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Anti-angiogenic, vascular-disrupting and anti-metastatic activities of vinflunine, the latest vinca alkaloid in clinical development

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ABSTRACT

The aim of this study was to investigate the anti-angiogenic, vascular-disrupting and anti-metastatic properties of vinflunine, the latest vinca alkaloid in phase III clinical development. The effects of vinflunine on *in vitro* endothelial cell functions relevant to the performance of an already formed vasculature and to the angiogenic process were evaluated. The *in vivo* anti-angiogenic properties of vinflunine were also investigated, as were its activity against a model of experimental metastasis. *In vitro* vinflunine induced a rapid change in the morphology of endothelial cells and disrupted the network of capillary-like structures, indicating potential vascular-disrupting activity. Furthermore, vinflunine showed anti-angiogenic properties, since it inhibited endothelial cell migration and the capacity of these cells to organise into a network of capillary-like structures. All these effects were observed under conditions that only marginally affect endothelial cell proliferation. *In vivo*, vinflunine inhibited bFGF-induced angiogenesis in Matrigel implants at doses 40–20-fold lower than its maximal therapeutic dose (MTD). Treatment of mice with vinflunine reduced the number of liver metastases induced by intrasplenic injection of LS174T cells, with significant effects also observed at low doses; i.e. 16-fold lower than the MTD. This study demonstrates that vinflunine expresses both vascular-disrupting and anti-angiogenic activities and induced marked effects against experimental metastases, all properties that support its ongoing clinical development.

1. Introduction

The original concept formulated in the 1970s,[1] that tumour cells are dependent on the establishment and the performance of a functional blood vessel system for survival, proliferation and metastatic dissemination, has led to major efforts to develop therapeutic tools indirectly to inhibit tumour growth and survival by impairing neovessel formation or function.[2] Targeting cells that support tumour growth, rather than cancer cells themselves, is a relatively novel approach to cancer therapy that is particularly promising because vascular cells are genetically stable and therefore less likely to accumulate mutations that allow them rapidly to develop drug resistance.[3,4] Two main classes of therapeutic agents

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targeting such processes have emerged: anti-angiogenic compounds, aimed at preventing new vessel formation; and vascular-disrupting agents, aimed at compromising the integrity and functionality of already existing tumour vessels.[2] Angiogenesis is a multi-step process that can be blocked by agents affecting any one of several events crucial to the whole process. The production and activity of angiogenic factors, their interaction with receptors on the surface of endothelial cells, the downstream signalling events, and the activities of endothelial cells induced by these stimuli are all targets for anti-angiogenic strategies.[2,4] On the other hand, tumour vasculature targeting is made possible by the structural, phenotypic and functional differences between vessels in tumour and normal tissue.[5–7] It is possible to exploit these differences for therapeutic purposes by directing therapeutic agents to endothelial cells within tumours or using agents that selectively affect endothelial cells.

Microtubules represent an interesting target for angiogenesis inhibitors and/or vascular-disrupting agents, since many crucial endothelial cell activities and characteristics, relevant to angiogenesis or the performance of a functional vasculature, including migration, proliferation, secretion, alignment, formation of a capillary-like structure, adhesion and cell morphology, require a functional cytoskeleton. Accordingly, microtubule/tubulin-interacting agents, including taxanes, colchicines, combretastatins and vinca alkaloids were among the first chemotherapeutics reported to have anti-angiogenic or vascular-disrupting properties.[8–12]

Vinca alkaloids have been widely used in many cancer chemotherapy protocols since the discovery of vincristine and vinblastine more than 30 years ago. The more recent introduction of vinorelbine into the clinic in the mid-1980s and the rapid recognition of its major clinical activities,[13–16] has resulted in renewed interest in this group of anti-cancer agents. Indeed, a newly identified derivative, vinflunine, with substitutions in the little-exploited region of the catharanthine moiety, obtained using super-acid chemistry and involving the selective introduction of two fluorine atoms at the 20′ position and the reduction of the 3′,4′ double bond,[17] is now undergoing phase III clinical trials. Differences in terms of the inhibitory effects of vinflunine on microtubule dynamics and its tubulin affinity have been identified, which appear to distinguish it from the other vinca alkaloids, and could be associated with reduced neurotoxicity.[18–20] Furthermore, pre-clinical studies have shown that vinflunine exhibits a high level of anti-tumour activity against a broad spectrum of different histological types. Concurrent experiments with vinorelbine have demonstrated the superiority of vinflunine, which is also apparent from historical comparisons with published data on vinblastine and vincristine.[18] Evidence has also been presented that the anti-tumour efficacy of vinflunine could, at least in part, be mediated by anti-vascular mechanisms.[21]

The purpose of the present study was to investigate the potential anti-angiogenic activity of vinflunine and to further characterise its anti-vascular properties. Therefore, the effects of vinflunine on endothelial cell functions *in vitro* and in an *in vivo* model of angiogenesis were assessed. Furthermore, since angiogenesis is a key event in tumour growth and metastases, the activity of vinflunine against an experimental liver metastasis model was also investigated.

2. Material and methods

2.1. Drug preparation

Vinflunine or 20′,20′-difluoro-3′,4′-dihydrovinorelbine sulphate from Pierre Fabre (France) was solubilised in sterile distilled water or in a saline solution (0.9% NaCl) for *in vitro* or *in vivo* assays, respectively. Cisplatin (Sigma) was solubilised in 0.9% NaCl.

2.2. Cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins and grown in M199 medium supplemented with 10% calf serum, 10% newborn serum, 50 μg/ml endothelial cell growth supplement (ECGF, crude extract from bovine brain), 100 μg/ml heparin and 20 mM HEPES (complete medium). Cells were used between the third and the fifth passage. The A-10 rat smooth muscle cells, purchased from the ATCC (American Type Cell Collection), were grown in RPMI medium supplemented with 10% foetal calf serum and 2 mM L-glutamine. LS174T (human colon carcinoma) tumour cell lines were purchased from the American Type Cell Collection (ATCC) and when tested at the International Council for Laboratory Animal Science (ICLAS), at the University Hospital of Nijmegen, The Netherlands, proved Mouse Antibody Production (MAP) test negative. LS174T cells were grown in minimal essential medium (MEM) supplemented with 5% heat-inactivated foetal bovine serum, and included 2 mM L-glutamine, 1.25 μg/ml fungizone and 100 μg/ml penicillin-streptomycin.

2.3. Proliferation assay

Cells (4×10^3 cells/well) were plated in 96-well plates in complete medium. After 24 h, vinflunine was added (10^{-9}–10^{-4} M) and left for 1 h or 4 h, then cells were washed and incubated for an additional 3 d. Alternatively, cells were exposed to vinflunine for the whole duration of the assay, i.e. 72 h. At the end of the incubation, cells were stained with 0.5% crystal violet in 20% methanol, rinsed with water and air dried. The stain was eluted with 1:1 solution of ethanol:0.1 M sodium citrate and absorbance at 540 nm was read with a Multiscan MC Titertek (Flow Laboratories, Milan, Italy). Results are expressed as the percentage of control proliferation and IC_{50} values (the drug concentration that causes 50% inhibition of cell proliferation).

