Potential Antagonism of Tubulin-Binding Anticancer Agents in Combination Therapies

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Abstract

ZD6126 is a vascular targeting agent, developed for the treatment of solid tumors. In vivo, ZD6126 is rapidly converted into the tubulin-binding agent N-acetylcobolin. We have previously reported that in vitro N-acetylcobolin disrupts microtubules and induces rapid changes in endothelial cell morphology, which in a tumor would lead to a rapid loss of tumor vessel integrity and subsequent extensive tumor necrosis. The aim of this study was to investigate the effect of cytotoxic antineoplastic drugs—cisplatin, doxorubicin, vincristine, paclitaxel, and docetaxel—on endothelial cell response to N-acetylcobolin. We found that cisplatin and doxorubicin did not interfere with the ability of N-acetylcobolin to cause morphologic changes in human umbilical vein endothelial cells, whereas vincristine showed additive effects. In contrast, the microtubule-stabilizing agents paclitaxel (1-10 μmol/L) and docetaxel (0.1-1 μmol/L) prevented the morphologic changes induced by N-acetylcobolin in human umbilical vein endothelial cells. The effect was observed when cells were exposed to paclitaxel and N-acetylcobolin together or when paclitaxel was given shortly before N-acetylcobolin. Paclitaxel and N-acetylcobolin interacted at the level of microtubule organization, as shown in immunofluorescence analysis of the cytoskeleton. The protective effect was reversible because 4 hours after paclitaxel wash out, cells recovered the sensitivity to N-acetylcobolin. In vivo, pretreatment of mice with paclitaxel inhibited the vascular targeting activity of ZD6126 on newly formed vessels in the Matrigel plug assay and ZD6126-induced necrosis in tumors. These findings indicate that paclitaxel, depending on the timing and schedule of administration, can affect the vascular targeting activity of ZD6126, which may have an effect on the optimal scheduling of therapies based on the combined use of microtubule-stabilizing and microtubule-destabilizing agents.

Vascular targeting agents, aimed at destroying the existing tumor vasculature, are being developed as antineoplastic compounds for the treatment of solid tumors (1, 2). Because solid tumors depend on a functional vasculature for survival, proliferation, invasion, and metastasis, disrupting vessel structure and function will potentially affect many aspects of tumor growth. Vessels in tumors have phenotypic and functional characteristics that distinguish them from the vasculature in normal tissues (3–6). Vascular targeting agents exploit these differences, causing direct and selective damage to vessels in tumors but sparing those in normal tissues. Strategies to damage the tumor vasculature include ligand-directed vascular targeting agents, cytokine-inducer flavonoids (flavone-8-acetic acid and 5,6-dimethylxanthenone-4-acetic acid), and tubulin targeting agents (reviewed in refs. 1, 2, 7). The latter base their activity on the different sensitivity of endothelial cells in tumors and normal tissues to microtubule cytoskeleton–destabilizing agents. Some of these compounds, which include ZD6126, combretastatin A-4 phosphate, and AVE8062A, are currently undergoing clinical trials.

ZD6126 is a water-soluble phosphate prodrug, rapidly converted in vivo into the tubulin-binding moiety ZD6126 phenol (N-acetylcobolin). In vitro, at sub-cytotoxic concentrations, N-acetylcobolin rapidly disrupts the tubulin cytoskeleton of endothelial cells, inducing actin stress fibers and membrane blebbing. In immature or proliferating endothelial cells, this disruption of the tubulin cytoskeleton is associated with a rapid alteration in cell shape, where cells retract and assume a rounded morphology (8). In vivo, retraction of tumor endothelial cells following ZD6126 treatment is thought to trigger a cascade of events, leading to loss of vessel integrity, reduction in tumor blood flow and vascular volume, and vessel occlusion (8–10). This results in massive central necrosis of the tumor, with a thin rim of viable tumor cells remaining at the periphery, a hallmark of the action of...
tubulin-binding vascular targeting agents (9, 11). However, these viable tumor cells can repopulate the tumor, which is able to resume its growth, unless treatment with the vascular targeting agent is repeated (9, 12). Alternatively, preclinical studies have shown an increased therapeutic effect when the compound is combined to other antineoplastic therapies (9). ZD6126, as well as other vascular targeting agents, has been shown to benefit from combination with radiotherapy and chemotherapy. ZD6126, for example, significantly enhances the antineoplastic efficacy of cisplatin (9, 13) and radiation (11, 14, 15).

