Antiangiogenic vinflunine affects EB1 localization and microtubule targeting to adhesion sites

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Abstract

The motile behavior of endothelial cells is a crucial event for neovascularization. We previously showed that non-cytotoxic concentrations of vinflunine inhibit capillary-like tube formation on Matrigel and endothelial cell migration with a concomitant increase in interphase microtubule dynamic instability. In this article, we further investigated the effects of vinflunine on migration and cytoskeleton interaction dynamics in HMEC-1 endothelial cells. We confirmed that vinflunine, at low and noncytotoxic concentrations (0.01–1 nmol/L), inhibited endothelial cell random motility by 54%. This effect was associated with a decrease in the percentage of stable microtubules and in the mean duration of pauses for dynamic ones. Moreover, we found that vinflunine altered adhesion site targeting by microtubules and suppressed the microtubule (+) end pause that occurs at adhesion sites during cell migration (from 151 ± 20 seconds in control cells to 38 ± 7 seconds in vinflunine-treated cells, P < 0.001). This effect was associated with the inhibition of adhesion site dynamics and the formation of long-lived stress fibers. Importantly, we found that vinflunine altered EB1 localization at microtubule (+) ends. These results highlight a new mechanism of action of vinflunine, which act by disrupting the mutual control between microtubule and adhesion site dynamics and strengthen the role of +TIPs proteins such as EB1 as key regulators of endothelial cell motility. [Mol Cancer Ther 2008;7(7):2080–9]

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Introduction

The formation of a functional vascular network through angiogenesis is a key event associated with tumor growth, cancer progression, and metastasis (1, 2). Microtubule-targeting agents (MTAs), which can be classically subdivided into microtubule-stabilizing (e.g., Taxanes) and microtubule-destabilizing agents (e.g., Vinca alkaloids), exert an antiangiogenic activity both in vitro and in vivo at very low and noncytotoxic concentrations (3–5). Vinflunine, the newest MTA of the Vinca alkaloid class in clinical development, shows antivascular effects (6) and a strong in vivo antiangiogenic activity at doses 40- to 20-fold lower than its maximal therapeutic dose (7). At noncytotoxic concentrations, MTAs affect angiogenesis by disturbing endothelial cell migration and differentiation into the capillary network, two crucial events during tumor angiogenesis (8, 9). Cell migration can be viewed as a periodically repeating sequence of events that includes the formation of pseudopodial protrusions, attachment and translocation of the cell body in the direction of new adhesion sites (10). Microtubules and actin filaments play a critical role in intracellular trafficking, cell polarity, and motility. These functions involve the ability of these polarized polymers to assemble and disassemble rapidly, and to interact with proteins that regulate their dynamic properties both temporally and spatially. Although the central role of the actin cytoskeleton in migration is largely known, more recent data supports a significant role for microtubules in this process (11). In fact, a coordinated regulation of microtubules, actin cytoskeleton, and adhesion sites is likely to be essential throughout the migration process (12). Several putative mechanisms by which MTAs could inhibit cell migration have been proposed. These include (a) the impairment of microtubule-organizing center reorientation toward the leading edge, (b) the inhibition of Rho GTPases and their downstream effectors by altering the cycle of microtubule polymerization and depolymerization, (c) the blockade of lamellipodia formation and cell polarization as a consequence of the inhibition of intracellular protein trafficking and vesicle transport, (d) the inhibition of microtubule-mediated integrin clustering and increased integrin avidity, and (e) HSP90 degradation (13–19). Within cells, most microtubule (+) ends are oriented toward and interact with the cell cortex, whereas the (−) ends are usually stably capped and often anchored at the centrosome, which is the main organelle responsible for the nucleation of new microtubules. Microtubule (+) ends exhibit a behavior called dynamic instability which is the switch between phase of growth and rapid shortening or pause (reviewed in ref. 20). We previously showed that low antiangiogenic and antimigratory concentrations of MTAs such as paclitaxel or vinflunine significantly increase interphase microtubule dynamic instability (21–23).
However, even if microtubule involvement in cell migration is now evident and require interactions with both adhesion sites and actin cytoskeleton (12), the relationship between microtubule dynamics and cell migration remains poorly understood (20, 24). In this view, a growing list of proteins that interact with the microtubule (+) ends called (+) end-tracking proteins (+ TIPs), are critically important for many microtubule-regulated processes including cell migration. Among them, EB1 regulate microtubule dynamic instability (25), the guidance of microtubules toward the leading edge through interaction with a microtubule-actin cross-linker such as ACF7, and the selective microtubule stabilization at the cell cortex during cell migration (26).