2.4. Analysis of cell morphology and adhesion

Non-tissue culture 96-well plates (Microtest, Becton Dickinson, Bedford, MA, United States of America (USA)) were coated with 5 μg/ml (625 ng/cm^2) fibronectin (Chemican, Temecula, CA, USA) or collagen IV (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) for 2 h at 37 °C. After washing in PBS, non-specific sites were blocked with 1% bovine serum albumin (BSA) in PBS (30 min at 37 °C).

1.5×10^{-4}–2.5×10^{-4} M HUVEC cells/well in DMEM-0.1% BSA were added and left to adhere for 4 h at 37 °C. Adherent cells were then exposed to vinflunine (10^{-8}–10^{-4} M) or to vehicle for 45 minutes. Wells were then washed gently with DMEM-0.1% BSA to remove detached cells, stained with crystal violet (0.5% in 20% methanol), rinsed with water and air dried. Cells were analysed by inverted light microscopy (IX70, Olympus Optical Co, Tokyo, Japan) and computer image analysis (Image Pro-Plus 4.5, Media Cybernetics, LP). The degree of cell spreading was evaluated as the cell area (area of the plate covered by the cells, normalised to the number of adherent cells). To evaluated the number of adhered cells, the stain was eluted with a 1:1 solution of ethanol:0.1 M sodium citrate and absorbance at 540 nm was read with a Multiscan MC Titertek.

2.5. Capillary-like structure disruption assay

HUVEC cells (2×10^4 cells/well) were plated in 96-well plates on a thick layer of Matrigel (Becton Dickinson, 10 mg/ml, 60 μl/well) and left to align for 24 h. Then, the formed capillary-like structures or cords were exposed for 1 h to vinflunine. Pictures were taken 1 h after addition of vinflunine.

2.6. Motility assay

Motility was assessed using a modified Boyden chamber, as described previously.[22] The supernatant of NIH3T3 cells was used as the attractant and was added to the lower compartment of the Boyden chamber. HUVEC cells were detached, washed in DMEM-0.1% BSA, resuspended in the same medium at a concentration of 10^6 cells/ml, and added to the upper compartment of the Boyden chamber. Vinflunine (10^{-8}–10^{-4} M) was added to the endothelial cells for a 4 h incubation. Filters were then stained with Diff-Quick (Marz-Dade, Dudingen, Switzerland) and the migrated cells in 10 high-power fields were counted. Results are expressed as the percentage of control migration (vehicle-treated cells) and as IC_{50} values.

2.7. Capillary-like structures formation assay

HUVEC cells (2×10^4 cells/well) were plated in 96-well plates on a thick layer of Matrigel (10 mg/ml, 60 μl/well) in the presence of either vehicle or vinflunine at the indicated concentrations. Pictures were taken 4 h (when endothelial cells are aligning) or 24 h later (when capillary-like structures had formed).

2.8. Mice

Female C57BL/6 (C57BL/6 NCrlBR) mice (Charles River, St Aubin-les-Elbeuf, France) were used for implanting the Matrigel. The homozygous female athymic nude mice (BALB/c/Ola/Hsd-*nu*, Harlan, UK) was used for implanting LS174T cells. Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the European Directive EEC/86/609, under the supervision of authorised investigators. All experiments were conducted in compliance with French regulations and Centre de Recherche Pierre Fabre (CRPF) ethical committee guidelines, based on the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia, as detailed previously.[23]

2.9. In vivo angiogenesis assay

The method described by Passaniti and colleagues[24] was used with some modifications. Briefly, bFGF (150 ng/ml) was embedded in a pellet of Matrigel (10 mg/ml, 0.5 ml) and injected subcutaneously (s.c.) in C57BL/6N mice. Mice received vinflunine at 0.63, 1.25, 2.5 or 5 mg/kg, intravenously (i.v.), immediately before and 2 d after Matrigel implantation. Control mice received the same volume of vehicle. After 4 d, the Matrigel pellet was removed and the haemoglobin content was measured using the Drabkin's procedure.[8] Each experimental control or vinflunine-treated group included 30 mice. For histological analysis, the Matrigel pellets were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Five-μm sections were then stained with H& E following standard procedures.

2.10. Liver metastasis assay: intrasplenic injection of LS174T human cancer colon cells

BALB/c Nude mice were anesthetised with isoflurane and the left flank was prepared for sterile surgery. A small abdominal incision was made in the left flank and the spleen was isolated and exteriorised. Viable LS174T tumour cells (1×10^6 cells / 40 μl / mouse) were injected into the spleen using a 27-gauge needle. The spleen was returned to the abdominal cavity and the wound was closed in one layer with metal wound clips. All mice survived this procedure. Vinflunine was administered i.v. on days 4, 7, 11, 14, 18 and 21 after tumour cell implantation. Mice were weighed 2–3 times weekly and were checked daily, with any adverse clinical reactions noted. On day 28 after tumour implantation, the experiment was stopped and mice were euthanised and then autopsied. Liver tumour burden was evaluated based on findings in terms of the size and number of lesions and classified in 5 categories, as follows: 0, (i.e. tumour free); '1–10' metastatic foci; '11–30' metastatic foci; '>30' metastatic foci; metastatic foci replacing the whole liver. Each experimental control or vinflunine-treated group included 20 mice.

3. Results

3.1. Vascular-disrupting effects of vinflunine

In vitro effects of vinflunine were investigated on endothelial cell morphology, on adhesion and on newly formed capillary-like structures, according to the experimental procedures of Micheletti and colleagues.[11]

3.2. Vinflunine affects endothelial cell morphology

Endothelial cells were left to adhere to two extracellular matrix components, fibronectin or type IV collagen, for 4 h and were then exposed for a short period, i.e. 45 min to vinflunine at 10^{-8}–10^{-4} M. Treatment of adherent cells with vinflunine

induced a rapid change in endothelial cell shape: cells retracted and assumed a rounded morphology, as reflected by the reduced cell area (Fig. 1A). Mean IC_{50} values were $9.9.10^{-5} \pm 1.5.10^{-5}$ M for fibronectin and $5.0.10^{-5} \pm 2.8.10^{-5}$ M for type IV collagen. The change in cell shape was not associated with detachment of the cells from their substrates, as judged by measurements of cell adhesion (Fig. 1B).

The modifications of endothelial cell morphology induced by 10^{-7} M vinflunine were rapidly reversible since cells reverted to their original shape within 2 h of removal of the compound by washing (Fig. 2C and G). Under these experimental conditions, cells treated with a higher concentration of vinflunine (10^{-5} M) only partially reverted (Fig. 2D and H), and their full recovery required more time (data not shown).

3.3. *Vinflunine affects newly formed capillary-like structures*

The final event during angiogenesis is the organisation of endothelial cells in a three-dimensional network of capillary-like structures. *In vitro*, endothelial cells, HUVEC seeded on a thick layer of Matrigel, rapidly align and within a few hours, form a network of cords, reminiscent of newly formed vessels, that is complete in 24 h. The addition of vinflunine (for 1 h) to newly formed capillary-like structures rapidly disrupted the integrity of the network, with this effect being observed from 10^{-7} M vinflunine (Fig. 3). After the addition of concentrations higher than 10^{-6} M, the cells appeared retracted and distinct cords were no longer observed.

