# Inhibition of Endothelial Cell Function *in Vitro* and Angiogenesis *in Vivo* by Docetaxel (Taxotere): Association with Impaired Repositioning of the Microtubule Organizing Center[1]

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## Abstract

**A number of cancer chemotherapeutic drugs designed to have cytotoxic actions on tumor cells have recently been shown to also have antiangiogenic activities. Endothelial cell migration and proliferation are key components of tumor angiogenesis, and agents that target the microtubule cytoskeleton can interfere with these processes. In this study, the effect on endothelial cell functions of the microtubule-stabilizing drugs Taxotere and Taxol were evaluated in three *in vitro* assays: a chemokinetic migration assay, an angiogenesis factor-mediated chemotactic migration assay, and a three-dimensional Matrigel tubule formation assay, using rat fat pad endothelial cells (RFPECs) and/or human umbilical vein endothelial cells (HUVECs). Taxotere was active in all three assays at concentrations that were not cytotoxic and did not inhibit endothelial cell proliferation. In the RFPEC chemokinetic migration and *in vitro* tubule formation assays, the $IC_{50}$ values were approximately $10^{-9}$ M for both Taxotere and Taxol. HUVEC migration, however, was more sensitive to Taxotere, with an observed $IC_{50}$ of $10^{-12}$ M in a chemokinetic assay. In a Boyden chamber assay, HUVEC chemotaxis stimulated by either of two angiogenic factors, thymidine phosphorylase or vascular endothelial growth factor, was inhibited by Taxotere with an $IC_{50}$ of $10^{-11}$ M and was ablated at $10^{-9}$ M. Taxotere was also up to 1000-fold more potent than Taxol in inhibiting either chemokinetic or chemotactic migration. When the microtubule cytoskeleton was visualized using immunofluorescence staining of $\alpha$-tubulin, there were no gross morphological changes observed in HUVECs or RFPECs treated with Taxotere at concentrations that inhibited endothelial cell migration but not proliferation. The effects of Taxotere on migration were associated with a reduction in the reorientation of the cell's centrosome, at concentrations that did not affect gross microtubule morphology or proliferation. Reorientation of the centrosome, which acts as the microtubule organizing center, in the intended direction of movement is a critical early step in the stabilization of directed cell migration. These data indicate that endothelial cell migration correlates more closely with changes in microtubule plasticity than with microtubule gross structure. The antiangiogenic activity of Taxotere *in vivo* was assessed in a Matrigel plug assay. In this assay, the angiogenic response to fibroblast growth factor 2 was inhibited *in vivo* by Taxotere with an $ID_{50}$ of 5.4 mg/kg when injected twice weekly over a 14-day period, and angiogenesis was completely blocked in mice that received 10 mg/kg Taxotere. The *in vivo* data further suggested that Taxotere had selectivity for endothelial cell migration and/or microvessel formation because infiltration of inflammatory cells into the Matrigel plug was much less sensitive to inhibition by Taxotere. In conclusion, Taxotere is a potent and potentially specific inhibitor of endothelial cell migration *in vitro* and angiogenesis *in vitro* and *in vivo*.**

## Introduction

Docetaxel (Taxotere) is a microtubule-binding drug with significant clinical antitumor activity, most notably in advanced ovarian, breast, and prostate cancers (1–6). Activity has also been observed in a number of tumors that are generally refractory to other chemotherapeutic agents, including lung, head and neck, bladder, and esophageal cancers, as well as in metastatic breast and lung cancers that have progressed despite previous chemotherapy. The spectrum of clinical and *in vitro* activities for Taxotere is similar to that of the related drug paclitaxel (Taxol). Although Taxotere shares a binding site similar to or the same as that of Taxol, it has a 2-fold greater affinity for the site and facilitates microtubule assembly at a greater initial rate and at a lower concentration than does Taxol (7, 8). Taxotere is also a more potent an inhibitor of tumor cell growth *in vitro* and *in vivo* than Taxol (9, 10).

Recent attention has focused on the role of the vasculature in promoting tumor progression and the role of microtubule-altering agents in perturbing endothelial cell function. The cytoskeleton has well-described roles in cell division and migration and has been proposed to participate in other aspects of endothelial cell biology (11–13). The integrity of the microtubule system is necessary for protein trafficking, and agents that regulate microtubule plasticity, such as

Taxol and colchicine, decrease the frequency and velocity of vesicle transport (14, 15) and alter plasma membrane composition through their direct effects on membrane trafficking pathways. Such effects include the inhibition of endothelial cell transcytosis of plasma constituents (15) and the inhibition of tumor necrosis factor $\alpha$ and G-protein-coupled receptor trafficking (14, 16). Finally, the release of key endothelial cell metabolites, such as von Willebrand factor (17, 18) and the vasoprotective prostanoids, prostaglandin $E_2$ and prostacyclin (19), requires an intact microtubule system. These findings, coupled with the observation that tubulin-depolymerizing agents increase vascular permeability, suggest that the tubulin cytoskeleton is critical for the maintenance of endothelial barrier function (20).

Microtubule-altering drugs inhibit the growth not only of tumor epithelium but also of endothelial cells, most likely as a consequence of their disruption of the reorganization of the microtubule network required for the $G_2$-M transition (21–23). The microtubules also play a role in the locomotion of most, but not all, cell types, and data suggest they may be involved in the coordination of the direction of cell movement (24, 25). Accordingly, microtubule-disrupting and microtubule-stabilizing agents have been shown to inhibit endothelial cell migration *in vitro* and angiogenesis *in vivo* (21–23, 26, 27). Interestingly, in one of these studies, Taxol was found to inhibit angiogenesis in a corneal neovascularization assay under conditions in which colchicine and vincristine were inactive (23). Because agents that only inhibited endothelial cell proliferation *in vitro* did not affect angiogenesis *in vivo*, it is likely that the antiangiogenic actions of Taxol were not due solely to its cytotoxic activity (22). Centrosomes are the main site of microtubule nucleation in most cells, and during endothelial cell migration, their location within the cell redistributes toward the side of the nucleus closest to the direction of migration (28–30). Stabilization of the microtubule cytoskeleton by Taxol resulted in a reduction of cell migration and an associated decrease in the proportion of cells with centrosomes redistributed toward the direction of migration (31), suggesting the two observations may be linked. Because Taxotere has effects on microtubules that are similar but not identical to those of Taxol (32), the objective of this study was to determine whether Taxotere can interfere with endothelial cell function and therefore potentially have antiangiogenic actions.

## Materials and Methods

**Materials.** Docetaxel (Taxotere) was kindly provided by Aventis Pharmaceuticals (Parsippany, NJ), and Taxol (paclitaxel) was from Sigma (St. Louis, MO). Recombinant human TP,[3] human VEGF$_{165}$, and carrier-free human FGF-2 (basic fibroblast growth factor) were obtained from R&D Systems (Minneapolis, MN). All other reagents and chemicals were from Sigma unless otherwise stated.

