Trimodal Cancer Treatment: Beneficial Effects of Combined Antiangiogenesis, Radiation, and Chemotherapy

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Abstract

It has been suggested that chemotherapy and radiotherapy could favorably be combined with antiangiogenesis in dual anticancer strategy combinations. Here we investigate the effects of a trimodal strategy consisting of all three therapy approaches administered concurrently. We found that in vitro and in vivo, the antiendothelial and antitumor effects of the triple therapy combination consisting of SU11657 (a multitargeted small molecule inhibitor of vascular endothelial growth factor and platelet-derived growth factor receptor tyrosine kinases), Pemetrexed (a multitargeted folate antimetabolite), and ionizing radiation were superior to all single and dual combinations. The superior effects in human umbilical vein endothelial cells and tumor cells (A431) were evident in cell proliferation, migration, tube formation, clonogenic survival, and apoptosis assays (sub-G1 and caspase-3 assessment). Exploring potential effects on cell survival signaling, we found that radiation and chemotherapy induced endothelial cell Akt phosphorylation, but SU11657 could attenuate this process in vitro and in vivo in A431 human tumor xenografts growing s.c. on BALB/c nu/nu mice. Triple therapy further decreased tumor cell proliferation (Ki-67 index) and vessel count (CD31 staining), and induced greater tumor growth delay versus all other therapy regimens without increasing apparent toxicity. When testing different treatment schedules for the A431 tumor, we found that the regimen with radiotherapy (7.5 GY single dose), given after the institution of SU11657 treatment, was more effective than radiotherapy preceding SU11657 treatment. Accordingly, we found that SU11657 markedly reduced intratumoral interstitial fluid pressure from 8.8 ± 2.6 to 4.2 ± 1.5 mm Hg after 1 day. Likewise, quantitative T2-weighted magnetic resonance imaging measurements showed that SU11657-treated mice had reduced intratumoral edema. Our data indicates that inhibition of Akt signaling by antiangiogenic treatment with SU11657 may result in: (a) normalization of tumor blood vessels that cause requisite physiologic conditions for subsequent radio/chemotherapy, and (b) direct resensitization of endothelial cells to radio/chemotherapy. We conclude that trimodal cancer therapy combining antiangiogenesis, chemotherapy, and radiotherapy has beneficial molecular and physiologic effects to emerge as a clinically relevant antitumor strategy. (Cancer Res 2005; 65(9): 3643-55)

Introduction

The concurrent or sequential combination of radiotherapy and chemotherapy is now considered a standard therapy regimen for the treatment of many tumors (1–4). The underlying rationale for combining different treatment modalities is to broaden the therapeutic index. It is assumed that different treatment modalities have overlapping anticancer effects but a decreased overlapping spectrum of side effects. Antiangiogenesis is one of the most promising new therapy principles which has recently gained strong impetus from successful clinical studies with an anti–vascular endothelial growth factor (VEGF) antibody (4–7). It has been suggested in experimental and clinical studies that angiogenesis inhibitors may be favorably combined with either chemotherapy (1, 4, 5, 8) or radiotherapy in dual combinations (3, 9–17). Therefore, the idea pursued in this paper of investigating a trimodal therapy regimen seems logical.

The rationale for combining radiation with angiogenesis inhibitors is derived, in part, from findings that irradiation induces expression of proangiogenic cytokines such as VEGF or platelet-derived growth factor (PDGF), resulting in protection of vessels from radiation-induced cell damage (9, 11, 18). Clinically, elevated expression of these growth factors correlates with higher vessel density and negative prognosis in various tumors (4, 10). Moreover, such tumors are often relatively resistant to radiation therapy (4, 10). In addition to promoting the expression of proangiogenic cytokines, radiation has also been reported to kill endothelial cells (9). Thus, the rationale for this combination involves both killing the endothelial cells with radiation and preventing their regrowth with the angiogenesis inhibitor. Similarly, the favorable combination of chemotherapeutic agents and angiogenesis inhibitors may be due to the sustained antiangiogenic effects of the chemotherapeutic agents (3, 4, 19).

Therefore, the question arises of whether the combination of all three modalities would have beneficial cellular or physiologic effects that could provide a rationale for trimodal therapy. The purpose of the present study was to evaluate the molecular and physiologic effects of a trimodal anticancer regimen consisting of the angiogenesis inhibitor SU11657, the chemotherapeutic agent Pemetrexed, and ionizing radiation, on human endothelium and A431 human epidermoid cancer cells in vitro and A431 human tumor xenografts on BALB/c mice in vivo.