Little is known of the possible interaction of chemotherapeutic agents with the activity of tubulin-binding vascular targeting compounds. The aim of this study was to investigate the effect of chemotherapeutic agents with different mechanisms of action (paclitaxel, docetaxel, vincristine, cisplatin, and doxorubicin) on in vitro endothelial cell responses to N-acetylcolchinol and the in vivo vascular targeting activity of ZD6126. In particular, we were interested in the combination of paclitaxel and ZD6126/N-acetylcolchinol because the two compounds bind the same target, tubulin, though on different sites, exerting opposite effects on microtubule polymerization.

Materials and Methods

Compounds. ZD6126 (MW 437) is a water-soluble phosphate prodrug, rapidly converted in vivo into the active compound N-acetylcolchinol (MW 357). Both molecules were supplied from AstraZeneca (Alderley Park, Macclesfield, United Kingdom). For the in vitro experiments, N-acetylcolchinol was dissolved in DMSO (0.1 mol/L stock solution) and further diluted in test medium immediately before use. For in vivo administration, ZD6126 was dissolved in PBS with 0.5% NaCO3.

Paclitaxel and docetaxel (kindly provided by Indena, Milan, Italy) were dissolved in DMSO and further diluted in test medium before use. For the in vivo assays, paclitaxel was dissolved in 50% Cremophor EL (Sigma, Milan, Italy) and 50% ethanol and further diluted with saline before injection. Doxorubicin, cisplatin, and vincristine (Sigma) were dissolved in saline.

Cells. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins and grown on 1% gelatin–coated glass coverslips for 2 to 3 days in culture medium. Cells were then washed to remove the compounds and incubated in culture medium for additional 3 days. At the end of the incubation, the cells were fixed and stained with crystal violet and the degree of proliferation was measured as absorbance at 540 nm, as indicated for the adhesion assay.

Immunofluorescence analysis of the cytoskeleton. HUVECs were grown on 1% gelatin–coated glass coverslips for 2 to 3 days in culture medium. Cells were then incubated for 1 hour with vehicle, N-acetylcolchinol (1 µmol/L), paclitaxel (1 µmol/L), or the two compounds together in DMEM-0.1% bovine serum albumin, and then fixed and permeabilized with cold methanol for 10 minutes at −20°C. Fixed cells were washed thrice with PBS and incubated at room temperature for 1 hour with antibodies against β-tubulin (T4026, Sigma) 1:200 in PBS-0.1% bovine serum albumin. After washing, cells were incubated with FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Coverslips were then washed and mounted with antifading medium containing 1:5 glycerol and 0.1 mol/L Tris-HCl (pH 8). Samples were analyzed by fluorescence microscopy (IX70, Olympus Optical) with U-HNIBA filter.

In vivo vascular targeting assay. The Matrigel plug assay was used. This assay was previously used to show that a single treatment with ZD6126 caused, after 2 hours, complete occlusion of newly formed vessels (8). Briefly, basic fibroblast growth factor (500 ng/pellet) was embedded in a pellet of Matrigel (Becton Dickinson; 12.5 mg/mL, 0.5 mL) and injected s.c. into C57BL/6N mice (Charles River, Calco, Italy). On day 7, when functional, perfused vessels are formed and evident within the Matrigel plug, mice were pretreated with paclitaxel (40 mg/kg i.v.) or vehicle 2 hours before ZD6126 (200 mg/kg i.v.) or vehicle, followed 2 hours later by ZD6126 injection. Fluorescent images of isolectin-B4–perfused positive vessels were obtained by excitation of the FITC fluorophore at 488 nm using an argon-ion laser of an Olympus FV500 laser scanning confocal microscope.

