Vascular-targeting Activity of ZD6126, a Novel Tubulin-binding Agent

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ABSTRACT

The tubulin-binding agent ZD6126 is a novel vascular-targeting agent in clinical development for the treatment of solid tumors. *In vivo*, ZD6126 is rapidly converted into N-acetylcolchinol (ZD6126 phenol). In this study, we have explored the antivasculature property of N-acetylcolchinol in *vivo* and ZD6126 in *vivo*. In cell culture, N-acetylcolchinol induced rapid changes in the morphology of human umbilical vein and lung microvessel endothelial cells. Within 40 min, the compound induced endothelial cell contraction, destabilization of the tubulin cytoskeleton, induction of actin stress fibers, and membrane blebbing. These effects occurred at noncytotoxic concentrations and were rapidly reversed on removal of the drug. Nonconfluent endothelial cells were more sensitive than confluent, quiescent cells. Among different cell types, endothelial cells were the most sensitive to the induction of morphological changes, whereas smooth muscle cells were not affected. *In vitro*, N-acetylcolchinol rapidly disrupted a network of newly formed cords. *In vivo*, ZD6126 caused shut down of newly formed vessels in the Matrigel plug assay, shortly after injection. This study indicates that rapid alteration of endothelial cell morphology may be responsible for the loss of tumor blood vessel integrity, vessel shut down, and extensive tumor necrosis induced by ZD6126 in experimental tumor models.

INTRODUCTION

The interest in vascular-targeted anticancer therapy arises from the dependence of tumor cells on a functional blood vessel system for survival, proliferation, and metastatic dissemination. Hence, the possibility to indirectly inhibit tumor growth and survival by impairing neovessel formation or function (1–3).

Unlike antiangiogenic agents, aimed at preventing vessel formation, the vascular-targeting agents aim to compromise the integrity and functionality of already existing tumor vessels, leading to shutdown of the tumor vascular system and consequent tumor cell death (4). Vascular targeting is made possible by the structural, phenotypic, and functional differences between vessels in tumor and normal tissues (5–7). Endothelial cells in tumors are exposed to a peculiar environment (low oxygen tension, low pH, presence of cytokines, and growth factors) and are undergoing angiogenesis. Hence, they differ from quiescent endothelial cells in terms of functional status and gene expression, including the expression of angiogenic determinants. It is possible to exploit these changes for therapeutic purposes in two distinct vascular-targeting approaches by: (a) directing therapeutic agents to endothelial cells within tumors or (b) using agents that selectively affect tumor endothelial cells *(i.e.*, tumor necrosis factor α, flavone acetic acid and its analogue dimethylxanthone acetic acid, and tubulin-binding agents; Refs. 4 and 8–10).

Microtubules are an interesting target for vascular-targeting agents. Compounds that affect microtubule dynamics act as antiangiogenic agents, because they affect endothelial cell functions crucial for this process (motility, invasion, attachment, alignment, and proliferation; Refs. 11–13). Microtubule depolymerizing agents, including colchicine, podophyllotoxin, and vincristine, cause damage to the existing vasculature of tumors (4). However, the narrow therapeutic window of these agents (vascular-targeting activity is achieved only at doses approaching or exceeding their maximum tolerated dose) has prevented their development as vascular-targeting agents. Recently, microtubule-stabilizing agents such as combretastatin A-4 (14) have been described, which have activity against tumor vasculature at doses significantly lower than the maximum tolerated dose, and, therefore, these agents have the potential to exploit this effect therapeutically.

The colchicine derivative ZD6126 is a novel water-soluble phosphate pro-drug. It is converted *in vivo* into N-acetylcolchinol (ZD6126 phenol), which binds to the colchicine-binding site on tubulin, and causes disruption of microtubules. In animal models, ZD6126 selectively induces tumor vascular damage and massive tumor necrosis at well-tolerated doses (15). ZD6126 is currently in early phase clinical trial. The aim of this study was to investigate the effects of this vascular-targeting antitumor agent on endothelial cells *in vitro* and on neo-formed vessels *in vivo*.

MATERIALS AND METHODS

Compounds. ZD6126 (MW 437) is a water-soluble phosphate pro-drug rapidly converted, *in vivo*, into the active compound N-acetylcolchinol (MW 357). Both molecules were from AstraZeneca (Alderley Park, Macclesfield, United Kingdom). For the *in vitro* experiments, they were dissolved in DMSO (0.1% stock solution) and freshly diluted in test medium before use.