SU11657 is a multitargeted inhibitor of class III/V receptor tyrosine kinases with potency and selectivity similar to SU11248,
which has been studied in preclinical animal models (20–25). SU11248 has also shown promise in its phase I/II trials in patients (20–26). Both SU11248 and SU11657 exert their antiangiogenic effects via potent inhibition of VEGF receptor 1 (VEGFR1), VEGFR2, and PDGF receptor (PDGFR; refs. 21, 22, 24). Additionally, both compounds may have direct antitumor effects by inhibition of c-kit and fetal liver tyrosine kinase 3 (flt3) expressed on tumor cells (21, 23, 25).

In contrast with the widely used classic antifolates such as 5-fluorouracil, the novel folate antimetabolite Pemetrexed inhibits several key enzymes of thymidylate and purine synthesis, like thymidylate synthase, dihydrofolate reductase, and glycaminidine ribonucleotide formyl transferase, as well as of other folate-requiring enzymes (27). Pemetrexed exhibits significant antitumor activity in a broad spectrum of human tumors, including mesothelioma, pancreatic, colorectal, gastrointestinal, lung, head and neck, breast and cervix cancers, and has currently entered Phase III clinical trials (28). Antifolates have been established in cancer treatment for many years and are widely used in combination with radiotherapy (29). The ability of Pemetrexed to sensitize cells to ionizing radiation was reported in preclinical studies (30–32).

In this study, we have examined several variables, in vitro and in vivo, as a function of therapy with different combinations of SU11657, Pemetrexed, and radiation. For example, apoptosis has been suggested to influence tumor response of radiotherapy, chemotherapy and antiangiogenesis. Evading apoptosis has been shown to promote drug resistance (33) and survival signaling by Akt (also known as protein kinase B; refs. 34, 35). Furthermore, it has been shown that radiation induces the phosphorylation of Akt via phosphatidylinositol 3'-kinase signaling (36, 37). Thus, the prosurvival effect of Akt activation in endothelial cells provides a key escape mechanism against radiation damage (36, 37). We therefore investigated treatment-related apoptosis induction and the activation of Akt in endothelial cells. To test the antiangiogenic effects of the treatments, we also did functional angiogenesis assays in human endothelial cells. In A431 tumor xenographs growing s.c. on nude mice, we examined tumor growth and immunohistopathologic changes. In addition to cellular sensitivity, we were interested in the altered tumor physiology associated with combinations of SU11657 and radio/chemotherapy. To investigate the physiologic effects, we measured intratumoral interstitial fluid pressure (IFP). It is assumed that elevated IFPs, a hallmark of solid tumors, may be associated with reduced tumor blood flow and impaired delivery of therapeutic drugs (3, 38–41) and should thus influence the results of our combination therapy. The intratumoral IFP data were correlated with functional T2-weighted magnetic resonance imaging (MRI) scans which depict tumor morphology as well as tumor physiologic variables such as intratumoral edema. Our results integrate cellular and physiologic effects of trimodal therapy, and argue for the beneficial combined effects of a trimodal antitumor strategy.

Materials and Methods

Cell culture, drug exposure, and irradiation. Human epidermoid carcinoma cells (A431) were obtained from the Tumorbank of the German Cancer Research Center (DKFZ, Heidelberg) and were grown as monolayers in DMEM medium with 10% FCS. Primary isolated human umbilical vein endothelial cells (HUVEC, Promocell, Heidelberg, Germany) were cultured up to passage 6, 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 basic fibroblast growth factor (Promocell; refs. 9, 42). Pemetrexed was obtained from Lilly Research Laboratories (Indianapolis, IN) and was dissolved in an aqueous buffer [150 mmol/L phosphate (pH 7.4)]. Cells were treated with Pemetrexed at various concentrations for 2 hours followed by a medium change to remove the drug. SU11657 was provided by Sugen, Inc. (South San Francisco, CA). Cells were treated with SU11657 for 2 hours in growth factor–reduced modified Promocell medium. Then the same volume of modified Promocell medium with 2-fold standard growth factor concentrations was added and cells were incubated under standard conditions (9, 42). If not indicated otherwise, for combination experiments, we chose 0.1 μmol/L SU11657 for proliferation and clonogenic survival assays, and 1 μmol/L SU11657 for other assays involving Matrigel. These doses were determined in pilot experiments as small doses showing significant effects as monotherapy. Photon irradiations from 0 to 8 Gy were done immediately after antiangiogenic and/or chemotherapy treatment using a linear accelerator at 6 MV (Mevatron, Siemens, Erlangen, Germany) at a dose rate of 2.5 Gy/minute.