In vivo analysis of tumor necrosis. The human carcinoma MDA-MB-435 (5 × 10⁶ cells) was xenografted s.c. into the flanks of female NCr-nu/nu mice (Animal Production Colony, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Tumor size was measured twice a week with calipers, and when the tumor reached a median weight of 480 mg (calculated from the formula: length x width ² / 2) mice were injected with paclitaxel (20 or 40 mg/kg i.v.) or vehicle, followed 2 hours later by ZD6126 (200 mg/kg i.p.) or vehicle (n = 5 mice/group). Tumors were removed 24 hours later, fixed in formalin, processed, and stained with H&E following standard procedures. Sagittal 5-μm sections were cut and necrosis was analyzed by computerized image analysis (Image Pro-Plus 4.5, Media Cybernetics) as the difference in stain intensity among vital and necrotic tissues. Area of necrosis was expressed as the percentage of total tumor area. Presence of necrosis was also confirmed by histopathologic analysis.

Statistical analysis. Two-tailed Mann Whitney U test was used to assess statistical differences. Statistical significance was set at P ≤ 0.05.
**Results**

Paclitaxel and docetaxel inhibit N-acetylcolchinol–induced changes in human umbilical vein endothelial cell morphology in vitro. As previously reported, 1-hour exposure to non-cytotoxic concentrations of N-acetylcolchinol induced a rapid retraction and rounding up of HUVECs in culture (Fig. 1; ref. 8). To investigate the effect of different cytotoxic compounds on endothelial cell sensitivity to N-acetylcolchinol, HUVECs were exposed for 1 hour to N-acetylcolchinol in the presence of different concentrations of the cytotoxic drugs.

Non-tubulin-binding cytotoxic compounds, cisplatin (up to 100 μmol/L) and doxorubicin (up to 17 μmol/L), had no effect on endothelial cell morphologic changes induced by N-acetylcolchinol (Fig. 1 shows the highest tested concentrations). Combination of N-acetylcolchinol with the microtubule-depolymerizing agent vincristine (0.01-0.1 μmol/L) gave an additive effect (Fig. 1 shows the lowest active concentration, whereas at higher concentrations, vincristine itself was active in inducing endothelial cell retraction). In contrast, the presence of microtubule-stabilizing agents paclitaxel (1-10 μmol/L) and docetaxel (0.1-1 μmol/L) inhibited the morphologic changes induced by N-acetylcolchinol in HUVECs (Fig. 1 shows concentrations of 10 and 1 μmol/L, respectively). The antagonizing effect was similarly evident when cells were exposed to paclitaxel for 1, 4, and 72 hours before the addition of N-acetylcolchinol, although at long incubation times (72 hours) some toxic effects of the high concentrations of paclitaxel per se occurred on endothelial cells (not shown). The effect was concentration dependent, with 10 μmol/L paclitaxel maximally preventing HUVEC shape change induced by 1 μmol/L N-acetylcolchinol (Fig. 2A and B). The protective effect of paclitaxel on endothelial cell response to N-acetylcolchinol was reversible; within 4 hours of paclitaxel removal, HUVECs were again sensitive to N-acetylcolchinol–induced cell shape change (Fig. 3).

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**Fig. 1.** Effects of different cytotoxic agents on the morphologic changes induced by N-acetylcolchinol in HUVECs. HUVECs adherent to fibronectin were exposed to the indicated concentrations of N-acetylcolchinol and one of the following: paclitaxel (10 μmol/L, ◦), doxorubicin (1 μmol/L, ◊), cisplatin (100 μmol/L, □), or vincristine (0.01 μmol/L, ▲). After 1 hour of incubation, cells were gently washed and stained with crystal violet. The degree of cell spreading was evaluated as cell area and expressed as percentage of control (cells exposed to vehicle). Points, mean values from at least two experiments; bars, SE. At N-acetylcolchinol concentration of 1 μmol/L, paclitaxel and doxorubicin significantly reduced HUVEC response to the compound. Vincristine had a significant additive effect (P < 0.05 at 1 μmol/L N-acetylcolchinol).