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[3] The abbreviations used are: TP, thymidine phosphorylase; FGF-2, fibroblast growth factor 2; HUVEC, human umbilical vein endothelial cell; RFPEC, rat fat pad endothelial cell; VEGF, vascular endothelial growth factor.

**Isolation and Culture of Endothelial Cells.** All tissue culture materials and reagents were from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise stated. Microvascular RFPECs were maintained in Medium 199 with Earle's salts (M199) supplemented with 15% (v/v) fetal bovine serum (Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, 5 units/ml penicillin G, and 5 $\mu$g/ml streptomycin sulfate. RFPECs were grown to a confluent cell monolayer for the chemokinetic migration assay, and the *in vitro* tube forming assay was performed on RFPECs at 70–80% confluence, as described below.

HUVECs were isolated from umbilical cords as described previously (33). After cannulation and rinsing, the vein was digested with 100 units/ml type 2 collagenase (Worthington Biochemical Corporation, Freehold, NJ) in M199 for 10 min at 37°C. Veins were flushed with warm M199, and the resulting endothelial cell suspension was centrifuged for 5 min at $250 \times g$. Primary cultures of HUVECs were seeded into 25-cm$^2$ flasks (Costar, Cambridge, MA) precoated with 0.02% (w/v) gelatin. Culture medium consisted of M199 supplemented with 20% (v/v) newborn calf serum, 5% (v/v) pooled human serum (Gemini Bio-Products), 2 mM L-glutamine, 5 units/ml penicillin G, 5 $\mu$g/ml streptomycin sulfate, 10 units/ml heparin (Sigma), 7.5 $\mu$g/ml endothelial cell growth supplement (Sigma), and 4.5 $\mu$g/ml bovine brain extract (Bio Whittaker Inc., Walkersville, MD). Cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$, and the medium was changed after 24 h and every 2 days thereafter until confluent. Primary cultures of HUVECs were passaged with 0.05% trypsin/0.02% EDTA and collected by centrifugation, and cell number was determined using a dual threshold cell counter (Coulter Electronics, Luton, United Kingdom). Confluent HUVEC monolayers (passages 2–4) were used in the chemokinetic and Boyden chamber migration assays as described below.

**Chemokinetic Endothelial Cell Migration Assay.** The migration assay performed was a monolayer denudation assay as described previously (33). Confluent RFPECs or HUVECs in gelatin-coated 6-well plates (Costar) were mechanically "wounded" by scraping with a pipette tip and denuding a strip of the monolayer 300-$\mu$m wide. Variation in the wound width within experiments was approximately 5%. Endothelial monolayers were washed twice with PBS and incubated in media supplemented with Taxotere or Taxol. Control RFPEC or HUVEC cultures received media alone. The extent of wound closure was observed and photographed after 24 h using a solid state TV camera (COHU Electronics, CA) attached to an inverted phase-contrast microscope. Images of the denuded endothelial monolayer were captured using a video graphics system (Sony Electronics). The effect of Taxotere or Taxol on the progression of endothelial cell migration was quantitated by calculating the difference in the denuded area after 24 h using the Scion Image program (version 1.61; Scion Corp., Frederick, MD). Data are expressed as a percentage of the migration in untreated endothelial cells (mean ± SE).

**Boyden Chamber Assay of Chemotactic Endothelial Cell Migration.** Confluent HUVEC monolayers were harvested with cell dissociation solution and suspended at 1 ×

$10^6$ cells/ml in M199 supplemented with 1% serum. HUVECs ($10^5$) were seeded into 8.0-$\mu$m-pore transwell inserts (Costar) precoated with 10 $\mu$g/ml fibronectin. Inserts containing HUVECs were placed into a 24-well plate (Costar) containing M199 supplemented with 1% serum and incubated for 1 h at 37°C. HUVEC migration was stimulated by addition of the chemotactic factors, TP (100 ng/ml) or VEGF (10 ng/ml), to the lower well of the Boyden chamber. The effect of Taxotere or Taxol on endothelial migration was observed by inclusion of either agent in the lower chamber. After 5 h, HUVECs were fluorescently stained with 10 $\mu$M cell tracker green (Molecular Probes, Eugene, OR), and the upper surface of the insert was swabbed to remove nonmigrated cells. Inserts were washed three times with PBS, fixed in 3.7% formaldehyde, and mounted on microscope slides. HUVEC migration was quantitated by counting the number of cells in three random fields ($\times$100 total magnification) per insert. Data are expressed as cells/field (mean $\pm$ SE) as a percentage of TP- or VEGF-stimulated migration in the absence of Taxotere or Taxol.

**Assay of *in Vitro* Tubule Formation.** The spontaneous formation of capillary-like structures by RFPECs on a basement membrane matrix preparation, Matrigel (Becton Dickinson, Bedford, MA), was used to assess angiogenic potential. Twelve-well plates (Costar) were coated with Matrigel (10 mg/ml) according to the manufacturer's instructions. RFPECs ($1.5 \times 10^5$ cells/well) were seeded on Matrigel-coated plates and incubated at 37°C for 60 min. The indicated concentrations of Taxotere or Taxol were added, and the cultures were incubated at 37°C for 24 h. *In vitro* endothelial tubule formation was observed and photographed after 24 h as described above. The degree of tubule formation was determined by counting the number of tubes contained in two random fields from each well. Data are expressed as a percentage of the number of tubes in untreated control wells (mean $\pm$ SE).

**Endothelial Cell Proliferation Assay.** RFPECs and HUVECs were seeded into gelatin-coated 24-well plates (Costar) at $2 \times 10^4$ cells/well and allowed to attach for 24 h. Cells were washed twice with PBS, and RFPECs were treated with media containing $10^{-12}$, $10^{-10}$, or $10^{-8}$ M Taxotere. HUVECs were treated with media containing $10^{-14}$, $10^{-11}$, or $10^{-8}$ M Taxotere. Control RFPEC or HUVEC cultures received media alone. Cells were harvested with trypsin/EDTA every 24 h for 3 days, and cell number was determined in a dual threshold cell counter (Coulter Electronics). Data are expressed as cells/well (mean $\pm$ SD).

**Immunofluorescence Staining of Endothelial Microtubules.** HUVECs or RFPECs were seeded on fibronectin-coated glass coverslips in 12-well plates (Costar) and grown to confluence. HUVECs were treated for 20 h with media containing Taxotere at $10^{-14}$, $10^{-11}$, or $10^{-8}$ M. RFPECs were treated with media containing Taxotere at $10^{-12}$, $10^{-10}$, or $10^{-8}$ M. Control HUVEC and RFPEC cultures received media alone. After 20 h, cells were washed twice with PBS and rendered permeable by treatment with 0.5% Triton X-100 in PEM buffer [100 mM PIPES, 2 mM EGTA, and 2 mM MgCl$_2$ (pH 6.8)] for 4 min. Cells were washed twice with PEM buffer, fixed in 3% formaldehyde in PEM buffer, washed four times with PEM buffer, and blocked with 5% BSA in PBS.

