

# Antiangiogenic Concentrations of Vinflunine Increase the Interphase Microtubule Dynamics and Decrease the Motility of Endothelial Cells

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## Abstract

**Angiogenesis is a key event in tumor progression and metastasis. This complex process, which constitutes a potent target for cancer therapy, is inhibited by very low concentrations of microtubule-targeting drugs (MTD). However, the intimate mechanisms of the antiangiogenic activity of MTDs remain unclear. Recently, we have shown that low antiangiogenic and noncytotoxic concentrations of paclitaxel induced an unexpected increase in microtubule dynamics in endothelial cells. In this study, we showed that vinflunine, the newest *Vinca* alkaloid, increased microtubule dynamic instability in human endothelial cells after 4-hour incubation at low concentrations (29% and 54% at 0.1 and 2 nmol/L). The growth and shortening rates were increased, and the percentage of time spent in pause and the mean duration of pauses were decreased, as previously observed with paclitaxel. As opposed to paclitaxel, the transition frequencies were not significantly disturbed by vinflunine. Moreover, low concentrations of vinflunine did not affect mitotic index and anaphase/metaphase ratio. Interestingly, these low vinflunine concentrations that increased microtubule dynamics exhibited an antiangiogenic effect through the inhibition of both morphogenesis and random motility. Capillary tube formation on Matrigel was decreased up to 44%. The cell speed and the random motility coefficient were decreased (13% and 19% and 13% and 33% at 0.1 and 2 nmol/L, respectively) and the persistent time was statistically increased. Altogether, our results confirm that the increase in microtubule dynamics is involved in MTD antiangiogenic activity and highlight the crucial role of interphase microtubule dynamics in angiogenesis.** (Cancer Res 2006; 66(6): 3256-63)

## Introduction

Microtubules, key components of the cytoskeleton, are dynamic polymers of tubulin that form a well-organized network of polarized tube filaments. Microtubule dynamics are highly regulated both spatially and temporally and this regulation is crucial for mitosis, cell migration, cell signaling, and trafficking (1). Microtubule-targeting drugs (MTD), e.g., *Vinca* alkaloids and taxanes, are major cancer chemotherapeutic drugs largely used in many human tumoral diseases. Vinflunine, the newest *Vinca*

alkaloid, currently in phase III clinical trials (2), is a fluorinated compound obtained by superacid chemistry (3). Similar to other MTDs, vinflunine suppresses microtubule dynamics both *in vitro* (4, 5) and in living cancer cells (4, 6). It classically slows down the metaphase-to-anaphase transition, blocks cancer cells in mitosis, and induces apoptosis (6–9).

In the last few years, the targeting of the tumor-associated blood vessels, required for invasive tumor growth and metastasis, has become a valuable new approach for cancer therapy (10, 11). Angiogenesis, the sprouting of new blood vessels from preexisting ones, enables tumor vasculature formation. This is a complex process that encompasses a series of cellular events of endothelial cells, such as migration, proliferation, and differentiation. Human endothelial cells have been shown to be very sensitive to MTDs compared with epithelial cells or other anticancer drugs (12, 13). In fact, angiogenesis can be inhibited by very low concentrations of MTDs (13–16) without any mitotic cell block or apoptosis induction (17, 18). Endothelial cells, seeded on specific substrate, such as Matrigel, spread and align with each other to form branching anastomosing tubes with multicentric junctions that give rise to a meshwork of capillary-like structures. This morphogenesis is strongly inhibited by low concentrations of MTDs as effectively *in vitro* (13, 14, 16–18) as *in vivo* (15, 19). Moreover, noncytotoxic concentrations of MTDs can also inhibit endothelial cell migration and chemotaxis (12, 14, 19, 20). However, the intimate mechanisms of these antiangiogenic perturbations in endothelial cell functions remain unclear. Recently, we showed that low and noncytotoxic concentrations of paclitaxel induced an unexpected increase in microtubule dynamics in endothelial cells and strongly inhibited angiogenesis (21).

To determine whether this observation may be extended to other MTDs, we investigated the effect of the newest *Vinca* alkaloid, vinflunine, on microtubule dynamics in living human dermal microvascular endothelial cells (HMEC-1) and their involvement on cellular processes that are required for angiogenesis (i.e., proliferation, migration, and morphogenesis). We found that low concentrations of vinflunine increased microtubule dynamic instability. On one hand, vinflunine increased the growth and shortening rates and decreased both the time spent in pause and the mean duration of pauses, as did paclitaxel. On the other hand, vinflunine did not significantly affect transition frequencies as opposed to paclitaxel. We also showed that, in addition to its antivasculature effect at cytotoxic concentrations (22), vinflunine, at low concentrations, exhibits an antiangiogenic effect through the inhibition of both migration and morphogenesis of HMEC-1. Altogether, our results confirm that the increase in microtubule dynamics is involved in the antiangiogenic activity of MTDs and highlight the crucial role of interphase microtubule functions in angiogenesis.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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## Materials and Methods

**Cell culture and drug.** HMEC-1 cell line was obtained from the Cell Culture Laboratory in the Hôpital de la Conception (Assistance Publique, Hôpitaux de Marseille, Marseille, France). Cells were routinely maintained at 37°C and 5% CO<sub>2</sub> in MCDB-131 medium (Gibco, Cergy-Pontoise, France) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin and streptomycin (all from Life Technologies, Paisley, United Kingdom), 1 µg/mL hydrocortisone (Pharmacia&Upjohn, St-Quentin-Yvelines, France), and 10 ng/mL epithelial growth factor (R&D Systems, Minneapolis, MN). HMEC-1 cells were used between passages 3 and 12. For all experiments, exponentially growing cells were seeded on culture plates coated with 0.1% gelatin (Sigma-Aldrich, Steinheim, Germany) and the ratio of drug molar concentration to cell number was the same.