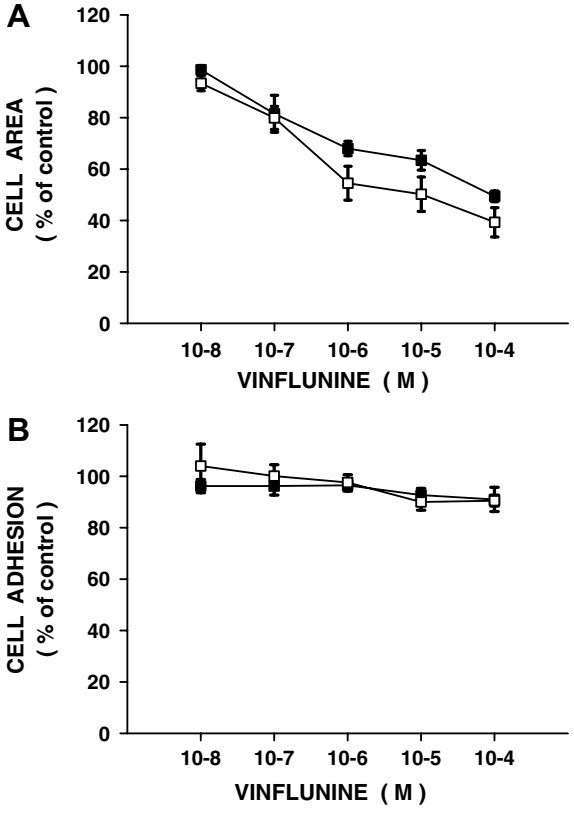

Fig. 1 – Effects of vinflunine on (A) endothelial cell morphology/spreading and (B) cell adhesion. Human umbilical vein endothelial cells (HUVEC) adherent to fibronectin (filled squares) or type IV collagen (open squares) were exposed to the indicated concentrations of vinflunine for 45 min, washed, and stained with crystal violet. The degree of cell spreading (evaluated as the cell area) and adhesion (absorbance) were expressed as the percentage of control (vehicle-treated cells). Data are means ± standard error (SE) of values from 3–4 independent experiments.

3.4. *Anti-angiogenic effects of vinflunine*

In vitro and *in vivo* experiments were performed to study the possible anti-angiogenic properties of vinflunine by investigating its effects on endothelial cell functions related to motility and capillary-like structures formation. *In vivo*, the anti-angiogenic activity of vinflunine was evaluated using the Matrigel plug assay.

3.5. *Vinflunine inhibits endothelial cell motility*

A short 4 h exposure of endothelial cells to vinflunine at 10^{-8}–10^{-4} M resulted in an inhibition of endothelial cell motility response to NIH3T3 cells-derived angiogenic factors (Fig. 4). Inhibition was dose dependent, with a mean IC_{50} value of $7.1 \times 10^{-7} \pm 0.85 \times 10^{-7}$ M.

3.6. *Vinflunine prevents the alignment of endothelial cells to form capillary-like structures*

The activity of vinflunine against the formation of capillary-like structures on the layer of Matrigel *in vitro* was investigated. Addition of vinflunine, at the time of HUVEC cell seeding on Matrigel, blocked the ability of endothelial cells to align, preventing capillary-like structures formation, with incomplete capillary-like structures observed at 10^{-7} M and complete inhibition being reached at 10^{-6} M, either 4 h or 24 h after cell seeding (Fig. 5).

3.7. *Effect of vinflunine on endothelial cell proliferation in vitro*

HUVEC endothelial cells were exposed to vinflunine for either 1, 4 or 72 h at 10^{-9}–10^{-4} M (Fig. 6). After a short exposure of cells to vinflunine, i.e. 1 h, no real inhibition of their proliferation was observed. Concentrations of vinflunine higher than 10^{-6} M are necessary to induce a significant inhibition of endothelial cell proliferation, when cells were exposed for 4 h, with an IC_{50} value of 8.4×10^{-6} M. In contrast, when cells were treated with vinflunine for a long period, i.e. 72 h, a marked dose-dependent reduction of endothelial cell proliferation was observed, with an IC_{50} value of 6.1×10^{-7} M.

3.8. *Specificity of the effects of vinflunine on endothelial cell functions*

In order to determine the specificity of the alterations of endothelial cell functions induced by vinflunine, its effects on the A-10 rat smooth muscle cells, used as a model of normal non-endothelial cells were assessed. Under the experimental conditions that have shown to affect endothelial cell

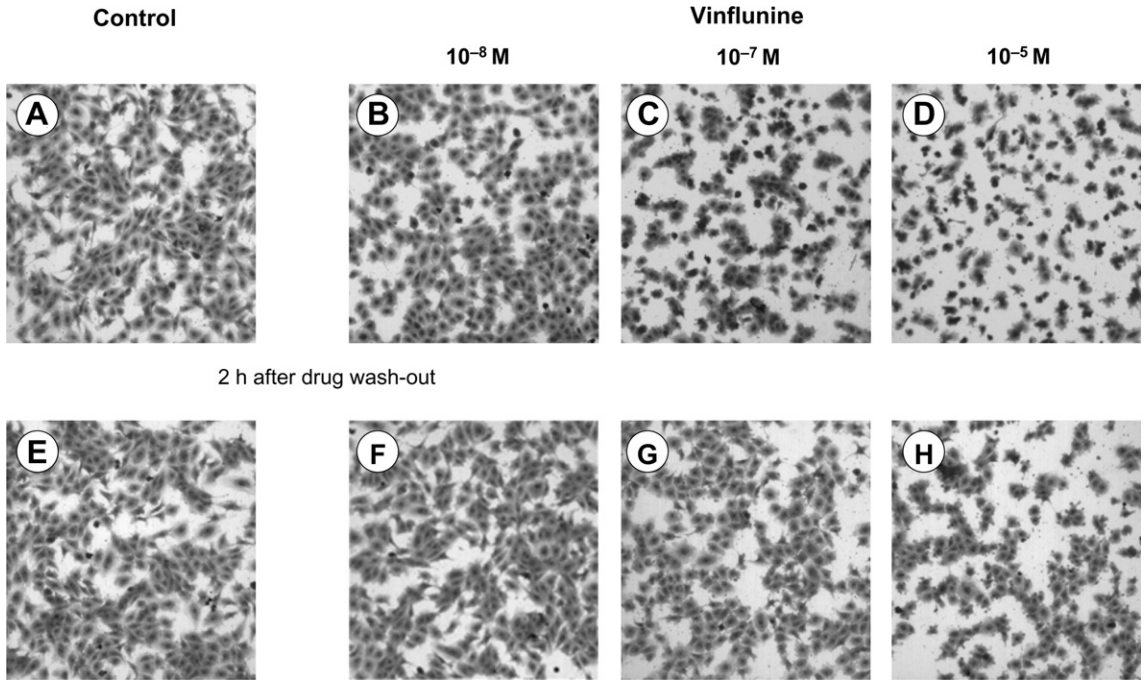

Fig. 2 – Representative images of human umbilical vein endothelial cells (HUVEC) exposed (A, E) to vehicle, or to vinflunine (B, F) 10^{-8} M (C, G) 10^{-7} M and (D, H) 10^{-5} M. Cells adherent to fibronectin were exposed for 45 min to vinflunine (B–D) or to the vehicle (A). To analyse the reversibility of the effect, after the 45-min incubation with vinflunine, parallel cell cultures were washed and incubated with vinflunine-free or vehicle-free medium for 2 h. Images were taken 2 h after drug or solvent wash-out (E–H). Magnification × 10.