Cell proliferation and clonogenic assay. Cell proliferation/cell viability was measured as described previously (9, 42). Briefly, a suspension of 50,000 endothelial or tumor cells was seeded (collagen I-coated flasks for endothelial cells) and incubated for 24 hours under standard conditions. The cells were then treated as indicated and counted after another 72 hours of incubation. To measure the clonogenic survival of endothelial or tumor cells were plated in triplicate to yield 50 to 100 colonies per culture flask and were incubated for 14 to 21 days. Colonies of more than 50 cells, as assessed by microscopic inspection, were scored as survivors, as described earlier (9, 31). For graphical representation of the combination experiments, the mean values of the measured surviving fractions were multiplied with the averaged surviving fraction after drug exposure alone. Radiation doses that reduced cell proliferation to 60% (D60 in Gy) following exposure to X-rays alone and in the combination treatments were derived by linear interpolation between measured data pairs. Enhancement ratios were calculated as the quotient of D60 with irradiation alone and D60 of the respective dual or triple combination. Sensitivity variables for clonogenic survival were calculated accordingly, but using radiation doses that decreased survival to 5% (D5).

Endothelial cell migration and tube formation. The endothelial cell ability to form tubular structures was assessed as previously described (9). Briefly, 24-well plates were coated with Matrigel (Becton Dickinson, Heidelberg, Germany). Endothelial cells (48,000 cells per well) undergo differentiation into capillary-like tube structures when plated on Matrigel. Cells were incubated with Pemetrexed (1.06 μmol/L) and/or SU11657 (1 μmol/L). Then samples were irradiated with a single dose of 4 Gy, and incubated for 6 hours on the Matrigel at 37°C/5% CO₂. After 6 hours of incubation, the media was aspirated, the cells were fixed and stained with Diff-Quik II reagents (Dade Behring AG, Germany). Tubes were counted by counting the number of anastomoses per field on at least five wells per group.

The migration of HUVEC after exposure to different treatment regimens was tested in a migration assay as described previously, with minor modifications (9). Briefly, Matrigel-coated transwell inserts (8 μmol/L pore size; Becton Dickinson) were used. Cells were incubated with Pemetrexed (1.06 μmol/L) and/or SU11657 (1.0 μmol/L) for 2 hours and respective samples were irradiated with a single dose of 4 Gy. Then a cell suspension of 200 μL (3 × 10⁵ cells/mL) per condition was added in triplicate transwells. After 18 hours of incubation, endothelial cells that had invaded the underside of the membrane were fixed, stained in thiazine and eosin solution by using Diff-Quick II solution (Dade Behring) and sealed on slides. Migrated cells were counted by microscopy.

Apoptosis in endothelial cells. HUVEC were treated with single modalities (0, 2, or 5 Gy irradiation; 1.06 μmol/L Pemetrexed; 1.0 μmol/L SU11657) or combinations. 24 hours after the end of therapy, cells were harvested and prepared for analysis of sub-G₁ DNA by flow cytometry (FACScan, Becton Dickinson) with propidium iodide staining (9, 43).
Additionally, a metronomic application of Pemetrexed (Pemetrexedmetron) 4 consecutive days (days 0-3), which we defined as a conventional schedule.

Immunocytochemistry. To analyze Akt phosphorylation, HUVEC were exposed to single treatment modalities (irradiation, 0, 2, or 5 Gy; Pemetrexed, 1.06 μmol/L; SU11657, 1.0 μmol/L) or combinations. Cells were grown on glass coverslips and fixed 45 minutes after treatment in 3.7% paraformaldehyde. Cells were incubated with a rabbit anti-Akt, phospho-specific (Ser473) primary antibody (Santa Cruz, Heidelberg, Germany) followed by incubation with the Alexa-488 conjugated anti-mouse secondary antibody (Molecular Probes, Leiden, the Netherlands). Cells were counterstained with propidium iodide for nuclear staining. Finally, the cells were washed and mounted with Mowiol on microscope slides, observed on a Zeiss Axiophot (Carl Zeiss, Jena, Germany) microscope with a 20× objective. Images were acquired using a cooled CCD camera (Photometrics, CH250) using fluorescence excitation with an FITC filter set and an acquisition time of 5 seconds, and then stored as TIFF files on a Sun SparStation 20 Unix workstation. Image processing and analysis was done with programs written for the Khoros Software package. Averaged intensity ± SD was analyzed for 10 fields on five slides.

