Combined Therapy with Direct and Indirect Angiogenesis Inhibition Results in Enhanced Antiangiogenic and Antitumor Effects

Amir Abdollahi, Kenneth E. Lipson, Axel Sckell, Heike Zieher, Frank Klenke, Daniel Poerschke, Alexandra Roth, Xiaohong Han, Martin Krix, Marc Bischof, Philip Hahnfeldt, Hermann-Josef Grone, Juergen Debus, Lynn Hlatky, and Peter E. Huber

ABSTRACT

The multifaceted nature of the angiogenic process in malignant neoplasms suggests that protocols that combine antiangiogenic agents may be more effective than single-agent therapies. However it is unclear which combination of agents would be most efficacious and will have the highest degree of synergistic activity while maintaining low overall toxicity. Here we investigate the concept of combining a “direct” angiogenesis inhibitor (endostatin) with an “indirect” antiangiogenic compound [SU5416, a vascular endothelial growth factor receptor 2 (VEGFR2) receptor tyrosine kinase (RTK) inhibitor]. These antiangiogenic agents were more effective in combination than when used alone in vitro (endothelial cell proliferation, survival, migration/invasion, and tube formation tests) and in vivo. The combination of SU5416 and low-dose endostatin further reduced tumor growth versus monotherapy in human prostate (PC3), lung (A459), and glioma (U87) xenograft models, and reduced functional microvessel density, tumor microcirculation, and blood perfusion as detected by intravital microscopy and contrast-enhanced Doppler ultrasound. One plausible explanation for the efficacious combination could be that, whereas SU5416 specifically inhibits vascular endothelial growth factor signaling, low-dose endostatin is able to inhibit a broader spectrum of diverse angiogenic pathways directly in the endothelium. The direct antiangiogenic agent might be able to suppress alternative angiogenic pathways up-regulated by the tumor in response to the indirect, specific pathway inhibition. For future clinical evaluation of the concept, a variety of agents with similar mechanistic properties could be tested.

INTRODUCTION

Without vascularization, solid tumors grow only to 1–2 mm (1). Thus, tumor growth and metastasis are angiogenesis dependent, and microvascular endothelial cells (ECs) recruited by a tumor have become an important second target in cancer therapy (2). As a result, angiogenesis inhibitors have been developed to target vascular ECs and block tumor angiogenesis. Targeting ECs that support tumor growth is particularly promising because these cells are genetically stable and, therefore, less likely to accumulate mutations that would allow them to develop drug resistance (3). A paramount challenge for antiangiogenic therapy is to design combination protocols that can counteract the diverse angiogenic stimuli produced by the tumor and its microenvironment. Studies suggest that the addition of antiangiogenic agents to conventional therapeutic strategies, e.g., chemotherapy (4–7), radiation (8–10), or other tumor-targeting agents (11), will increase clinical efficacy (12–15). For relapsed multiple myeloma, the angiogenic agent thalidomide has become an important treatment option (16), and its combination with dexamethasone is now being advocated for newly diagnosed multiple myeloma (17).