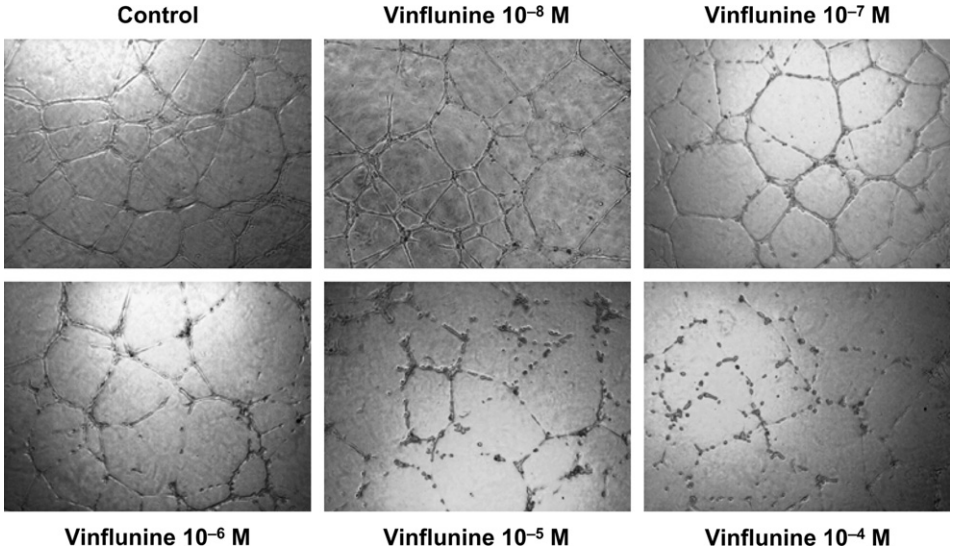

Fig. 3 – Effects of vinflunine on neoformed capillary-like structures. Vehicle (control) or vinflunine at the indicated concentrations were added to cords formed by endothelial cells on Matrigel 24 h after seeding. Images were taken 1 h after vinflunine addition. Magnification × 40.

morphology, vinflunine had no effect on smooth muscle cell shape or attachment (Fig. 7A). Cells remained adherent and fully spread even at the highest tested concentration, i.e. 10^{-4} M. In contrast, vinflunine, inhibited A-10 cell motility in a dose-dependent manner and this effect was comparable to that observed with endothelial cells, as reflected by IC_{50} values of 7.2×10^{-7} M and 7.1×10^{-7} M, respectively (Fig. 7B). However, the inhibition of A-10 smooth muscle cell motility was observed at vinflunine concentrations that also inhibit A-10 cell proliferation, as reflected by an IC_{50} value of 7.8×10^{-7} M, whereas the inhibition of endothelial cell motility occurred at concentrations 10-fold lower the concentration inhibiting their proliferation, i.e. 7.1×10^{-7} M versus 8.4×10^{-6} M. To exclude the possibility that any cytotoxic drug

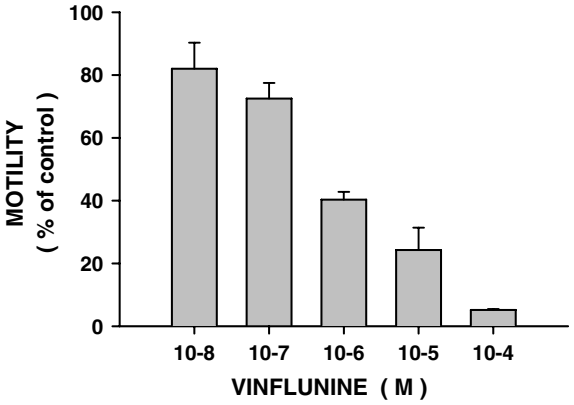

Fig. 4 – Effects of vinflunine on endothelial cell motility. Motility was assessed in the Boyden chamber, using the supernatant of NIH-3T3 cells as the attractant. Human umbilical vein endothelial cells (HUVEC) cells were exposed to 10^{-8}–10^{-4} M vinflunine for 4 h. Data, expressed as the percentage of control migration, are means ± standard error (SE) of values from three independent experiments.

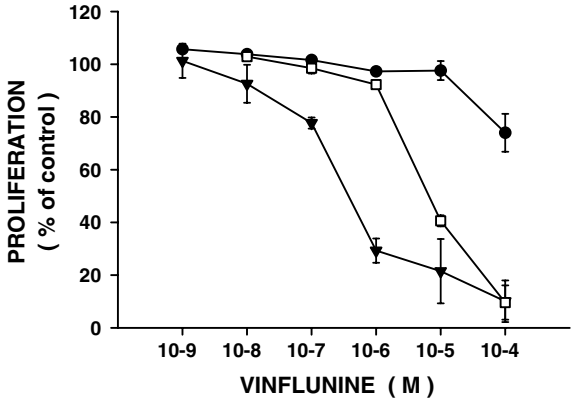

Fig. 6 – Effects of vinflunine on endothelial cell proliferation. Human umbilical vein endothelial cells (HUVEC) were exposed to the indicated concentration of vinflunine for either 1 h (filled circles), 4 h (open squares) or 72 h (filled triangles). Data are presented as the percentage of control proliferation (vehicle-treated cells), expressed as means ± standard error (SE) of values from two independent experiments.

had the same effect as vinflunine on endothelial cell morphology and motility, the effects of cisplatin were also investigated. Cisplatin did not affect endothelial cell morphology nor motility at concentrations up to 10^{-4} M (Fig. 8A and B), whereas it inhibited endothelial cell proliferation after a 1 h, 4 h or 72 h incubation period, with IC_{50} values of 8.8×10^{-5} M, 2.7×10^{-5} M or 5.9×10^{-6} M, respectively. Overall, these data suggest that the effect of vinflunine on endothelial cells is not a mere consequence of its anti-proliferative properties.

3.9. *Vinflunine inhibits angiogenesis in vivo*

The effect of vinflunine on angiogenesis *in vivo* was evaluated using the Matrigel plug assay, a relatively simple and rapid *in vivo* model of induced neovascularisation. Indeed, subcutaneous injection of bFGF embedded in a pellet of Matrigel induced a strong angiogenic response in 4 days, with a 2–4-fold increase in the haemoglobin content compared with pellets containing Matrigel alone (Fig. 9). Intravenous treatment of mice with vinflunine, immediately before and 2 d after Matrigel implantation, resulted in a dose-dependent inhibition of the bFGF-induced angiogenic response, compared with vehicle-treated animals. Inhibition of haemoglobin content was significant ($P < 0.01$) at 1.25, 2.5 and 5 mg/kg, with no effect at 0.63 mg/kg ($P > 0.05$). An ID_{50} value (dose which inhibited 50% of bFGF-induced neovascularisation) was calculated as 1 mg/kg (Fig. 9). Histological analyses of the Matrigel pellets confirmed the anti-angiogenic activity of vinflunine. Four days after injection, bFGF-containing Matrigel in vehicle treated mice (Fig. 10B) presented a strong angiogenic response, with a high degree of cellularity surrounding and invading the pellets of Matrigel. Cells were scattered singly throughout the Matrigel or organised in thin cords, tubules and blood vessels.