Endothelial microtubules were visualized ($\times$1000 magnification) by immunofluorescence staining using a monoclonal antibody to $\alpha$-tubulin (1:100; Sigma) and a Cy3-conjugated second antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA), and the coverslips were mounted on microscope slides. Alterations in HUVEC and RFPEC gross microtubule morphology were assessed by comparison of photomicrographs of control and Taxotere-treated endothelial cells from three individual experiments.

Analysis of centrosome orientation in migrating endothelial cells at the open edge of a confluent monolayer was performed on HUVEC monolayers that were mechanically scraped, treated with Taxotere, and immunostained for $\alpha$-tubulin, as described above. The microtubule organizing center (or centrosome) was readily visualized in cultured endothelial cells as the region with the densest microtubule staining and from which most of the cytoplasmic microtubules emanated (29). The position of the centrosome in the first row of HUVECs adjacent to the denuded area was quantitated as described previously (29). The centrosome was classified as being either between the nucleus and toward the open wound edge or between the nucleus and the rest of the monolayer away from the wound edge. Data are expressed as the percentage of migrating endothelial cells with their centrosome orientated toward the wound edge (mean $\pm$ SD).

**Assay of *in Vivo* Angiogenesis.** A Matrigel plug assay was used to assess the antiangiogenic activity of Taxotere in mice *in vivo*. Growth factor-reduced Matrigel was supplemented with FGF-2 to a final concentration of 0 (control plug) or 1 $\mu$g/ml, and 500 $\mu$l were injected s.c. in the ventral midline of 6–8-week old C57Bl/6 mice. The following day, the mice received a 100-$\mu$l i.p. injection of Taxotere (0–20 mg/kg) in 10% ethanol, 5% glucose. Mice received injections every third day as described, for a total of four injections over a 14-day period, at which time the Matrigel plugs and underlying musculature were removed. The plugs were fixed overnight in 10% neutral buffered formalin and processed for paraffin embedding. Sections of 5 $\mu$m from embedded plugs were stained with H&E and examined for blood vessel formation. The effect of Taxotere on the angiogenic response to FGF-2 in Matrigel plugs was quantitated by counting the number of blood vessels/field ($\times$200 magnification) for the entire cross-sectional area of each Matrigel plug, and the data were expressed as vessels/field (mean $\pm$ SD). Vessel numbers were confirmed by CD31 immunostaining (BD Biosciences, San Diego, CA), and infiltration of inflammatory cells was evaluated by staining with CD45 (leukocyte common antigen Ly-5; PharMingen). The Matrigel plug assay was performed in duplicate, each with 5 mice/dose of Taxotere or vehicle.

**Statistical Analysis.** Statistical analysis of pooled data was performed using the Mann-Whitney $u$ test.
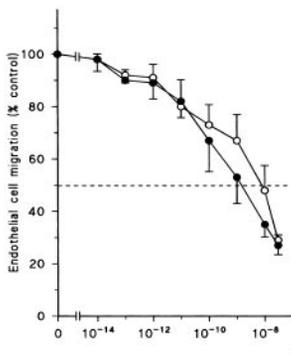
## Results

**Taxotere Was More Potent than Taxol at Inhibiting Endothelial Cell Migration *in Vitro*.** Chemokinetic and chemotactic models of migration were used to compare the effects of Taxotere and Taxol on endothelial cell migration. In

## A RFPEC

**Unwounded**

**Control, 0 h**

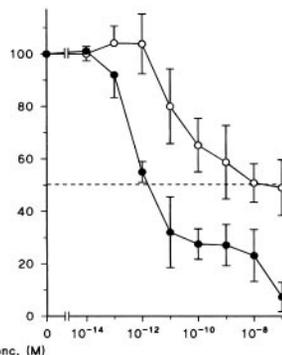**Control, 24 h**
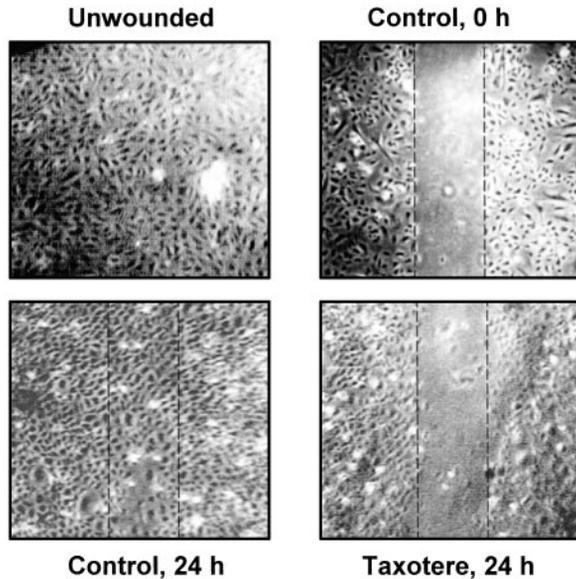
**Taxotere, 24 h**



**Fig. 1.** Taxotere and Taxol inhibited endothelial cell migration in a chemokinetic assay. *A,* photomicrographs ($\times$40 magnification) showing confluent RFPECs before (unwounded) and after (control, 0 h) mechanically scraping with a pipette tip and the migration of RFPECs into the scraped area after 24 h when treated with media alone (control, 24 h) or with Taxotere or Taxol ($10^{-8}$ M). *Dotted lines* indicate the denuded area occupied by the initial scraped area. *B* and *C,* quantitation of the inhibition of RFPEC (*B*) or HUVEC (*C*) migration by Taxotere (●) or Taxol (○) after 24 h. Data are expressed as a percentage of the migration in untreated endothelial cells (mean $\pm$ SE).
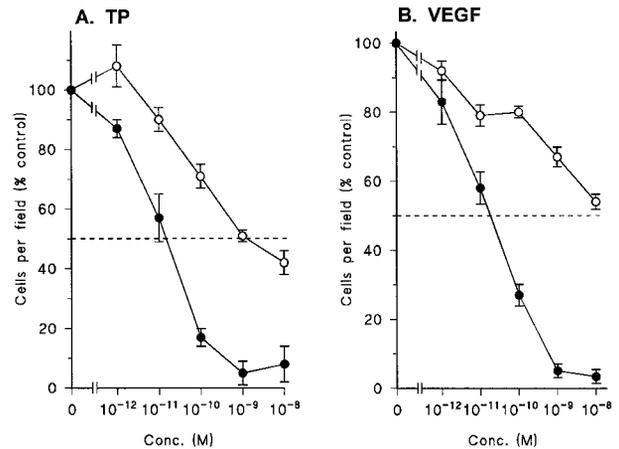


