Progressive tumor growth requires angiogenesis, which occurs mainly by neovascularization and/or cooption of existing blood vessels. Angiogenesis is a stepwise process regulated by angiogenic factors and their inhibitors (1–3). In particular, vascular endothelial growth factor (VEGF) is associated with the vascularization of normal and neoplastic tissue (4). It is a mitogen of endothelial cells and also a permeability factor (5). The two major VEGF receptors (VEGFR), flt-1 (VEGFR-1) and flk-1/KDR (VEGFR-2), are primarily expressed on vascular endothelial cells (4). Other proangiogenic factors, known to be implicated in the neovascularization of growing tumors, are basic fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor (PDGF; ref. 6). Receptor tyrosine kinases (RTK) for these factors are expressed by endothelial cells as well as by other cells (e.g., fibroblasts, smooth muscle cells, and pericytes) in the tumor bed, which can be induced by tumor cells to elaborate proangiogenic proteins. In particular, pericytes act as stabilizing cells of newly formed immature vessels and thus

**Abstract**

**Purpose:** Different antiangiogenic approaches have been proposed in cancer treatment where therapeutic efficacy has been shown with the addition of cytotoxic agents. Here, we used SU6668, a small-molecule receptor tyrosine kinase inhibitor, to investigate the combinatorial effect with paclitaxel on the cellular populations of the developing vasculature.

**Experimental Design:** The effect of this combination was evaluated in vitro in a 72-hour proliferation assay on human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells derived from lungs, endothelial cells, aortic smooth muscle cells, and human ovarian carcinoma cells sensitive (1A9) and resistant (1A9-PTX22) to paclitaxel. Combination data were assessed by isobologram analysis. Cell survival was determined by terminal deoxyribonucleotide transferase–mediated nick-end labeling and Annexin V staining. The activity of the combination in vivo was evaluated in fibroblast growth factor-2–induced angiogenesis in Matrigel plugs s.c. implanted in mice. The 1A9-PTX22, paclitaxel-resistant xenograft model was used to evaluate tumor response.

**Results:** Combination index values and isobologram analysis showed synergy in inhibition of proliferation of HUVEC, human microvascular endothelial cells derived from lungs, and aortic smooth muscle cells. The combination induced greater apoptosis in HUVEC than the single agents. The addition of paclitaxel to the treatment with SU6668 significantly decreased the hemoglobin content and the number of CD31-positive vessels in Matrigel plugs in vivo. The combination of the drugs was more active than either single agent against 1A9-PTX22 xenografts; the tumor growth delay was accompanied by a significant reduction of vascular density.

**Conclusions:** These findings show that the activity of angiogenesis inhibitors on vascular cells could be potentiated when administered in combination with chemotherapeutic agents that themselves have vascular targeting properties.
decrease vascular permeability (1). Pericytes, initially recruited to microvessels independent of PDGF, require PDGF to further proliferate and migrate along angiogenic sprouts (2).

During tumor progression, the simultaneous orchestration of various growth factors plays a relevant role, where tumor cells may switch from the production of one growth factor to another. It is likely that the inhibition of a single factor will cause only partial control of tumor growth. Several agents, mainly small molecular weight compounds targeting the above-mentioned receptors, have recently entered the developmental pipeline (7). The targeted inactivation of RTK is often pursued with ATP-competitive inhibitors that block the enzymatic activity and thereby interfere with phosphorylation of cellular substrates. RTKs that share a relatively conserved ATP-binding site can also be targeted by multikinase inhibitors. There are several examples of such agents with broad-spectrum activity under clinical development, all showing inhibition of multiple kinases in addition to VEGFR (8, 9). Their effect on angiogenesis and consequently tumor growth has been extensively reported in preclinical studies. Major findings emerging from these studies are that these compounds given as monotherapy cause a cytostatic response with an incomplete and transient effect on tumor growth and that their combination with conventional therapy is essential to improve responses (10). This is supported by recent clinical studies, where, in particular, bevacizumab (Avastin), an anti-VEGF antibody, increased the average survival time of patients with metastatic colorectal cancer and is now in combination trials with standard chemotherapy in different tumor types (11, 12). A variety of clinical studies are under way with small-molecule RTK inhibitors in combination with chemotherapeutic agents.

Although cytotoxic agents are believed primarily to target tumor cells, they might affect other proliferating populations, including vascular cells. The antiangiogenic effects mediated by conventional anticancer drugs have been known for some time (13, 14). Recent studies by Kerbel and Kamen (15) have proposed a strategy of rescheduling the administration of classic cytotoxic drugs to target tumor endothelial cells. The administration of these chemotherapeutic agents at low doses on a frequent and continuous schedule (metronomic regimen) has been proposed to optimally exploit their antiangiogenic activity (15, 16). The combination of such cytotoxic regimens with angiogenesis inhibitors has been shown to dramatically inhibit the growth of solid tumors resistant to conventional therapy (17, 18). Although these studies implicate the tumor vasculature as the major target of these treatments, the relative contribution of endothelial cells, supporting vascular cells, or even tumor cell damage to the observed therapeutic response is not completely understood. Furthermore, little is known about the mechanism of action of the different class of compounds affecting the vascular compartment.