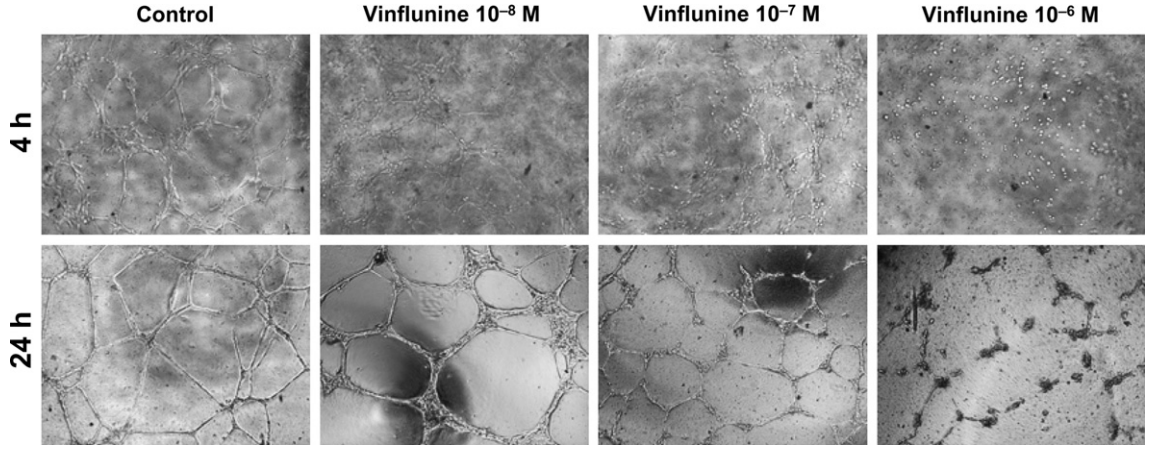

Fig. 5 – Effects of vinflunine on the formation of capillary-like structures by endothelial cells. Human umbilical vein endothelial cells (HUVEC) were plated onto a thick layer of Matrigel (10 mg/ml) in the presence of vehicle (control) or 10^{-8}–10^{-6} M vinflunine. Images were taken 4 h after plating (when cells begin to align) or 24 h (when the network of cords is formed). Magnification × 40.

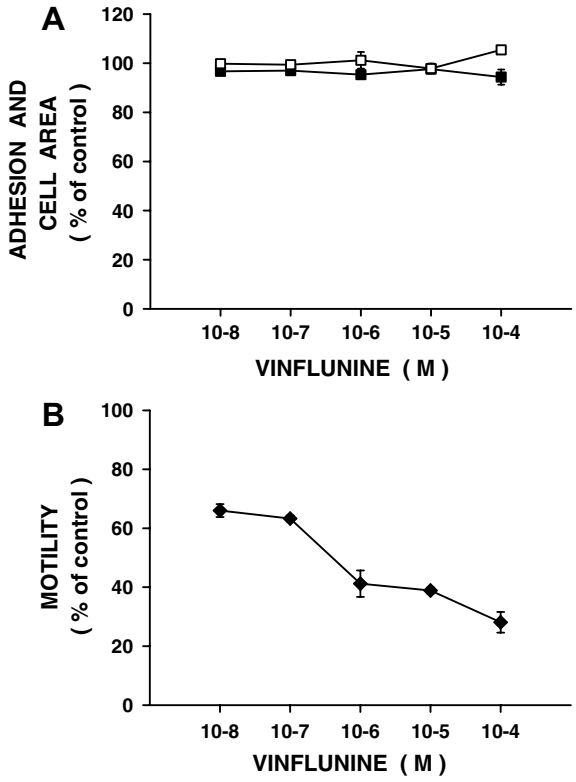

Fig. 7 – Effects of vinflunine on (A) the A-10 rat smooth muscle cell morphology/spreading and adhesion, as well as (B) on cell motility. A-10 rat smooth muscle cells adherent to fibronectin were exposed to the indicated concentrations of vinflunine for 45 min, washed, and stained with crystal violet. The degree of cell spreading (evaluated as the cell area/open squares) and adhesion (absorbance/filled squares) were expressed as the percentage of control response (vehicle-treated cells). Data are means ± standard error (SE) of values from two independent experiments (A). Motility was assessed in the Boyden chamber, using the supernatant of NIH-3T3 cells as the attractant. A-10 smooth muscle cells were exposed to 10^{-8}–10^{-4} M vinflunine for 4 h. Data, expressed as the percentage of control migration, are means ± SE of values from two independent experiments (B).

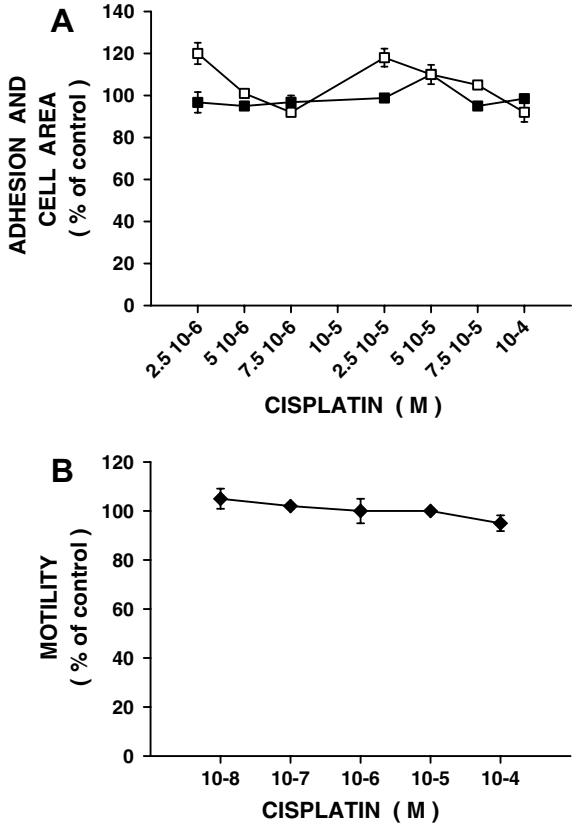

Fig. 8 – Effects of cisplatin on endothelial cell morphology/spreading and adhesion (A), as well as on cell motility (B). Human umbilical vein endothelial cells (HUVEC) adherent to fibronectin were exposed to the indicated concentrations of cisplatin for 45 min, washed, and stained with crystal violet. The degree of cell spreading (evaluated as the cell area/open squares) and adhesion (absorbance/filled squares) were expressed as the percentage of control response (vehicle-treated cells). Data are means ± standard error (SE) of values from two independent experiments (A). Motility was assessed in the Boyden chamber, using the supernatant of NIH-3T3 cells as the attractant. HUVEC cells were exposed to 10^{-8}–10^{-4} M cisplatin for 4 h. Data, expressed as the percentage of control migration, are means ± SE of values from two independent experiments (B).

A marked reduction of the angiogenic response was observed in the pellets of mice treated with 2.5 mg/kg vinflunine compared with vehicle treatment, since only a minimal endothelial cell recruitment was detected in these pellets (Fig. 10D).

3.10. *Anti-metastatic effects of vinflunine*

The activity of vinflunine against an experimental liver metastases model was investigated.

