Antiangiogenic activity of paclitaxel is associated with its cytostatic effect, mediated by the initiation but not completion of a mitochondrial apoptotic signaling pathway

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Abstract

Angiogenesis is a critical event in tumor growth and metastasis, which can be inhibited by conventional anticancer drugs such as the microtubule-damaging agent paclitaxel (Taxol). In this study, we investigate the mechanism of action of paclitaxel on human endothelial cells. We characterize two distinct effects of paclitaxel on human umbilical vein endothelial cell and human microvascular endothelial cell-1 proliferation according to drug concentration: a cytostatic effect at low concentrations and a cytotoxic effect at concentrations 10 nmol/L. The cytotoxic effect involves signaling pathways similar to those described in tumor cells (i.e., microtubule network disturbance, G2-M arrest, increase in Bax/Bcl-2 ratio, and mitochondrial permeabilization) that result in apoptosis. In sharp contrast, the cytostatic effect involves an inhibition of endothelial cell proliferation without apoptosis induction and without any structural modification of the microtubule network. This cytostatic effect is due to a slowing of the cell cycle rather than to an arrest in a specific phase of the cell cycle. In addition, paclitaxel, at cytostatic concentrations, early initiates an apoptotic signaling pathway associated with increases in the mitochondrial reducing potential, mitochondrial membrane potential, p53 expression, and Bax/Bcl-2 ratio. However, this apoptotic pathway is stopped upstream of mitochondria permeabilization and it does not lead to endothelial cell death. Finally, we found that paclitaxel inhibits endothelial cell morphogenesis on Matrigel at all tested concentrations. In conclusion, we describe the mechanism of action of low concentrations of paclitaxel related to the antiangiogenic properties of this drug. [Mol Cancer Ther 2004;3(10):1301–10]

Introduction

Angiogenesis, which is defined as the formation of a neo-vascular blood supply derived from preexisting blood vessels, has been reported to be a critical step in tumor growth since 1971 (1). At that time, several pieces of evidence showed that angiogenesis is essential for the growth of both primary tumors and metastasis (2–5). The discovery of the angiogenesis dependence of tumor growth and spread has created new research avenues and it has led to the reexamination of several chemotherapeutic agents. In particular, microtubule-damaging agents, and especially taxanes and Vinca alkaloids, seem to be among the most potent antiangiogenic agents in vitro and in vivo (6, 7). Moreover, several new microtubule-damaging agents such as combretastatin A4 and some derivatives are in clinical trials for antiangiogenic therapies, with promising preliminary results (8).

Paclitaxel (Taxol) is a taxane widely used in chemotherapy, and it is highly efficacious in the treatment of breast, ovarian, and lung malignancies (9, 10). It stabilizes the microtubule network and inhibits microtubule dynamics in tumor cells (11–13). In general, this action on microtubules leads to programmed cell death (i.e., apoptosis) after cell cycle arrest at the metaphase-anaphase transition (13). Paclitaxel, like other microtubule-damaging agents, activates the intrinsic mitochondrial apoptotic pathway (14–16). It induces the loss of the mitochondrial membrane potential (ΔΨm) and the opening of the permeability transition pore leading to the release of proapoptotic factors such as cytochrome c, caspase cascade activation, and DNA fragmentation. These effects are modulated by the members of Bcl-2 family composed of proapoptotic proteins (i.e., Bax-like proteins and BH3-only proteins) and antiapoptotic proteins (i.e., Bcl-2-like proteins). Microtubule-damaging agents turn the balance of proapoptotic and antiapoptotic proteins toward apoptosis often by translocation of Bax from cytosol to mitochondria and/or by phosphorylation-mediated inhibition of Bcl-2 (17, 18).

We have shown previously that paclitaxel and Vinca alkaloids specifically act on mitochondria isolated from tumor cells to induce cytochrome c release via the permeability transition pore opening (19). This effect on mitochondrial permeability has also been described in purified liver mitochondria leading to an increase in the...
cytochrome oxidase–mediated reactive oxygen species production (20). We have found that tubulin is an inherent component of mitochondrial membranes (15) and a direct interaction between microtubule-damaging agents and isolated mitochondria has been shown recently (21). This direct effect of paclitaxel on mitochondria has also been described in whole cells (16, 20, 22). Altogether, these data strengthen the key role played by mitochondria in the mechanism of action of microtubule-damaging agents.