In order to fully understand the functional implication of microtubule dynamics in the antiangiogenic activity of MTAs, we investigated the effect of vinflunine on the microtubule (+) end interaction with other cytoskeleton structures during cell migration. We showed that vinflunine, at subnanomolar and noncytotoxic concentrations, inhibited endothelial cell motility. Such an effect was associated with a decrease in microtubule pause at the cell cortex, and an inhibition of microtubule targeting to adhesion sites. This lack of adhesion site targeting by microtubules led to an inhibition of adhesion site dynamics and to the formation of long-lived stress fibers. Importantly, we found that these effects were associated with EB1 mislocalization at microtubule (+) ends. Therefore, our results reveal a new mechanism of action of a MTA, vinflunine, and highlight the role of microtubule (+) end interaction with other cytoskeleton proteins that interact with the microtubule (+) ends called (+ TIPs), are critically important for many microtubule-regulated processes including cell migration.

Materials and Methods

Drug and Cell Culture

Human dermal microvascular endothelial cell line (HMEC-1) were routinely maintained at 37°C and 5% CO₂ in MCDB-131 medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L of glutamine, 1% penicillin and streptomycin, 1 μg/mL of hydrocortisone (OHC; Pharmacia and Upjohn), and 10 ng/mL of epithelial growth factor (R&D Systems). HMEC-1 cells were used between passages 3 and 12. For all experiments, exponentially growing cells in endothelial growth medium 2 were seeded on 10 μg/mL of fibronectin-coated (Sigma-Aldrich) culture plates (8 × 10⁴ cells/cm²) and the ratio of drug molar concentration/cell number was strictly identical for all experiments. A stock solution of vinflunine (Pierre Fabre Oncology) was prepared in distilled water and maintained frozen at −20°C.

Random Motility Measurements

Cells were seeded in 24-well culture plates. One hour later, the cells were treated with vinflunine at various concentrations or were untreated and subjected to time-lapse videomicroscopy for 10 h. Random motility measurements were done as previously described (23, 27). Briefly, 90 cells from three independent experiments were tracked for 10 h. The average cell speed (S) and the coordinates of the nuclei centroids were computed by the Metamorph software (Universal Imaging Corporation). For each cell, the mean square displacement \( <D^2> \) was determined from the formula:

\[
<D^2> = \sum_{i=1}^{M} d_i^2
\]

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

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

\[
<D^2> = 2n\mu t = nS^2Pt
\]

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

Transfection of HMEC-1 with GFP-α Tubulin, GFP-Actin, GFP-EB1, mcherry-Tubulin and DsRed-Paxillin Plasmids

The transfection of HMEC-1 cells was done as previously described (23). Briefly, 8 × 10⁴ cells were resuspended in 100 μL of the specific electroporation R buffer (Amaza). Four to eight micrograms of plasmid DNA (GFP-actin, mcherry-tubulin, GFP-EB1, and pEGFP-α-tubulin; ref. 23), and DsRed-paxillin provided by Alan Rick Horwitz (University of Virginia, Charlottesville, VA; ref. 28) were added to the cell suspension, transferred to a 2.0 mm electroporation cuvette, and nucleofected with Amaza Nucleofector (Amaza) using program number T-016. DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After transfection, cells were immediately transferred in endothelial growth medium 2. Twenty-four hours later, cells were then treated for 4 h with vinflunine at various concentrations. Time-lapse microscopy and measurements were then done.