**Fig. 2.** Effects of different concentrations of paclitaxel on endothelial cell retraction in response to N-acetylcolchinol. HUVECs were exposed to vehicle (■) or paclitaxel, 0.1 (○), 1 (▲), 10 (△), or 100 μmol/L (□) for 1 hour before the addition of the indicated concentration of N-acetylcolchinol. A, degree of cell spreading indicated by HUVEC area (expressed as percentage of control). Points, mean values from at least two experiments; bars, SE. At N-acetylcolchinol concentration of 1 μmol/L, the protective effect was significant (P < 0.05) for paclitaxel concentrations of 1 and 10 μmol/L. B, representative images of cells after incubation with N-acetylcolchinol (1 μmol/L) and/or the indicated amount of paclitaxel (>100).

**Paclitaxel inhibits N-acetylcolchinol–induced microtubule depolymerization in human umbilical vein endothelial cells in vitro.** Endothelial cell retraction caused by 1 μmol/L N-acetylcolchinol was associated with rapid alterations in the organization of the tubulin cytoskeleton and depolymerization of microtubules (8). Because paclitaxel is known to cause an opposite effect on microtubule organization, we investigated the effect of a concomitant treatment with these two compounds on endothelial cell microtubules. As expected, N-acetylcolchinol alone (1 μmol/L) caused the depolymerization of microtubules (Fig. 4B), whereas paclitaxel (1 μmol/L) induced the formation of microtubule bundles (Fig. 4C). In the presence of both compounds, the typical modifications induced by either compound alone were much less evident (Fig. 4D), indicating a functional reciprocal interference between the two agents.
N-Acetylcolchinol does not inhibit the antiproliferative effects of paclitaxel in human umbilical vein endothelial cells in vitro.

Following the observation that paclitaxel protected endothelial cells from the morphologic changes induced by N-acetylcolchinol, we also examined whether N-acetylcolchinol could interfere with the antiproliferative activity of paclitaxel on endothelial cells. In conditions of brief exposure to the drugs, N-acetylcolchinol alone (up to 10 μmol/L) did not affect endothelial cell proliferation (8), whereas paclitaxel alone inhibited endothelial cell proliferation with an IC50 of 1.9 μmol/L (Fig. 5). The addition of N-acetylcolchinol (0.1 or 10.0 μmol/L) did not modify endothelial cell proliferation in response to paclitaxel (Fig. 5), indicating that N-acetylcolchinol, even at high concentrations, does not antagonize the cytotoxic activity of paclitaxel. Similar results were obtained when cells were exposed to both compounds for 72 hours, although in this case, high concentrations of N-acetylcolchinol per se showed some antiproliferative effect (not shown).

Paclitaxel inhibits the effects of ZD6126-induced vessel shutdown in vivo. We next evaluated the effects of paclitaxel in an in vivo model of newly formed vasculature. We have previously reported that a single injection of ZD6126 rapidly caused the shutdown of newly formed vessels in the Matrigel plug assay (8). In this assay, a Matrigel plug containing basic fibroblast growth factor implanted s.c. in mice induces the formation of new vessels, with a maximal response observed 7 days after implantation. The use of an FITC-conjugated lectin allows the visualization of functional, perfused neo-vessels entering the plug (Fig. 6A; ref. 8). Treatment with ZD6126 (200 mg/Kg i.v.) at day 7 caused the shutdown of vessels that, 1.5 hours after treatment, were no longer perfused by FITC-isolectin B4 (Fig. 6B), whereas treatment with paclitaxel alone (40 mg/kg i.v.) did not cause major alterations in the structure or perfusion of vessels (Fig. 6C). In contrast, pretreatment of mice with paclitaxel, 2 hours before ZD6126, prevented ZD6126-induced vascular shutdown: vessels remained perfused, as they were labeled by the lectin (Fig. 6D).