A stock solution of vinflunine (Pierre Fabre Oncology, Toulouse, France) was prepared in distilled water and maintained frozen at -20°C.

**Transfection of HMEC-1 with green fluorescent protein- $\alpha$  tubulin plasmid.** For transfection of HMEC-1, we used a plasmid pEGFP-Tub (Clontech, Palo Alto, CA) encoding a fusion protein consisting of the human codon-optimized variant of green fluorescent protein and the gene encoding human  $\alpha$ -tubulin.

HMEC-1 ( $8 \times 10^5$  cells) were resuspended in 100 µL of the specific electroporation R buffer (Amaxa, Cologne, Germany). Eight micrograms of plasmid DNA were added to the cell suspension, transferred to a 2.0 mm electroporation cuvette, and nucleofected with Amaxa Nucleofector (Amaxa) using program number T-016. DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After electroporation, cells were immediately transferred into RPMI medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% penicillin and streptomycin, 1 µg/mL hydrocortisone, and 10 ng/mL epithelial growth factor. Cells were seeded in six-well plates. Twenty-four hours later, cells were incubated for 4 hours with vinflunine and microtubule dynamics measurements were done.

**Time-lapse microscopy and analysis of microtubule dynamic instability.** Transfected cells were placed in RPMI culture medium lacking sodium bicarbonate and supplemented with 25 mmol/L HEPES and 4.5 g/L glucose. Thirty microliters Oxyrase per milliliter (Oxyrase, Inc., Mansfield, OH) were added to reduce photodamage. Transfected cells were placed in a double coverslip chamber maintained at  $37 \pm 1^\circ\text{C}$ , and observed using a fluorescence microscope (Leica DM-IRBE, Leica Microsystems, Rueil-Malmaison, France),  $\times 100$  objective lens. Thirty-one images per cell were acquired at 4-second intervals using a digital camera (charge-coupled device camera coolsnap FX; Princeton Instruments, Trenton, NJ) driven by Metamorph software (Universal Imaging Corporation, Downingtown, PA) as previously described (6).

For analysis of microtubule dynamic instability, the positions of the plus ends of individual microtubules with time were recorded and analyzed using Metamorph software as previously described (23). Changes in length  $\geq 0.5$  µm were considered as growth or shortening events. Changes in length  $< 0.5$  µm were considered as phases of attenuated dynamics or pauses. The rates of growth and shortening events were determined by linear regression. Means and SEs were calculated per event. The catastrophe frequency based on time was calculated by dividing the number of transitions from growth or pause to shortening by the total time growing and paused for each individual microtubule. The rescue frequency based on time was calculated similarly, dividing the total number of transitions from shortening to pause or growth by the time spent shortening for each individual microtubule. Means and SE of transition frequencies were calculated per microtubule ( $n > 30$  for each experimental condition from three independent experiments). Dynamicity is the total length grown and shortened divided by the life span of microtubule population (24).

**Growth inhibition assay and cell cycle analysis by flow cytometry.** 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 mg/mL MTT (Sigma-Aldrich; ref. 17). The stain was eluted with 100 µL DMSO and absorbance was measured at 550 nm.

To perform cell cycle analysis, cells were harvested, fixed in 70% cold methanol, and incubated with 120 µg/mL propidium iodide immediately before analysis. DNA content was measured by flow cytometry (FACScan; BD Biosciences, San Jose, CA) as previously described (6).

**Mitotic index and metaphase to anaphase transition.** As previously described (21), exponentially growing HMEC-1 were seeded, 24 hours before drug treatment, on eight-well chamber slides (Labtek, Naperville, IL) previously coated with 0.1% gelatin. After 24-hour incubation with vinflunine, cells were fixed in 3.7% formaldehyde for 30 minutes. After fixation, cells were washed in PBS and incubated for 2 minutes with 0.25 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Finally, cells were observed using the video microscopy platform and 500 cells per experimental conditions were counted as previously described (6).

**In vitro capillary network formation on Matrigel.** A 24-well culture plate was coated with 250 µL of 5.25 mg/mL Matrigel (BD Biosciences, Pont de Claix, France) at 4°C. Matrigel was then allowed to solidify for 1 hour at 37°C before cell seeding. One hour later, vinflunine was added and photographs were taken after 4-hour incubation using our video microscopy platform. The formation of capillary networks was quantitatively evaluated by measuring the total capillary tube length in 20 view fields per well using Metamorph software as previously described (17).

**Random motility measurements.** Cells were seeded in 24-well culture plate and treated with vinflunine 1 hour later. Plates, maintained at 37°C, were positioned on the motorized stage on the inverted microscope (Leica DM-IRBE). The motorized stage advanced to preprogrammed locations and a photograph was taken for each place at 10-minute intervals. Twenty HMEC-1 cells were tracked for 4 hours. The average cell speed ( $S$ ), the coordinates of the nuclei centroids, and the maximal distance to origin reached by them ( $D_{\max}$ ) were computed by the Metamorph software. The persistent random-walk model was used to characterize cell motility (25, 26). For each cell, the mean square displacement ( $\langle D^2 \rangle$ ) was calculated from the following formula:

$$\langle D^2 \rangle = \sum_{i=1}^M d_i^2$$

where  $d_i$  is the displacement of the cell from its initial position at time level  $t = i\Delta t$ .

From this value, the random motility coefficient ( $\mu$ ) that reflects cell ability to migrate and to colonize a new area, and the persistence time ( $P$ ), which constitutes the average time between significant direction changes, were calculated as follows:

$$\langle D^2 \rangle = 2n\mu t = nS^2Pt$$

where  $t$  is time,  $S$  is the average cell speed, and  $n = 2$  in our two-dimensional walk model.

**Statistical analysis.** Sigma Stat software (Jandel Scientific, San Rafael, CA) was used to perform statistical analysis. Each experiment was done at least in triplicate, data were expressed as mean  $\pm$  SE, and the statistical test used was indicated in each figure legend and table. Statistically significant difference between conditions was retained for  $P < 0.05$ .