**Fig. 2.** Taxotere and Taxol inhibited human endothelial cell migration in response to a chemotactic gradient. HUVECs were harvested and seeded on 8.0-$\mu$m transwell inserts coated with fibronectin. Addition of a chemoattractant, TP (*A*) or VEGF (*B*), to the lower well of the Boyden chamber stimulated endothelial migration to the underside of the transwell membrane. Migrated cells were fluorescently stained after 5 h with cell tracker green. Inhibition of HUVEC chemotaxis by Taxotere (●) or Taxol (○) was quantitated from 3 random low-power fields/insert. Data are expressed as cells/field (mean $\pm$ SE), as a percentage of TP- or VEGF-stimulated migration in the absence of Taxotere or Taxol (percentage of control).

the chemokinetic model, confluent scrape-wounded RFPEC monolayers were incubated with either Taxotere or Taxol (0–30 nM), and the degree of closure of the "wound" was assessed after 24 h. Untreated RFPECs migrated into the denuded area, recolonizing 81 $\pm$ 7% of the original open area (Fig. 1*A*). In this model, Taxotere and Taxol inhibited cell migration with similar potencies, with $IC_{50}$s of approximately $10^{-9}$ M for the two agents (Fig. 1*B*). At the highest concentration tested, 30 nM, Taxotere and Taxol inhibited migration of microvascular RFPECs by 75 $\pm$ 6% ($P < 0.005$).

When migration was examined using human macrovascular endothelial cells (HUVECs), Taxotere was found to be a

more potent inhibitor of HUVEC chemokinesis than Taxol (Fig. 1*C*). Taxotere achieved maximal inhibition of migration at $10^{-7}$ M ($P \leq 0.001$) with an $IC_{50}$ of $2 \times 10^{-12}$ M. However, Taxol at the highest tested concentration ($10^{-7}$ M) suppressed migration in the chemokinetic assay by only 50% compared with untreated controls (Fig. 1*C*). Because HUVECs appeared to be more sensitive to Taxotere than RFPECs, we further evaluated the inhibitory actions of Taxotere and Taxol in a Boyden chamber assay, a chemotactic model of migration more representative of tumor-induced endothelial migration. Stimulation of HUVECs along a directional gradient of TP (Fig. 2*A*) or VEGF (Fig. 2*B*) resulted in migration to the underside of the membrane. Migration toward either chemotactic stimulus was inhibited by Taxotere and Taxol in a concentration-dependent manner. Taxotere ablated the migration of HUVECs at concentrations above $10^{-9}$ M ($P \leq 0.005$), with an $IC_{50}$ of $10^{-11}$ M (Fig. 2), values that were comparable with those observed for the chemokinetic migration of HUVECs (Fig. 1*C*). Similarly, Taxol also reduced migration ($P \leq 0.01$), although it was 100-1000-fold less potent than Taxotere at inhibiting chemotactic migration of HUVECs.

**The Antimigratory Effect of Taxotere Was Mediated at Concentrations That Did Not Affect Endothelial Cell Proliferation.** The taxanes have well-defined cytotoxic effects that arise as a result of their actions on microtubules and possibly other biochemical targets. An important consequence of these actions is the inhibition of cell proliferation by preventing $G_2$-M transition. To determine whether the observed inhibitory effect of Taxotere on endothelial cell migration may be attributed to the same cytotoxic actions that ultimately lead to mitotic arrest, the sensitivity of RFPECs and HUVECs to Taxotere-mediated growth inhibi-
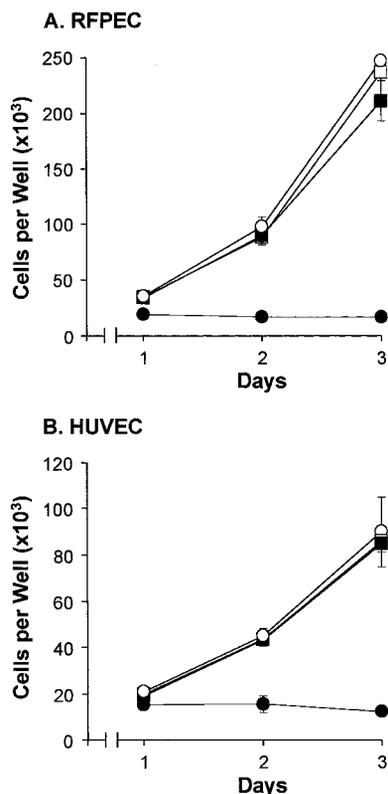
Fig. 3. Taxotere prevented endothelial cell migration and *in vitro* tubule formation at concentrations that did not affect proliferation. RFPECs and HUVECs were seeded into 24-well plates at $2 \times 10^4$ cells/well. *A,* RFPECs were treated with media containing Taxotere at $10^{-12}$ (■), $10^{-10}$ (○), or $10^{-8}$ (●) M. *B,* HUVECs were treated with media containing Taxotere at $10^{-14}$ (■), $10^{-11}$ (○), or $10^{-8}$ (●) M. Control RFPEC or HUVEC cultures received media alone (□). Cells were harvested with trypsin/EDTA every 24 h for 3 days, and cell number was determined in a dual threshold cell counter. Data are expressed as cells/well (mean ± SD).
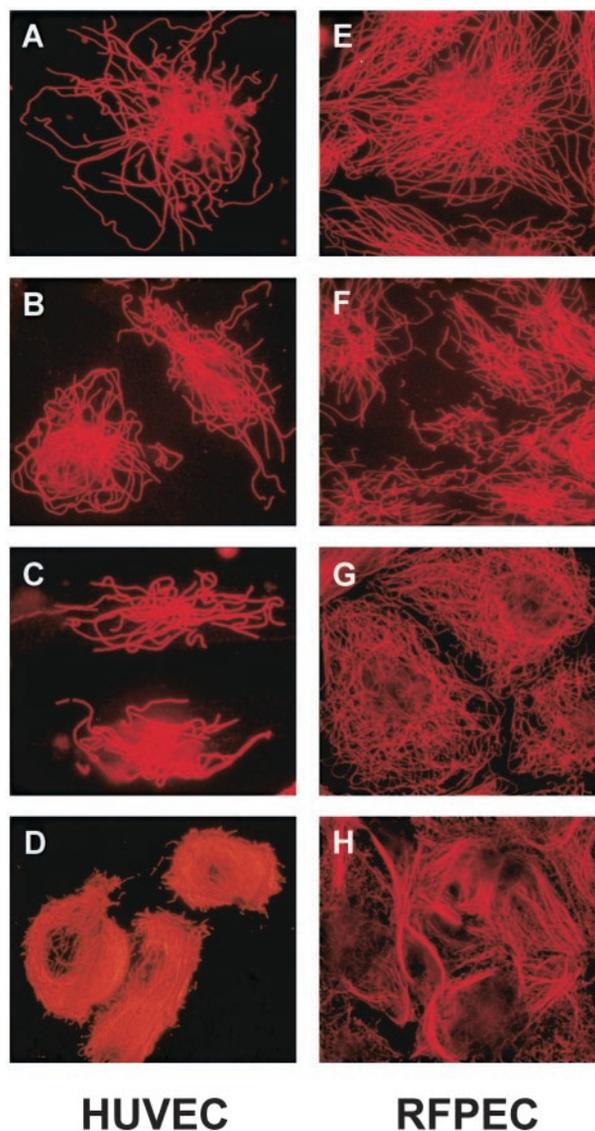