Paclitaxel inhibits several endothelial cell functions in vitro such as proliferation, migration, metalloprotease production, and morphogenesis (7, 23–25). In vivo, inhibition of these endothelial functions and direct cytotoxicity of paclitaxel abrogate tumor angiogenesis in multiple xenograft models (7, 24, 26–28). Interestingly, human endothelial cells seem to be more sensitive to paclitaxel than most of other cellular types (25, 28). Recently, Wang et al. (25) showed that paclitaxel inhibits endothelial cell growth at very low concentrations that induce neither mitotic block nor structural modification of the microtubule network. However, the mechanism responsible for this effect of low paclitaxel concentrations in endothelial cells has not been elucidated yet.

The aim of this study was to assess the effects of a wide range of paclitaxel concentrations on endothelial cell proliferation, microtubule network organization, capillary network formation, and apoptosis induction. Furthermore, we explored the behavior of mitochondria and the expression of the major proteins involved in the apoptotic intrinsic pathway. This study was done on two endothelial cell types, the immortalized human microvascular endothelial cell (HMEC-1), which display the phenotypical characteristics of both microvascular and macrovascular endothelial cells (29), and the human umbilical vein endothelial cells (HUVEC), which constitute the most studied macrovascular model for targeting antiangiogenic drugs (7, 23, 28, 30–34). We found that paclitaxel exerts two distinct effects on endothelial cell proliferation: a cytostatic effect at low concentrations and a cytotoxic effect at concentrations 10 nmol/L on both cell types. We also found that the mitochondrial apoptotic pathway is early initiated but fails to induce apoptosis at cytostatic concentrations. Altogether, our results point out the mechanism of action of low concentrations of paclitaxel related to the antiangiogenic properties of this drug.

Materials and Methods

Cell Culture and Drug

HUVEC and HMEC-1 were obtained from the Cell Culture Laboratory in the Hôtel de la Conception (Assistance Publique des Hôpitaux de Marseille, France). They were routinely maintained in culture at 37 °C and 5% CO2. HUVECs were grown in standard culture RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) containing 20% heat-inactivated fetal bovine serum (Life Technologies), 2 mmol/L glutamine (Life Technologies), 1% penicillin and streptomycin (Life Technologies), 50 IU/mL sodium heparin (Sanofi-Synthelabo, Paris, France), and 50 μg/mL endothelial cell growth supplement (BD Biosciences, Bedford, MA). HMEC-1 were grown in MCDB-131 medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin and streptomycin, 1 μg/mL hydrocortisone (Pharmacia & Upjohn, St-Quentin-Yvelines, France), and 10 ng/mL epithelial growth factor (R&D Systems, Minneapolis, MN). HUVECs were used between passages 2 and 5 and HMEC-1 between passages 3 and 12. Exponentially growing cells (2.5 × 105/mL) were seeded 24 hours before drug treatment on 0.1% gelatin-coated culture plates for all experiments, except for the Matrigel assay. The ratio of drug molar concentration to cell number was strictly identical for all experiments.

A stock solution of paclitaxel (Alexis Corp., Lausen, Switzerland) was prepared in DMSO and maintained frozen at −20°C. The highest final concentration of DMSO used in cell culture was 0.1%.

Growth Inhibition Assays

To measure growth inhibition using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded in 96-well plates and, after a 72-hour drug treatment, incubated 3 hours with 0.5 μg/mL MTT (35). The stain was eluted with 100 μL DMSO and absorbance was measured at 550 nm (MR 7-000, Dynatech, Denkendorf, Germany). Cell growth measurements were confirmed using the trypan blue exclusion method as described previously (15). Cell viability was expressed as the percentage of unstained (viable) cells divided by the total number of cells. Cell cycle time was determined as the time required to double viable cell number.

Detection of Apoptosis by Annexin V-FITC Staining

Following a 72-hour incubation, 5 × 105 cells were incubated with 4 μg/mL Annexin V-FITC and 5 μg/mL propidium iodide for 15 minutes (kit Annexin V-FITC, Bender Medsystems, Vienna, Austria) followed immediately by flow cytometry (FACScan, Becton Dickinson, Mississauga, Ontario, Canada). Cytogram analysis was done with Cell Quest Pro software (Becton Dickinson) as described previously (15).