Paclitaxel inhibits ZD6126-induced acute necrosis in tumors in vivo. Because acute shutdown of tumor vasculature is thought to be a prerequisite for the vascular targeting activity of ZD6126, we next investigated the effect of paclitaxel on ZD6126-induced tumor necrosis in vivo (Fig. 6E-H). In s.c. MDA-MB-435 tumors, a single injection of ZD6126 alone (200 mg/kg i.p.) induced central necrosis of the tumor mass, evident 24 hours after treatment (6.8-fold increase of necrotic area compared with vehicle; Fig. 6F and E). Paclitaxel alone was not relevant on the formation of necrosis in a short time (Fig. 6G).
However, paclitaxel (20 mg/kg i.v.), given 2 hours before ZD6126, largely prevented the induction of acute tumor necrosis caused by the vascular targeting agent (1.7-fold increase in necrosis; Fig. 6H). Similar findings were obtained with 40 mg/kg paclitaxel (not shown). These findings indicate that paclitaxel can inhibit the vascular targeting activity of ZD6126 in vivo.

**Discussion**

This study shows that cytotoxic antineoplastic agents may affect endothelial cell responses to ZD6126, depending on their mechanisms of action. Paclitaxel and docetaxel, microtubule-stabilizing compounds, acting on the same target of ZD6126 but with an opposite effect on microtubules, inhibited endothelial cell responses to the vascular targeting compound. This antagonizing effect was observed both in vitro, where taxane-treated endothelial cells were less sensitive to N-acetylcolchinol–induced shape change and microtubule depolymerization, and in vivo, where pretreatment with paclitaxel inhibited both ZD6126-induced shutdown of neovascularure and ZD6126-induced tumor necrosis.

Endothelial cell shape change induced by microtubule-depolymerizing agents, such as ZD6126, seems to be a direct consequence of their effect on the microtubular cytoskeleton, which may involve the activation of the small GTPase RhoA (17–19). It seems most likely that the inhibitory effect of paclitaxel on N-acetylcolchinol–induced cell shape changes is due to stabilization of the microtubules. This is supported by our finding that the inhibitory effect of paclitaxel on the HUVEC morphologic alterations induced by N-acetylcolchinol was paralleled by an inhibitory effect on N-acetylcolchinol–induced HUVEC microtubule depolymerization. Moreover, the non-tubulin-binding drugs cisplatin and doxorubicin had no effect on endothelial cell responses to the vascular targeting agent, whereas vincristine, which also causes microtubule destabilization, had an additive effect. Finally, the protective effect of paclitaxel on the HUVEC morphologic alterations induced by N-acetylcolchinol was observed at concentrations (≥1 μmol/L) compatible with an effect on the microtubule polymer mass. It is worth noting that both the concentrations of paclitaxel and N-acetylcolchinol are biologically relevant, compatible with the plasma concentrations achieved after administration of commonly used doses. A plasma concentration of 10 μmol/L N-acetylcolchinol was observed 60 minutes after injection of a single dose of 200 mg/kg ZD6126 to mice (10). Sixty minutes after injecting a single dose of 22.5 mg/kg paclitaxel, plasma concentration in mice was 60 μmol/L (20). Plasma concentrations of paclitaxel higher than 1 μmol/L are found in patients following a 3-hour infusion of 135 to 225 mg/m² paclitaxel for several hours following drug administration (21).

The protective effect of taxanes against ZD6126/N-acetylcolchinol has not been specifically investigated thus far. However, a recent study analyzed the ability of related tubulin-depolymerizing compounds to overcome the stabilizing effect of taxanes on purified tubulin (22). Paclitaxel-stabilized microtubules were 2 to 11 times less sensitive to the depolymerizing activity of N-acetylcolchinol.
in agreement with this hypothesis, we found that although N-acteycolchilln, even if antagonizing its microtubule-stabilizing activity, would not counteract antimitotic activity of microtubule-destabilizing agents, which also affect microtubule microtubule bundling (25, 26). It can be hypothesized that its ability to suppress the very rapid dynamics that characterizes N-acteycolchilln is indeed due to an antagonistic interaction of the two compounds at the level of tubulin/microtubules.