Fig. 4. Taxotere inhibited endothelial cell migration at concentrations that did not affect gross microtubule morphology. Immunofluorescence photomicrographs of confluent HUVECs (*A−D*) and RFPECs (*E−H*) monolayers stained for $\alpha$-tubulin. HUVECs were treated for 20 h with media alone (*A*) or media containing Taxotere at $10^{-14}$ (*B*), $10^{-11}$ (*C*), or $10^{-8}$ (*D*) M. RFPECs were treated for 20 h with media alone (*E*) or media containing Taxotere at $10^{-12}$ (*F*), $10^{-10}$ (*G*), or $10^{-8}$ (*H*) M. Cells were permeabilized and fixed, and microtubules were visualized (×1000 magnification) by immunofluorescence staining using a monoclonal antibody to $\alpha$-tubulin and a Cy3-conjugated second antibody. Photomicrographs are representative of three individual experiments.

tion was evaluated. RFPECs incubated with media alone increased 12-fold in number over the 3 days subsequent to seeding (Fig. 3*A*). Taxotere at $10^{-10}$ M, a concentration that inhibited RFPEC migration and *in vitro* tubule formation (Figs. 1*B* and 6*C*), was permissive for endothelial cell growth, and the rate of proliferation was comparable with that of untreated RFPECs over the same period (Fig. 3*A*). At a higher concentration, ($10^{-8}$ M), Taxotere inhibited both RFPEC migration and proliferation. Similar findings were also observed for HUVEC proliferation, with a concentration of Taxotere that significantly attenuated HUVEC migration ($10^{-11}$ M; Figs. 1*C* and 2) having no effect on HUVEC proliferation, and with inhibition of proliferation seen only at a higher concentration (Fig. 3*B*). Thus, the curves for the inhibitory effect of Taxotere on endothelial cell migration and *in vitro* tubule formation were not coincident with that for inhibition of cell proliferation, indicating that the antiangiogenic effect of Taxotere was not likely mediated through nonspecific cytotoxicity.

**Taxotere Inhibited Endothelial Cell Migration at Concentrations That Did Not Affect Gross Microtubule Morphology but Were Associated with Reduced Intracellular Centrosome Reorientation.** The cytotoxic effects of Taxotere result from the stabilization of the microtubule cytoskel-

eton by preventing the dissociation of tubulin dimers. To determine whether the observed inhibitory effect of Taxotere on endothelial cell migration may be a consequence of alterations in gross microtubule structure, the morphology of tubulin networks in HUVECs and RFPECs was examined by immunofluorescence staining using an antibody to $\alpha$-tubulin (Fig. 4). The tubulin cytoskeletons in untreated HUVECs (Fig. 4*A*) and RFPECs (Fig. 4*E*) are morphologically similar, with cytoplasmic tubulin filaments radiating from a central point
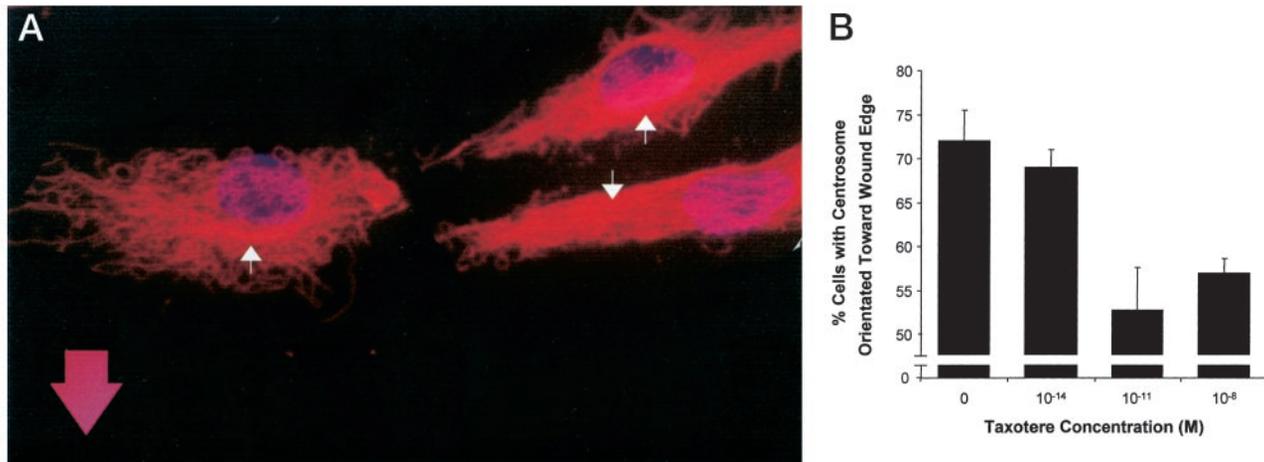
Fig. 5. Taxotere inhibited endothelial cell migration by preventing centrosome reorientation at concentrations that did not affect gross microtubule morphology. *A,* immunofluorescence photomicrograph of migrating HUVECs at the scraped edge, stained for $\alpha$-tubulin. The cell nucleus was identified using a 4′,6-diamidino-2-phenylindole counterstain (*blue*). The position of the centrosome (*small arrows*) can be visualized as the densest region of microtubules, from which most of the cytoplasmic microtubules emanate. The *large arrow* is perpendicular and points to the open edge of the cell monolayer, indicating the presumed direction of cell movement into the denuded area. *B,* quantitation of the effect of Taxotere on centrosome reorientation in migrating HUVECs after scraping. Confluent HUVEC monolayers were mechanically scraped and treated for 20 h with media containing Taxotere at 0, $10^{-14}$, $10^{-11}$, or $10^{-8}$ M. Cells were stained for $\alpha$-tubulin ($\times 600$ magnification) by immunofluorescence as described in Fig. 4. The position of the centrosome in the first row of HUVECs adjacent to the scraped area was judged as being either on the side of the nucleus toward or away from the direction of migration. Cells in which the centrosome could not be clearly visualized were not included in the quantitation. The position of the centrosome in the first row of HUVECs immediately subsequent to scraping was random relative to the nucleus (*i.e.,* 50% toward and 50% away), and this represents the baseline value on the Y axis. Data are the average of three individual experiments and are expressed as the percentage of migrating endothelial cells with the centrosome orientated toward the wound edge (mean ± SD).