Among cytotoxic agents, we and others have shown that the taxanes, tubulin-targeting compounds, are potent “angiogenesis inhibitors” (19–21) at subcytotoxic concentrations (reviewed in refs. 13, 15). This activity has been associated with the crucial role of microtubules in cell functions, including those that characterize endothelial cells in the developing tumor neovasculature (22). Furthermore, we have recently shown that the combination of paclitaxel (Taxol) with SU6668, a potent inhibitor of signal transduction mediated by VEGF, FGF-2, and PDGF-BB RTKs (23), inhibited ovarian carcinoma xenograft progression in the peritoneal cavities of nude mice. We found that the combination, even at low doses of paclitaxel, effectively blocked tumor spread and increased the survival of mice (24).

The purpose of this study was to determine the combined activity of paclitaxel with SU6668 on the cellular populations of the developing vasculature. We found that paclitaxel and SU6668 synergistically inhibited the proliferation and increased apoptosis of endothelial cells. Furthermore, the treatment of mice with the two drugs in combination was more efficacious than monotherapy in inhibiting neoangiogenesis in s.c. Matrigel plugs at doses that were also shown to delay the growth of a paclitaxel-resistant ovarian carcinoma xenograft.

Materials and Methods

Drugs and reagents. Paclitaxel (Taxol, kindly provided by Indena S.p.A., Milan, Italy) and SU6668, [(Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid (Sugen, Inc., South San Francisco, CA) for in vitro use were prepared as a 1,000 × stock solution in DMSO, then diluted in test medium. For in vivo use, paclitaxel was prepared as a stock solution of 12.5 mg/mL in 50% polyoxyethylated castor oil (cremophor EL) and 50% ethanol and freshly diluted in 0.9% saline to the indicated concentrations before inoculation. Paclitaxel was administered i.v. at doses of 6 mg/kg daily or 20 mg/kg every 4th day as detailed in Results.

SU6668 was supplied as 5% w/v SU6668, 16.92% 1 N sodium hydroxide solution, 24.60% cremophor EL, 1.56% benzyl alcohol, 35.14% PEG 400, 16.79% deionized water, and administered by oral gavage at the dose of 100 mg/kg daily. We have previously shown these doses to be pharmacologically active and nontoxic (24, 25). Control groups received the corresponding vehicles without the addition of the drugs.

PDGF-BB (MP Biomedicals GmbH, Verona, Italy), VEGF, and FGF-2 (R&D Systems Minneapolis, MN, obtained through the National Cancer Institute Biologics Resources Branch, Preclinical Repository, Frederick, MD) were used at a final concentration of 10 ng/mL.

Cells. Human umbilical vein endothelial cells (HUVEC) were isolated as described (26). The cells were cultured in Medium 199 Hanks (Biochrom AG, Berlin, Germany), 20 mmol/L HEPES, 100 units/mL penicillin and streptomycin, 2 mmol/L L-glutamine (Life Technologies, Paisley, United Kingdom), and supplemented with 20% FBS (Life Technologies). Human microvascular endothelial cells (HMEC) derived from lung and human aortic smooth muscle cells (AoSMC) were obtained from Cambrex, Co. (Caravaggio, Italy) and cultured in endothelial basal medium (Cambrex) supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin and streptomycin, 20 mmol/L HEPES, and 15 units/mL heparin (Pfizer, Milan, Italy). 1 μg/mL hydrocortisone (Sigma Aldrich, Milan, Italy), and 10% newborn calf serum (Life Technologies).

IA9 ovarian cancer cell line and the paclitaxel-resistant variant IA9-PTX22 (25) are subclones of the A2780 human ovarian carcinoma cell line (27). The tumor cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS.

Mice. Female NCr-nu/nu mice (nude mice) were obtained from the animal production colony of the National Cancer Institute (Biological Testing Branch, Developmental Therapeutics Program), Frederick Cancer Research and Development Centre (Frederick, MD), Patho-rogen-free, female C57BL/6N mice were from Charles River (Calco, Italy). Mice were housed following institutional guidelines and national (D.L. no. 116, G.L. Suppl. 40, February 18, 1992, Circolare no. 8, G.U., July, 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).
Growth inhibition assay. HUVEC, HMVEC, and AoSMC were plated (4 × 10^3 cells per well) in 96-well plates (Falcon, Bedford, MA) in 50 μL of their medium containing 2.5%, 1%, and 0.5% FBS, respectively. 1A9 tumor cell variants (4 × 10^5 per well) were plated in 50 μL RPMI 1640 without serum. After 24 hours, paclitaxel (10^{-6}, 10^{-7}, and 10^{-8} mol/L) and SU16668 (10^{-6}, 10^{-7}, 10^{-8} mol/L) were added as single agents or in combination. Following 1-hour incubation, HUVEC and HMVEC were exposed to 10 ng/mL VEGF (or FGF-2, where specified) and AoSMC to PDGF-BB (10 ng/mL), whereas tumor cells were serum (10%) stimulated. After 72 hours, the cell proliferative response was measured by the amount of 490 nm absorbance with a Multiscan (Titerteck, Flow Laboratories, Milan, Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 46 μg/mL phena-methesulfate (CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay, Promega Corporation, Madison, WI). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was converted into the aqueous soluble formazan product that was then measured by the amount of 490 nm absorbance with a Multiscan MC (Tietterick, Flow Laboratories, Milan, Italy).