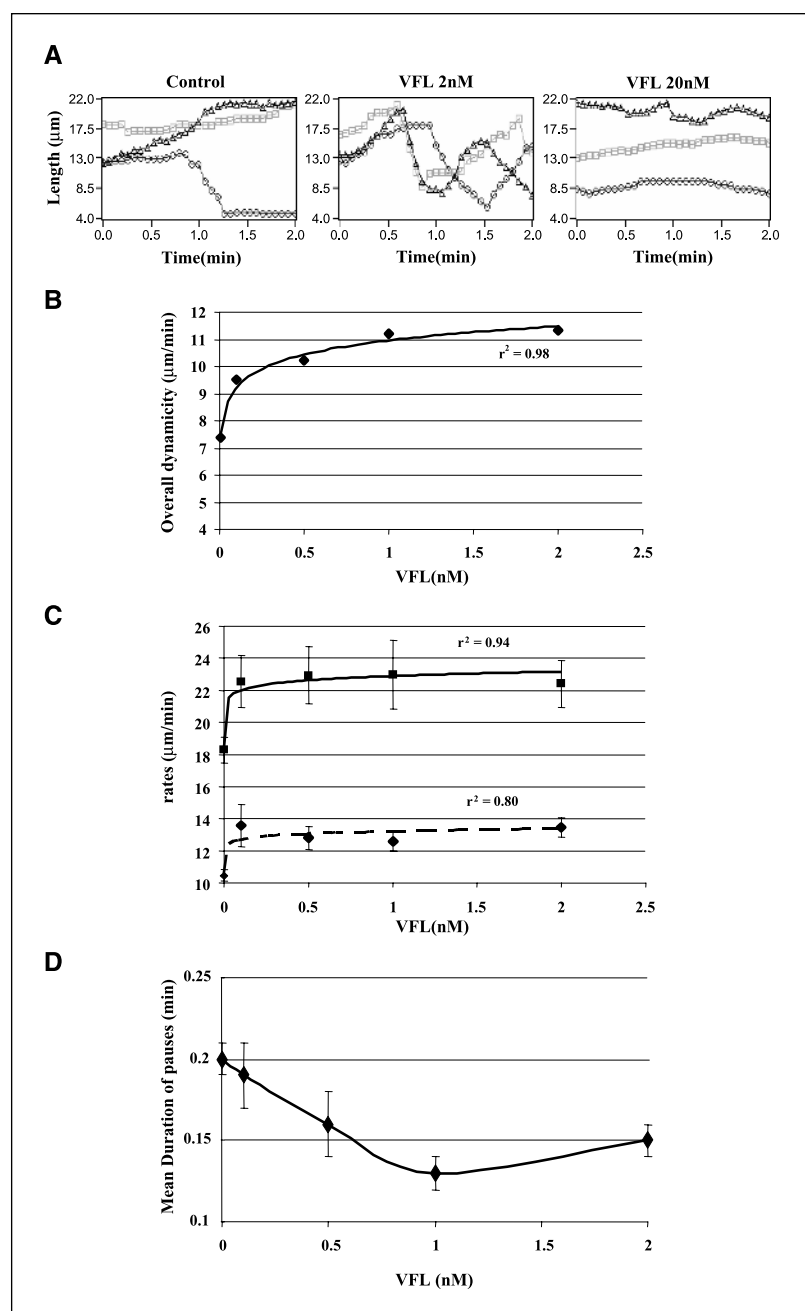
## Results

**Low concentrations of vinflunine increased microtubule dynamics in living human endothelial cells.** We have previously shown that paclitaxel, at very low concentrations, increased microtubule dynamics in endothelial cells (21). Consequently, we first determined the effects of a wide range of vinflunine concentrations (0.1-20 nmol/L) on microtubule dynamic instability in living endothelial cells.

HMEC-1 cells were transiently transfected by electroporation with green fluorescent protein- $\alpha$  tubulin and the dynamic properties of their microtubules were analyzed by time-lapse fluorescence microscopy following a 4-hour vinflunine incubation.

In control cells, microtubules alternated between phases of growth, shortening, and pause (a state of attenuated dynamic instability; Fig. 1A, left; Supplementary Data). As shown in Table 1, the plus ends of control microtubules grew at a mean rate of  $10.3 \pm 0.3 \mu\text{m}/\text{min}$ , slower than their mean shortening rate of  $18.8 \pm 1.0 \mu\text{m}/\text{min}$ . The mean lengths grown and shortened were  $1.82 \pm 0.09$  and  $2.73 \pm 0.18 \mu\text{m}$ , respectively. Microtubules spent almost half of their time (45.4%) in a paused state neither growing nor shortening to a detectable extent. The overall microtubule dynamicity was  $7.4 \mu\text{m}/\text{min}$ . Interestingly, the overall dynamicity of microtubules was increased by vinflunine concentrations  $<5 \text{ nmol}/\text{L}$  compared with control cells (+29%, +39%, +52%, and +54% for 0.1, 0.5, 1, and 2 nmol/L, respectively; Fig. 1A, middle; Supplementary Data; Table 1). This increase in overall dynamicity varied logarithmically accord-

ing to the vinflunine concentration (Fig. 1B). Several specific variables were affected. First, vinflunine increased logarithmically both the growth and shortening rates (Fig. 1C) and decreased both the percentage of time spent in pause from 45.4% to 34.8% (Table 1) and the duration of pauses from 0.20 to 0.15 minutes (Fig. 1D; Table 1). Low concentrations of vinflunine failed to significantly disturb the time-based transition frequencies, except for 2 nmol/L that increased the catastrophe frequency (Table 1). The increase in microtubule overall dynamicity was confirmed in living HMEC-1 cells microinjected with rhodamine-labeled tubulin and treated with 2 nmol/L vinflunine (+64%, data not shown). In addition, the different dynamic instability variables were similarly affected in both microinjected and transfected HMEC-1 (no significant difference, data not shown).