Immunofluorescence Microscopy of Microtubule Network

Cells were grown on eight-well chamber slides (Labtek, Naperville, IL), incubated 24 hours with paclitaxel, fixed in formaldehyde 3.7% (15 minutes), and permeabilized with 0.1% saponin (30 minutes). Microtubule network was stained using an α-tubulin antibody (1:400, mouse monoclonal, Sigma-Aldrich, Steinheim, Germany) and a FITC-conjugated secondary antibody (Jackson ImmunoResearch, Baltimore, MD) as described previously (16). Finally, cells were observed using a DM-IRBE microscope (Leica, Bensheim, Germany) coupled with a digital camera (CCD camera coolscope FX, Princeton Instruments, Trenton, NJ).

Cell Cycle Analysis and Bromodeoxyuridine Incorporation Assay

To perform cell cycle analysis, cells were harvested, fixed in cold 70% methanol, and incubated with 120 μg/mL
propidium iodide immediately before analysis. DNA content was measured by flow cytometry (FACSscan, Becton Dickinson). Cytogram analysis was done with Mod-Fit software (Becton Dickinson) as described previously (36). For bromodeoxyuridine (BrdUrd) incorporation study, cells were pulsed with 20 μmol/L BrdUrd for 30 minutes at 37°C before drug treatment or after a 24-hour treatment for analysis at 24 and 48 hours, respectively. Twenty-four hours later, cells were fixed in cold 70% methanol and denatured in HCl as described previously (37). Cells resuspended in 0.5% PBS-Tween were incubated with a primary anti-BrdUrd antibody (Becton Dickinson) and then with a secondary FITC-conjugated antibody (Jackson ImmunoResearch). Finally, cells were resuspended in PBS containing 5 μg/mL propidium iodide and analyzed by flow cytometry.

Mitochondrial Function Analysis
For analysis of mitochondrial reducing potential, we did MTT assays after short times of treatment as described previously (33) with slight modifications. Solutions of paclitaxel were prepared in culture medium containing 0.5 mg/mL MTT and added on cells for 4 hours. Absorbance at 550 nm was measured as described above. For analysis of ΔΨm, cells were incubated with paclitaxel for 4 or 24 hours. Then, they were harvested, incubated with 100 nmol/L 5,5’-dihexyloxacarbocyanine iodide (DiOC6w, Molecular Probes, Leiden, Netherlands) for 30 minutes, and analyzed by flow cytometry (14). To ensure that DiOC6 uptake was specific for ΔΨm, we also treated cells with 50 μmol/L carbonyl cyanide m-chlorophenylhydrazone, which is a protonophore that dissipates the ΔΨm.

Western Blotting
After a 4- or 24-hour incubation with paclitaxel, cells were lysed for 10 minutes at 95°C in a lysis buffer [62.5 mmol/L Tris-HCl (pH 6.8), 0.5% SDS, 5% β-mercaptoethanol, 10% glycerol] and sonicated. Equal amounts of proteins were separated by using 8% to 15% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Membranes were blocked in low-fat milk and then probed with different antibodies. The primary antibodies used were anti-p53 (1:100, mouse monoclonal, DAKO, Glostrup, Denmark), Bax (1:50, mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (1:100, mouse monoclonal, DAKO), and α-tubulin antibody (1:1,000, mouse monoclonal, Sigma-Aldrich). Peroxidase-conjugated goat anti-mouse antibodies were used as secondary antibodies (Jackson ImmunoResearch). Visualization of proteins was accomplished using an enhanced chemiluminescence detection kit (Pharmacia Biosciences, Little Chalfont, United Kingdom) as described previously (38) and densitometric quantitation was done using Metamorph software (Universal Imaging Corp., Downingtown, PA) and the same microscope device as for immunofluorescence experiments. The formation of capillary networks was quantitatively evaluated by both measuring the total capillary tube length and counting the number of capillary tubes in 15 view fields per well using Metaview software.

Statistical Analysis
Sigma Stat software (Jandel Scientific, San Rafael, CA) was used to perform statistical analysis. Each experiment was done at least in triplicate and data were expressed as mean ± SD. Statistically significant difference between two conditions was retained for P < 0.05.