Time-lapse Microscopy and Image Acquisition

Transfected cells were placed in endothelial growth medium 2 containing the same concentration of vinflunine and supplemented with ascorbic acid (0.1 μg/mL) to reduce photodamage, in a double coverslip chamber maintained at 37 ± 1°C. Time-lapse acquisitions for microtubule dynamics experiments were done on a Leica DM-IRBE fluorescence microscope (Leica), 100× objective lens. Thirty-one images per cell were acquired at 4-s intervals using a digital camera (CCD camera coolsnap FX; Princeton Instruments) driven by Metamorph software (Universal Imaging Corporation) as previously described (29). Double fluorescence time-lapse acquisitions were done on a Nikon TE 2000 microscope (Nikon) equipped for multифluorescence time-lapse analysis at 60× objective lens. Cells were imaged every 10 s for 5 to 45 min using a
Results

Antiangiogenic Concentrations of Vinflunine Inhibit Endothelial Cell Random Motility

The cell motility behaviors of HMEC-1 cells were analyzed by time-lapse videomicroscopy by determining four variables: the average cell speed of migration, the percentage of time spent in a stationary state, the persistence time, which is a measure of the average time between two significant direction changes, and the random motility coefficient. The average migration speed of HMEC-1 cells was \(0.42 \pm 0.07 \mu m/min\). HMEC-1 cells spent \(~43\%\) of the time in a nonmigrating phase that we termed “stationary state.” The mean persistence time, which is a measure of the average time between two significant direction changes, was \(16 \pm 2.7\) minutes. Finally, the random motility coefficient, which reflects the capacity of a cell population to migrate into a new area was \(1.32 \pm 0.34 \mu m^2/min\).

We previously showed that vinflunine inhibited capillary-like tube formation on Matrigel at nontoxic concentrations of \(\leq 2\) nmol/L (23). Antiangiogenic concentrations of vinflunine (0.01–2 nmol/L) decreased by \(50\%\), the mean migration speed of HMEC-1 cells (Fig. 1A). For concentrations >2 nmol/L, the migration speed was reduced to a lesser extent. Importantly, the decrease in the average migration speed was mainly due to an increase in the percentage of time that the cells spent in a stationary state, from \(43\%\) to a maximum of \(70\%\) for control cells and cells treated with 1 nmol/L of vinflunine, respectively (Fig. 1B). In addition, the persistence time was increased by up to \(100\%\), reaching 35 minutes at vinflunine concentrations ranging from 0.01 to 1 nmol/L (\(P < 0.05\); Fig. 1C). Finally, the random motility coefficient was decreased by \(54\%\) in this range of concentrations (Fig. 1D). The increase in persistence time and the decrease in random motility were lower at concentrations \(\geq 2\) nmol/L of vinflunine. These results indicate that the antimigratory effect of vinflunine optimally occurs at nontoxic concentrations (0.01–1 nmol/L).

Vinflunine Inhibits Microtubule Pauses at the Cell Periphery

One of the most sensitive effects of MTAs in cells is the alteration of microtubule dynamics. We previously showed that antiangiogenic concentrations of vinflunine increase microtubule dynamic instability (i.e., growth and shortening rates and overall dynamicity) of dynamic microtubules in living HMEC-1 cells (23). In this study, we further examined the effect of vinflunine on microtubule pauses at the cell periphery. At antiangiogenic concentrations (0.01–2 nmol/L), the percentage of microtubules that were paused at the cell periphery during the time course of the experiment (2 minutes) was decreased from \(49\%\) in control cells to \(<15\%\) in vinflunine-treated cells (Fig. 2A). In addition, the mean duration of pauses of dynamic microtubules was decreased by \(~30\%\) (Fig. 2B). These results show that vinflunine significantly decreases microtubule pauses at the cell periphery in living HMEC-1 cells.