Data were expressed as a percentage of the control (vehicle-treated cells) and the IC_{50} was extrapolated from the data.

Combination data analysis by the isobologram method. The data obtained from the growth inhibition assays in response to the combinations of SU16668 with paclitaxel were subjected to isobologram analysis. The isobologram method relies on the calculation of the combined concentrations of SU16668 and paclitaxel that cause a given effect, like 30%, 50%, or 70% of growth inhibition (reviewed in ref. 28). Briefly, for each experimental concentration of SU16668, the concentration of paclitaxel causing 30%, 50%, or 70% of growth inhibition in combination was found by nonlinear fitting of the concentration-effect relationship of paclitaxel with the given SU16668 concentration and vice versa. In this way, multiple combinations of corresponding drug concentrations that achieved the same effect (either 30%, 50%, or 70% growth inhibition) were found, normalized with respect to the IC_{50, A}, IC_{50, B}, or IC_{50, T} value of single drugs, and plotted in the isobologram. Additive combinations fall along the diagonal line connecting IC_{50, A}, IC_{50, B}, or IC_{50, T} of the single drugs, synergistic combinations fall below the line, and antagonistic above (see Fig. 1A).

For each combination of drug concentrations (\(D_{SU16668 \times \ D_{paclitaxel}}\)), producing in combination the effect X, the combination index (CI) was calculated as follows: CI = \(D_{SU16668 \times D_{paclitaxel}} / IC_{SU16668 \times D_{paclitaxel}}\), where IC_{SU16668} and IC_{paclitaxel} are the concentrations of each individual drug that would produce the effect X if given alone.

Each experiment generated a set of CI values for a particular effect level. The CI values obtained from all experiments with a given cell line were pooled and the mean and variance were calculated at the 30%, 50%, and 70% cell survival levels. A confidence band was calculated around each mean using a \(\tau\) distribution at the 90% probability level. Additivity was claimed when the value CI = 1 was inside the confidence band (with the band included in the 0.8-1.2 interval), synergism when the CI with its confidence band was <1, and antagonism when the CI with its confidence band was >1.

Quantification of apoptosis. To determine cell survival, 1 × 10^5 HUVEC were plated on 1% gelatin-coated glass slides in a six-well plate with culture medium. After 24 hours, medium was replaced with medium plus paclitaxel (10^{-8} and 10^{-7} mol/L), SU1668 (10^{-8} and 10^{-7} mol/L), or the combination of these concentrations. VEGF (10 ng/mL) was added after 30 minutes and the cells were further incubated with the drugs or vehicles. Control for apoptosis was serum-starved HUVEC. The addition of VEGF without drugs was used as control for survival. After 16-hour exposure to the drugs, apoptotic cells were evaluated by terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL; In situ Cell Death Detection kit, Fluorescein, Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. Nuclei of live cells were labeled with 4’,6-diamidino-2-phenylindole (1 μg/mL) for 20 minutes at 37°C. The number of apoptotic cells (TUNEL) and live cells (4’,6-diamidino-2-phenylindole) were quantified in 10 random fields. Representative images were obtained by LSM 510 meta–confocal scanning laser microscope (Carl Zeiss, Jena, Germany). Nuclear staining with 4’,6-diamidino-2-phenylindole was captured with 405 nm and emission with band pass at 420 to 480 nm. TUNEL was evaluated at excitation of argon laser at 488 nm and emission with band pass 505 to 550 nm.

The presence of apoptotic cells was also evaluated by Annexin V staining. After 48-hour exposure to the drugs, the cells were harvested by incubation with trypsin/EDTA (0.025%/0.01% w/v). After two washes with PBS, the cells were exposed at room temperature for 15 minutes to 5 μL Annexin V-FITC and 5 μL propidium iodide (Apoptosis Detection kit I, BD Bioscience, Milan, Italy) following the instructions of the manufacturer. The cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Analysis was carried out by FACSort (Becton Dickinson & Co., Mountain View, CA) at 488 nm and emission in the range of 520 to 530 nm.

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Ten thousand cells (events) for each condition were evaluated, such that Annexin V–positive cells indicate cells undergoing apoptosis, whereas Annexin V and propidium iodide–positive cells are scored as necrotic.
Angiogenesis assay in Matrigel. Chilled plugs of Matrigel (12.5 mg/mL, 0.5 mL; BD Bioscience, Bedford, MA) mixed with 300 ng/plug FGF-2 were injected s.c. in 4-week-old female C57BL/6N mice as previously described (29). Negative control plugs of Matrigel were obtained by injecting Matrigel without FGF-2. Mice received daily treatments with paclitaxel (6 mg/kg, i.v.) SU6668 (100 mg/kg, orally), or the combination. Control mice received the corresponding volume of vehicles. Treatments began the same day as the Matrigel injection and continued for 6 days. Twenty-four hours after the last treatment, mice were euthanized by carbon dioxide inhalation. The Matrigel plugs were harvested and analyzed for hemoglobin content (Drabkin reagent kit, Sigma) or fixed in zinc fixative (BD Bioscience) for histologic examination. The angiogenic response was subjectively graded based on the number of infiltrating cells and the presence of cords and erythrocyte-containing vessels as described previously (29). Immuno-histochemical analysis for CD31 antigen was done as described below.