Results
Paclitaxel Exhibits Cytostatic and Cytotoxic Effects on Human Endothelial Cells
Paclitaxel inhibited growth of HUVEC and HMEC-1 in a time- and concentration-dependent manner. After a 72-hour incubation, the IC50 values for paclitaxel (i.e., the concentrations inhibiting 50% of cell growth) were 2 and 5 nmol/L for HUVEC and HMEC-1, respectively (Fig. 1A). The maximal effect (IC95) was obtained with 25 nmol/L paclitaxel in both cell types. To investigate whether the paclitaxel-induced growth inhibition was due to apoptosis, we did Annexin V-FITC staining. After a 72-hour incubation, paclitaxel induced apoptosis at concentrations 10 nmol/L for both HUVEC and HMEC-1 (Fig. 1B). However, lower concentrations of paclitaxel (<10 nmol/L) did not induce apoptosis at concentrations <10 nmol/L. Exponentially growing cells were incubated with a range of concentrations of paclitaxel for 72 hours. Following drug treatment, cell growth was assessed by using MTT test (A) and apoptosis was assessed by Annexin V-FITC staining and flow cytometry analysis (B). *, P ≤ 0.05, one-way ANOVA test.
not induce apoptosis (Fig. 1B) or necrosis (data not shown), although they significantly inhibited cell proliferation (Fig. 1A). In particular, the IC_{50} failed to induce apoptosis in both cell types. In addition, same experiments done after 96- and 120-hour incubation showed that apoptosis induction was not delayed and confirmed that paclitaxel concentrations <10 nmol/L failed to induce endothelial cell death (data not shown). These results suggested that paclitaxel exerted two distinct effects on human endothelial cell growth. At concentrations 10 nmol/L, paclitaxel had a cytotoxic effect with apoptosis induction. In sharp contrast, at concentrations <10 nmol/L, it induced a cytostatic effect defined as an inhibition of cell proliferation without apoptosis.

**Cytostatic Effect of Paclitaxel Is Associated with a Slowing of the Cell Cycle**

Microtubule-damaging agents generally induce modification of microtubule network and G2-M arrest in proliferating cells. Thus, we investigated whether the cytostatic and cytotoxic effects of paclitaxel could result from a structural modification of microtubule network and/or a G2-M arrest. After a 24-hour incubation with cytotoxic concentrations of paclitaxel, immunofluorescence experiments showed extensive modifications of the cell shape and microtubule network organization. We found in HMEC-1 (Fig. 2A, right) and HUVEC (data not shown) that most of cells incubated with 100 nmol/L paclitaxel exhibited mitotic spindles (arrows). By flow cytometry and Mod-Fit analysis, we found that 46% and 75% of HUVEC and HMEC-1, respectively, were arrested in G2-M phase after a 24-hour incubation with 10 nmol/L paclitaxel (Fig. 2B), confirming that cytotoxic concentrations of paclitaxel induced G2-M arrest of endothelial cells.

Interestingly, no modification of microtubule network organization was observed when cells were incubated with cytostatic concentrations (Fig. 2A, middle). Furthermore, there was no increase in G2-M arrested cells at cytostatic concentrations, as shown for 5 nmol/L in Fig. 2B. Similar experiments done after 48-hour treatment confirmed the absence of G2-M arrest at cytostatic concentrations and its presence at cytotoxic concentrations (data not shown).

BrdUrd incorporation experiment is a specific test of DNA synthesis and thus of cell transition through S phase. We did BrdUrd incorporation to analyze putative modifications of cell cycle progression, which could be responsible for the cytostatic effect of paclitaxel. As shown in Fig. 3A, paclitaxel induced a concentration-dependent increase in the percentage of BrdUrd-unlabeled cells after 24- and 48-hour incubation. This increase was observed for both HMEC-1 (Fig. 3A) and HUVEC (data not shown) at similar levels. The maximal effect was reached after 48-hour incubation with 5 nmol/L paclitaxel, with an increase in BrdUrd-unlabeled cells of 83 ± 4.1% and 68 ± 3.0% for HUVEC and HMEC-1, respectively, as compared with control cells (P < 0.05).

To determine whether this increase in BrdUrd-unlabeled cells was due to a G1 arrest or to a global slowing in cell cycle progression, we did propidium iodide complementary staining that enables the distinction between cells in G1 phase (DNA content = 2n) and cells in S or G2-M phase (DNA content > 2n). As shown in Fig. 3B, cytostatic concentrations of paclitaxel increased the percentage of BrdUrd-unlabeled HMEC-1 in G1 phase after both 24- and 48-hour incubation. These concentrations of paclitaxel also increased the percentage of BrdUrd-unlabeled HMEC-1 in S or G2-M phase after both 24- and 48-hour incubation (Fig. 3C). In addition, the concentration-dependent increases in the percentage of BrdUrd-unlabeled cells in G1 and S or G2-M phase were similar (P > 0.05) and no accumulation was noticed between 24- and 48-hour incubation (P > 0.05). Similar results were obtained with HUVEC (data not shown). Altogether, these results show that paclitaxel-induced cytostatic effect was due to a global slowing of the cell cycle rather than to an arrest in a specific phase of the cell cycle. The biological significance of these increases in BrdUrd-unlabeled cells...
was confirmed by the increase in the cell cycle time as determined by trypan blue staining and cell counting (24, 26, and 30 hours for control, 1 and 5 nmol/L paclitaxel-treated cells, respectively).