Angiogenesis inhibitors have been divided into two classes, namely, “direct” and “indirect” (18). Direct angiogenesis inhibitors such as endostatin target the microvascular ECs and prevent them from responding to various proangiogenic stimuli. In contrast, indirect angiogenesis inhibitors interfere with the proangiogenic communication between the tumor–cell and endothelial–cell compartments. This indirect inhibitory effect can be achieved by (a) inhibiting the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) in the tumor; (b) blocking the activity or neutralizing the proangiogenic proteins in circulation [e.g., using solubilized VEGF receptor 2 (VEGFR2)-Fc-fragments]; or (c) blocking the expression or activation of their receptor on ECs (e.g., VEGFR2). Because of the nature of indirect angiogenesis inhibition, tumors are thought to be able to circumvent the inhibitory effect by switching the production of proangiogenic factors (e.g., from VEGF to bFGF). Indeed, recent studies have shown that tumors produce different angiogenic factors over the course of their growth (19). Because there is the potential of tumor “escape” when specific, indirect antiangiogenic agents e.g., anti-VEGF are delivered individually, appropriate combination protocols involving these agents are needed to achieve the greatest benefit (20). We considered that endostatin plus a VEGF receptor antagonist would be a good combination for further study because of the pervasive role of VEGF in tumor growth and the direct action of endostatin. Another rationale for investigating the combination of endostatin and VEGF inhibition is their closely balanced relationship under physiological conditions, which may become imbalanced as the cancer or inflammatory disease progresses (21, 22). Circulating endostatin levels have been shown to be elevated in patients with renal cell carcinoma (23) and hepatocellular carcinoma (24), and to be correlated with circulating VEGF levels. Additionally, effects of exogenous endostatin have been suggested to be mediated, at least in part, by their ability to down-regulate VEGF expression within the tumor (25). Endostatin decreases VEGF-induced vascular leakage and suppresses VEGF-induced retinal neovascularization and retinal detachment (26). Elevated VEGF levels with an imbalance between proangiogenic cytokines and endostatin are associated with human metastatic liver tumors (27). The extent of the imbalance between VEGF and endostatin was a predictive marker for the outcome of vitreous surgery in patients with proliferative diabetic retinopathy (28).

In the present report, we evaluate the concept of combining a direct (endostatin) and an indirect (SU5416, an inhibitor of VEGFR2 signaling) angiogenesis inhibitor on human ECs in vitro and on human xenograft tumors in vivo. We show that the therapeutic efficacy of SU5416 can be enhanced by the addition of a low dose of the endogenous angiogenesis inhibitor endostatin. The combined therapy with SU5416 and endostatin enhanced the antiangiogenic effects in human ECs in vitro and enhanced tumor growth delay of human xenografts (prostate adenocarcinoma PC3, non-small cell lung cancer (NSCLC) A549, and glioblastoma U87). Additionally, tumor angiogenesis was quantified and visualized using a new cranial window model (A549), and blood perfusion was detected by contrast-
enhanced Doppler ultrasound. The data confirm that combined treatment with direct and indirect inhibitors of angiogenesis may result in synergistic antiangiogenic activity, improving the overall antitumor efficacy of these agents.

MATERIALS AND METHODS

Reagents and Cell Culture. Primary isolated human umbilical vein ECs (HUVECs; Promocell, Heidelberg, Germany) and human dermal microvascular ECs (HDMECs; Promocell) were cultured up to passage 9. Cells were maintained in culture at 37°C with 5% CO₂ and 95% humidity in serum-reduced (5% FCS) modified Promocell medium, supplemented with 2 ng/ml VEGF and 4 ng/ml bFGF (Promocell). This combination of growth factors (VEGF and bFGF) optimized growth kinetics. Human adenocarcinoma prostate tumor cells (PC3) and human glioblastoma multiforme cells (U87; Tumorbank DKFZ, Heidelberg, Germany), were cultured in DMEM (10% FCS). Human recombinant VEGF and bFGF proteins were purchased from Promocell.

The angiogenesis inhibitor SU5416 was synthesized at SUGEN Inc., as described previously (29). SU5416 is an ATP-competitive inhibitor of the VEGFR2 receptor tyrosine kinase, with a Ki value of ~160 nM. It also inhibits platelet-derived growth factor receptor β tyrosine kinase, with a Ki value of 320 nM (30, 31). Endostatin treatment was performed with soluble recombinant human (in vitro) and mouse (in vivo) endostatin (Pichia pastoris; Calbiochem, Schwalbach, Germany).

Endothelial and Tumor Cell Proliferation Assay. Proliferation assay was performed as described previously by O’Reilly et al. (32) with minor modifications. Briefly HUVEC and HDMEC passages 6–9 were grown to confluence in modified Promocell medium supplemented with 5% FCS, containing 2 ng/ml VEGF and 4 ng/ml bFGF (endothelial standard condition). PC3 and U87 cells were cultured in DMEM supplemented with 10% FCS (tumor cell standard condition). Cells were harvested by trypsinization at 37°C and neutralized with trypsin-neutralizing solution (TNS). A suspension of 50,000 cells in modified Promocell medium/DMEM was added to 25-cm² flasks (Becton Dickinson, Heidelberg, Germany). The cells were incubated for 24 h under standard conditions, incubated with angiogenesis inhibitors for 1 h in media with no additional growth factor supplementation and thereafter were incubated for another 72 h in standard condition. Cells were then dispersed in trypsin, were resuspended, and were counted in a Coulter counter.