Vinflunine Disturbs Microtubule–Adhesion Site Crosstalk

One of the major interphase functions of microtubules during cell migration is to regulate adhesion site dynamics and/or turnover. This process involves microtubule (+) end targeting of adhesion sites and microtubule pauses at adhesion sites (30). Because vinflunine inhibits microtubule pauses at the cell cortex, we investigated the effect of vinflunine on the targeting of adhesion sites by microtubule (+) ends. For this purpose, HMEC-1 cells were
cotransfected with DsRed-paxillin and GFP-tubulin to visualize both microtubules and adhesion sites. Double fluorescence time-lapse acquisition every 10 seconds over a period of 10 minutes clearly showed that microtubule (+)

ends specifically target paxillin-containing adhesion sites at the cell periphery in control cells (Fig. 3A). In contrast, in vinflunine-treated cells, the microtubule network was disorganized with curved microtubules which did not reach the peripheral adhesion sites (Fig. 3B). Indeed, the percentage of microtubules that targeted adhesion sites during the time course of the experiment (10 minutes) was significantly decreased by vinflunine (1 nmol/L) from 88% to 62% (*, P < 0.05; Student’s t test).

**Figure 2.** Vinflunine suppressed microtubule pauses at the cell periphery. HMEC-1 cells transiently transfected with GFP-tubulin were incubated with various concentrations of vinflunine for 4 h. Microtubule dynamics were then analyzed by time-lapse video microscopy. The percentage of microtubules that were paused at the cell periphery during the time course of the experiment (2 min; A) and the microtubule mean duration of pauses of dynamic microtubules (B) were determined by tracking peripheral microtubule (+) ends over time. Note that 90% and 60% of cellular microtubules targeted the cell periphery in control cells and in vinflunine-treated cells, respectively. Points, mean; bars, SE (*, P < 0.05; Student’s t test).

90% and 60% of cellular microtubules targeted the cell periphery in control cells and in vinflunine-treated cells, respectively. Points, mean; bars, SE (*, P < 0.05; Student’s t test).

**Figure 1.** Vinflunine inhibited HMEC-1 random motility. HMEC-1 cells were seeded on fibronectin-coated plates (10 μg/mL) and incubated with various concentrations of vinflunine 1 h later. Cells were then tracked for 10 h and random motility variables were calculated using the random-walk model. Motility variables were the cell speed (A), the percentage of time spent in a stationary state (B), the persistence time (C), and the random motility coefficient (D). Points, mean; bars, SE (*, P < 0.05; Student’s t test).
Vinflunine strongly decreased the mean duration of microtubule pauses at adhesion sites from 151 ± 20 seconds in control cells to 38 ± 7 minutes in vinflunine-treated cells (P < 0.001; Fig. 4C). Altogether, these results show that vinflunine strongly inhibits the microtubule–adhesion site crosstalk that occurs during endothelial cell migration.

**Vinflunine Inhibits Adhesion Site Dynamics and Induces Stress Fiber Formation**

To better understand the consequences of the alteration of the microtubule–adhesion site crosstalk by vinflunine, we analyzed its effect on actin and adhesion site organization and dynamics, which are crucial for cell migration. For this purpose, HMEC-1 cells were cotransfected with DsRed-paxillin and GFP-actin plasmids. Migrating control cells displayed large lamellipodia with more intense actin staining at the leading edge, where new adhesion sites were formed, concomitantly with the appearance of actin-rich membrane ruffles. Cells also displayed adhesion site sliding at the retracting edge (Fig. 5A; Supplementary Movie 3). In contrast, in vinflunine-treated cells, the cortical actin network and membrane ruffles disappeared, and many thick and long-lived stress fibers were formed.
between static focal adhesions (Fig. 5B; Supplementary Movie 4). These effects on adhesion site dynamics were more readily observed on longer time course experiments (Supplementary Movies 5 and 6). Vinflunine-induced stress fiber formation was further confirmed by direct immunofluorescence staining on fixed cells using Phalloidin-TRITC (Supplementary Fig. S1). These results show that vinflunine alters adhesion site and actin dynamics in endothelial cells at antiangiogenic concentrations.

Vinflunine Alters EB1 Localization at Microtubule (+) Ends

Several lines of evidence indicate that microtubule + TIPs, including EB1, are critically important for microtubule "search and capture" mechanism and for the stabilization of microtubules at adhesion sites.