Cells. HUVECs, isolated and cultured as described (12), were used between the third and fifth passages. HMVEC-Ls from Clonetics (BioWhitaker, Walkersville, MD) were cultured in Endothelial Basal Medium. NIH-3T3 (murine fibroblasts) and MDA-MB-435 (human breast carcinoma cells) were cultured in DMEM 10% fetal bovine serum, SKOV-3 (human ovarian carcinoma cells) and A-10 (rat smooth muscle cells) were cultured in RPMI 1640 10% fetal bovine serum, 2 mM L-glutamine, and 2 mM sodium pyruvate.

Analysis of Cell Morphology. Nontissue culture 96-well plates (Microtest; Becton Dickinson, Bedford, MA) were coated with 5 μg/ml (625 ng/cm²) fibronectin (Chemicon, Temecula, CA), collagen I (Becton Dickinson), and collagen IV (Sigma, St. Louis, MO) in PBS or with gelatin 1% (Life Technologies, Inc., Paisley, Scotland) for 2 h at 37°C. After washing in PBS, nonspecific sites were blocked with 1% BSA in PBS (30 min at 37°C). Cells (2.5 × 10⁴ cells/well, unless indicated) in DMEM 0.1% BSA were added and let adhere for 4 h at 37°C. Adherent cells were then exposed to ZD6126, N-acetylcolchinol, or vehicle for 40 min (three to six wells for each condition). Wells were washed 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 analyzed 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, normalized to the number of adherent cells). To evaluate the number of adhered cells, the stain was eluted with a 1:1 mixture of 20% methanol and water, washed, and air dried.

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**Proliferation Assay.** Cells (4 × 10³ cells/well) were plated in 96-well plates in complete medium. After 24 h, N-acetylcolchinol was added (0.001–
100 μM). After 1 h, cells were washed and incubated for an additional 3 days. Alternatively, the cells were incubated with the compound for 72 h. Cells were stained with crystal violet, and absorbance was measured as for the adhesion assay. Data are the percentage of control proliferation and the IC50, the drug concentration that causes 50% inhibition of cell proliferation.

**Immunofluorescence Analysis of the Cytoskeleton.** HUVECs were grown on 1% gelatin-coated coverslips for 3–5 days, incubated for 40 min with N-acetylcolchinol or vehicle in DMEM 0.1% BSA, and fixed and permeated with cold absolute ethanol for 10 min at −20°C. Fixed cells were washed and incubated at room temperature for 1 h with antibodies against β-tubulin (T4026; Sigma), 1:200. After washings, cells were incubated with 1:200 FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) together with the F-actin-binding rhodamine-phalloidin (25 IU/ml; Biomolecular Probes, Leiden, the Netherlands). Coverslips were then washed and mounted with N-propyl gallate in 50% (volume for volume) glycerol and 0.1 mM Tris-HCl (pH 8). Samples were analyzed at fluorescence microscopy (IX70; Olympus) with U-HNIBA filter for β-tubulin and U-HWIG2 for F-actin.

**Cord Disruption Assay.** HUVECs (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 for 48 h. N-acetylcolchinol or vehicle was added to the formed cords and left for 24 h. Pictures were taken at the indicated times (0.5–24 h) after compound addition.

**In Vivo Angiogenesis Assay.** The Matrigel plug assay was used (12). Briefly, basic FGF (500 ng/pellet) was embedded in Matrigel (12.5 mg/ml, 0.5 ml) and injected s.c. in C57BL/6N mice (Charles River, Calco, Italy). ZD6126 (200 mg/kg, i.p., single injection) or vehicle (PBS ± 5% NaCO3) was given to mice 1, 5, or 22 h before necropsy (day 7 after Matrigel injection, n = 6). One h before necropsy, mice were injected i.v. with 100 μl of FITC-conjugated Griffonia simplicifolia Isolcetin-B4 (0.25 mg/ml; Vector, Burlingame, CA). The Matrigel plug was removed and frozen. Perfused, FITC-positive vessels were analyzed with a laser confocal microscope (InSight Plus; Meridian Instruments, Inc., Okemos, MI) with Argon 488 nm emission source.

**RESULTS**

**N-acetylcolchinol and ZD6126 Affect Endothelial Cell Morphology.** The effects of the pro-drug ZD6126 and the active compound N-acetylcolchinol on endothelial cell morphology were investigated. In preliminary experiments, we observed that N-acetylcolchinol was ≥10 times more potent than the pro-drug ZD6126 (data not shown), suggesting a limited conversion of the pro-drug in the absence of serum. The subsequent *in vitro* experiments were performed only with N-acetylcolchinol.