Clonogenic Assay. ECs (HUVECs, HDMECs) and tumor cells (PC3, U87) were grown under standard conditions. To investigate the effects of endostatin and SU5416 on clonogenic survival, increasing numbers of cells (10³ to 5 × 10⁶) were plated in 25 cm² flasks. Cells were incubated with compounds and cultures were returned to the incubator for 14–16 days, after which they were stained with Giemsa (Sigma, Seelze, Germany), colonies were counted, and the surviving percentage was determined for plating efficiency and clonogenic survival.

Matrigel Invasion Assay. To assess the migration/invasion ability of ECs after treatment with endostatin and SU5416, a Matrigel invasion assay was used. Transwell inserts with an 8-µm pore size were coated with Matrigel (0.78 mg/ml; Becton Dickinson, Heidelberg, Germany). HUVECs and HDMECs were trypsinized, and 200 µl of cell suspension (3 × 10⁵ cells/ml containing different concentrations of endostatin and SU5416 were added in triplicate transwells. Chemoattractant media (500 µl) was added to the lower wells.

After 12 h of incubation, ECs that had invaded the underside of the membrane were fixed, were stained in thiazine and eosin solution using Diff-Quik II solution (Dade Behring, Marburg, Germany), and were sealed on slides. Migrating cells were counted by microscopy.

EC Morphogenesis Assay: Tube Formation. To examine the ability of HUVECs and HDMECs to produce tubular structures in vitro, 24-well plates were coated with 300 µl of Matrigel (Becton Dickinson, Heidelberg, Germany). This extract of the Engelbreth-Holm-Swarm (EHS) murine sarcoma, which contains basement membrane components, is liquid at 4°C and forms a gel when warmed to 37°C. When plated on Matrigel, HUVECs (48,000 cells/well) undergo differentiation into capillary-like tube structures in modified Promocell medium (10% FCS) supplemented with VEGF (2 ng/ml) and bFGF (4 ng/ml). Angiogenesis inhibitors were added, and, after a 6-h incubation
the first appearance of newly formed vessels and the onset of perfusion in these vessels. On days 7, 14, 21, and 28 after tumor implantation, the tumor growth, i.e., the two-dimensional tumor surface was documented with standardized microscopic digital photography of the cranial window using bright field microscopy at ×10 and was measured with a computer-based analysis program (AnalySIS V3.0; Soft Imaging System, Münster, Germany). Additionally, the functional vessel density ([FVD]), the length of perfused microvessels per tumor surface area as a measure for angiogenesis was assessed by intravital fluorescence video microscopy. FITC-labeled dextran (Sigma; M, 2,000,000; 0.1 ml of a 5% solution in 0.9% NaCl) as a plasma marker was injected into a tail vein. For off-line analysis, a custom-made computer program was used (CapImage; Engineer Office Dr. Zeintl, Heidelberg, Germany). Animals were treated from day 15 on, after randomization into four groups: endostatin (n = 5; 0.75 mg/kg/day in twice daily injections s.c. in 100 μl of PBS), SU5416 (n = 5; 100 mg/kg/week s.c. in 100 μl of carboxymethylcellulose every other day), combined endostatin with SU5416, and control (n = 5; 100 μl of PBS daily s.c.). On day 28 after implantation, experiments were terminated for ethical reasons.