(the microtubule organizing center or centrosome) to the cell periphery. As expected, the morphology of the tubulin cytoskeleton was comparable with that of untreated cells in HUVECs (Fig. 4*B*) and RFPECs (Fig. 4*F*) treated with Taxotere at concentrations ($10^{-14}$ and $10^{-12}$ M, respectively) that did not affect endothelial migration or proliferation. Gross morphological changes in the tubulin cytoskeleton were observed in HUVECs (Fig. 4*D*) and RFPECs (Fig. 4*H*) at concentrations of Taxotere ($10^{-8}$ M) that ablated both migration and proliferation, when compared with control cells (Fig. 4, *A* and *E*). These morphological changes manifested in HUVECs (Fig. 4*D*) as an interwoven fabric of highly concentrated filaments and manifested in RFPECs (Fig. 4*H*) as dense peripheral banding of tubulin filaments. However, no comparable gross morphological changes were observed in HUVECs (Fig. 4*C*) or RFPECs (Fig. 4*G*) at $10^{-11}$ and $10^{-10}$ M Taxotere, respectively, concentrations that were chosen because they inhibited migration but not proliferation of the cells.

The microtubule organizing center (the centrosome) is critical in stabilizing a chosen direction of movement in the initial steps of cell migration. A change in the direction of cell migration ensues after a reorientation of the centrosome in the provisional direction of movement (24). In *in vitro* and *in vivo* models of endothelial cell denudation, the centrosome of migrating endothelial cells is repositioned from a random site in relation to the nucleus to a location between the leading edge of the cell and the nucleus (20, 21). We determined whether the observed inhibitory effect of Taxotere on endothelial cell migration might be associated with an impeded centrosome reorientation. Confluent HUVEC monolayers were mechanically scraped to

stimulate unidirectional migration, and the position of the centrosome in relation to the nucleus was visualized after 20 h in the first row of cells adjacent to the open edge of the monolayer by immunofluorescent staining of $\alpha$-tubulin (Fig. 5*A*). The position of the centrosome in the first row of HUVECs immediately subsequent to scraping was random relative to the nucleus (*i.e.*, approximately 50% toward the open edge and 50% away), and this represents the baseline value. As a response to the stimulation to migration, 72 ± 3% ($P < 0.001$) of centrosomes in untreated HUVECs in the first row of cells adjacent to the open area were reoriented to the side of the nucleus closest to the direction of cell movement (Fig. 5*B*). These observations were consistent with those reported in similar studies (21, 23). Similarly, Taxotere at concentrations ($10^{-14}$ M) that did not affect endothelial function in other assays also did not affect centrosome reorientation. As expected, Taxotere at concentrations ($10^{-8}$ M) that inhibited endothelial function and altered gross microtubule morphology also prevented centrosome repositioning (57 ± 2%; $P < 0.01$ *versus* untreated HUVECs). However, Taxotere at concentrations ($10^{-11}$ M) that inhibited endothelial migration but did not affect proliferation or gross microtubule morphology also reduced centrosome reorientation as a response to a stimulus to migration (53 ± 5%; $P < 0.01$). These data indicated that endothelial cell migration correlated more closely to changes in microtubule plasticity/dynamics than to changes in gross structure and that plasticity of microtubules was highly sensitive to the effects of Taxotere.
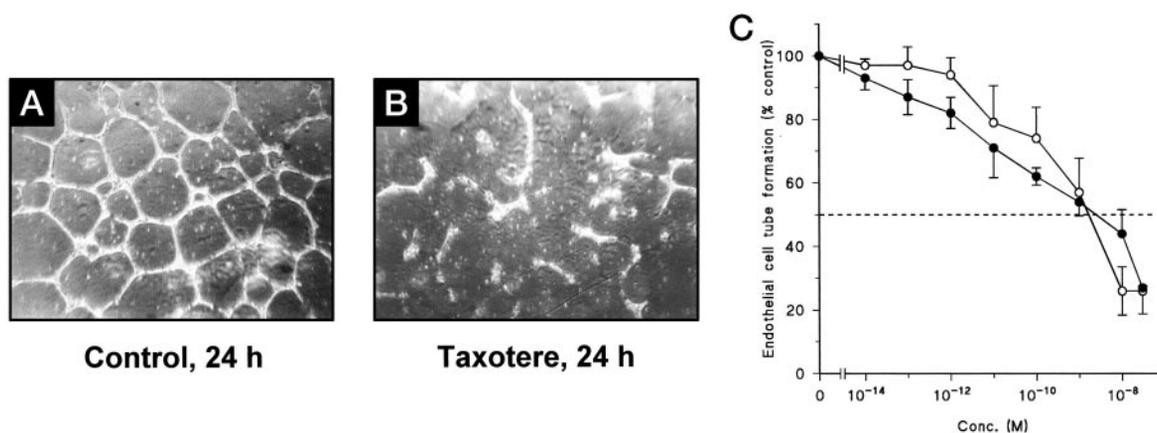
Fig. 6. Taxotere and Taxol inhibited *in vitro* endothelial tubule formation. The spontaneous formation of capillary-like structures by RFPECs on Matrigel was used to assess angiogenic potential. *A* and *B,* photomicrographs of RFPECs seeded on Matrigel-coated wells after 24 h. Untreated RFPECs (control, *A*) migrated to form connected tubular networks. Taxotere (*B*) or Taxol ($10^{-8}$ M) significantly attenuated network formation. *C,* effect of Taxotere or Taxol on network formation. Inhibition of *in vitro* endothelial tubule formation by Taxotere (●) or Taxol (○) was quantitated from photographs after 24 h by counting the number of tubes contained in two random fields from each well. Data are expressed as a percentage of the number of tubes in untreated wells (mean ± SE).

**Taxotere and Taxol Were Equipotent Inhibitors of Endothelial Tubule Formation in Three-dimensional Cultures *in Vitro*.** Endothelial differentiation in three-dimensional cultures is dependent on cell migration. The inhibition of RFPEC and HUVEC migration by Taxotere and Taxol suggested that these agents might also inhibit endothelial *in vitro* tubule formation. RFPECs on a basement membrane matrix (Matrigel) underwent spontaneous alignment into cords, which established the pattern for further tubule formation. These cords fused into continuous tubes with complete lumen to form capillary-like structures over 24 h (Fig. 6*A*). Taxotere and Taxol ($10^{-8}$ M) reduced the formation of tubules by RFPECs to $25 \pm 7\%$ ($P < 0.005$) of that observed in untreated cultures (Fig. 6*B*), and the two agents were equipotent inhibitors ($IC_{50}$ of $10^{-9}$ M; Fig. 6*C*). These concentrations were higher than those required for some of the other effects of Taxotere on endothelial cells. Because cells in this assay were embedded in Matrigel, it is possible that the physical or chemical nature of this matrix could have contributed to this difference. In agreement with its other observed effects on cell migration, however, Taxotere also inhibited *in vitro* tubule formation at concentrations that did not affect endothelial cell proliferation (Fig. 3*A*).