**Paclitaxel, at Cytostatic Concentrations, Disturbs Mitochondrial Functions Only at Early Time of Treatment**

Besides its effect on microtubules, paclitaxel directly acts on isolated mitochondria and can target mitochondria in intact cancer cells (16, 19–22). Here, we investigated both the early and the late effects of paclitaxel on mitochondria of endothelial cells. MTT can be reduced in formazan by mitochondrial dehydrogenases, especially by succinate dehydrogenase, thus being a marker of the mitochondrial reducing potential (33). Therefore, we did MTT reduction assays on both HUVEC and HMEC-1 after 4-hour incubation with paclitaxel to evaluate mitochondrial reducing potential in endothelial cells. As shown in Fig. 4A, paclitaxel induced significant increases in the reducing potential of mitochondria, with extents between 3.8 ± 0.9% and 9.7 ± 2.3% for HUVEC (P < 0.05) and between 9 ± 1.4% and 15.8 ± 3.9% for HMEC-1 (P < 0.05).

Then, we evaluated the ΔΨm by DiOC6 staining and flow cytometry analysis. After a 4-hour incubation with paclitaxel, the increase in ΔΨm uptake indicated a ΔΨm increase as compared with untreated cells (Fig. 4B). This hyperpolarization of mitochondrial membrane was observed at both cytostatic and cytotoxic concentrations, with increases in extent between 8.75 ± 5.1% and 19.9 ± 4.9% for HUVEC (P < 0.05) and between 10.2 ± 4.2% and 19.2 ± 5.3% for HMEC-1 (P < 0.05). As expected, treatment with m-chlorophenylhydrazone induced a significant decrease in ΔΨm as shown in Fig. 4B, indicating that DiOC6 was localized in mitochondria and that its uptake really reflected ΔΨm.

This early increase in ΔΨm is described as transient and it is generally followed by the loss of ΔΨm (39). This disruption is usually associated with the opening of the mitochondrial permeability transition pore and with the subsequent release of apoptotic factors such as cytochrome c (39). To investigate whether the early increase in ΔΨm detected after 4-hour incubation was followed by its disruption, we did DiOC6 staining after 24-hour incubation with paclitaxel. As shown by the appearance of depolarized cells on Fig. 4C, cytotoxic concentrations of paclitaxel induced the loss of ΔΨm for both HUVEC and HMEC-1. In sharp contrast, cytostatic concentrations failed to induce ΔΨm collapse as shown by similar profiles obtained with control and 5 nmol/L paclitaxel-treated cells. As for cells incubated during 4 hours, m-chlorophenylhydrazone addition induced a significant decrease in ΔΨm as shown in Fig. 4B, confirming that DiOC6 uptake reflected ΔΨm.

In conclusion, at both cytostatic and cytotoxic concentrations, paclitaxel disturbed mitochondrial functions at early time points. However, mitochondrial disturbance led to ΔΨm disruption and thus apoptosis only at cytotoxic concentrations.

**Paclitaxel, at Cytostatic Concentrations, Induces Modifications in Bcl-2 and p53 Expression Only at Early Time of Treatment**

To understand why early disturbances of mitochondria were insufficient to initiate apoptosis at cytostatic concentrations, we evaluated changes in the expression of Bcl-2 and Bax, which are significantly involved in the balance of proapoptotic and antiapoptotic signals at the mitochondrial level (40). Western blot quantitative analysis was done after 4-hour incubation, when mitochondrial hyperpolarization was observed for all tested concentrations, and after 24-hour incubation, when extensive mitochondrial depolarization was induced only by the cytotoxic concentrations. As shown in Fig. 5A, after 4-hour incubation, Bcl-2 expression was decreased by 62% (P < 0.05) at cytostatic concentrations (1 and 5 nmol/L) and became almost undetectable (94% decrease; P < 0.05) at the cytotoxic