**Figure 1.** In living HMEC-1, microtubule dynamics were increased and suppressed by 2 and 20 nmol/L vinflunine, respectively. Life history plots of the changes in length of three representative microtubules ( $\Delta$ ,  $\circ$ ,  $\square$ ) in the absence of drug (left) or after 4-hour incubation with 2 nmol/L (middle) or 20 nmol/L (right) vinflunine (VFL) show the differences between the three conditions (A). Concentration dependence for the effects of low concentrations of vinflunine on the overall dynamicity (B), on the growth ( $\blacklozenge$ ) and shortening ( $\blacksquare$ ) rates (C), and on the mean duration of pauses (D). Points, mean for at least 30 microtubules for each condition; bars, SE.

**Table 1.** Effects of vinflunine on microtubule dynamic instability variables in living HMEC-1

Variables	Control	0.1 nmol/L (%)	0.5 nmol/L (%)	1 nmol/L (%)	2 nmol/L (%)	5 nmol/L (%)	20 nmol/L (%)
Mean rates ( $\mu\text{m}/\text{min}$ )							
G	10.3 $\pm$ 0.3	13.6 $\pm$ 1.3 (+31.5)*	12.8 $\pm$ 0.7 (+24.0)*	12.6 $\pm$ 0.6 (+22.0)*	13.5 $\pm$ 0.6 (+30.3) <sup>†</sup>	8.9 $\pm$ 0.5 (-13.2) <sup>‡</sup>	8.4 $\pm$ 0.8 (-18.6) <sup>‡</sup>
S	18.8 $\pm$ 1.0	22.6 $\pm$ 1.7	22.9 $\pm$ 1.8 (+22.2) <sup>‡</sup>	23.0 $\pm$ 2.1 (+22.5) <sup>‡</sup>	22.4 $\pm$ 1.4 (+19.4) <sup>‡</sup>	12.5 $\pm$ 0.9 (-33.6) <sup>†</sup>	16.2 $\pm$ 1.6
Mean duration (min)							
G	0.18 $\pm$ 0.01	0.19 $\pm$ 0.02	0.19 $\pm$ 0.02	0.18 $\pm$ 0.02	0.15 $\pm$ 0.01 (-16.7) <sup>‡</sup>	0.15 $\pm$ 0.01	0.18 $\pm$ 0.01
S	0.15 $\pm$ 0.01	0.14 $\pm$ 0.01	0.16 $\pm$ 0.02	0.14 $\pm$ 0.01	0.14 $\pm$ 0.01	0.11 $\pm$ 0.01 (-26.7) <sup>†</sup>	0.17 $\pm$ 0.02
P	0.20 $\pm$ 0.01	0.19 $\pm$ 0.02	0.16 $\pm$ 0.02 (-20.0) <sup>‡</sup>	0.13 $\pm$ 0.01 (-35.0) <sup>†</sup>	0.15 $\pm$ 0.01 (-25.0)*	0.18 $\pm$ 0.02	0.21 $\pm$ 0.02
Mean length ( $\mu\text{m}$ )							
G	1.82 $\pm$ 0.09	2.54 $\pm$ 0.20 (+39.4) <sup>‡</sup>	2.47 $\pm$ 0.22 (+35.9) <sup>‡</sup>	2.33 $\pm$ 0.19	2.02 $\pm$ 0.10 (+10.9)*	1.37 $\pm$ 0.13	1.51 $\pm$ 0.07
S	2.73 $\pm$ 0.18	3.09 $\pm$ 0.31	3.78 $\pm$ 0.37	3.32 $\pm$ 0.31	3.24 $\pm$ 0.19 (+18.3) <sup>‡</sup>	1.37 $\pm$ 0.12 (-49.9) <sup>†</sup>	2.71 $\pm$ 0.19
Percentage time spent							
G	34.0	34.8	41.1	44.1	36.8	32.6	31.8
S	20.6	21.2	21.6	24.5	28.4	16.9	19.5
P	45.4	44.0	37.3	31.4	34.8	50.5	48.7
Catastrophe frequency ( $\text{min}^{-1} \pm \text{SE}$ )	2.22 $\pm$ 0.18	2.85 $\pm$ 0.52	3.12 $\pm$ 0.96	3.05 $\pm$ 0.60	3.37 $\pm$ 0.45 (+51.8) <sup>‡</sup>	2.13 $\pm$ 0.28	2.31 $\pm$ 0.469
Rescue frequency ( $\text{min}^{-1} \pm \text{SE}$ )	6.02 $\pm$ 0.55	7.40 $\pm$ 0.86	5.55 $\pm$ 0.85	5.37 $\pm$ 0.79	5.66 $\pm$ 0.67	8.99 $\pm$ 0.75 (+49.3)*	6.21 $\pm$ 0.76
Dynamicity ( $\mu\text{m}/\text{min}$ )	7.4	9.5 (+29)	10.2 (+39)	11.2 (+52)	11.3 (+54)	5.0 (-32)	5.8 (-21)

NOTE: The percentage change from control values is shown for the variables that were significantly different from controls.

Abbreviations: G, growth events; S, shortening events; P, pauses.

\* $P < 0.01$ , Student's  $t$  test.

<sup>†</sup> $P < 0.001$ , Student's  $t$  test.

<sup>‡</sup> $P < 0.05$ , Student's  $t$  test.

In sharp contrast, high concentrations of vinflunine (5 and 20 nmol/L) reduced microtubule dynamic instability compared with control cells (-32% and -21%, respectively; Fig. 1A, right; Supplementary Data; Table 1).