**Taxotere Is a Potent Inhibitor of Angiogenesis *in Vivo*.** Because Taxotere was observed to be both antimigratory and antiangiogenic *in vitro* (Figs. 1, 2, and 6), its effect on angiogenesis *in vivo* was investigated using a Matrigel plug assay. In this assay, liquid Matrigel, either by itself or containing an angiogenic factor, was injected s.c. into C57Bl/6 mice. The Matrigel hardened to form a plug, which was removed after 2 weeks and processed to assess and quantitate the angiogenic response. There was little angiogenesis or cell migration into control plugs that had not been supplemented with FGF-2 (Fig. 7, *A* and *D*). In contrast, FGF-2-supplemented Matrigel plugs elicited a robust angiogenic response, which was also accompanied by an inflammatory cell and/or connective tissue infiltrate (Fig. 7, *B*, *E*, and *G*).

This observation is consistent with FGF-2 being a chemoattractant for multiple cell types of neuroectoderm and mesodermal origin (34–36). In fact, a pan-leukocyte antibody identified the majority of the cells infiltrating the plug as inflammatory cells (Fig. 7, *G* and *H*). The angiogenic response to FGF-2 was inhibited in a dose-dependent manner in mice that received i.p. Taxotere injections every third day (Fig. 7, *C*, *F*, and *H*); the $ED_{50}$ for the inhibition of angiogenesis on this schedule was approximately 5 mg/kg Taxotere, with complete inhibition observed at 10 mg/kg (Fig. 7*I*). In contrast, Taxotere had little apparent effect on the extent of invasion of inflammatory cells into the Matrigel plug (Fig. 7, *G versus H*). Thus, Taxotere showed specificity *in vivo* for the inhibition of migration of endothelial cells, compared with its effect on connective tissue and inflammatory cells.

**Discussion**

Tumor angiogenesis is a critical component of tumor growth and metastasis, and the targeting of the vascular supply of tumors is an intense field of interest, with many promising preclinical trials highlighting the potential effectiveness of this form of therapy. The present study reports that the cytotoxic drug Taxotere inhibited endothelial cell migration and angiogenesis *in vitro* and *in vivo*. Proliferating endothelial cells were highly sensitive to the cytotoxic and apoptosis-inducing actions of a range of chemotherapeutic drugs, including alkylating agents (cyclophosphamide), antimetabolites (5-fluorouracil), and antitumor antibiotics [doxorubicin (37, 38)]. In the present study, however, Taxotere inhibited endothelial cell migration and angiogenesis (tubule formation) *in vitro* at concentrations substantially below those that inhibited the proliferation of the cells (Fig. 3) or caused alterations in their shape or viability (data not shown). These observations were consistent with those reported for Taxol, which inhibited HUVEC capillary formation at >100-fold lower concentrations than those required for growth inhibi-
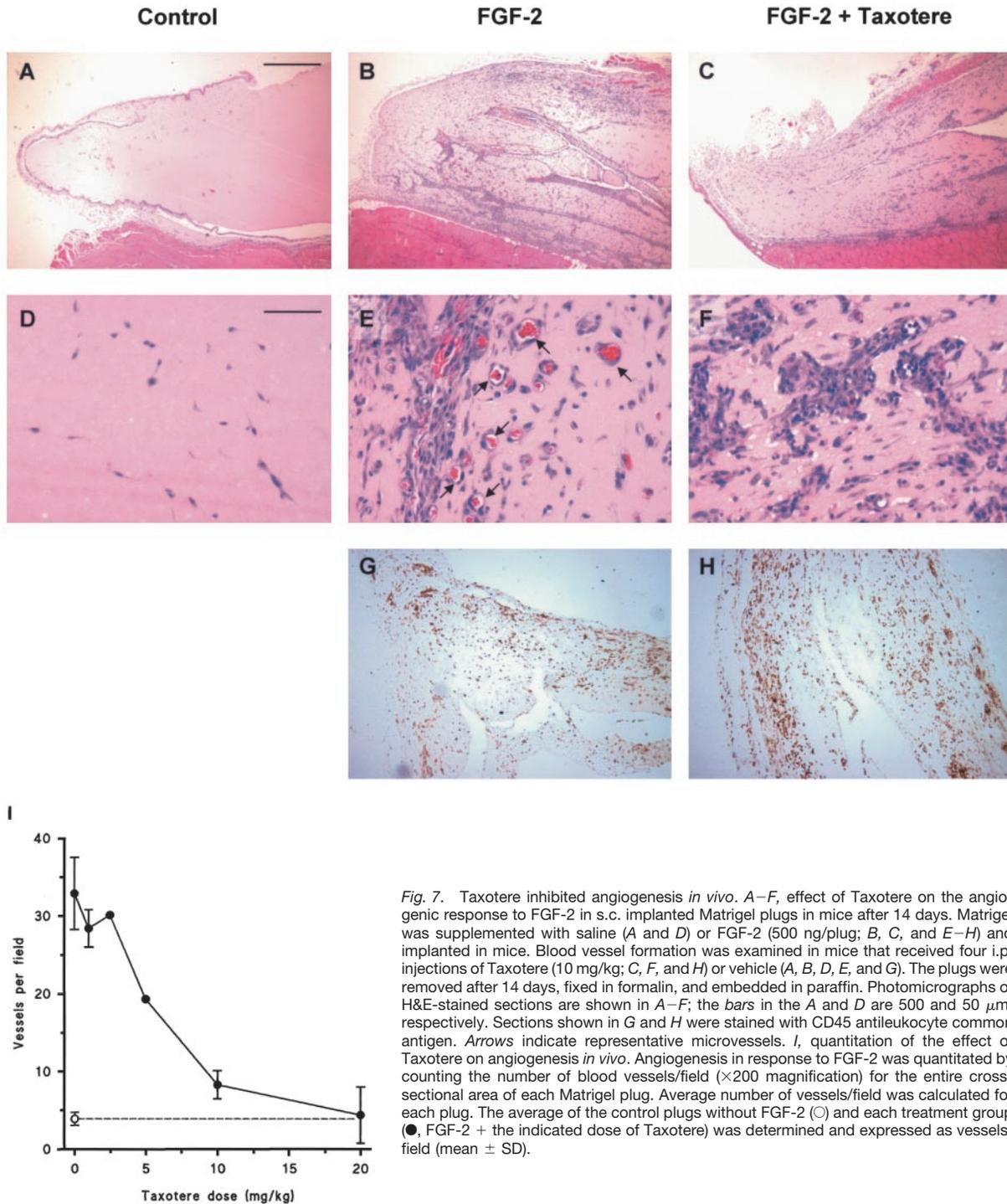
Fig. 7.   Taxotere inhibited angiogenesis *in vivo*. *A–F*, effect of Taxotere on the angiogenic response to FGF-2 in s.c. implanted Matrigel plugs in mice after 14 days. Matrigel was supplemented with saline (*A* and *D*) or FGF-2 (500 ng/plug; *B, C,* and *E–H*) and implanted in mice. Blood vessel formation was examined in mice that received four i.p. injections of Taxotere (10 mg/kg; *C, F,* and *H*) or vehicle (*A, B, D, E,* and *G*). The plugs were removed after 14 days, fixed in formalin, and embedded in paraffin. Photomicrographs of H&E-stained sections are shown in *A–F*; the *bars* in the *A* and *D* are 500 and 50 $\mu$m, respectively. Sections shown in *G* and *H* were stained with CD45 antileukocyte common antigen. *Arrows* indicate representative microvessels. *I,* quantitation of the effect of Taxotere on angiogenesis *in vivo*. Angiogenesis in response to FGF-2 was quantitated by counting the number of blood vessels/field (×200 magnification) for the entire cross-sectional area of each Matrigel plug. Average number of vessels/field was calculated for each plug. The average of the control plugs without FGF-2 (○) and each treatment group (●, FGF-2 + the indicated dose of Taxotere) was determined and expressed as vessels/field (mean ± SD).

