotherapeutics**

Compounds	IC_{50} (pM)
Paclitaxel	0.1
5-FU	5000
Camptothecin	10000
Cisplatin	5000000
Doxorubicin	100000

HMVEC were grown in a 96-well plate with EGM2 medium containing growth factors and FBS. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

Cell growth inhibition by paclitaxel at ultra low concentrations is selective for EC type

Human ECs of other origins and non-EC types of primary cells were tested against paclitaxel in 3-day proliferation assays. The IC_{50} of paclitaxel inhibition of growth of these cells is listed in Table 2. All human ECs, including HMVECs, HUVECs and HUAECs, were inhibited by ultra low concentrations of paclitaxel with an IC_{50} of 0.1 pM. Transformed HUVECs were similarly sensitive to the ultra low paclitaxel. However, non-EC types of human cells, including NHAs, NHDFs, NHEKs, HMEpCs, PrEpCs and UASMCs, were all significantly less sensitive to the inhibition of paclitaxel. The IC_{50} values for these cells were near 1 nM, which was in line with the anti-mitotic activity of paclitaxel. It was striking that proliferation of non-EC cell types was 6000- to 55 000-fold less sensitive to paclitaxel compared to human EC types tested. Selective inhibition of EC proliferation is one of the approaches to inhibit angiogenesis. The high potency and selectivity of paclitaxel against ECs makes it an exceptional agent for anti-angiogenic therapy.

Table 2 **Inhibition of proliferation of human primary cells by pacilitaxel**

Cell types	IC_{50} (pM)
HMVEC	0.1
HUVEC	0.1
HUAEC	0.1
NHA (astrocytes)	5500
NHDF (fibroblasts)	700
MepC (mammary epethelial cells)	1000
PrEpC (prostate epithelial cells)	650
UASMC (smooth muscle cells)	1000

Cells were grown and proliferations were performed as described in Materials and methods. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

Table 3 **Inhibition of proliferation of EC of various species by pacilitaxel**

EC types	IC_{50} (pM)
Human	
HMVEC	0.1
HUVEC	0.1
HUAEC	0.1
HUVEC (transformed)	0.1
Mouse	
BEND3	1000
SVEC4-10	10000
MBMEC	300

Cells were grown and proliferations were performed as described in Materials and methods. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