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**Figure 3.** Paclitaxel induced a cell cycle slowing at cytostatic concentrations. Exponentially growing HMEC-1 were pulsed with 20 µmol/L BrdUrd for 30 minutes before paclitaxel incubation or after a 24-hour incubation for measurements at 24- and 48-hour incubation, respectively. Percentage of BrdUrd-unlabeled cells ([BrdUrd (-)]) was determined by flow cytometry. Incubation with cytostatic concentrations of paclitaxel (24 and 48 hours) induced an increase in the total percentage of BrdUrd-unlabeled HMEC-1 (A), percentage of BrdUrd-unlabeled HMEC-1 in G1 phase (B), and percentage of BrdUrd-unlabeled HMEC-1 in S or G2-M phase (C). **, P < 0.05, one-way ANOVA test.
concentration (100 nmol/L). At this time of treatment, Bax expression remained unchanged. Thus, through Bcl-2 down-regulation, paclitaxel induced an early increase in the Bax/Bcl-2 ratio in favor of apoptosis induction at both cytostatic and cytotoxic concentrations. The p53 protein has been described to be involved in the mechanism of action of paclitaxel (41, 42). In addition, it has been shown to down-regulate Bcl-2 and to transactivate Bax gene (43), leading us to investigate putative modifications of its expression. As shown in Fig. 5A, paclitaxel induced a 2-fold increase in p53 expression at concentrations 1 nmol/L as early as 4-hour treatment. Altogether, the increase in Bax/Bcl-2 ratio, p53 expression, mitochondrial reducing potential, and ΔΨm indicated that paclitaxel rapidly initiated mitochondrial apoptotic signaling pathway at both cytostatic and cytotoxic concentrations.

Similar experiments done after 24-hour incubation (Fig. 5B) showed that Bcl-2 expression was recovered at cytostatic concentrations because it was no longer different as compared with control cells (P > 0.05). In contrast, at cytotoxic concentration, Bcl-2 expression level remained low (48% decrease as compared with control cells; P < 0.05). Bax expression was not modified at any tested concentrations (data not shown). As a consequence, a high Bax/Bcl-2 ratio in favor of apoptosis was maintained only at cytotoxic concentration. In parallel, p53 expression was no longer induced after 24-hour incubation with paclitaxel at any tested concentrations.

Paclitaxel Exhibits In vitro Antiangiogenic Activity at Cytostatic Concentrations

To determine which paclitaxel concentrations display a potent antiangiogenic activity, we did in vitro Matrigel assay on HUVECs. Photographs were taken and analyzed after 6- and 24-hour incubation with a wide range of paclitaxel concentrations. As shown in Fig. 6A, paclitaxel inhibited morphogenesis of HUVEC in a concentration-dependent manner as soon as 6-hour incubation. In vitro angiogenesis inhibition was confirmed by counting total

Figure 4. Paclitaxel differently disturbed mitochondrial functions at cytostatic and cytotoxic concentrations. Exponentially growing cells were incubated with a range of paclitaxel concentrations for 4 hours and mitochondrial reducing potential was assessed by using MTT test (AU, arbitrary unit; A). Paclitaxel induced an increase in the reducing potential of mitochondria at cytostatic and cytotoxic concentrations. ΔΨm was also assessed by DiOC6 staining followed by flow cytometry analysis (AU) after 4-hour (B) or 24-hour (C) incubation with paclitaxel. Paclitaxel induced an hyperpolarization of mitochondrial membrane at cytostatic and cytotoxic concentrations (B). m-Chlorophenylhydrazone (CCCP; 50 μmol/L) was used as a negative control. *, P < 0.05, one-way ANOVA test. Paclitaxel induced depolarization of mitochondrial membrane only at cytotoxic concentration (C). Percentage of depolarized cells (with disrupted ΔΨm) is noticed.
capillary tubes and by measuring their total length in several random view fields. As shown in Fig. 6B and C, paclitaxel reduced the number of closed capillary tubes as well as their length. Interestingly, paclitaxel antiangiogenic activity appeared from 0.1 nmol/L (25 ± 7% and 20 ± 3% decreases for vessel count and capillary tube length, respectively; \( P < 0.05 \)) and the maximal effect was reached at 10 nmol/L (77 ± 4% and 52 ± 3% decreases, respectively; \( P < 0.05 \)). Similar results were obtained after 24-hour incubation (data not shown), confirming that paclitaxel exhibited potent antiangiogenic activity at both cytostatic and cytotoxic concentrations.