tion (26). In our studies, TP- and VEGF-mediated human endothelial cell chemotaxis was inhibited by Taxotere at concentrations up to 1000-fold lower than those observed to inhibit breast cancer cell proliferation and 4000-fold lower than that reported to inhibit VEGF-stimulated human endothelial cell proliferation (26, 39). VEGF has been reported to antagonize the inhibitory effect of Taxol on HUVEC proliferation (26), and thus the ability of Taxotere to inhibit VEGF-

mediated migration in our studies suggests there are potential differences in the roles the microtubules play in proliferation and cell migration.

The difference in the concentration of Taxotere required to inhibit endothelial cell migration *versus* proliferation indicates that the movement of endothelial cells may be more sensitive to changes in microtubule plasticity than gross structure. An early event in directed cell migration that is dependent on the

maintenance of microtubule plasticity is the reorientation of the centrosome in the intended direction of movement. Extension of a new pseudopod in a migrating cell precedes centrosome reorientation to the new leading edge of the cell. If reorientation does not occur within a 30-s period, the new pseudopod is retracted (24). Thus, centrosome reorientation has been postulated to be required for the maintenance of leading pseudopods and stabilization of a chosen direction of movement, and the microtubule cytoskeleton mediates the communication at the cell periphery, where microtubules interact with the cortical actin filament network (40). In our study, Taxotere at $10^{-11}$ M inhibited directed endothelial migration, possibly by preventing stabilization of pseudopod extension and potentially as a consequence of the impeding of centrosome reorientation; this occurred at concentrations that did not affect gross microtubule morphology. These data imply that drug-mediated effects on microtubule plasticity/dynamics rather than on gross microtubule organization or expression are sufficient for inhibition of cell locomotion, a conclusion supported by studies with other microtubule-altering agents (25, 31). The molecular nature of these actions of Taxotere are not known, including whether they are a direct consequence of its binding to tubulin or result from effects on other cellular components or pathways.

Several aspects of the *in vivo* antiangiogenic actions of Taxotere reported here are noteworthy. The dose and schedule of Taxotere used in the *in vivo* experiments were selected based on previous studies of the experimental antitumor activity of Taxotere in mouse models (41). However, recent reports suggest that drug schedules designed to maximally inhibit tumor cell proliferation may not achieve an optimal antiangiogenic effect (37, 38, 42). Thus, a schedule in which lower doses are given less frequently but at regular intervals and for more extended period of times has been proposed to more effectively target tumor neovascularization. This "metronomic" dosing schedule of cytotoxic drugs may have a greater impact on the more slowly proliferating endothelial cell than would a high, intermittent dosing regimen, which would be a less effective antiangiogenic regimen due to the low frequency of endothelial cells undergoing cell division at any particular time (43). Thus, it is possible that optimization of the schedule for Taxotere administration *in vivo* would result in antiangiogenic effects at even lower doses than those used in this study. The antiangiogenic effects of Taxotere also appeared to have some selectivity for endothelial cells *in vivo*. The migration of inflammatory cells, such as leukocytes and macrophages, into the Matrigel plug was less sensitive to inhibition by Taxotere, and the basis for this selectivity is not known. In contrast, no evidence for selectivity was reported in a similar study, in which endothelial cell and fibroblast infiltration into the Matrigel plug were similarly reduced by Taxol (22). It is also possible that the effect of Taxotere observed *in vivo* is not due solely to a direct effect on endothelial cells but is rather a consequence of effects on other cell types that can modulate angiogenesis. The importance of these *in vivo* actions to the clinical antitumor activity of Taxotere remains to be determined.

The effectiveness of Taxotere may depend on the nature of the vascular bed from which the blood supply is derived. It is curious to note that the microvascular endothelial cell line (RFPEC) used in these studies was less sensitive to the antiangiogenic effects of Taxotere and Taxol compared with macrovascular endothelial cells (HUVECs). Evidence supports the concept that the blood supply of a tumor is derived from the venous side of the circulation (44). If this is indeed the case, then the use of Taxotere as an antiangiogenic agent would be advantageous because the migration of macrovascular endothelial cells appears to be more susceptible to inhibition by Taxotere.

Taxotere inhibited human endothelial cell migration stimulated by two angiogenic factors *in vitro*. Because chemotactic gradients are used by tumors to induce angiogenesis, it is possible that the antitumor activities of Taxotere observed clinically may include antiangiogenic actions. This is supported by data from our studies demonstrating that Taxotere can inhibit angiogenic factor-stimulated blood vessel formation *in vivo*, along with previous reports of similar observations for Taxol (22, 23). Furthermore, the observation that the inhibition of endothelial cell migration by Taxotere was associated with an inhibition of centrosome reorientation at concentrations that did not affect endothelial cell proliferation or gross microtubule morphology suggests that Taxotere may be inhibiting endothelial cell migration by a mechanism of action that differs from those that are thought to contribute to its effect on mitosis. In conclusion, Taxotere is a potent, effective, and specific inhibitor of endothelial cell migration *in vitro* and angiogenesis *in vitro* and *in vivo*, and its antiangiogenic activity may contribute to its well-documented clinical activity.

## Acknowledgments

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# Molecular Cancer Therapeutics

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