Statistical Analysis. Tumor growth experiments were designed to assess the response to treatment by using the reduction in tumor growth. For this purpose, the tumor volume V was normalized to V_0 at the onset of treatment (day 0) to compare tumor growth. Statistical evaluation of tumor growth was undertaken by comparing the standardized volumes for each day. In addition, the general response to treatment was assessed on the basis of the time, T_50 required to reach four times the initial tumor volume. For multiple comparisons in tumor growth experiments, histological quantitation, and in vitro experiments, we used the Kruskall-Wallis ANOVA for nonparametric variables. For parametric variables, ANOVA was used along with Fisher’s least-significant-difference (LSD) method using the software program Statistica 5.0 (StatSoft, Hamburg, Germany). All of the analyses were two-tailed. Data of intravital microscopy were analyzed statistically using the software program SigmaStat for Windows (Version 2.03; SPSS AG, Zurich, Switzerland). Depending on parametric or nonparametric distribution of data, one-way repeated measures ANOVA or Friedman repeated measures ANOVA on ranks was used with Turkey test for all pairwise multiple comparison procedures. A P of 0.05 was considered significant.

RESULTS

Endostatin and VEGF Inhibition Have Synergistic Antiproliferative Effects on ECs. EC proliferation is an important step in tumor angiogenesis. We first investigated the effects of endostatin in combination with SU5416 on endothelial and tumor cell proliferation in cultures. Fig. 1, A and B, show dose-response curves, demonstrating that each compound alone significantly inhibited EC proliferation. To determine whether combinations exhibited increased antiproliferative activity, isobolographic analysis was performed. Different combinations of concentrations were added to HUVECs. From the dose-response curves, the concentration at which HUVEC proliferation was inhibited to 60% of control (IC_60) was calculated. Fig. 1C shows the isobologram generated from these data (36). When compared with the theoretical (diagonal) line representing (zero interaction) additive effectivity, all of the values from the combination treatment fell below the line indicating synergistic activity for this end point.

For additional in vitro combination experiments, an endostatin dose of 100 ng/ml (IC_60) and a SU5416 dose of 1 μM (IC_70) were chosen. Using these drug concentrations, the combination of endostatin and SU5416 appeared to have synergistic antiproliferative effects in HUVEC and also in a second human (HDMEC) EC (HUVEC: expected proliferation, 80% × 70% = 56%; measured proliferation, 38%; P < 0.01; HDMEC: expected combined inhibition from single effects 72% × 62% = 44%; measured combined inhibition, 28%; P < 0.01; Fig. 1D).

The combination using these drug concentration (100 ng/ml endostatin, 1 μM SU5416) also exhibited some weak antiproliferative activity in U87 glioma and PC3 tumor cells (P < 0.05), but it was modest and appeared to be only additive (Fig. 1E).

Combined Endostatin and VEGF Inhibition Enhances Reduced Clonogenic Survival in ECs. Clonogenic survival is an important criterion of cell survival in response to antitumor agents because the final cell death may occur only after additional cell divisions. A single application of endostatin or of SU5416 reduced the clonogenic survival of ECs significantly (P < 0.05; Fig. 2). The combination of endostatin and SU5416 induced a synergistic inhibition of clonogenic
survival in HUVECs (expected survival fraction, 0.75; measured, 0.32) and HDMECs (expected, 0.68; measured, 0.29). There was also a modest reduction of clonogenic survival in U87 and PC3 tumor cells after treatment with the angiogenesis inhibitors alone, but this was statistically significant only for SU5416. As expected, the combinations were more effective than single agents but, in contrast to the effect on ECs, appeared to be only additive.

**Endostatin and SU5416 Enhance Apoptosis in ECs.** To investigate potential mechanisms of the antiproliferative effect of endostatin and SU5416, we analyzed the apoptosis fraction of HUVECs and HDMECs. Fig. 3A demonstrates that both endostatin and SU5416 used alone induced apoptosis. The combination of endostatin and SU5416 appeared to be additive for the induction of apoptosis. Qualitatively similar results were obtained with Hoechst staining of HUVEC cytospins and counting the apoptotic cells under the microscope (data not shown). Fig. 3B demonstrates that neither endostatin nor SU5416 alone induced significant apoptosis in PC3 and U87 cells in vitro. The combination of endostatin and SU5416 weakly induced apoptosis in the tumor cells.