Treatment of human tumor xenografts. 1A9-PTX22 human ovarian carcinoma (10 × 10^6 cells per inoculum) were transplanted s.c. into the flanks of female nude mice (mean body weight of 21 ± 2 g). Mice were randomized with tumors of ~300 mg and treatment was given accordingly to two protocols (P1 and P2). Mice in P1 received 10 daily treatments (Q1 × 10) of SU6668 (100 mg/kg, orally) or paclitaxel (6 mg/kg, i.v.) or a combination of the two agents, given at the same time. Mice in P2 received three treatments every 4th day (Q4 × 3) of paclitaxel at 20 mg/kg (i.v.) 10 daily treatments of SU6668 at 100 mg/kg (orally), or a combination of the two. In both experiments, control animals received corresponding volumes of both vehicles.

The diameters of the tumor masses were measured every 2nd day in two dimensions with Vernier calipers. The estimates of tumor weights (g = cm^3) were calculated with the following formula: tumor weight = length × width × length / 2. Tumor weights were normalized in the different groups by obtaining the relative tumor weight (RTW), calculated with the following formula: RTW = Wt / Wc × 100%, where Wt is the tumor weight at any day of measurement (day t) and Wc is the tumor weight at the day of initiation of treatment. The medians of these values (median RTW) evaluated for each observation day for all of the tumors in control and treated groups were used to calculate the treatment efficacy. Response to therapy was expressed as percentage of treated versus control (%T/C) = (median RTW of the treated mice / median RTW of controls) × 100; growth delay (T / C) was calculated as difference in days for T/C tumors to reach a RTW of 5-fold. Mice were weighed twice weekly and the percentage body weight loss (body weight of treated mice relative to their weights at start of treatment) was calculated.

Seven to nine mice were observed until tumors reached a median weight of 1.5 g and used for tumor growth evaluation. Four tumors per group were harvested 24 hours after the last treatment, dissected, and one half of the tumors were fixed in 10% phosphate-buffered formalin for routine H&E analysis and the second half were fixed in a solution of zinc fixative and paraffin-embedded for immunohistochemical analysis.

Immunohistochemistry. To evaluate microvessel density (MVD), zinc-fixed samples were immunostained with rabbit monoclonal anti-mouse CD31 antibody MEC 13.3 (kindly supplied by A. Vecchi, Department of Immunology, Mario Negri Institute, Milan, Italy; ref. 30). The reaction was revealed by incubating the sections with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) and further hematoxylin counterstaining was done. Vascular hotspots (three to four fields in each sample) were selected at ×40 to ×100 magnification and microvessels were counted at high magnification (×400). Each section was examined independently by two investigators in a blinded fashion and the mean of the results was used for analysis. The median value for each sample was reported. MVD of each group was calculated as the mean value of the medians of each sample.

Statistical analysis. The data on apoptotic cell (TUNEL and Annexin V staining) MVD and tumor weight were subjected to ANOVA and the significance of multiple pairwise comparisons was determined with the Tukey-Kramer correction defined as P < 0.05. The Mann-Whitney U test at P < 0.05 was done to evaluate effects on hemoglobin content in Matrigel plugs.

Results

Synergistic effects of SU6668 and paclitaxel on endothelial and smooth muscle cells. The effects of SU6668 and paclitaxel as single agents were evaluated on macrovascular (HUVEC) and microvascular (HMVEC) endothelial cells, smooth muscle (AoSMC) cells, and human ovarian cancer cells (1A9 and 1A9-PTX22) in response to growth factors. SU6668 inhibited the proliferation of HUVEC and HMVEC activated by VEGF (IC50 of 5.7 × 10^−7 and 4.5 × 10^−7 mol/L, respectively) and of HUVECs stimulated with FGF-2 (IC50 of 3.5 × 10^−4 mol/L).

SU6668 inhibited PDGF-BB-induced proliferation of AoSMC with an IC50 of 2.2 × 10^−6 mol/L. The ovarian carcinoma cell lines 1A9 and its paclitaxel-resistant variant, 1A9-PTX22, did not respond to VEGF, FGF-2, and PDGF-BB, and their proliferation (induced by FBS) was not affected by SU6668 at concentrations up to 10^−6 mol/L.

HUVEC and HMVEC responded to paclitaxel with an IC50 of 2.2 × 10^−6 and 2.7 × 10^−6 mol/L, respectively, whereas the IC50 for AoSMC was 5.5 × 10^−8 mol/L. Paclitaxel inhibited the proliferation of the 1A9 and the 1A9-PTX22 tumor cell line with an IC50 of 2 × 10^−6 and 3 × 10^−8 mol/L, respectively.

HUVEC grown on diverse proteins of the extracellular matrix, such as fibronectin, laminin, collagen IV, and collagen I, did not show differences in response to either of the two drugs (data not shown); therefore, these substrates were not included in further studies.

For qualitative assessment of antagonism or synergy, we evaluated the isobolograms (Fig. 1A) of the combinations of SU6668 with paclitaxel obtained at the IC30, IC50, and IC70 levels for HUVEC, HMVEC, and AoSMC cell lines. Figure 1B to D shows all the cell types at the IC50 level where the data points fall under the additivity line, thus indicating synergy at all combinations tested. Because 1A9 and 1A9-PTX22 tumor cell lines responded only to paclitaxel, the combination with SU6668 did not improve the cytotoxic effects (data not shown).