Figure 4. Vinflunine inhibited microtubule pauses at adhesion sites. Time-lapse video sequence of the peripheral region of HMEC-1 cells cotransfected with GFP-tubulin and DsRed-paxillin (A) and treated with 1 nmol/L of vinflunine for 4 h (B). Time is indicated in seconds ("'). In the control cell (A), microtubule (+) ends specifically targeted the peripheral adhesion sites and remained stabilized to these adhesion sites for long periods of time (>200 s). Video frames showing the indicated microtubule (+) ends stabilized at adhesion sites are labeled with an "S". Note that most of these adhesion sites were small in size (focal complexes). In sharp contrast, in the vinflunine-treated cells (B), peripheral adhesions were not targeted and were larger in size (focal adhesions; arrowheads). In this condition, when a microtubule–adhesionsite targeting event occurred (arrows on the time-lapse sequence), the duration of the microtubule stabilization at the adhesion site was much shorter than in control cells (<30 s; frames labeled with an "S'"). Bars, 3 μm. C, mean microtubule duration of pauses at adhesion sites (bars, SD; ***, P < 0.001; Student’s t test).
microtubules within the cell cortex (31). Thus, we examined the effect of vinflunine on EB1 localization. For this purpose, HMEC-1 cells were cotransfected with GFP-EB1 and mcherry-tubulin to visualize both microtubules and EB1 dynamics. In control cells, video microscopy showed that GFP-EB1 concentrated at growing microtubule (+) ends and appeared as comets moving towards the cell periphery (Supplementary Movie 7). In contrast, no comets were visible in vinflunine-treated cells (1 nmol/L; Supplementary Movie 8). To further confirm this effect of vinflunine on EB1 localization, HMEC-1 cells were immunostained for endogenous EB1 and α-tubulin. In control cells, endogenous EB1 displayed a comet-like distribution at microtubule (+) ends. Upon vinflunine treatment, EB1 comets were strongly altered and more likely appeared as dots at microtubule (+) ends (Fig. 6).

**Discussion**

In this article, we revealed how microtubules participate in the mechanism responsible for the antimigratory effect of vinflunine on endothelial cells that we described previously (23). Inhibition of endothelial cell motility by vinflunine was associated with (a) a decrease in the percentage of stable microtubules and in the mean duration of pauses for dynamic microtubules; (b) a decrease in adhesion site targeting by microtubule (+) ends, and in microtubule pauses at adhesion sites; (c) a decrease in adhesion site dynamics and an increase in stress fibers; (d) an alteration of EB1 localization at microtubule (+) ends.

One of the most critical functions of interphase microtubules during cell migration is to target adhesion sites, a process guided by the actin cytoskeleton, thus regulating adhesion site dynamics and turnover (30, 32–35). The mechanism of targeting includes the growth of microtubules toward the adhesion site followed by a short association of the microtubule (+) end with the adhesion site. Then, the microtubule can either continue to grow, undergo catastrophe and shorten, or be stabilized at the adhesion site. Thus, the pause of microtubule (+) ends in interphase cells seems to be dependent on their capture and transient stabilization by adhesion sites (30, 33, 35). Such a process is recognized to promote cell relaxation by...
inhibiting focal complex maturation into focal adhesions and the subsequent actomyosin contractility, thus decreasing the adhesion forces to the substrate to promote cell migration (32, 34). Altogether, our results suggest that the antimigratory effect of vinflunine results from an inhibition of microtubule–adhesion site crosstalk, thus inhibiting adhesion site dynamics and leading to the formation of long-lived stress fibers. Alternatively, the formation of stable stress fibers and the lack of adhesion site dynamics could be a downstream secondary effect of inhibition of migration. However, because vinflunine is a MTA, we expect that the primary mechanism of its antimigratory action is related to disturbance in microtubule function. This is underscored by the fact that vinflunine significantly reduced the localization of EB1 at microtubule (+) ends.