Animal studies. All in vivo experiments were approved by institutional and governmental animal protection committees (Regierungspraesidium, Karlsruhe, Germany). Athymic female mice (BALB/c, nu/nu, 8 weeks, 20 g) were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were maintained under clean room conditions in sterile rodent microisolator cages (VentilRack, Heidelberg, Germany). Human A431 epidermoid carcinoma cells were injected s.c. into the right hind limb (1 × 10^6 cells in 100 μL PBS). Two sets of experiments were done. The first experiment set out to ask the question whether the order of administration of single dose radiotherapy and continuous angiogenesis inhibition influenced tumor growth. Mice with established tumors (~200 mm^3 (222 ± 39 mm^3) mean ± SD) were stratified into five groups (n = 10-12 each) receiving either vehicle as control, radiotherapy alone, SU11657 alone, or two regimens of combined radiotherapy and SU11657. Radiotherapy was given either 1 day before start of antiangiogenic therapy or 1 day after start of antiangiogenic therapy, as indicated.

For the second set of experiments, three modalities, antiangiogenesis, chemotherapy, and radiotherapy were combined in a trimodal schedule, as indicated. Animals were randomized into nine groups (n = 12-15 each) when tumor volume reached ~200 mm^3 (208 ± 42 mm^3, mean ± SD). Mean tumor volume was determined thrice weekly by calipers and calculated by volume V = length × width × thickness × 0.5. Chemotherapy with Pemetrexed was administered in the trimodal experiment at 150 mg/kg (total dose, 600 mg) in 200 μL PBS i.p. for 4 consecutive days (days 0-3), which we defined as a conventional schedule. Additionally, a metronomic application of Pemetrexed (Pemetrexedmetron) with a lower daily dosage (100 mg/kg) was given on days 0, 1, and 2, and on days 7, 8, and 9 (same total dose of 600 mg).

SU11657 was given s.c. in 100 μL carboxymethylcellulose as vehicle thrice weekly at 100 mg/kg starting on day 0 or day 1, as indicated, and was given until the end of observation. In combination regimen, SU11657 was given 30 minutes before Pemetrexed injection. Radiotherapy was delivered using a Co-60 source (Gammatron, Siemens) with a single dose of 7.5 Gy. When given on day 0, radiotherapy was given 24 hours before antiangiogenic therapy. In the trimodal experiment, radiotherapy was given on day 1, 4 hours after SU11657 and Pemetrexed administration.

Measurement of interstitial fluid pressure in vivo. IFP in A431 tumors was measured to determine the influence of the different therapy modalities and its association with tumor growth and therapy outcome. The IFP is thought to be nearly uniform throughout the central volume of an idealized spherical tumor (12, 19). Here, the intratumoral IFP was measured using a modified pressure sensor (SAMBA 3000, Samba Sensors, Gothenburg, Sweden) consisting of a laser-optic fiber. The pressure measurement is not based on the often used principle of a hydrostatic difference between a glass capillary filled with fluids and the interstitium, but is instead based on a pressure-sensitive optical Fabry-Perot interferometer. According to the manufacturer, the fiber (diameter, 0.25 mm inside a 0.3 mm cannula) was calibrated between 0.95 and 1.15 bar with a resolution of 0.2 mbar (±2% accuracy). Thus, we measured the IFP relative to the ambient atmospheric pressure, as assessed with the built-in barometer of the SAMBA 3000. When inserted in the tumor tissue, the pressure signal typically rose up to a plateau within 30 seconds. This equilibrium pressure at 1 minute was considered the IFP. In prior experiments, we found that the IFP was independent of the specific localization of the pressure sensor inside the tumors, thus confirming the uniformity of the pressure distribution in this experimental tumor system (data not shown).

Functional magnetic resonance imaging. MRI is a noninvasive method to visualize tumor size and morphology. Two randomly chosen animals from each therapy group were examined on day 10. The animals were examined in a 2.35 T scanner (BiSpec 24/40, Bruker Medizintechnik, Ettlingen, Germany, ref. 45). An actively shielded gradient coil with a 120-mm inner diameter was used. This coil was driven by the standard 150 V/100 A gradient power supply. In this configuration, 180 mT/m could be reached in 180 milliseconds. As RF-coil, we used a resonator with a 90 mm inner diameter. T2-weighted scans were acquired using a multispin echo imaging sequence (field of view 4 × 4 cm, 128 × 96 matrix, 2.2 mm slice thickness). The tumor was determined as a region of interest in each scan for further evaluation. The T2-relaxation time (range 0 to >200 milliseconds) was measured in T2-map images to assess changes in tumor tissues after treatment. It is known that necrosis and edema both have long T2 times, with necrosis even being higher than edema. Therefore, the multispin echo sequence was used (TE = 8, 16, 24, … 96 milliseconds). T2 was then calculated from these data. Tissue with T2 times between 140 and 180 milliseconds were defined as edema, tissue with T2 times >180 milliseconds were defined as necrosis (46). The limits were set via T2 measurements in defined regions of interest. Then, a histogram analysis was used to quantify the relative portions of tumor edema (T2-relaxation time = 140-180 milliseconds) and necrosis (>180 milliseconds) within the entire tumor, corresponding to the measured pixels in the regions of interest.