The strength of drug interaction was evaluated using the CI value, calculated on the pairs of concentrations giving 30%, 50%, or 70% growth inhibition. The average CI values for HUVEC, HMVEC, and AoSMC were all significantly lower than 1, thus demonstrating synergy at all of the inhibitory concentrations tested (Table 1).

SU6668 and paclitaxel in combination increase apoptosis in endothelial cells. Apoptosis in HUVEC in response to different combinations of paclitaxel and SU6668 was determined and representative results are shown in Fig. 2. After 16 hours of exposure to paclitaxel (10^−8 mol/L) and SU6668 (10^−6 mol/L) in the presence of VEGF, TUNEL staining of nuclear fragmentation (Fig. 2A) showed a marginal effect on HUVEC (7% and 3% apoptotic cells, respectively). Serum starvation was used as control for apoptosis (7% apoptosis) and VEGF alone as survival factor (1% apoptosis). The combination showed a significant (P < 0.05) increase of apoptotic HUVEC (23%) in respect to the VEGF control or the single treatments with paclitaxel or SU6668, an ~3-fold increase in apoptosis compared with paclitaxel, and an ~8-fold increase compared with SU6668 (Fig. 2A).
SU6668 and Paclitaxel on Vascular Cells

Serum-starved HUVEC exhibited apoptosis marked by morphologic changes, such as membrane rupture and DNA fragmentation (Fig. 2B). Such changes were not found in the VEGF-treated cells, which were protected from apoptosis. Few signs of apoptosis were observed in HUVEC treated with SU6668. Distinct phenotypes of cells undergoing mitotic stress (also termed mitotic catastrophe) were observed compared with the controls. This phenomenon was also observed in cells treated with the combination of paclitaxel and SU6668, where the cells undergoing mitotic stress resulted in an ~3-fold increase in multinucleation compared with paclitaxel as monotherapy (Fig. 2B). This observation correlated with the significantly higher increase in apoptotic cells treated with the combination (Fig. 2A).

HUVEC apoptosis was further evaluated with Annexin V staining after 48-hour exposure to SU6668 (10^{-6} mol/L), paclitaxel (10^{-6} mol/L), or the combination (Fig. 2C). As reported above, SU6668 (32% Annexin V–positive cells) and paclitaxel (30% positive cells) induced increased apoptosis compared with VEGF alone (18% positive cells). The combination of the two agents significantly increased (P < 0.05) the number of apoptotic cells (45% positive cells; Fig. 2C).

**SU6668 combined with paclitaxel affects the angiogenic response in Matrigel.** The effect of daily treatments of paclitaxel (6 mg/kg, Q1 C0 = 13 days. In P2, the higher dose of paclitaxel in combination with SU6668 resulted in T/C = 30% and T – C = 12 days.

No signs of toxicity in P1 or P2, evaluated by body weight loss, were observed with the combination treatments.

To evaluate whether the beneficial effect of the combination could have been indeed mediated via the tumor vasculature, histologic analyses of tumor samples were done at the end of the treatment (Fig. 4). The CD31-positive staining at day 13 (beginning of treatment) showed that the tumors were vascularized (MVD = 12.40 ± 3.86). After 10 treatments (day 22), tumors from control mice showed intense vascular sprouting and the mean MVD count was 13.93 ± 6.96, no different from that on day 13, thus indicating a consistent pattern of vascularization independent of tumor size. The administration of paclitaxel did not affect the angiogenesis in 19-PTX22 tumors (MVD = 12.06 ± 3.86) compared with the control group. At the same time point, angiogenesis in mice treated with SU6668 was moderately affected (MVD = 9.00 ± 3.72). The combination of paclitaxel

Seven days after implantation, FGF-2 (positive control) induced a strong angiogenic response with a distinct increase in cell infiltration, cord formation, and the number of blood-containing vessels. Many strongly CD31 positive vessels were detectable in plugs stimulated by FGF-2 (Fig. 3B). The vessel number was estimated to be 0.25 ± 0.55 in the control plugs (Matrigel alone) and 29.75 ± 10.48 in the FGF-2-supplemented plugs (Fig. 3C). Administration of paclitaxel resulted in marked inhibition of cellular infiltration, and cord and blood vessel formation. The antiangiogenic effect of paclitaxel was confirmed by CD31 immunostaining where the vessel number was significantly decreased to 9.12 ± 9.27 (P = 0.002).

Morphologic analysis of Matrigel plugs treated with SU6668 showed marginal effects because no evident decrease in cell infiltration was detectable. However, there was a decrease in cord formation, confirmed by CD31-positive vessels that were significantly decreased (3.50 ± 2.32, P < 0.001).

Treatment with SU6668 in combination with paclitaxel caused a profound decrease in cellularity and cord formation and the number of CD31-positive vessels was strongly and significantly (P < 0.001) reduced to 1.06 ± 1.66.