3.11. *Low doses of vinflunine reduces the number of experimental liver metastases by human LS174T colon cancer cells*

The activity of vinflunine against experimental liver metastases, generated by injection of human LS174T colon cancer cells into the spleen of nude mice, was evaluated. Liver metastatic foci were detected in all vehicle-treated mice (control), and 55% of which exhibited a liver completely replaced by the metastatic foci (Table 1 and Fig. 11A and D1). Treatment of mice with multiple i.v. doses of vinflunine on days 4, 7, 11, 14, 18 and 21 after intrasplenic injection of LS174T cells induced a dose-dependent reduction in the number of metastatic foci at the surface of the liver of these animals, compared with the vehicle-treated group (Table 1). A slight overall decrease in liver metastatic foci was already observed at the very low dose of 0.16 mg/kg vinflunine, although maximal overall inhibition was reached at the maximal tolerated dose (MTD) of 20 mg/kg. This reflects a wide therapeutic window of vinflunine against this experimental metastasis model. More specifically, at 1.25 mg/kg, a dose that is 16-fold

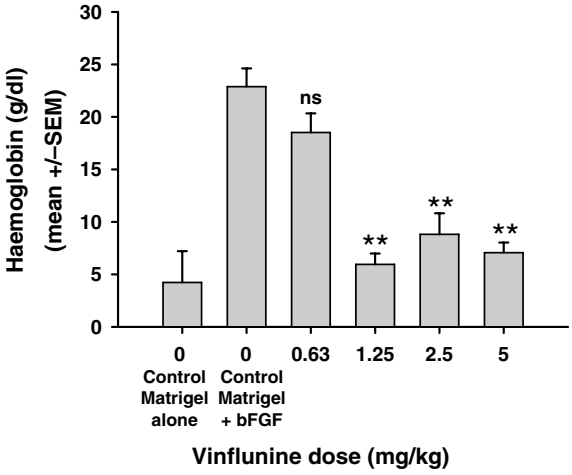

Fig. 9 – Effects of vinflunine on angiogenesis *in vivo*. bFGF (150 ng/ml) was embedded in Matrigel and implanted subcutaneously (s.c.) into C57BL/6N mice on day 0. Mice were treated intravenously (i.v.) on days 0 and 2 at the indicated doses of vinflunine or with vehicle (controls), and the angiogenic response was evaluated 4 d later in terms of the haemoglobin content of the pellet. Data are means ± standard error (SE) from three independent experiments ($n = 30$); ns, $P > 0.05$; **, $P < 0.001$ (Mann-Whitney test).

Table 1 – Inhibition of experimental liver metastases by vinflunine

VFL dose (mg/kg)	Number of liver metastatic foci				
	0	1–10	11–30	>30	Liver replaced by metastatic foci
20	60	35	5	0	0
10	30	70	0	0	0
5	30	50	0	20	0
1.25	20	25	25	30	0
0.16	0	20	10	40	30
0.08	0	5	10	20	65
0 Control	0	2	10	33	55

For each dose of vinflunine, data are expressed as the percentage of mice having numbers of liver metastatic foci of '0', '1–10', '11–30', '>30' or with a liver completely replaced by metastatic foci. The grey area highlights the doses of vinflunine reducing the overall liver metastatic burden.

lower than vinflunine MTD, a significant decrease in the number of metastases was observed. None of the mice treated with this dose of vinflunine had a liver completely replaced by tumour burden, and complete inhibition of macroscopically detectable tumour foci was observed in 20% of the group of mice (Table 1). In comparison, such a low dose of vinflunine did not result in marked anti-tumour efficacy in experimental tumour xenograft models, where vinflunine MTD was around 20–40 mg/kg.[23,25] Macroscopic evaluation and histological analyses revealed that complete inhibition of liver metastases was obtained in 60% of the mice treated with 20 mg/kg vinflunine (Table 1 and Fig. 11B). Furthermore, in the liver of the remaining 40% of this group, a few residual tumour foci were

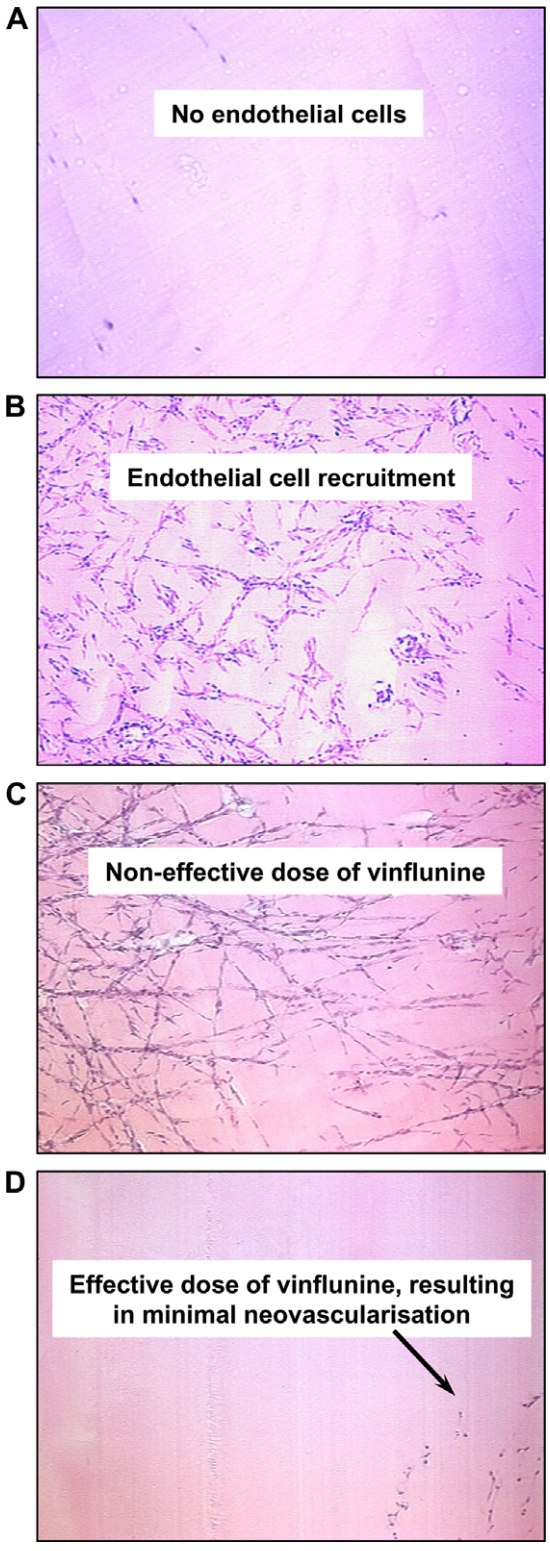

Fig. 10 – Inhibition of angiogenesis *in vivo* by vinflunine: histological analyses of the Matrigel. On day 4 after Matrigel implantation, pellets were removed, fixed and embedded in paraffin. Five-μm sections of (A) Matrigel alone, (B) bFGF-containing Matrigel from vehicle-treated mice (control), (C) bFGF-containing Matrigel from mice treated with 0.63 mg/kg vinflunine, or (D) bFGF-containing Matrigel from mice treated with 2.5 mg/kg vinflunine were stained with haematoxylin and eosin (H& E). Magnification × 400.