These observations suggest that EC apoptosis can contribute to the potent suppression of EC proliferation and clonogenic survival observed on treatment with endostatin and SU5416.

**Endostatin and SU5416 Reduce EC Migration and Invasion.** EC migration and invasion are critical for tumor angiogenesis. We examined the invasion of HUVECs and HDMECs through Matrigel-coated transwell inserts (Fig. 4). Endostatin (100 ng/ml) or SU5416 (1 μM) significantly reduced HUVEC and HDMEC migration/invasion. Interestingly, a combination of endostatin and SU5416 showed a more-than-additive reduction of EC invasion (HUVEC: expected, 47% migrated cells, observed, 23% migrated cells; HDMEC: expected, 40%, observed, 21%).

**Combined Endostatin and SU5416 Reduce EC Tube Formation.** The production of tubular structures is another important step in angiogenesis. We, therefore, investigated the effects of endostatin and SU5416 on HUVEC and HDMEC EC tube formation. As shown in Fig. 5, control ECs, plated on Matrigel and incubated with control medium, aligned to form lumen-like structures and anastomosing tubes with multicentric junctions. EC cultures, treated with either endostatin or SU5416 alone, formed fewer tubes as well as fewer and weaker anastomoses. Endostatin not only quantitatively reduced the number of tubes but also altered the morphology of the cells. Cells treated with endostatin appeared to be contracted and condensed. When both treatments, endostatin and SU5416, were combined, the effects were greater than after each treatment alone, suggesting at least an additive inhibition of tube formation. Few single cells remained...
and were morphologically highly altered, and no capillary-like network could be detected.

**Tumor Growth in Human Prostate and Glioblastoma Tumor Models in Mice.** Next we investigated whether the findings of anti-angiogenic activity in vitro translated to in vivo efficacy in s.c. xenografts in nude mice. For tumor growth quantification, we used the human PC3 prostate adenocarcinoma and the human glioblastoma U87 cell lines. After tumor cell injection, tumors were allowed to grow until established (7 days for U87 and 21 days for PC3). Then mice were randomized and were divided into therapy groups. Fig. 6 shows relative tumor growth for control and for endostatin, SU5416, and their combination. We found that tumor growth was significantly delayed by each therapy alone. For the PC3, tumor growth delay was significant from day 10 after the beginning of therapy onward (P < 0.01). For the U87, the growth delay was significant from day 5 onward. Interestingly, the combination of endostatin and SU5416 resulted in a significantly (P < 0.01) greater tumor growth delay than was produced by either therapy alone, starting on day 20 for PC3 and day 12 for U87, until the end of observation. Aside from that day-by-day comparison, the general response to treatment was additionally assessed on the basis of the time T⁰ required to reach four times the initial tumor volume (Table 1). This analysis confirmed that each treatment alone induced significant tumor growth delay (P < 0.01), and the combination of endostatin and SU5416 further reduced tumor growth versus each monotherapy (P < 0.05). For the PC3, tumor growth delay caused by the combination endostatin and SU5416 was 22.1 days, which was 3–4-fold that caused by either therapy alone. In the case of U87, the growth delay was 11.6 days for the combination, which was 2–3-fold that caused by either therapy alone. All of the treatments were tolerated well, and no difference in animal behavior or weight was found between groups, which indicated low general toxicity.

**Histological Examination.** Histological examination of PC3 xenografts at day 10 after treatment started showed marked differences in tumors from animals that received endostatin, SU5416, or the combination therapy versus controls (Fig. 7). Extensive tumor necrosis with less mitotic figures were observed in H&E sections after endostatin, SU5416, and the combination therapy. Tumor cellular proliferation (% Ki-67 positivity) was markedly reduced after endostatin, SU5416 and the combination of both. Quantitative analysis of the tumor cell proliferation (% Ki-67 positivity) showed a significant 1.8-fold reduction after SU5416 (P < 0.01 versus control) and a 2.6-fold reduction after endostatin (P < 0.001 versus control) treatment. Combination treatment resulted in a 3.5-fold decrease in tumor cell proliferation (P < 0.001 versus control), which was sig-