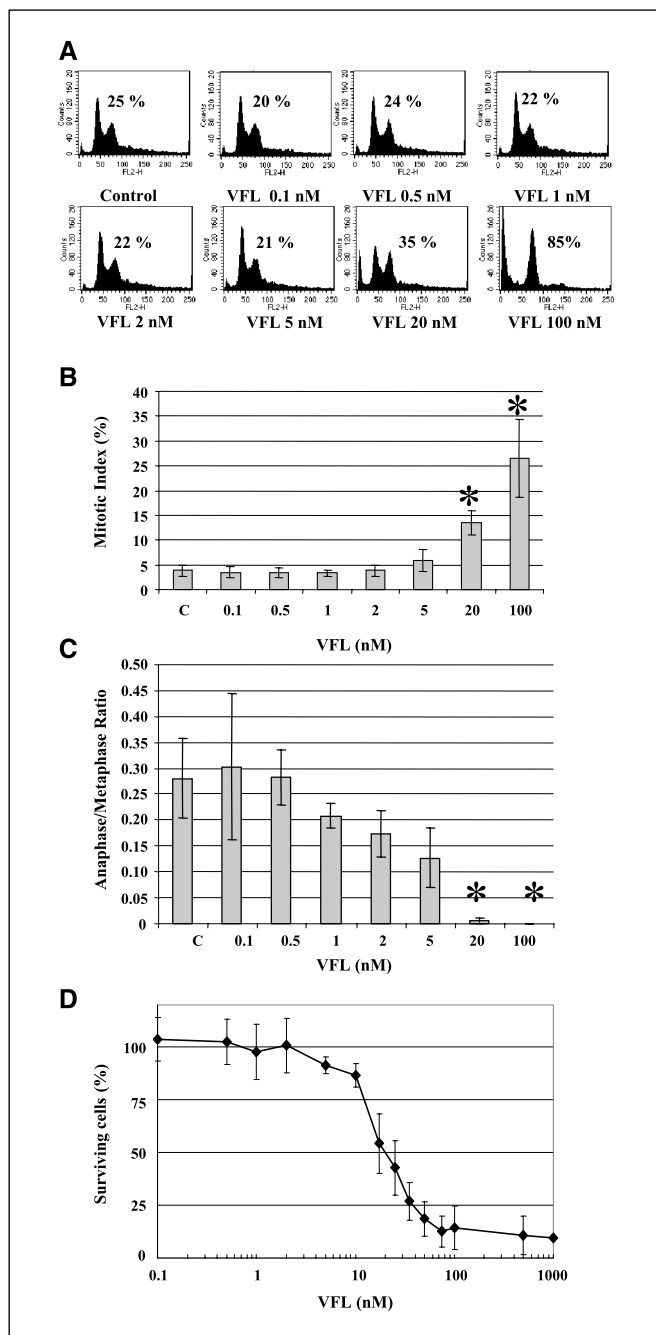
We thus showed a biphasic effect of vinflunine on microtubule dynamics of HMEC-1: concentrations  $\geq 5$  nmol/L suppressed microtubule dynamics, whereas concentrations  $< 5$  nmol/L increased it.

**Vinflunine concentrations that increased microtubule dynamics failed to disturb mitotic progression and cell proliferation.** Regulation of microtubule dynamics plays a key role in mitotic progression, chromosome segregation, and finally cell division and proliferation (1). Suppression of microtubule dynamics by MTDs is responsible for slowing down mitotic progression, increasing mitotic index, and blocking cells in mitosis (4). To evaluate the effect of vinflunine-mediated increase in microtubule dynamics on mitosis, we first searched for a putative modification in cell cycle progression of HMEC-1 after a 24-hour incubation with vinflunine. As shown in Fig. 2A, concentrations of vinflunine  $\leq 5$  nmol/L failed to disturb the cell cycle profiles, whereas higher concentrations induced a canonical G<sub>2</sub>-M block. To further investigate the vinflunine effects on mitosis, we quantified

the mitotic index and the metaphase-to-anaphase transition by DAPI staining. Interestingly, vinflunine concentrations that increased microtubule dynamics had no effect on the mitotic index and the anaphase/metaphase ratio, in contrast to higher concentrations (Fig. 2B and C). Moreover, this effect on mitosis was correlated with HMEC-1 proliferation inhibition. Whereas the high concentrations of vinflunine strongly inhibited cell growth, low vinflunine concentrations had no effect on cell proliferation as shown by MTT assay (Fig. 2D).

Thus, low concentrations of vinflunine that increased microtubule dynamics are ineffective on mitotic progression and cell proliferation.

**Vinflunine concentrations that increased microtubule dynamics disturbed endothelial cell motility.** To better understand vinflunine effects on endothelial cells, we investigated cell motility that constitutes a key process in angiogenesis. Endothelial cell random motility is of critical importance for capillary growth rate and network structure. A significant degree of randomness in cell migration direction is required for vessel branching, anastomosis, and capillary loop formation (27). To study the effect of low concentrations of vinflunine on random cell motility, we analyzed the motility behavior of HMEC-1 using the persistent random-walk



**Figure 2.** Vinflunine concentrations that increased microtubule dynamics failed to disturb mitotic progression and proliferation. Cell cycle profiles represent DNA distribution in HMEC-1 treated with various concentrations of vinflunine for 24 hours (A). The percentages of cells in  $G_2$ -M phase, calculated with Modfit software, are indicated on each profile. Percentage of cells in mitosis, quantified after DAPI staining, after 24-hour incubation with vinflunine (B) and the ratio of cells in anaphase to cells in metaphase, which is a reflection of the time required to pass from metaphase to anaphase (C). Columns, mean; bars, SE. \*,  $P < 0.05$ , one-way ANOVA. Concentration dependence for growth of HMEC-1 in the presence of vinflunine after a 72-hour incubation was analyzed using MTT assay (D). Points, mean; bars, SE.

model (25, 26). We used time lapse video microscopy to observe and track the moving cells and then to determine their mean square displacements. Figure 3A shows the representative trajectories of two cells, control and treated with 2 nmol/L vinflunine.