Although paclitaxel protected cells from the activity of N-acteycolchilln, the opposite was apparently not true, as N-acteycolchilln did not interfere with the cytotoxicity of paclitaxel. The cytotoxic activity of paclitaxel is associated with its ability to suppress the very rapid dynamics that characterizes mitotic spindle microtubules, rather than with the induction of microtubule bundling (25, 26). It can be hypothesized that microtubule-destabilizing agents, which also affect microtubule dynamics, would not counteract antimitotic activity of paclitaxel, even if antagonizing its microtubule-stabilizing activity. In agreement with this hypothesis, we found that although N-acteycolchilln seemed to reduce paclitaxel-induced microtubule bundling, it did not affect paclitaxel-induced cytotoxicity. This is in accordance with previous studies showing that microtubule-depolymerizing agents can indeed synergize with paclitaxel in inhibiting tumor cell proliferation (23, 24, 26). The finding that N-acteycolchilln does not affect the cytotoxic activity of paclitaxel has important implications for the clinical use of the two agents in combination because it would seem to rule out a possible inhibitory activity of ZD6126 against the main activity of paclitaxel on tumor cells.

The rationale of combining vascular targeting therapies with chemotherapeutics is threefold. First, the two types of drugs target different cell types within the tumor tissue, [i.e., immature/proliferating endothelial cells (vascular targeting agents) and tumor cells (chemotherapeutics)]. Therefore, the effect of a combination therapy would simultaneously affect two different cellular compartments within the tumor mass. Second, vascular targeting agents are known to cause central necrosis of the tumor without affecting the viability of tumor cells at the periphery of the tumor. In contrast, cytotoxic chemotherapeutic agents are likely to preferentially affect the highly proliferative, well-perfused tumor cells at the tumor periphery, with relative sparing of tumor cells in the poorly perfused inner areas of the tumor mass. Therefore, a combination of the two strategies would be expected to achieve a more complete targeting of the whole tumor, hitting both tumor cell populations at the periphery and in the central area of the tumor. Our findings suggest that administration of paclitaxel before ZD6126 might potentially interfere with the activity of ZD6126 on the tumor vasculature, whereas the antitumor activity of paclitaxel would not be affected. Finally, vascular targeting agents, by altering blood flow, can modulate the local pharmacokinetics of the chemotherapeutic agent. Indeed, it has been proposed that the use of vascular targeting agents, administered after the cytotoxic compound, may “trap” the cytotoxic compound within the tumor, therefore increasing the time of exposure of the tumor cells. Several studies have shown improved therapeutic results when using a schedule administering the vascular targeting agents (27, 28) and, in particular, ZD6126 (9, 13, 28), within a few hours after giving the chemotherapeutic agent. However, the present study indicates that in the case of combinations of paclitaxel with ZD6126, and conceivably with other tubulin-depolymerizing agents, this strategy may be less effective.

Our findings are apparently in contrast with a previous study indicating an increased therapeutic effect of the paclitaxel/ ZD6126 combination when paclitaxel was given 15 minutes before ZD6126 (10). However, in that study, paclitaxel was administered i.p., a suboptimal administration route for this agent (20), and there was no indication that active, potentially inhibitory doses of paclitaxel had indeed reached the tumor before ZD6126 acted on the tumor vasculature.

Our in vitro observation that the antagonizing effect of paclitaxel reversed within a few hours indicates that it should be feasible to optimize the timing of administration of paclitaxel and ZD6126 to show at least additive antitumor activity. Preclinical studies with different schedules are ongoing to verify this hypothesis.

In conclusion, this study reports that microtubule-stabilizing agents may inhibit the vascular targeting activity of ZD6126, suggesting that a careful design of the sequence and timing of administration of the two agents may be critical to avoid potential antagonism while optimizing the potential therapeutic effects. It is worth noting that our study was focused on the effect of the two compounds on only one cellular compartment, endothelial cells, whereas in the in vivo setting several cell populations can be exposed to and differently affected by these compounds. Indeed, combinations of microtubule-stabilizing and microtubule-destabilizing drugs have shown a synergistic effect in preclinical studies and are currently under clinical investigation for cancer therapy (reviewed in ref. 26), therefore indicating the potential usefulness of such combinations regimens. Based on these findings, further investigations are warranted to optimize the design of therapeutic approaches combining microtubule-destabilizing vascular targeting agents with microtubule-stabilizing cytotoxic agents in in vivo tumor models.

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