Mouse ECs show responses only to higher concentrations of paclitaxel

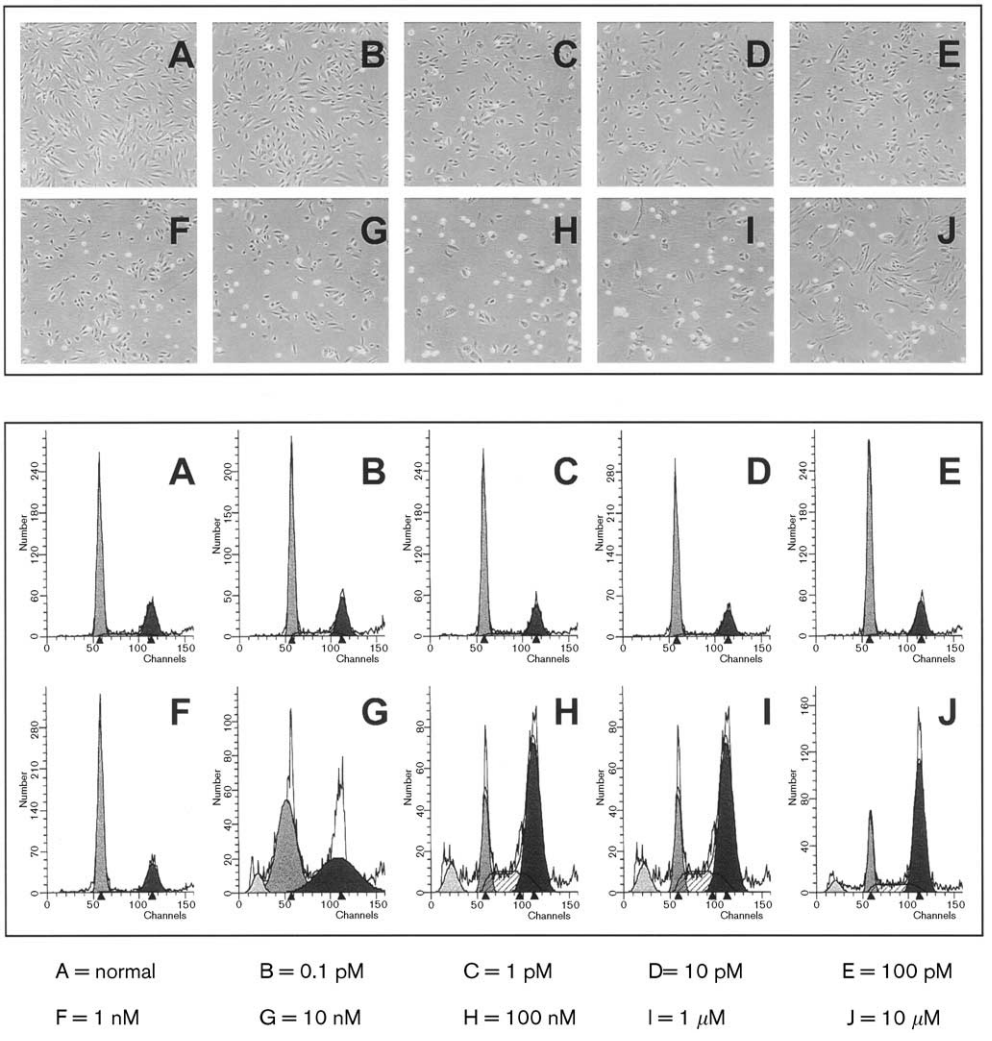
It is widely appreciated that ECs from different species have significant differences in response to angiogenesis inhibitors. We were interested in the sensitivity of mouse ECs to paclitaxel as anti-angiogenic agents would be tested in mouse models before going to the clinic. Three sources of mouse ECs were tested—BEND3 was an immortalized mouse brain capillary EC line [12], SVEC4-10 was an EC line derived by SV40 transformation of ECs from mouse auxiliary lymph node vessels and MBMECs were primary mouse brain microvascular ECs derived from normal mouse brain tissues. Proliferation of these mouse ECs was inhibited by paclitaxel at higher concentrations (Table 3), sensitivity being much lower as compared to that of human ECs. The primary mouse ECs (MBMECs) were inhibited with $IC_{50} = 0.3$ nM, similar to that for human non-EC primary cells. The immortalized mouse ECs, BEND3 and SVEC4-10, were even less responsive to the inhibition of paclitaxel. The lack of sensitivity of mouse ECs to ultra low concentrations of paclitaxel makes it less attractive to test the anti-angiogenic activity of ultra low paclitaxel in mouse angiogenesis and tumor models.

Paclitaxel at ultra low concentrations does not arrest HMVECs at the G_2/M phase of the cell cycle and does not affect cellular microtubules

To explore the mechanism of HMVEC growth inhibition by paclitaxel at ultra low concentrations, we studied cell morphology, cell cycle progression and cellular tubulin of HMVECs treated with paclitaxel. The cells treated with 0.1–1000 pM paclitaxel showed reduced cell density, consistent with the data as in the MTS quantification in Fig. 1. The cell morphology did not appear different from that of untreated cells (Fig. 2, upper panel). Flow cytometry analysis of cell cycle distribution of HMVECs treated with paclitaxel showed G_1 arrest of cells exposed to ultra low drug concentrations (Fig. 2, lower panel). In contrast, paclitaxel at higher concentrations (above 1 nM) caused the cells to detach from the culture plate, and induced cell cycle arrest at the G_2/M phase and apoptosis, consequences of paclitaxel affecting microtubules.