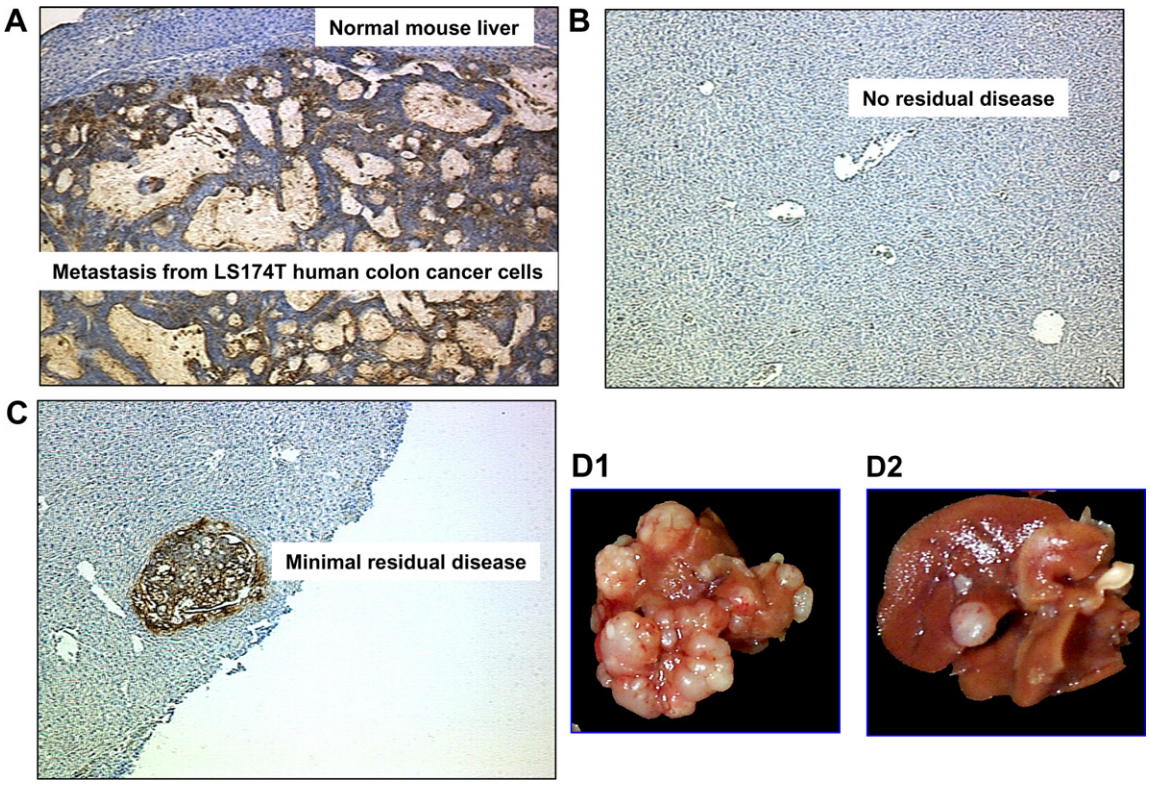

Fig. 11 – Inhibition of experimental liver metastases by vinflunine: histological analyses. On day 28 after intrasplenic injection of LS174T cells, livers were removed, fixed and embedded in paraffin. Five-μm sections of livers from (A) vehicle-treated mice or (B and C) mice treated with 20 mg/kg vinflunine were stained with haematoxylin and eosin (H& E). Complete inhibition of liver metastases was obtained in 60% of the mice treated with 20 mg/kg vinflunine (B) and in the liver of the remaining 40% of this population, a marked reduction in both the number and the size of metastatic foci was observed (C). Magnification × 160. Example of macroscopic aspect of livers of control mice (D1) or mice having been treated with 20 mg/kg vinflunine (D2).

observed, compared with the vehicle-treated group, as illustrated in Fig. 11A, C and D2 and in Table 1. Vinflunine treatment was well tolerated, as reflected by body weight measurements with a maximal median body weight loss of only 10% being recorded at the highest dose of 20 mg/kg, a figure below the National Cancer Institute (NCI) toxicity criteria of 15–20%.[26]

4. Discussion

Tumour-induced angiogenesis, the formation of new capillaries from existing blood vessels, is critical for tumour growth and metastatic spread. Among the many compounds capable of inhibiting angiogenesis and/or disrupting tumour vasculature are the conventional chemotherapeutic drugs. This study reports that vinflunine, the latest vinca alkaloid in clinical development and currently ongoing phase III clinical trials, not only affects endothelial cell morphology and disrupts newly formed blood vessels, but is also able to inhibit endothelial cell motility and the formation of new vessels. These effects were noted using *in vitro* experimental conditions that only marginally affect endothelial cell proliferation. This study also reports that low sub-therapeutic doses of vinflunine inhibit bFGF-induced neovascularisation in Matrigel implants *in vivo* and that vinflunine markedly reduces the number of experimental liver metastatic foci in mice, with significant effects also observed at low doses; i.e. 16-fold lower than the MTD.

In vitro, vinflunine caused a rapid retraction of endothelial cells. Indeed, this effect was obtained after a short 45 min exposure. The ability of tubulin-binding agents to induce rapid endothelial cell retraction is thought to reflect vascular-disrupting activity. Endothelial cell shape changes may lead to the shutdown of already formed vessels. Accordingly, vinflunine disrupted the network of newly formed capillary-like structures *in vitro*. These results add support to a previously published study that identified *in vivo* anti-vascular activity of vinflunine.[21] Using a well-vascularised tumour model, the murine MAC15A colon adenocarcinoma, these authors showed that a single intraperitoneal (i.p.) dose of vinflunine, lower than its MTD, resulted in tumour growth delay, associated with a vascular shutdown over a minimum period of 24 h. These data suggested that an anti-vascular mechanism might, at least in part, contribute to the anti-tumour activities of vinflunine.

Furthermore, studying the effects of vinflunine on *in vitro* endothelial cell functions relevant to the angiogenic process, it has been shown to inhibit endothelial cell motility in response to NIH3T3-derived chemotactic factors, again suggestive of anti-angiogenic activity. This was further supported by

the finding that vinflunine prevented capillary-like structures formation *in vitro*, using an assay that mimics the final events during angiogenesis, when endothelial cells become organised into a three-dimensional network of capillaries. More importantly, vinflunine inhibited the angiogenic response induced by bFGF in Matrigel implants *in vivo*, suggesting an anti-angiogenic activity *in vivo*.