HUVECs were left to adhere to different extracellular matrix components for 4 h and then exposed for 40 min to N-acetylcolchinol. Treated cells retracted and assumed a rounded morphology, indicated by the reduced cell area (Fig. 1, A and B). Similar effects were observed when cells were plated on fibronectin, gelatin, type I collagen, and type IV collagen. After 40 min, treatment with N-acetylcolchinol caused only ≤20% cell detachment (data not shown). When added to endothelial cells while they were adhering to the substrate, the compound only marginally impaired attachment (data not shown), excluding an effect on cell–substrate recognition/interaction.

Endothelial cell retraction was associated with alterations in the organization of both the tubulin and actin cytoskeleton (Fig. 1C–H). N-acetylcolchinol (1 μM) caused complete depolymerization of microtubules in 40 min and induced the formation of actin stress fibers across the cell body (Fig. 1). Many cells retracted and exhibited blebbing of the plasma membrane (Fig. 1). The effect of N-acetylcolchinol on endothelial cell morphology was more prominent on scattered, nonconfluent cells (in three experiments, mean IC50 was 0.07 ± 0.01 μM), whereas confluent, quiescent cells were less sensitive (IC50 = 0.62 ± 0.2 μM).

Alteration of cell morphology occurred at concentrations of N-acetylcolchinol that did not impair endothelial cell proliferation. When HUVECs were exposed for 1 h to N-acetylcolchinol, inhibition of cell proliferation was observed only at concentrations much higher (100–1000-fold) than those required to alter cell shape (Table 1). Only at longer exposure times (72 h) was an antiproliferative effect observed (data not shown).

N-acetylcolchinol also affected the morphology of endothelial cells isolated from microvessels, HMVEC-Ls, causing their retraction at concentrations that did not affect their proliferation (Table 1). Nonendothelial cells were generally less sensitive to the compound in terms of cell shape changes compared with endothelial cells (Table 1). N-acetylcolchinol caused marginal changes in the morphology of smooth muscle cells A10, which were also less sensitive to the antiproliferative activity of the compound (Table 1). NIH-3T3 fibroblasts showed an intermediate behavior between HUVEC and A10 (Table 1). Even though the morphology of the human tumor cell lines tested (SKOV-3 and MDA-MB-435) was only partially affected by N-
acetylcolchinol (41 and 37% reduction in cell area, respectively), although the compound had an antiproliferative activity comparable with endothelial cells (Table 1).

The modifications induced by N-acetylcolchinol on endothelial cell morphology (both HUVECs and HMVEC-Ls) were reversible, and cells reverted to the original shape by 3 h after removal of the compound. Recovery of cell shape was faster (1 h) when cells were treated with low concentrations of the compound (0.1–1 μM), but even after treatment with higher concentrations, no significant permanent modification of the cell shape was apparent (Fig. 2). Colchicine, a related tubulin-targeting agent, induced similar changes in endothelial cell morphology, although in this case, changes were not reversible (data not shown).

**N-acetylcolchinol and ZD6126 Affect Newly Formed Blood Vessels.** We next evaluated whether the compounds had activity against newly formed vessels, in *in vitro* and *in vivo* models. *In vitro*, HUVECs, seeded on a permissive thick layer of the reconstituted basement membrane Matrigel, rapidly align and form a network of cords, reminiscent of newly formed vessels. The addition of ≥1 μM N-acetylcolchinol to formed cords rapidly disrupted the integrity of the network (Fig. 3, A and B). The effect was reversible because, 24 h later, cells appeared to have re-established the original structures (Fig. 3, C and D).

*In vivo*, we evaluated the effect of ZD6126 on newly formed capillaries induced by basic FGF in a pellet of Matrigel. Seven days after injection, FGF-containing Matrigel in vehicle-treated mice (Fig. 3E) presented a strong angiogenic response, with numerous functional, perfused neo-vessels, particularly abundant and presenting a larger caliber at the periphery of the pellet. Treatment of mice with ZD6126 (200 mg/kg, i.p.) caused an almost complete shutdown of the vessels, which, 1 h after treatment, were no longer perfused by FITC-isoelectin B4 (Fig. 3F), H&E staining and immunostaining for the endothelial cell-specific antigen CD31 showed that vascular structures were still present in parallel pellets (data not shown). Similar to our observations on *in vitro* cords, also in this *in vivo* model, the effect was reversible, and 22 h after treatment, vessels in Matrigel were again perfused (Fig. 3H). The phenomenon of reperfusion appeared primarily at the periphery of the pellet.