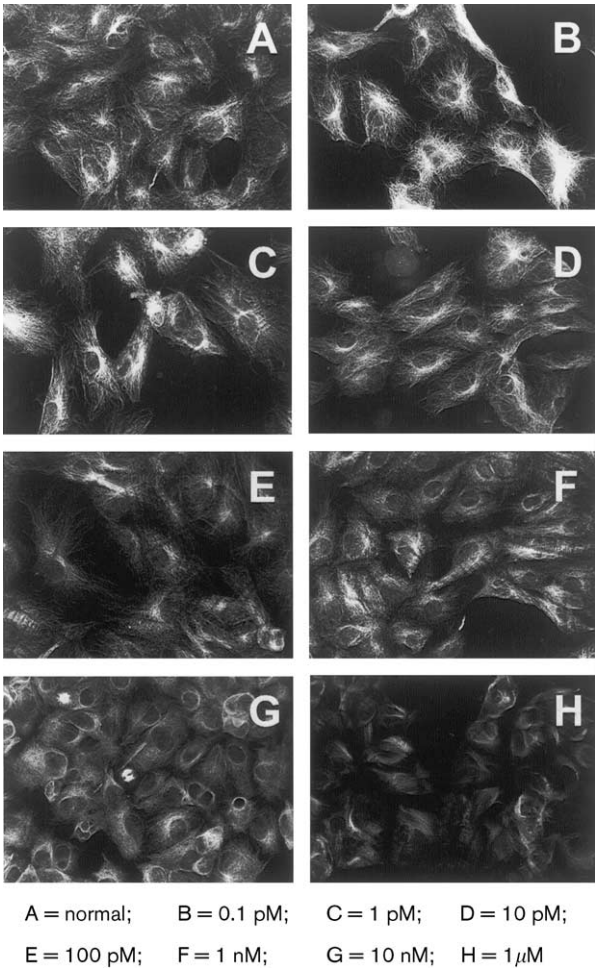
The morphology and cell cycle analysis data indicated that cell growth inhibition by paclitaxel at ultra low concentrations was not through disrupting cell microtubules. To confirm this hypothesis, we performed microtubule staining in cells treated with various concentrations of paclitaxel. The fine cellular microtubule network in cells treated with 0.1–100 pM paclitaxel was similar to that of untreated cells (Fig. 3). However, paclitaxel at higher concentrations (above 1 nM) showed

Fig. 2



Effect of paclitaxel on HMVEC morphology and the cell cycle. HMVECs were grown in T75 flasks with EGM2 medium with 5% FBS (A, normal) and with paclitaxel added at various final concentrations as shown (B–J). After 48 h incubation, cells treated with ultra low concentrations (0.1–100 pM) of paclitaxel showed lower cell density as compared to control (upper panel). These treated cells were all collected and subjected to flow cytometry analysis of DNA profiles (lower panel). Cells treated with paclitaxel concentrations above 1 nM (G–J) were mostly rounded up and not attached to flasks, and they were blocked at the G_2/M phase of the cell cycle and went to apoptosis.

Fig. 3



Effect of paclitaxel on HMVEC microtubules. HMVECs were grown in eight-well chamber slides in normal culture medium containing various concentrations of paclitaxel for 48 h. The cells were then fixed and stained for cellular tubulins. Cellular microtubules were observed with immunofluorescent microscopy. Paclitaxel at ultra low concentrations (0.1–100 pM, B–E) did not affect the cellular microtubule assembly.

the expected cellular effect, i.e. interference with microtubule assembly in HMVECs.

Paclitaxel at ultra low concentrations inhibits angiogenesis *in vitro*

To show paclitaxel at ultra low concentrations has anti-angiogenic activity, we tested it in an EC sprouting and tube formation angiogenesis model *in vitro*. HMVECs attached to microcarrier beads imbedded in fibrin matrix were able to sprout and form tubule structures (Fig. 4). This EC property was dependent on the presence of angiogenesis stimulators VEGF and bFGF. Paclitaxel at ultra low concentrations (0.1–100 pM) inhibited the HMVEC tube formation in this three-dimensional fibrin matrix. Mouse EC BEND3 were studied in a similar fashion, but paclitaxel did not show any inhibition at concentrations below 10 nM (data not shown). These data further show paclitaxel at ultra low concentrations is a specific inhibitor of human ECs.