How does vinflunine reduce the size of EB1 comets? As EB1 localize at growing microtubule (+) ends, the inhibition of microtubule dynamics by vinflunine, and particularly growing events, which is the main mechanism of action of MTAs in vitro and at cytotoxic concentrations in cells (36), may be a cause of the loss of EB1 at microtubule (+) ends. However, in our experiments, vinflunine was used at low noncytotoxic and antiangiogenic concentrations that do not suppress microtubule dynamic instability but rather

Figure 6. Vinflunine altered EB1 localization at microtubule (+) ends. HMEC-1 cells were incubated with various concentrations of vinflunine for 4 h and then immunostained for α-tubulin and EB1. In control cells, EB1 appeared as long comets at the peripheral microtubule (+) ends (arrowheads). In contrast, in cells incubated with vinflunine, the length of the EB1 comets was significantly reduced and more likely appeared as dots at the (+) end of microtubules (arrows). Bars, 10 μm.
increase it (23). In these conditions, we did not observe a complete loss of endogenous EB1 at the tip of the microtubules. Instead, a significant reduction in the size of EB1 comets was evidenced such that EB1 appeared as dots at the (+) end of microtubules. This is consistent with the fact that in vinflunine-treated endothelial cells, microtubules continued to grow beyond adhesion sites with similar rates compared with noncaptured microtubules in untreated cells (data not shown). Thereby, tubulin and/or microtubule conformational changes induced by vinflunine, which like other Vinca alkaloids preferentially binds to the microtubule (+) end (36), may alter EB1 interaction and/or decrease the number of EB1 binding sites at the microtubule (+) end. Thus, the reduction of EB1 at microtubule (+) ends is more likely a consequence of a direct inhibition of its binding than a consequence of any changes in microtubule dynamic instability. Besides these direct effects of vinflunine on EB1-microtubule interaction, we cannot exclude the activation of a signaling pathway that posttranslationally modifies EB1. The transition from tip-specific binding to microtubule decoration can be mimicked for p150<sub>glued</sub>, which directly binds to EB1 by using kinase inhibitors and site-directed mutants (37, 38). This suggests that kinase activity is responsible for the (+) tip specificity of tip trackers. Interestingly, EB1 phosphorylation by the COP9 signalosome protects it from degradation by the ubiquitin system and the EB1 COOH terminus is sufficient for interaction with COP9 (39); therefore, vinflunine may inhibit EB1-COP9 interaction and accelerate EB1 degradation which would occur at the rear of EB1 comets when EB1 dissociates from microtubules and would result in dot-like EB1 distribution at microtubule tips.

How does the reduction of EB1 comets affect endothelial cell migration? EB1 has been proposed as the core regulator of microtubule (+) end functions because all +TIPs shown to interact with microtubules directly bind to EB1 (26). Microtubule targeting and its subsequent capture at adhesion sites would involve proteins for which a minimum steady state concentration of EB1 is necessary for direct binding. Several proteins compete for binding to the COOH terminus of EB1, such as those containing a CAP-Gly domain similar to p150<sub>glued</sub>, and are known to participate in microtubule targeting at the cell cortex. Moreover, the possible lower affinity of protein complexes involved in microtubule anchoring/stabilization at adhesion sites and/or steric hindrance at microtubule tips may require a sufficient set of free EB1 at the rear of the comet to interact for a minimum amount of time. Overexpression of the EB1 COOH terminus in fibroblasts inhibits their migration (40), which would be consistent with a buffering of such low-affinity protein complexes. Moreover, the model of force generation by cytoskeleton-end-tracking proteins developed by Dickinson et al. (41) suggests that regulated cycles of EB1 interaction with tubulin are critical. Interestingly, it has been recently shown that EB1 suppresses microtubule dynamic instability and increases microtubule pause in <i>vitro</i> (25). Thus, the effect of vinflunine on EB1 at microtubule (+) ends may be directly involved in the decrease in microtubule pauses and in the increase in microtubule dynamic instability we previously evidenced (23).

Altogether, our observations suggest that low antiangiogenic concentrations of MTAs inhibit endothelial cell migration by preventing microtubule targeting of adhesion sites through the disruption of normal EB1 turnover at microtubule +ends. A proteomic approach is in progress to identify and characterize microtubule (+) end protein complexes that are involved in endothelial cell migration. These complexes may lead to biomarkers with potential diagnostic values and to new therapeutic targets.

Disclosure of Potential Conflicts of Interest

A. Kruczynski: Pierre Fabre Medicadent employee. The other authors reported no potential conflicts of interest.

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