**Discussion**

In the current study, we show that the antiangiogenic activity of paclitaxel is associated with a cytostatic effect characterized by the initiation but not completion of an apoptotic mitochondrial pathway. We describe this phenomenon on both an established cell line and a primary culture of human endothelial cells to extend our results to the whole endothelial phenotype.

We found that paclitaxel exerts, between 1 and 10 nmol/L, a cytostatic effect characterized by a strong inhibition of proliferation (up to 70%) without any detectable modification of the microtubule network organization, without G2-M arrest, and without apoptosis induction. To our knowledge, it has never been described in cancer cells that a high inhibition of cell proliferation induced by microtubule-damaging agents involves only a cytostatic effect. Indeed, in cancer cells, microtubule-damaging agents generally induce G2-M arrest and subsequent apoptosis (44). Moreover, when they do not induce mitotic block (14), cells seem to be blocked in another phase of the cell cycle, particularly in G1 phase (37, 41).

Interestingly, these cytostatic concentrations prevent angiogenesis *in vitro*, arguing for a relationship between inhibition of proliferation and antiangiogenic effects of paclitaxel. This link was recently suggested by Wang et al. (25), but they concluded in a G1 arrest of cells treated with cytostatic concentrations. Although we obtained similar profiles by flow cytometry, our complementary BrdUrd incorporation experiments lead us to another conclusion. In particular, the absence of accumulation of BrdUrd-unlabeled cells in G1 phase, between 24- and 48-hour treatment, strongly suggest a global slowing of the cell cycle responsible for cytostatic effect rather than a G1 arrest. In addition, this slowing of the cell cycle was confirmed by the paclitaxel-induced increase in the cell cycle time.

Elsewhere, because we and others have shown previously that mitochondria play a key role in the mechanism of action of microtubule-damaging agents (16, 19, 20, 37), we investigated their behavior in paclitaxel-treated endothelial cells. We showed early disturbance of mitochondrial functions (i.e., increases in the mitochondrial reducing potential and \( \Delta \Psi_m \)) associated with the cytostatic effect of paclitaxel. These modifications of mitochondrial activity are transient and they are described to be rapidly followed by the \( \Delta \Psi_m \) collapse and the subsequent release of proapoptotic factors from mitochondria to cytosol (39). However, this \( \Delta \Psi_m \) disruption did not occur at cytostatic concentrations in our study. Thus, although the mitochondrial apoptotic pathway leads to endothelial cell death at cytotoxic concentrations, the same signaling pathway is initiated at cytostatic concentrations but stopped upstream of mitochondrial permeabilization.

We can propose several mechanisms responsible for this incomplete mitochondrial apoptotic pathway. On one hand, the absence of structural modification of microtubule network organization might prevent the activation of factors required for the fulfillment of apoptotic pathways. For instance, disruption of microtubule cytoskeleton is necessary for microtubule-sequestered proapoptotic proteins such as Bim to translocate to mitochondria and fully activate mitochondrial endogenous effectors (45). Similarly, it has been shown that disturbances of microtubule network integrity induce various signals, including c-Jun NH2-terminal kinase and Raf-1 activation, which can promote apoptosis (46, 47). On the other hand, the absence of cell cycle arrest could also be involved. In fact, cell cycle arrest triggers a sustained activation of cyclin-dependent kinases, such as p34<sub>cdc2</sub>, capable of inducing activation of proapoptotic proteins and subsequent apoptosis (48). Our

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Paclitaxel early modified Bax/Bcl-2 ratio and p53 expression at cytostatic and cytotoxic concentrations. Exponentially growing HUVECs were incubated with a range of concentrations of paclitaxel for 4 hours (A) or 24 hours (B); then p53, Bcl-2, and Bax expression was assessed by Western blotting. Bcl-2 expression was decreased, whereas p53 expression was increased at cytostatic and cytotoxic concentrations after 4-hour incubation (A). α-Tubulin expression was used as a control. Bcl-2 expression was recovered at cytostatic concentration and remained low at cytotoxic concentrations, whereas p53 expression was no longer induced after 24-hour incubation (B).
results support the idea that antiangiogenic activity of paclitaxel arises, at least in part, from modulation of mitochondrial functions in endothelial cells as described previously for subapoptotic concentrations of a peptide trivalent arsenical (49).