**DISCUSSION**

This study shows that the vascular-targeting agent ZD6126, through its active metabolite N-acetylcolchinol, affects endothelial cell morphology, destabilizes the tubulin cytoskeleton, and disrupts newly formed vessels.

*In vitro*, N-acetylcolchinol causes retraction of endothelial cells, disrupting the tubulin cytoskeleton and inducing actin stress fibers and membrane blebbing. Cooperation between microtubules and the actin cytoskeleton is crucial in the control of cell shape, adhesion, contraction, and motility (16). Microtubules directly control actin filament organization by locally modulating the activity of the small GTPases of the Rho family. In particular, microtubule-disrupting agents induce guanine nucleotide exchange factor GEF-H1-mediated activation of Rho, which triggers stress fiber formation (17). Combretastatin A4 causes Rho-mediated endothelial cell retraction, membrane blebbing, and assembly of stress fibers (18). Therefore, it is possible that
morphological changes and cytoskeletal modifications caused by N-acetylcolchinol might involve GEF-H1/Rho activation.

The ability of tubulin-binding agents to cause rapid endothelial cell retraction is thought to underlie their vascular-targeting activity. In vivo, in a tumor setting, endothelial cell retraction may lead to the increased permeability, exposure of the basement membrane, platelet activation, and coagulation. Accordingly, in a panel of tumor models in vivo, ZD6126 induced rapid (30–60 min) changes in central tumor capillaries with focal loss of endothelial cells, exposure of the basal lamina, accumulation of platelets and fibrin, thrombus formation, and congestion of tumor vessels (15). By 24 h, extensive central necrosis of the tumor, with only a thin viable rim of tumor cells at the periphery, is observed (15, 19).

After injection of a single, well-tolerated dose (61 mg/kg) to mice, ZD6126 is rapidly converted to N-acetylclocolin (peak concentrations at 10 min). Thereafter, the plasma concentration of N-acetylclocolin declines (half-life of ~1 h), and 1 h after administration, plasma concentrations are ~2 μg/ml (5.6 μM; Ref. 15). The plasma profile of N-acetylclocolin in mice (consistent with the conditions active in vitro on endothelial cell morphology), together with the rapid elimination of the compound from the plasma, may further rule out a significant effect on cell proliferation, which requires higher concentrations and/or longer times of exposure.

N-acetylcolchinol alters more effectively the shape of nonconfluent endothelial cells than confluent, quiescent endothelial cells. This might depend on differences in the stability of the actin cytoskeleton, in the strength of cell–cell interactions or in the expression of tubulin isotypes, tubulin post-translational modifications, or microtubule-associated proteins between confluent and nonconfluent cells. This difference in sensitivity suggests that the compound is more likely to affect immature endothelial cells (such as those in tumor vessels) rather than mature, quiescent cells of vessels in normal tissues. In agreement, preclinical studies showed activity of ZD6126 on tumor vessels without similar effects on vessels in normal tissues (15).

The effect of N-acetylclocolin on endothelial cell morphology is rapid and reversible, because within 1–3 h after removal of the compound, the cells reverted to their original shape. Although several mechanisms might contribute to the reversibility of the compound activity, this behavior is in agreement with the tubulin-binding kinetics of this class of compounds: (a) a rapid binding to tubulin and (b) a very short dissociation half-life (20). This property represents an improvement compared with other microtubule-stabilizing agents, such as colchicine, whose narrow therapeutic window has been ascribed to the pseudo-irreversible binding to tubulin (4, 14).

The reversibility of the effect of ZD6126/N-acetylclocolin on endothelial cell morphology, in vitro cord disruption, and in vivo vessels occlusion in the Matrigel model suggests a potential therapeutic margin in the use of this compound. However, these findings also suggest that the damage to tumor vascular induced by this compound may not be permanent and that, especially at the periphery of a tumor, vessels may rapidly recover their functionality. This observation parallels the in vivo evidence that single treatment with ZD6126, although causing central tumor necrosis, may induce only a modest tumor growth delay in animal models (15). Repeated single agent administration or combination with conventional therapies offer promising therapeutic potential for ZD6126. Indeed, recent preclinical studies have confirmed the increased antitumor activity of multiple doses of ZD6126 (15) and of combinations of this compound with chemotherapy and radiotherapy regimens (15, 19).

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