Cells treated by vinflunine appeared to stay focused around the same area whereas control cells moved farther. In addition, the maximal distance to origin raised by cells ( $D_{max}$ ) during the time course experiment was decreased up to 30% (data not shown).

We then calculated the three main random motility variables, i.e., cell speed ( $S$ ), persistence time ( $P$ ), and random motility coefficient ( $\mu$ ). As shown in Fig. 3B, HMEC-1 migrated at a mean speed of 0.61  $\mu$ m/min. Low concentrations of vinflunine (0.1-2 nmol/L) significantly reduced the cell speed from 13% to 19% ( $P < 0.05$ ). The persistence time (i.e., the average time between significant direction changes) was slightly, but statistically, increased between 6.3% and 19.5% in the same concentration range ( $P < 0.05$ ; Fig. 3C). Interestingly, cell ability to migrate and to colonize a new area, as estimated by the random motility coefficient, was suppressed by vinflunine in a concentration-dependent manner (from 13% at 0.1 nmol/L to 33% at 2 nmol/L; Fig. 3D;  $P < 0.05$ ). This suppression can be illustrated by direct observation of cell trajectories (Fig. 3A). Thus, low concentrations of vinflunine that increased microtubule dynamics may exhibit an antiangiogenic effect through the inhibition of endothelial cell random motility.

**Vinflunine concentrations that increased microtubule dynamics inhibited capillary-like tube formation on Matrigel.** Matrigel assay is an *in vitro* specific test for angiogenesis that permits the morphogenesis of endothelial cells in capillary-like tubes. We have previously shown that low and noncytotoxic concentrations of paclitaxel increased microtubule dynamics and inhibited capillary-like tube formation on Matrigel (17, 21). To determine whether vinflunine produces a similar effect on *in vitro* angiogenesis, we quantified the total capillary tube length following vinflunine treatment. As shown in Fig. 4A, 2 nmol/L vinflunine strongly inhibited morphogenesis of HMEC-1. Interestingly, for vinflunine concentrations ranging from 0.1 to 5 nmol/L, the total tube length decreased (-19% to -44%, respectively), more significantly than from 5 to 20 nmol/L (-44% to -52%, respectively; Fig. 4B). In other words, maximal effect was reached for vinflunine concentrations that increased microtubule dynamics.

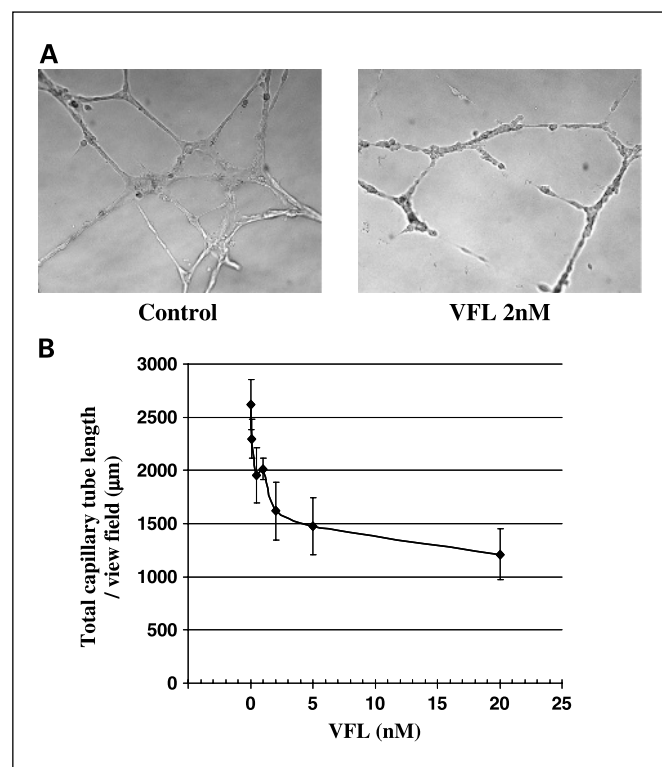
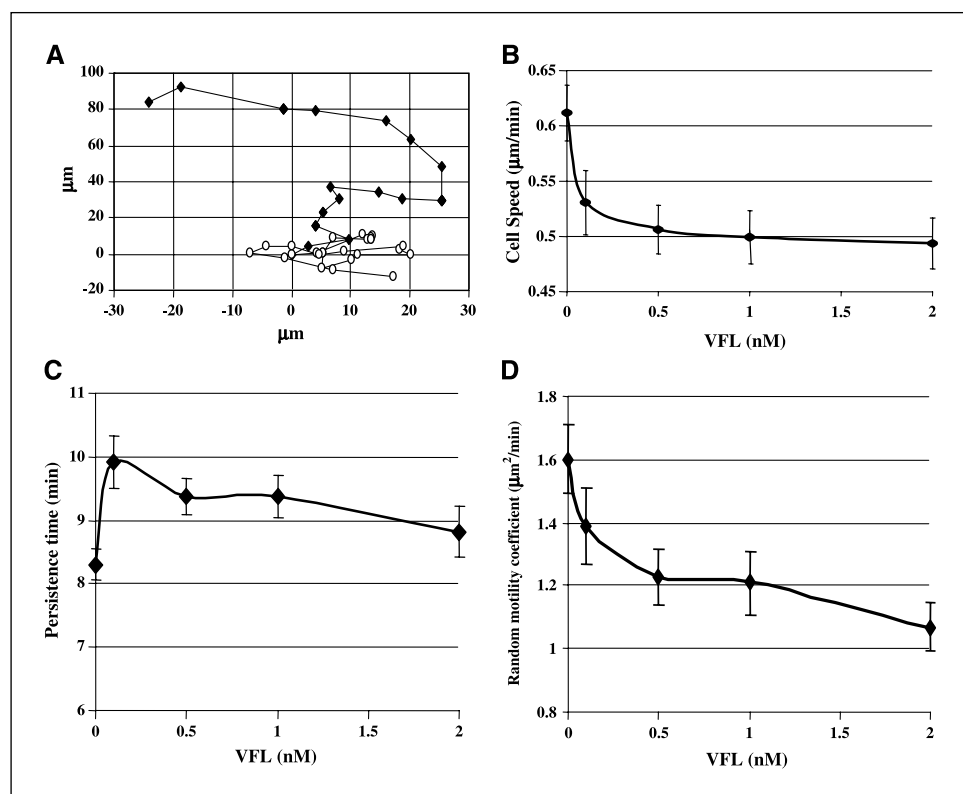
Thus, concentrations of vinflunine that increased microtubule dynamics may exhibit an antiangiogenic effect through the inhibition of endothelial cell morphogenesis.