Interestingly, coinciding with the increase in DC\textsubscript{m}, we found that Bcl-2 expression was decreased at early times of treatment, which is normally associated with apoptosis initiation (50). The simultaneous induction of p53, known to down-regulate Bcl-2, could be responsible for this Bcl-2 level modulation (43). Moreover, p53 induction and $\Delta$V\textsubscript{m} fluctuations observed under paclitaxel treatment could be related through reactive oxygen species (51). Thus, paclitaxel-induced p53 induction could be responsible for the early increase in both Bax/Bcl-2 ratio and $\Delta$V\textsubscript{m}. However, after 4-hour incubation with 100 nmol/L paclitaxel, the discrepancy between the induction of p53 and the decrease in Bcl-2 expression level suggests that p53 is not the sole factor responsible for Bcl-2 down-regulation. Interestingly, after 24-hour incubation with cytostatic concentrations, Bcl-2 expression remains 48% lower than in control cells. Moreover, it has been largely shown that Bcl-2 is inactivated by phosphorylation when cells are arrested in G\textsubscript{2}-M (52). Thus, at cytotoxic concentration, the remaining Bcl-2 protein may be inactivated by phosphorylation keeping the Bax/Bcl-2 balance in favor of apoptosis. These modifications of mitochondrial functions and Bax/Bcl-2 ratio are responsible for the mitochondrial permeabilization and thus for the $\Delta$V\textsubscript{m} disruption leading to apoptosis. Altogether, our data show that, at cytotoxic concentrations, paclitaxel acts on endothelial cells through a “classic” mechanism that was described previously in tumor cells (13, 14, 16, 20).

By comparison with astrocytes, fibroblasts, keratinocytes, epithelial cells, and smooth muscle cells, Wang et al.

Figure 6. Paclitaxel inhibited in vitro capillary network formation. Paclitaxel inhibited morphogenesis of HUVEC on Matrigel in a concentration-dependent manner after 6-hour incubation (A). Bar, 20 $\mu$m. Mean of total capillary tube number per view field (B) and mean of total capillary tube length per view field (C) was decreased by paclitaxel at 6-hour incubation. Fifteen random view fields per well were examined. *, $P \leq 0.05$, **, $P \leq 0.001$, Student’s test.
showed that the cytostatic effect of paclitaxel was selective for the endothelial cell type. This distinct sensitivity of endothelial cells versus nonendothelial cells to low concentration of paclitaxel suggests the presence of specific targets in human endothelial cells (25). Interestingly, it has been recently shown that protracted low-dose regimens of chemotherapeutic drug induced an increase in thrombospondin 1 expression in human endothelial cells (53). Thrombospondin 1 is a well known, highly specific, and potent endogenous inhibitor of angiogenesis (54) and its induction could be responsible for the antiangiogenic effects of at least some low-dose metronomic chemotherapy regimens (52). Therefore, we could hypothesize a putative involvement of thrombospondin 1 in cytostatic effect of paclitaxel. Similarly, we can hypothesize the existence of specific signaling pathways (e.g., proangiogenic factors such as vascular endothelial growth factor) that could explain the cytostatic effect of paclitaxel on human endothelial cells. In fact, this cytostatic effect seems to be specific of paclitaxel as docetaxel induced G2-M arrest and subsequent apoptosis from IC50 for cell proliferation.4 In addition, one can propose that microtubule composition (i.e., tubulin isotypes and post-translational modifications as well as microtubule-associated and related proteins) could be specific for the endothelial phenotype. To answer this question, proteomic analysis of endothelial microtubule network is in progress. Elsewhere, one might surmise that even if no modification of microtubule network organization was observed after incubation with cytostatic concentrations, microtubule dynamic instability could be disturbed by such concentrations, as this variable is the most sensitive to microtubule-damaging agents (13). Therefore, the study of microtubule dynamics in living human endothelial cells is in progress.

Altogther, our results suggest a relationship of microtubule network functions, regulation of cell cycle progression, and mitochondrial integrity in the antiangiogenic activity of paclitaxel. Further investigation will be necessary to fully characterize the proteins of interest that can be potential targets for improved antiangiogenic therapeutics.

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Cytostatic Effect of Paclitaxel on Endothelial Cells


Antiangiogenic activity of paclitaxel is associated with its cytostatic effect, mediated by the initiation but not completion of a mitochondrial apoptotic signaling pathway

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