**SU6668 in combination with paclitaxel delays the growth of 1A9-PTX22 ovarian carcinoma xenografts.** The observed enhanced effect of the combination of SU6668 with paclitaxel in the Matrigel plug angiogenic assay encouraged us to investigate the effect of this combination on tumor vasculature in vivo. We have previously shown the additive effect of SU6668 in combination with paclitaxel on the survival of mice bearing human ovarian carcinoma (24). Here, we investigated the response of 1A9-PTX22, a paclitaxel-resistant tumor xenograft, to combinations of SU6668 with paclitaxel administered by two different schedules (Table 2). Daily doses (Q1 = 10) of SU6668 at 100 mg/kg (orally) were combined with a daily low dose of paclitaxel (6 mg/kg for 10 injections) or at an optimal dose of 20 mg/kg given thrice every 4th day. As shown in Table 2, the 1A9-PTX22 xenografts were resistant to treatment with paclitaxel in both protocols, whereas a marginal tumor growth inhibition with SU6668 was observed. The combinations showed a greater therapeutic effect. In P1, when low frequent doses of paclitaxel (same dose as in the Matrigel experiment) were combined with SU6668, the tumor response was as follows: T/C = 34% and T – C = 13 days. In P2, the higher dose of paclitaxel in combination with SU6668 resulted in T/C = 30% and T – C = 12 days.

No signs of toxicity in P1 or P2, evaluated by body weight loss, were observed with the combination treatments.

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### Table 1. CI values of SU6668 with paclitaxel on endothelial and smooth muscle cells

<table>
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<th>IC50</th>
<th>IC70</th>
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<td>0.34</td>
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<tr>
<td></td>
<td>SU6668 + PTX</td>
<td>0.40</td>
<td>0.47</td>
<td>0.58</td>
</tr>
</tbody>
</table>

NOTE: The CI was evaluated for the combination of SU6668 with paclitaxel at IC50, IC50, and IC70 of each drug for HUVEC/HMVEC/AoSMC after 72-hour exposure (experiment as in Fig. 1). The CI was calculated as described in Materials and Methods and evaluated such that CI < 1 is synergism, CI > 1 is antagonism, and CI = 1 is additivity. Data are expressed as mean ± SE of all available pairs of concentrations producing the indicated growth inhibition and are representative of one experiment, repeated at least thrice.

Abbreviation: PTX, paclitaxel.

with SU66668 significantly inhibited the vasculature of the tumor (MVD = 6.50 ± 1.59, P < 0.001).

**Discussion**

In the present study, we show that paclitaxel synergizes with SU66668, an inhibitor of the RTKs VEGFR-2, PDGFRβ, and FGFR-1, on the *in vitro* proliferation of vascular cells. These findings, together with the *in vivo* inhibition of angiogenesis in Matrigel plugs and the decreased vascularization of solid tumors, support the hypothesis that the enhanced effect exerted by the combination of paclitaxel and SU66668 on tumor growth is also mediated by an effect on the vasculature.

The use of inhibitors of angiogenesis in combination with conventional therapy is a common strategy in the development of investigational new drugs. Several preclinical studies have shown that treatment with inhibitors of growth factor receptor signaling in combination with chemotherapy potentiate the anticancer effects (24, 31–33). In the case of inhibitors of angiogenesis, these findings were often associated with a higher level of apoptosis in tumor cells and/or endothelial cells (34). Clinical benefit has recently been shown with combinations of bevacizumab, a humanized monoclonal antibody against VEGF, and chemotherapy (11). Direct evidence that bevacizumab has antivascular effects in human rectal cancer has also been shown (35), but whether these treatments improve the delivery of therapeutics to tumors or may also sensitize the endothelium to cytotoxic agents remains to be investigated. The activity of cytotoxic agents (including tubulin-binding agents such as paclitaxel) as strong inhibitors of endothelial cell functions related to angiogenesis has also been extensively reported (13, 19, 36, 37). However, the pharmacologic interactions between
paclitaxel in combination with inhibitors of angiogenesis have not been explored in detail.

In our study, SU6668 used in combination with paclitaxel potentiated inhibitory effects on endothelial (HUVEC and HMVEC) and smooth muscle (AoSMC) cell proliferation. Based on the isobologram analysis (38), the drug interaction could be defined synergistically in all cell populations. The increment of this vascular targeting potential of paclitaxel in combination

![Graph A](image)

**Fig. 3.** Effect of SU6668 in combination with paclitaxel on angiogenesis in Matrigel. Matrigel containing FGF-2 (300 ng/plug) was injected s.c. in C57BL/6N mice and paclitaxel (6 mg/kg, daily i.v.), SU6668 (100 mg/kg, daily orally), or the combination was administered from days 1 to 6. A, angiogenic responses evaluated by measuring the hemoglobin content of the plugs (n = 8) at day 7 following treatment. Points, hemoglobin content (g/dL) for each plug; bars, median. *, P < 0.001, compared with negative control (plugs without FGF-2, mice receiving vehicle); **, P < 0.05 compared with positive control (FGF-2-containing plugs, mice receiving vehicle), SU6668, and paclitaxel-treated plugs (Mann-Whitney U test). B, representative images of H&E (×100 magnification) and anti-CD31 (×200) immunostaining of Matrigel plugs. Paclitaxel induced reduction of cellular infiltration, cords, and vessel formation relative to the positive controls, and reduced CD31-positive vessels. SU6668 did not affect the cellularity of the plugs; however, a strong reduction of CD31-positive vessels was detected. The combination of the two agents induced a maximal reduction of cellular infiltration, cords, and blood vessel formation and CD31-immunostained vessels were absent. C, MVD of CD31-positive vessels counted (n = 5) in four different fields at ×400 magnification. Columns, mean number of vessels; bars, SD. *, P < 0.05 compared with positive vehicle (FGF-2-containing plugs; ANOVA with Tukey-Kramer correction). PTX, paclitaxel.
Indeed, cisplatin, used as a control "non-antivascular" drug in combination as a vascular targeting/angiogenesis inhibitor. Paclitaxel with SU6668 even at sublethal concentrations of proliferation (37). Here, we found synergistic effects of at concentrations lower than those required to affect tumor cell taxanes (e.g., paclitaxel) that inhibit endothelial cell functions those required to kill cancer cells. This is not the case for the (13, 14). However, the effect on endothelial cells has often been reported to have antiangiogenic activity factors.