Therefore, overall these studies suggest that vinflunine has both vascular-disrupting and anti-angiogenic properties *in vitro* as well as *in vivo*. Compounds that affect microtubule dynamics act as anti-angiogenic or vascular-disrupting agents because they impair endothelial cell functions, relevant to angiogenesis or to the performance of the tumour vasculature, that require a functional microtubule network. Tubulin-interacting compounds promote either microtubule polymerisation (e.g. taxanes and epothilones) or depolymerisation (e.g. colchicines and vinca alkaloids). However, despite their opposing final effects, the main activity of these compounds is likely to be alteration of microtubule dynamics.[27,28] Microtubule-stabilising agents, such as taxanes exhibit anti-angiogenic activity,[8,22] whereas microtubule-destabilising agents, like vinca alkaloids, colchicines derivatives and combretastatins, are described as vascular-disrupting agents.[6,11,29] However, anti-angiogenic activity has also been described using low concentrations of vinblastine, with a report of its suppression of endothelial cell migration and capillary-like structures formation.[12]

Interestingly, these anti-angiogenic and vascular-disrupting effects of vinflunine were observed *in vitro* under experimental conditions that only marginally affect endothelial cell proliferation. Indeed, no anti-proliferative effect of vinflunine was observed when endothelial cell proliferation was assessed after 1 h exposure to vinflunine, the treatment conditions similar to those used in the assays evaluating *in vitro* vascular-disrupting activities. Furthermore, after a 4 h incubation period with vinflunine, endothelial cell motility was inhibited at lower drug concentrations than was proliferation. This suggests that the effects of vinflunine on endothelial cell functions relevant to the performance of an already formed vasculature, and to the angiogenic process might be more important than that on proliferation in the overall alteration of endothelial cell functions *in vivo*. Furthermore, under these experimental conditions (cell exposure to vinflunine of 1 h or 4 h), vinflunine did not induce cytotoxic activity on tumour cells.[30] Another indication that the anti-angiogenic and vascular-disrupting properties of vinflunine might not merely be dependent on inhibition of proliferation is the lack of effect of cisplatin, a cytotoxic and non-tubulin-interacting agent, on endothelial cell morphology and motility. Therefore, vinflunine might conceivably affect tumour-induced angiogenesis or vasculature *in vivo* at local concentrations lower than those necessary to cause a cytotoxic effect on tumour cells. Such *in vitro* preferential effects on endothelial cell morphology or motility and the capacity to form new vessels, versus endothelial cell proliferation have also been described for taxanes[8,22] as well as for newer colchicine derivatives.[11] Furthermore, Pasquier and colleagues[28] showed that paclitaxel affected endothelial functions *in vitro* at non-cytotoxic concentrations by a mechanism different to that involved in anti-tumour activity. Indeed, anti-angiogenic, non-cytotoxic concentrations of paclitaxel induced an increase in microtubule dynamics in endothelial cells but not in cancer cells, where a suppression of microtubule dynamics was observed after paclitaxel treatment. These findings support the hypothesis that impairment of microtubule functions that contribute to cell shape, polarisation, migration and other processes might be responsible for specific anti-angiogenic or vascular-disrupting effects of certain microtubule/tubulin-interacting agents. Furthermore, the *in vivo* anti-angiogenic and vascular-disrupting activities of vinflunine were also obtained at doses lower than the MTD, i.e. the highest non-toxic dose inducing the highest anti-tumour effect. The dose of vinflunine inhibiting 50% of bFGF-induced neovascularisation in Matrigel implants *in vivo* was 1 mg/kg, which is 20–40-fold lower than the MTD, that induces optimal anti-tumour effects in a series of experimental tumour models.[18] Vinflunine-induced vascular shutdown in the murine MAC15A tumours occurred at 10 mg/kg, which is 5-fold lower than the MTD of vinflunine in this model.[21] However, in early studies with colchicine, podophyllotoxin or vinblastine, whilst definite *in vivo* vascular-disrupting activity was recorded, this was only achieved at doses approaching their MTDs, an adverse finding which has prevented their development as vascular-disrupting agents.[29] It is considered that the *in vivo* induction of anti-angiogenic effects or of vascular shutdown within tumours should occur at doses lower than the MTDs, thus providing a wide therapeutic window. This point is highlighted in *in vivo* pre-clinical studies of combretastatin A-4.[6,31] Accordingly, pre-clinical studies have shown vinflunine to exhibit such a wide therapeutic window in various experimental tumour models and in comparison with the other vinca alkaloids.[25,33] The *in vivo* dual anti-angiogenic and vascular-disrupting activities of vinflunine expressed at low sub-therapeutic doses have implications for the inclusion of vinflunine in combination therapies, since combining of drugs with different mechanisms of action at doses below their MTDs should result in a synergistic anti-tumour activity with minimal toxicity.

Anti-angiogenic activity is expected to result not only in control of tumour progression but also of metastases formation and growth. In support of this, vinflunine has been shown markedly to reduce the number of liver metastatic foci induced by the intrasplenic implantation of colon cancer cells. A slight overall decrease was even already observed at the very low dose of 0.16 mg/kg vinflunine, whereas maximal and complete inhibitory activity was reached at the highest tolerated dose of 20 mg/kg, reflecting a wide therapeutic window. It is noteworthy, the low dose of 1.25 mg/kg, that is 16-fold lower than the vinflunine MTD, resulted in a marked reduction of liver metastatic foci, whereas such low doses did not induce marked anti-tumour activity in experimental tumour xenografts models.[23] Obviously, more studies are thus needed to determine a potential involvement of anti-angiogenic mechanisms in this experimental anti-metastatic activity and to clarify the therapeutic relevance of these findings.

Furthermore, the intermittent schedule of administration of vinflunine used in these *in vivo* studies to reach the end-point of optimal tumour/metastasis-cell kill has to be optimised so as better to exploit the anti-angiogenic/vascu-

lar-disrupting properties of low doses of vinflunine. As discussed by Klement and colleagues,[32] in the clinic, chemotherapy is normally given acutely, usually in the form of bolus infusions at MTDs with long rest periods between successive drug exposures. Such rest periods provide the endothelial cell compartment of a tumour with an opportunity to repair some of the damage induced by the chemotherapy and so assist tumour regrowth. Indeed, the activity of several anti-angiogenic and vascular-disrupting agents has been shown to be readily reversible.[11] This is also the case with vinflunine in terms of its effects on cell morphology, since within 2 h of washing out the lowest concentration tested, cells reverted to their original shape. So it has been proposed that this could be partially compromised by administering compounds frequently at doses significantly below the MTD, with no prolonged drug-free breaks.[33] This concept, called 'metronomic' chemotherapy, was tested in pre-clinical models and proved to be effective in treating experimental tumours in which the cancer cells have developed resistance to the same chemotherapeutics, with the advantage of being less acutely toxic, therefore making more prolonged treatments possible.[34] The efficacy of metronomic chemotherapy can also be significantly increased when administered in combination with anti-angiogenic drugs, such as antibodies against vascular endothelial growth factor (VEGF) or VEGF receptor 2.[33] Clinical trails are currently under way to test several combinations of metronomic chemotherapy and anti-angiogenic drugs, including, for example, daily low doses of cyclophosphamide plus the recently approved bevacizumab (humanised anti-VEGF monoclonal antibody).[33] Of course, this concept needs to be confirmed using different chemotherapeutic agents. Therefore, in this context, the challenge is to be able to find the optimal dose and frequency of administration of vinflunine that results in sustained anti-angiogenic/vascular-disrupting effects and to determine whether such a metronomic chemotherapy-based schedule could potentiate its *in vivo* activity in combination with other standard cytotoxic agents or certain specific angiogenesis inhibitors, which could greatly extend the potential clinical usage of this novel vinca alkaloid.

In conclusion, this study has demonstrated that vinflunine exhibits both anti-angiogenic and vascular-disrupting activity at doses below its MTD, and induces marked inhibition of experimental metastases, also at doses significantly below the MTD, thus opening up new possibilities for its clinical development.

Conflict of interest statement

The following authors: Anna Kruczynski, Eric Chazottes, Géraldine Berrichon, Christel Ricome and Bridget T. Hill are employed by Pierre Fabre Medicaments, where the product vinflunine was synthesised. There are no other interests with regard to this paper that might be construed as a conflict of interest. The other authors have nothing to declare.

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