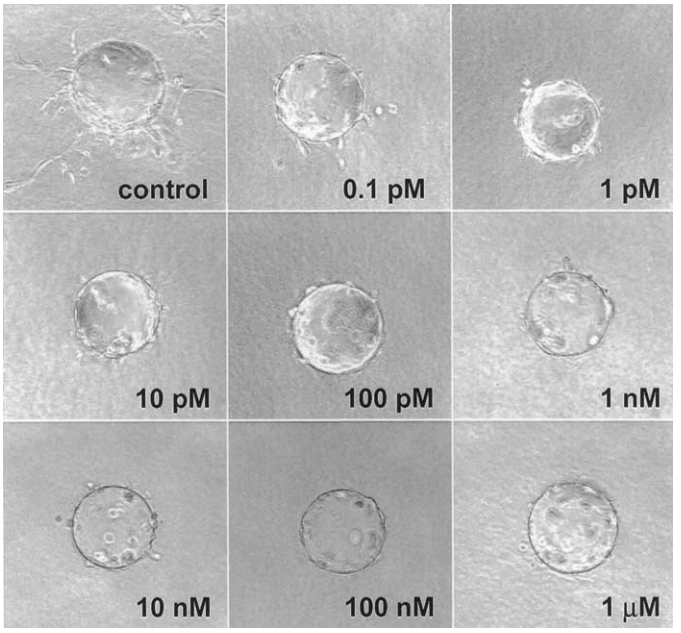
Discussion

Conventional chemotherapeutics inhibit tumor cell growth by blocking topoisomerase II activity (e.g. camptothecin), alkylating DNA (cisplatin), interfering with cellular microtubules (vinblastine and paclitaxel) or inhibiting metabolism (5-FU and methotrexate). The non-selective cell killing mechanism of action of these agents dictates they may inhibit EC growth as well and can be used as 'accidental' anti-angiogenic inhibitors when dosed appropriately. Application of chemotherapeutics for anti-angiogenesis cancer therapy was proposed in 1991 [15] and has been recently reviewed [2,7]. Recent studies have shown continuous low-dose application of chemotherapeutics has resulted in regression of large established tumors and inhibition of angiogenesis in animal models, without major toxicities or signs of acquired drug resistance during the course of treatment in mice [8,9]. Therefore, further analysis and evaluation of chemotherapeutics as antiangiogenic agents should uncover more benefits of these agents in cancer therapy.

Microtubule-interfering agents were among the first chemotherapeutics reported to have an anti-angiogenic effect [6]. In that report, Baguley *et al.* showed that vinblastine inhibited the growth of a drug-resistant colon adenocarcinoma and linked the efficacy to vascular collapse. Recently, the anti-angiogenic activity of paclitaxel has been described [16–18]. The angiogenesis in matrigel implants induced by tumor-derived stimulators was strongly inhibited in a mouse model with i.v. injections of 20–28 mg/kg paclitaxel at day 0 and 4 [16]. The dose was similar to conventional cytotoxic therapy regimen and not far away from the maximum tolerated dose of paclitaxel. In more recent studies, paclitaxel at 3–6 mg/kg daily dosing was found to be effective in inhibiting mouse cornea angiogenesis and intratumoral angiogenesis in a mouse model [17,18].

In the current investigation, we found that paclitaxel had direct effects on human ECs at ultra low concentrations (0.1–100 pM). Proliferation of human ECs was completely blocked by low picomolar concentrations of paclitaxel. This effect was seen in several commonly used human ECs, including HMVECs, HUVECs and HUAVECs. A direct effect of paclitaxel at a concentration as low as 10 pM on EC migration was reported before [16]. The potent EC inhibitory activities of paclitaxel were further demonstrated in our three-dimensional tube formation assay. Paclitaxel at 1 pM completely prevented HMVECs from sprouting and forming tubular structures in fibrin matrix, a process closely mimicking angiogenesis *in vivo* which requires ECs to migrate and proliferate.

Fig. 4



Inhibition of HMVEC sprouting and tube formation in the fibrin matrix. HMVECs grown on microcarrier beads sprouted and formed tubes when imbedded in fibrin gel with growth factors and FBS (EGM2 medium). Paclitaxel was able to completely suppress this endothelial function at ultra low concentrations, further indicating the anti-angiogenic activity of ultra-low-dose paclitaxel.