## Discussion

The mechanisms mediating the antiangiogenic activity of MTDs have not been clearly defined. In this work, we found that vinflunine, the newest *Vinca* alkaloid, increased microtubule dynamic instability in living human endothelial cells. This increase was observed at concentrations that inhibited angiogenesis, disturbing both morphogenesis and migration of endothelial cells, but that failed to affect cell proliferation and mitotic progression.

**MTDs increase interphase microtubule dynamics in endothelial cells.** Here, we showed that low concentrations of vinflunine increased microtubule dynamic instability up to 54% in human interphase endothelial cells. Vinflunine increased both the growth and shortening rates, and decreased both the percentage of time spent in pause and the duration of pauses, in the same way as paclitaxel (21). These results show that the increase in microtubule dynamics constitute a common effect of MTDs on endothelial cells. However, in contrast to paclitaxel, vinflunine did not significantly alter the time-based transition frequencies. Paclitaxel was a more potent inducer of microtubule dynamicity than vinflunine and it increased the time-based rescue

**Figure 3.** Vinflunine concentrations that increased microtubule dynamics inhibited endothelial cell motility. HMEC-1 cells were seeded on 0.1% gelatin-coated plates and incubated with various concentrations of vinflunine 1 hour later. Cells were then tracked for 4 hours and random motility variables were calculated using the random-walk model. A low vinflunine concentration (2 nmol/L) modified endothelial cell trajectory (○) compared with control cell (◆, A). Affected variables were the cell speed (B), the persistence time (C), and the random motility coefficient (D). Points, mean; bars, SE ( $P < 0.05$ , one-way ANOVA).



**Figure 4.** Vinflunine concentrations that increased microtubule dynamics inhibited capillary-like tube formation. HMEC-1 cells were seeded on Matrigel and, 1 hour later, vinflunine was added for 4 hours. Then, the formation of capillary networks (A) was quantified by measuring the total capillary tube length (B). Points, mean; bars, SE ( $P < 0.05$ , one-way ANOVA).

frequency in HMEC-1 (21). This difference between the two drugs may be of crucial importance for their effect on mitosis, as Rusan et al. (28) have shown the importance of transition frequency regulation during mitosis. The lack of effect on transition frequencies by low concentrations of vinflunine may be responsible for both the lower increase in microtubule dynamics and the normal mitosis completion in contrast to paclitaxel (21). In addition, this result suggests that transition frequencies may not be of crucial importance for cell motility in comparison with mitosis. Indeed, the key role of transition frequencies in the mitotic process was recently reviewed (1) and is also sustained by our previous results (29). We have thus shown that the overall dynamicity in A549-T12 and A549-T24, two Taxol-resistant cell lines, was increased up to 167% in the absence of paclitaxel. This increase in microtubule dynamics was associated with disturbances in transition frequencies and an increase in mitotic index. Thus, vinflunine, which increased microtubule dynamicity without disturbing transition frequencies, failed to affect spindle microtubule functions. Vinflunine may thus exhibit its antiangiogenic effect by selectively affecting interphase microtubule functions. These results confirm the prominence of interphase endothelial cell functions in the angiogenic process compared with mitotic ones, as previously suggested for the lowest antiangiogenic concentration of paclitaxel (21). Elsewhere, it is also thinkable that paclitaxel and vinflunine may exhibit complementary effects on mitosis and interphase functions to target angiogenesis.

**Putative mechanisms of MTD-induced increase in microtubule dynamics.** At low concentrations, vinflunine increased microtubule overall dynamicity in a logarithmic fashion as did paclitaxel (21). This observation sustains the hypothesis of a modulating effect of a small number of binding sites, probably

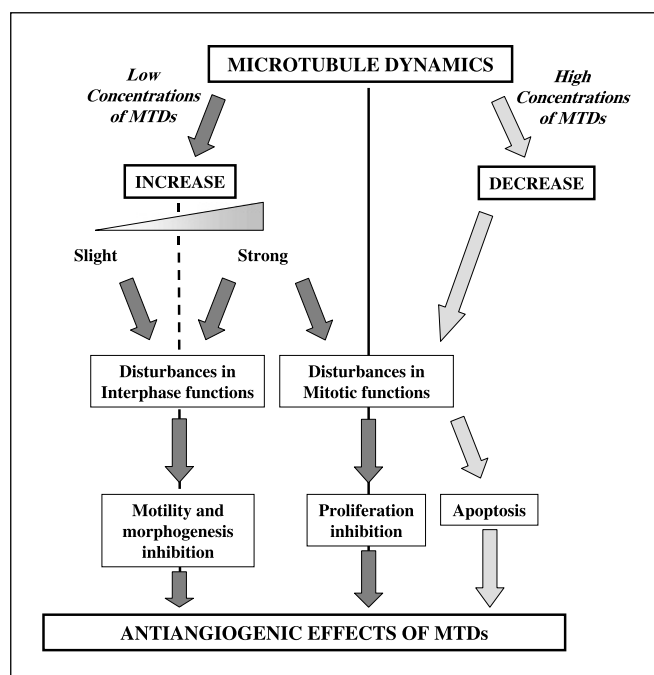
located at the microtubule plus ends. Such increase in microtubule dynamics was previously described *in vitro* with microtubules composed by a mixture of  $\alpha\beta$ II and  $\alpha\beta$ III tubulin isotypes (30). In this study, a low concentration of paclitaxel was able to slightly increase both the growth rate and dynamicity and to decrease the percentage of time spent in pause. Thus, the increase in microtubule dynamics might result from a direct effect of MTDs on microtubules. Alternatively, drug binding at the end of microtubules may also disturb the equilibrium of plus-end binding proteins (+TIPS), also involved in microtubule dynamics regulation (31).