The vascular targeting activity of paclitaxel by stabilization of microtubules and blocking mitotic spindles in G2-M phase of the cell cycle has been well documented (44, 45). By enabling cells to exit from the mitotic cycle, paclitaxel can induce apoptosis and induce cell death by the phenomenon termed mitotic stress (or catastrophe; ref. 46). Accordingly, paclitaxel induced endothelial cell death through apoptosis as indicated by DNA fragmentation (TUNEL) or plasma membrane rupture (Annexin V staining). Moreover, evaluation of HUVEC exposed to paclitaxel revealed morphologic changes, such as multinucleation, and the cells were blocked in abnormal metaphase (Fig. 2). In contrast, a mechanism of induction of apoptosis by SU6668 in endothelial cells has not been well documented (42, 47). Although, in this study, we have shown only marginal induction of HUVEC apoptosis by SU6668, this was significantly increased by the addition of SU6668 to paclitaxel. Also, more cells exposed to the drug combination showed mitotic stress. It is, therefore, possible that the proapoptotic effect of SU6668 on HUVEC was sufficient to potentiate the activity of paclitaxel. However, more detailed studies are necessary to investigate the mechanisms of cell death that underlie the combinatorial potency of RTK inhibitors and paclitaxel.

The antiangiogenic/vascular targeting effect of the combination was shown in vivo in Matrigel plugs transplanted s.c. in mice. Treatments of the single agents had a marginal effect, whereas combination of the two agents showed significant decreases in the hemoglobin content and all the endothelial cell morphogenesis variables evaluated. In fact, immunohistochemical analysis showed that CD31-positive vessels were absent in the treated Matrigel plugs, and total cellularity was decreased to levels similar to those in the negative controls. These results strongly support our in vitro data on endothelial cells that the two drugs can indeed target the developing vasculature. In the current study, the effect on angiogenic responses was shown in Matrigel plugs where mice received daily treatments with low doses of paclitaxel. It

with SU6668, evaluated by the CI values, was always <1, thus demonstrating a strong synergy. The synergistic inhibitory effect of the combination of SU6668 and paclitaxel seen in HMVECs indicates that this effect can indeed occur in neoangiogenesis. Interestingly, we have observed that when combining paclitaxel with SU6668, synergistic inhibition is exerted on the proliferation of AoSMCs. The effect was related to the growth dependence of AoSMC on PDGF-BB, the receptor for which is inhibited by SU6668. In fact, SU5416 (a selective inhibitor of VEGFR-2) did not affect the proliferation of AoSMC, and when added to paclitaxel did not exert a greater effect than paclitaxel alone (data not shown). On the other hand, the proliferation of tumor cell lines (1A9 and 1A9-PTX22), which do not respond to paclitaxel revealed morphologic changes, such as multinucleation, and the cells were blocked in abnormal metaphase (Annexin V staining). Moreover, evaluation of HUVEC exposed to paclitaxel revealed morphologic changes, such as multi

### Table 2. Evaluation of the response of 1A9-PTX22 tumor xenografts to paclitaxel and SU6668 treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule (× no. treatments)</th>
<th>n*</th>
<th>%T/C</th>
<th>T – C</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>SU6668</td>
<td>100</td>
<td>Q1 × 10</td>
<td>9</td>
<td>41 (22)</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>6</td>
<td>Q1 × 10</td>
<td>9</td>
<td>74 (22)</td>
</tr>
<tr>
<td></td>
<td>PTX + SU6668</td>
<td>6</td>
<td>Q1 × 10</td>
<td>9</td>
<td>34 (22)</td>
</tr>
<tr>
<td>P2</td>
<td>SU6668</td>
<td>100</td>
<td>Q1 × 10</td>
<td>8</td>
<td>40 (20)</td>
</tr>
<tr>
<td></td>
<td>PTX</td>
<td>20</td>
<td>Q4 × 3</td>
<td>7</td>
<td>81 (17)</td>
</tr>
<tr>
<td></td>
<td>PTX + SU6668</td>
<td>20</td>
<td>Q4 × 3</td>
<td>7</td>
<td>30 (20)</td>
</tr>
</tbody>
</table>

NOTE: In two separate protocols (P1 and P2), 1A9-PTX22 was transplanted s.c. in nude mice and treatments began when tumors reached ~300 mg. P1 shows the response of tumors where mice received daily treatments of SU6668 (100 mg/kg orally, Q1 × 10), paclitaxel (6 mg/kg i.v., Q1 × 10), or the combination. P2 shows the response of xenografts to SU6668 (100 mg/kg orally, Q1 × 10), paclitaxel (20 mg/kg i.v., Q4 × 3), or the combination. No significant body weight loss was observed in the treated groups. PTX, paclitaxel.