The lack of inhibition of mouse ECs by ultra low concentrations of paclitaxel is intriguing, and indicates it may not be possible to show *in vivo* efficacy of ultra low paclitaxel in mouse models. However, paclitaxel was active against mouse ECs at low nanomolar concentrations, so it is still one of the most potent among all anti-angiogenic agents. The efficacy of low-dose 6 mg/kg daily administration of paclitaxel inhibiting mouse cornea and tumor angiogenesis strongly supports the anti-angiogenic activity of paclitaxel. Human ECs are 10 000-fold more sensitive to the inhibition of paclitaxel than mouse ECs. This ultra sensitivity indicates that paclitaxel can be used at a much lower dose continuously in humans to achieve anti-angiogenic effects and that it may cause minimal toxicity considering many human non-EC cell types are not affected by ultra low concentrations of paclitaxel.

The mechanism of paclitaxel's anti-mitotic effect has been shown to be the inhibition of microtubule depolymerization and blockade of cell division in the G_2/M phase of the cell cycle [14]. Paclitaxel blocks mitosis and induces multi-nucleation of cells during interphase. These are associated with formation of an incomplete metaphase plate of chromosomes and an altered arrangement of spindle microtubules. These cellular effects of paclitaxel are achieved at low nanomolar drug concentrations in cell culture. Human ECs are inhibited by paclitaxel at ultra low concentrations (0.1–100 pM)—5000-fold lower than the concentrations needed for anti-mitotic effect. The mechanism of ultra low paclitaxel is not known. Our data suggest a novel mechanism of action that does not rely on disrupting microtubule assembly. First, inhibition of EC proliferation at pM concentrations of paclitaxel was cytostatic and no cell apoptosis was detected either by flow cytometry or by caspase-3 measurement (data not shown). The cells were arrested mostly at the G_1 phase of the cell cycle, whereas cells treated with higher concentrations of paclitaxel showed G_2/M arrest and apoptosis. Next, the morphology of human ECs treated with picomolar concentrations of paclitaxel was not different from untreated cells except for lower density, but cells exposed to higher paclitaxel concentrations became round and not well attached. Finally, cellular microtubule staining of human ECs treated with paclitaxel at concentrations below 1 nM did not show an abnormal microtubule network. Only in cells treated with higher concentrations was the condensed microtubule observed. Thus, the direct effect on the microtubule structure was not caused by ultra low concentrations of paclitaxel. Distinct sensitivity of ECs versus non-ECs to ultra-low-dose paclitaxel suggests the presence of specific targets in human ECs. We are currently investigating the mechanism at the molecular level, by examining alterations

in signal transduction in ECs treated with ultra low concentrations of paclitaxel.

'Metronomic' dosing, or anti-angiogenic scheduling, of cancer chemotherapeutics has been increasingly recognized to be a potential application of these agents in cancer therapy [2,7]. Data presented in this study, along with published observations [16–18], suggest that paclitaxel is a candidate of choice for clinical exploration. Paclitaxel at ultra low concentrations is not only potent at inhibiting angiogenesis, but also selective against ECs, as non-EC types are not inhibited by such ultra low concentrations of paclitaxel. Other chemotherapeutic agents, such as 5-FU, camptothecin and doxorubicin, are non-selective, and they inhibit ECs and non-ECs with similar IC_{50} (5–100 nM, Table 1 and data not shown). The unique properties of paclitaxel make it a powerful tool in cancer therapy. It can be envisaged that paclitaxel can be administered to maintain continuous drug concentrations of 100–1000 pM in plasma for long-term control of cancer following conventional treatment, such as surgery, radiation or chemotherapy. The suggested plasma drug concentrations of 100–1000 pM is higher than that needed for *in vitro* activity in consideration of the high plasma protein binding of this agent (89–98%) and paclitaxel at this concentration range still possesses cell-type selectivity. Clinical experience of paclitaxel accumulated over the last decade should guide us to deliver this agent to achieve the desired blood drug concentrations. Acquired drug resistance of continuous ultra-low-dose paclitaxel is not expected because ECs are the intended targets, which are considered genetically stable. Since normal non-EC types are not inhibited by paclitaxel at concentrations below 1000 pM, toxicity of ultra-low-dose paclitaxel is expected to be minimum. Angiogenesis-suppressed tumors are deprived of nutrients and growth and survival factors/signals from vessels and ECs, and are prone to apoptosis and more sensitive to radiation [19]. This continuous ultra-low-dose regimen may also be used in combination with radiation or surgery to enhance efficacy. Finally, combination of continuous ultra-low-dose paclitaxel with other novel angiogenesis inhibitors may also provide added clinical benefits.

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