The increase in microtubule dynamics was not detectable in cancer cells and seemed to be specific to endothelial cells (21), suggesting that it could be due to a specific tubulin isotype composition. Moreover, previous studies have shown that *Vinca* alkaloids could disturb transition frequencies in cancer cells (4). Here, the lack of effect of vinflunine on transition frequencies suggests that there may be microtubule dynamics regulatory proteins that are different in endothelial and cancer cells whose presence or absence would protect endothelial cell microtubules against disturbances in transition frequencies induced by vinflunine. Another hypothesis, as previously proposed (21), may involve the regulation of microtubule dynamics by the activation of an endothelial specific signaling pathway by MTDs. An early increase in reactive oxygen species production induced by very low concentrations of both paclitaxel (17) and vinflunine (personal data), concomitant with an increase in microtubule dynamics, is under investigation. These hypotheses are not exclusive and whatever the mechanism responsible for the increase in microtubule dynamics, higher concentrations of MTDs abolish it and finally suppress microtubule dynamics, corresponding to the biphasic effect observed with both vinflunine and paclitaxel.

**Microtubule dynamics in interphase endothelial cells, a key in MTD antiangiogenesis.** We show that angiogenesis inhibition by low concentrations of vinflunine is due to disturbances of both endothelial cell migration and morphogenesis and it is associated with a concomitant increase in interphase microtubule dynamics. These results highlight a potential link between these events.

Microtubules are able to regulate cell motility through three main mechanisms that modulate the actin cytoskeleton behavior: (a) the regulation of Rho family GTPases activation, (b) the polarization of migrating cells, and (c) the turnover of focal adhesions. Specific phases of microtubule dynamic instability (i.e., growth and shortening) modulate the activity of Rho family GTPases and, subsequently, actin dynamics and organization (32). In our model, growth and shortening rates are both similarly strongly modified by vinflunine, and the equilibrium between them remains stable. Thus, the hypothesis relying on an inhibition of cell migration through modification in Rho family GTPases activity could be excluded.

Cell polarization, which is necessary for migration, requires microtubule stabilization at the leading edge (33) to allow local delivery of motility-required components. The kinetic destabilization of microtubules induced by vinflunine may prevent their stabilization, the directed vesicular transport, and finally cell polarization at the leading edge. This default in cell polarization may explain the increase in the mean time between significant direction changes observed following vinflunine treatment.



**Figure 5.** Antiangiogenic effects of MTDs and microtubule dynamics. Low concentrations of MTDs increase microtubule dynamics to disturb interphase endothelial cell functions and thus inhibit angiogenesis. A stronger increase in microtubule dynamics disturbs the mitotic process, resulting in endothelial cell proliferation inhibition. As described in cancer cells, high concentrations of MTDs classically suppress microtubule dynamics, inhibit the mitotic progression and cell proliferation, and induce apoptosis in endothelial cells.

The multiple targeting of focal adhesions by microtubules induces their dissociation to promote cell migration (34, 35). In our model, we could potentially explain the inhibition of cell migration by the inhibition of focal adhesion targeting. The high microtubule dynamicity may prevent the accurate targeting of focal adhesions. A decrease in pause events, suggested by the decrease in the percentage of time spent in pause and the duration of pauses, could disturb the local interaction between actin and/or focal adhesions and microtubules.

For capillary tube formation, cells have to adopt a specific shape to be appropriately connected to each other. Moreover, microtubule dynamics are essential for both changes in endothelial cell shape during early morphogenesis (36) and protrusion of cell membranes, necessary for capillary tube initiation (37). Thus, in our model, we could explain morphogenesis inhibition by the disturbances of microtubule dynamics. Vinflunine, promoting a kinetic destabilization of microtubules, may inhibit the formation and the stabilization of capillary tubes.

Morphogenesis of endothelial cells may be compared with neuritogenesis (36, 38) and it has been shown that suppression of microtubule dynamics could result in an inhibition of axonal growth cone movement (39). Moreover, microtubule dynamics suppression may inhibit cell migration (40). Thus, a precise regulation of microtubule dynamics is required to ensure their optimal interphase functions and, similar to the mitotic process (1), a single disturbance in interphase microtubule dynamics, either an increase or a decrease, seems to be sufficient to alter microtubule-dependent interphase cell functions, such as cell motility.

In conclusion, we showed here for the first time that vinflunine exhibits an antiangiogenic activity through an unexpected increase in interphase microtubule dynamics, which seems to be an effect common to different MTDs. This increase in microtubule dynamic instability has no consequence on cell proliferation but is associated with disturbances of the major endothelial cell functions (i.e., morphogenesis and migration), probably by disturbing the crosstalk between microtubules and other cytoskeletal components. These results and our previous studies (17, 21) allowed us to build a model that connects the MTDs antiangiogenic effects and the disturbances in endothelial

cell microtubule dynamics (Fig. 5). Further studies are now required to identify the molecular determinants linking the increase in microtubule dynamics and the interphase endothelial cell functions.

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