### Table 1 Response of PC3 and U87 xenograft tumors growing s.c. after treatment with endostatin, SU5416, and the combination endostatin + SU5416

<table>
<thead>
<tr>
<th>Tumor/Treatment</th>
<th>Days required for tumors to grow four times the original volume ((T_0) ± SE)</th>
<th>Growth delay (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3 control</td>
<td>15.5 ± 1.5</td>
<td>4.6</td>
</tr>
<tr>
<td>PC3 SU5416</td>
<td>20.1 ± 1.8⁰</td>
<td>7.8</td>
</tr>
<tr>
<td>PC3 Endostatin</td>
<td>23.3 ± 2⁰</td>
<td>22.1</td>
</tr>
<tr>
<td>PC3 SU5416 + Endostatin</td>
<td>37.6 ± 2²⁰</td>
<td>3.5</td>
</tr>
<tr>
<td>U87 control</td>
<td>3.2 ± 0.8</td>
<td>7.1</td>
</tr>
<tr>
<td>U87 SU5416</td>
<td>6.7 ± 1.1²⁰</td>
<td>11.6</td>
</tr>
<tr>
<td>U87 Endostatin</td>
<td>10.3 ± 1.3²⁰</td>
<td></td>
</tr>
<tr>
<td>U87 SU5416 + Endostatin</td>
<td>14.8 ± 1.3²⁰</td>
<td></td>
</tr>
</tbody>
</table>

a P < 0.01 versus control.
b P < 0.05 versus each monotherapy.
nificantly higher than that induced by each therapy alone ($P < 0.05$ for each comparison against a single therapy). Qualitatively similar results were observed for U87 tumors.

Thus, for both PC3 and U87, the combination therapy endostatin and SU5416 was significantly more effective than each therapy alone, which suggested at least an additive tumor growth-inhibitory capacity for the combination.

**Tumor Blood Perfusion.** To analyze tumor blood perfusion, we used intermittent-power Doppler ultrasound as a noninvasive method to determine blood flow in the s.c. growing U87 tumors. Ten days after the start of antiangiogenic therapy, a total of 12 animals were examined immediately after i.v. injection of 100 μl of Levovist 300 ultrasound contrast agent. An example of the ultrasonographic visualization of tumor blood flow is demonstrated in Fig. 8. A reduction of blood perfusion was observed after treatment with either endostatin or SU5416. The combination treatment of endostatin and SU5416 resulted in a further reduction in tumor blood perfusion.

**Intravital Microscopy of A549 Lung Carcinoma Cranial Window Model.** Intravital microscopy in conjunction with the cranial window system can be used as an experimental in vivo model to...
characterize tumor angiogenesis, microcirculation, and tumor growth with high temporal and spatial resolution. This model was initiated by implanting chunks of A549 NSCLC into holes in the calvaria (Fig. 9). In the first week after implantation, tumors were examined every 24 h by bright field microscopy to observe the newly formed vessels and the onset of perfusion in these vessels. On days 7, 14, 21, and 28 after tumor implantation, functional microvessel density was additionally analyzed by intravital fluorescence using FITC-labeled dextran. The onset of perfusion in newly formed vessels occurred within 24 h after the first appearance of the new vessels. After 7 days, all of the tumors were established and had functional microcirculation. Animals were then randomized into four groups: control, endostatin, SU5416, and the combination of endostatin and SU5416. Tumor growth analysis showed a typical growth for solid tumors in the two-dimensional tumor diameter up to day 14, the day that treatment started. From that time on, all of the treatment groups showed inhibited tumor growth reaching ~50% of the control group. Starting at 1.0 ± 0.2 mm² on the day of implantation, the tumors reached a final two-dimensional tumor surface by day 28 of 4.3 ± 2.8 mm² (control), 2.1 ± 0.9 mm² (SU5416), 2.5 ± 0.7 mm² (endostatin), and 2.4 ± 1.4 mm² (SU5416 and endostatin). The differences between the treatment groups and the control did not reach statistical significance (P > 0.05 for each comparison). Only if the three treatment groups were pooled and considered as one treatment group was the tumor growth reduction statistically significant (P < 0.05). In contrast, the analysis of FVD as a measure of angiogenesis revealed highly statistically significant differences (P < 0.001) over the time of investigation within all of the groups, and between groups at the same time point of investigation. The data of FVD are presented in detail in Table 2. Intravital fluorescence microscopy showed that, in all of the treatment groups, FVD decreased significantly with the beginning of treatment, compared with the controls. This effect was most distinct in the group treated with the combination of SU5416 and endostatin. Endostatin and SU5416, as well as the combination treatment, were able to significantly decrease FVD versus control and versus days 7 within the same experimental group. The strongest antiangiogenic response was observed in the group treated with the combination of SU5416 and endostatin: Here on days 21 and 28 after tumor implantation, the decrease in FVD was significant not only versus control but also versus SU5416 and endostatin alone. Thus, the combination of endostatin and SU5416 demonstrated strong antiangiogenic activity and reduced microcirculation and tumor growth in the cranial tumor window model.