* n = number of mice evaluated.
† %T/C ≤ 40%.
‡ P < 0.01.
has been shown that paclitaxel, when administered at low concentrations to endothelial cells, does not affect the classic cytotoxic-mediated signaling pathways similar to those described in tumor cells, but possibly could have cytostatic effects on vascular cells through different signaling pathways (44). Interestingly, when treatment started at an advanced stage of the angiogenesis process (day 4 after Matrigel implantation), when tubes and cords were already formed, treatment with paclitaxel blocked vessel development; the addition of SU6668 to the combination caused a greater inhibition of vessel maturation, as shown by a decrease in tubes and vessels (data not shown).

Several studies have shown that combination therapy based on cytotoxic agents and antiangiogenic compounds potentiates the antitumor activity (reviewed in ref. 10); however, the relative involvement of the host compartment versus the tumor compartment as targets for these kinds of treatments is far from clear. The working hypothesis of our study was that by using a paclitaxel-resistant tumor model and excluding the tumor compartment, the effect of the treatments was mainly on the host compartment. In our xenograft model of ovarian carcinoma resistant to paclitaxel (1A9-PTX22), we showed that the combination was more active than either treatment alone. Although the effect was limited, the tumor growth

---

**Fig. 4.** Histology of the response of 1A9-PTX22 tumor xenografts to the combination of SU6668 with paclitaxel. Representative images of H&E and anti-CD31 immunostaining of 1A9-PTX22 tumor sections obtained 24 hours after the last treatment (day 22) with SU6668, paclitaxel, or the combination as described in Table 2 (P1). The vehicle-treated group showed intense angiogenesis. In paclitaxel-treated tumors, reduction of the number of vessels was not observed. The stain for CD31-positive vessels was decreased in the SU6668-treated group and more so in tumors from mice treated with the combination of the two drugs. Multifocal to coalescing areas of coagulate necrosis are evident in the tumors of mice treated with the combination (arrows). PTX, paclitaxel. Original magnification, \( \times100 \).
delay was accompanied by a significant reduction of vascular density. As in the Matrigel plug, the addition of paclitaxel to the SU6668 therapy caused a significant decrease in the number of CD31-positive vessels. Interestingly, this effect seemed not to be due to the smaller size of the tumor, as the MVD at the beginning and the end of treatment did not change in vehicle-treated mice, thus indicating a stable angiogenic phenotype in the appropriate tumor size range. These results suggested that SU6668 and paclitaxel could potentiate the effect of each other on the tumor vasculature and act on a common target.

Drug-resistant tumor models have been used to show the activity of therapeutics on the tumor vasculature (17, 18). In these studies, using a dosing schedule of chemotherapeutic agents that induced more sustained apoptosis of endothelial cells together with antibody blocking VEGF receptor, a persistent regression of established tumors occurred (17). Here, no difference in tumor growth delay was observed by combining SU6668 with high-dose paclitaxel (20 mg/kg, Q4 × 3) or the same total dose but split (6 mg/kg daily, Q1 × 10). Interestingly, in the sensitive tumor xenograft 1A9 (data not shown) and another tumor model (24), the SU6668-based combination therapy was more effective with paclitaxel given at the optimal dose than at the same total dose split into fractions. Overall, 1A9 (sensitive tumor) was more responsive than the 1A9-PTX22 (resistant) xenograft (data not shown). We believe that in the case of 1A9-PTX, the response of the tumor relies mainly on the effect of the combination therapy on the host compartment, probably vascular cells. In this case, paclitaxel is equally active given at either dose schedules and its activity complements the effect of angiogenic inhibitors. In contrast, in the paclitaxel-sensitive tumor model, the combination affects both the tumor and host compartment, and the magnitude of the effect on the tumor dose schedule dependent and related to the sensitivity of cells to paclitaxel.

We cannot exclude the possibility that more complex cell interactions occur in vivo. It has recently been shown that the inhibition of PDGF-mediated signaling in tumor stroma can enhance the antitumor activity of paclitaxel and this has been associated with an increase of paclitaxel uptake in tumors (48). Further studies are needed to study the pharmacologic interaction of these types of combinations on tumor tissue in vivo.

In conclusion, we have shown that the effect of SU6668 on endothelial vascular cells could be enhanced when in combination with paclitaxel, which also exerts vascular targeting activity.

An aspect that has recently been increasingly appreciated is that sustainable tumor regression and long-term survival could possibly come from rationally designed combination therapies. Multitargeted therapies aimed at the different cell types that contribute to tumor progression, including tumor cells themselves, and cancer-associated host cells could be potentiated by chemotherapeutics (31). Examples are provided by the preclinical studies and early clinical trials of newly developed broad-spectrum RTK inhibitors that show a strong additive/synergistic activity in combination with conventional cytotoxic agents (49). These trials support studies such as those described here, which are necessary to elucidate the nature of pharmacologic interactions in the tumor environment and the mechanisms of tumor growth inhibition in the presence of antiangiogenic agents in combination with chemotherapeutics.

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References


The Vascular Targeting Property of Paclitaxel Is Enhanced by SU6668, a Receptor Tyrosine Kinase Inhibitor, Causing Apoptosis of Endothelial Cells and Inhibition of Angiogenesis

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