**DISCUSSION**

For antiangiogenic therapy to be successful, it must inhibit diverse angiogenic stimuli produced by the tumor and its microenvironment while limiting overall toxicity. Thus, it has been suggested that combination antiangiogenesis protocols may be more effective than monotherapies. To date, it is unclear which agent combinations would be most efficacious and have the highest degree of synergistic activity. Here we demonstrate that this bimodal therapy of direct and indirect angiogenesis inhibition resulted in marked enhancement of anti-

![Fig. 9. Intravital microscopy as an in vivo model to characterize tumor angiogenesis, microcirculation, and tumor growth with high temporal and spatial resolution (see Table 2). A549 human NSCLC xenograft tumors were grown in a cranial window of severe combined immunodeficient mice until day 15, when tumors and microvessels were established. Bright field microscopy shows tumor diameter, and intravital fluorescence microscopy using FITC-labeled dextran revealed functional microvessel density and tumor microcirculation.](image)
angiogenic effects in vitro and in vivo. In vitro, the combination of endostatin and SU5416, a synthetic, small-molecule, ATP-competitive inhibitor of the VEGFR2 receptor tyrosine kinase, showed higher activity than each therapy alone with respect to EC proliferation, clonogenic survival, migration, apoptosis induction, and cell cycle blockade (data not shown). In vivo, in human prostate cancer (PC3), NSCLC (A549), and glioblastoma (U87) xenograft tumor models, the combination therapy resulted in enhanced tumor cellular proliferation reduction, enhanced tumor growth delay, and reductions in tumor cell proliferation, microvascularature, and blood flow.

In the PC3 prostate cancer model, tumors treated with SU5416 in combination with low-dose endostatin [compared with doses applied, e.g., in the study by O’Reilly et al. (32)] were found to be only 4.5-fold of the size of control tumors at 40 and 60 days. A regression was induced in some tumors, which was not achieved with either therapy alone. The U87 glioblastoma model was used as an example of an aggressive tumor that is associated with poor prognosis in the clinic (1-year survival, <10%) and that exhibits high resistance to conventional therapies. The combined therapy of U87 xenografts with SU5416 and endostatin not only resulted in significant tumor growth delay but also in reduced tumor blood perfusion, as shown by contrast-enhanced Doppler ultrasound. In vivo angiogenesis was further investigated through the application of a novel cranial window model in which the microvascular development in NSCLC (A549) could be assessed using intravital microscopy. One aim of this model was to mimic the bone microenvironment as a common site of NSCLC metastasis. In contrast to the traditional cranial window models in which the tumor is directly implanted in the cranium, we implanted the tumor into a hole in the calvaria allowing it to grow in the bone microenvironment. Using this intraossear tumor window, we observed results similar to those seen in the s.c. tumor xenografts of PC3 and U87. Tumor growth, angiogenesis, and microcirculation were strongly impaired after treatment with SU5416, endostatin, or the combination of the two. The antiangiogenic effects in the combination group were significantly greater than after each monotherapy, as seen by the strong reduction of FVD in the cranial window model of A549 lung carcinoma. Our results in tumor growth inhibition can be explained in part by the assumption that tumor growth inhibition occurs after the impairment of angiogenesis, as described in Jain et al. (37).

Because of the ability of endostatin to suppress angiogenesis under a variety of circumstances [high ambient concentrations of bFGF, VEGF, interleukin-8, or platelet-derived growth factor (20)] and the strength of SU5416 against the important VEGF receptor target in particular, we show that the combination of endostatin and VEGFR inhibition increased the antiproliferative activity in ECs and tumors in vivo. Endostatin as a single agent has shown measurable antiangiogenic activity in its first two Phase I studies (38). With increasing doses of endostatin, generally decreased tumor blood flow and metabolism was measured by positron emission tomography (PET) scans. Further tumor biopsy analysis revealed a significant increase in tumor cell apoptosis and EC apoptosis after 8 weeks of therapy (39). It is thought that in conjunction with other antiangiogenics or chemotherapeutics, the efficacy of endostatin will be improved (40, 41) using a versatile repertoire of activities for endostatin that contribute to its antiangiogenic function (42).

Using genome-wide expression profiling, we recently demonstrated that many key regulators of proangiogenic pathways, including several genes related to VEGF signaling (such as, e.g., Ap1 transcription factors, Thrombin receptors, Stats, and HIF-1α), are directly down-regulated after endostatin treatment in human microvascular ECs in vitro. Thus, endostatin may act cooperatively with the VEGF receptor inhibitor, because of the ability of endostatin to down-regulate VEGF upstream and downstream signaling components on the phosphorylation and transcriptional levels.

Perhaps more importantly, endostatin may additionally abrogate the angiogenic escape mechanisms in activated endothelium, which could circumvent the blockade of VEGF signaling. Thus, the notable therapeutic enhancement observed may occur for two reasons: (a) the endostatin-induced down-regulation of angiogenic pathways that are turned on by the high-intratumoral- or developmental-stage-specific angiogenic heterogeneity (44); and (b) the ability of endostatin to suppress the alternative angiogenic mechanisms up-regulated by the blockade of VEGF signaling.

Taken together, our results suggest that angiogenic inhibitors that possess presumed distinct modes of action may, in fact, show increased therapeutic effectiveness when these agents are combined. Enhanced antiangiogenic effectiveness has been reported for the combination of endostatin with angiostatin (44, 45) and for endostatin with chemotherapeutic agents (46). In contrast, the dual blockade of VEGFR and epidermal growth factor receptor (EGFR) using small molecules that block receptor tyrosine kinases of the same family did not result in enhanced antitumor activity (11). In fact, only when VEGFR blockade or epidermal growth factor receptor blockade was combined with gemcitabine, were supra-additive therapeutic effects observed.

Our chosen models, lung cancer, prostate cancer, and glioblastoma, are common malignant diseases. Patients suffering from lung cancer or glioblastoma, in general, share a poor prognosis, with the exception of the rare cases of early-stage lung cancer, despite continuing efforts to improve survival rates using combinations of surgery, radiotherapy, and chemotherapy (47, 48). In the case of prostate cancer, effective therapies exist, but the severe side effects of local and systemic tumor therapy rationalize improved therapies.

In summary, the strategy of combining direct and indirect angiogenesis inhibition to achieve a synergistic effect warrants clinical investigation. A variety of agents with mechanistic properties that are similar to the properties of the specific drugs used here could be evaluated in humans.

ACKNOWLEDGMENTS

We thank Drs. P. Peschke and F. Kiessling for suggestions and support with the animal work and Thuy Trinh for excellent in vitro work, as well as Professor M. Wannenmacher for support of the entire study.

REFERENCES


8897
Combined Therapy with Direct and Indirect Angiogenesis Inhibition Results in Enhanced Antiangiogenic and Antitumor Effects


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/24/8890

Cited articles
This article cites 42 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/24/8890.full.html#ref-list-1

Citing articles
This article has been cited by 22 HighWire-hosted articles. Access the articles at:
/content/63/24/8890.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.