Immunohistochemistry. For histologic analysis, tumors were harvested from three additional animals per treatment group, 11 days after the start of therapy and at the end of the observation period, fixed in buffered formalin and embedded in paraffin (42). Tissue slices (5 μm) were stained with H&E and general tissue morphology was visualized and photographed with a camera (Nikon Super Coolscan ED 4000, Tokyo, Japan) mounted on a Zeiss microscope (Carl Zeiss, Jena, Germany). Tumor cell proliferation was assessed by the percentage of Ki-67-positive cells determined by immunohistochemical staining with the MIB-1 monoclonal mouse anti-human Ki-67 antibody (Dako, Hamburg, Germany). Sections were counterstained with H&E. Ki-67 staining was quantified by counting the number of positively stained nuclei of 200 to 250 cells in 10 randomly chosen fields at ×100 magnification. To quantify tumor vessel counts, frozen sections were fixed and stained with primary antibody to CD31 (Becton Dickinson) and 10 random fields at ×100 magnification were chosen. To detect the phosphorylated Akt (phospho-Akt or p-Akt) status in vivo, paraffin-embedded tissue sections were stained using rabbit anti phospho-Akt (Ser473) antibody (HIC specific, Cell Signaling Technology, Beverly, MA) and Signal Stain phospho-Akt (Ser473) IHC detection Kit (Cell Signaling Technology) according to the manufacturer’s instructions (47).
**Statistical analysis.** The tumor volumes $V$ were calculated as $V = 0.5 \times a \times b^2$. Statistical evaluation of tumor growth was undertaken by daily comparisons of the volumes. In addition, the general response to treatment was assessed on the basis of the time, $T_n$, required to reach $n$ times the initial tumor volume. For multiple comparisons the Kruskall-Wallis ANOVA was used for nonparametric variables. For parametric variables, ANOVA was used along with Fisher's least-significant-difference. All analyses were two-tailed. A $P$ value of 0.05 was considered statistically significant.

**Results**

**Endothelial and tumor cell proliferation and clonogenic survival.** We first investigated the effects of SU11657 alone on HUVEC and A431 cell proliferation and viability in the 72-hour proliferation/viability test. SU11657 reduced the cell number with an IC$_{50}$ of 0.2 ± 0.05 μmol/L for HUVEC and an IC$_{50}$ of 1.5 ± 0.3 μmol/L for A431 (Fig. 1A). We then did combination treatments on proliferation and clonogenic survival of HUVEC and A431 tumor cells using SU11657 (0.1 μmol/L) and Pemetrexed (1.06 μmol/L) doses that had only a modest effect on proliferation inhibition (for Pemetrexed; see ref. 44). Radiation alone reduced cell proliferation and clonogenic survival in HUVEC and tumor cells in a dose-dependent manner. The addition of either SU11657 or Pemetrexed significantly increased the antiproliferative effects of irradiation ($P < 0.05$; Fig. 1B; Table 1) in both A431 and endothelial cells. Importantly, the triple combination resulted in a pronounced antiproliferative effect that was more than additive and statistically significant versus both dual combinations ($P < 0.05$) in A431 and HUVEC. This supra-additive effect is reflected by the calculated enhancement ratios of >1 (Table 1). Interestingly, the radiosensitizing drug effects were more pronounced in HUVEC than in A431 cells.

Similarly, for clonogenic survival as an end point, SU11657 and Pemetrexed sensitized both A431 cells and HUVEC to radiation ($P < 0.05$; Fig. 1C; Table 2). As observed for proliferation, the combined effects were more toxic in HUVEC than in A431 cells. For example, the surviving fractions for A431 and HUVEC were 0.6 versus 0.4 (at 2 Gy), 0.46 versus 0.19 (2 Gy + SU), 0.44 versus 0.21 (2 Gy + Pem), 0.35 versus 0.12 (2 Gy + SU + Pem = triple therapy). Furthermore, the more than additive antiproliferative effect of triple therapy was present over the entire radiation dose (0-8 Gy) in endothelial cells, whereas supra-additivity was
only observed for high radiation doses (>4 Gy) in A431 cells. These data suggest a high primary inherent sensitivity of endothelial cells to trimodal treatment, but tumor cell proliferation also seems to be sensitive to triple therapy.

**Endothelial cell migration/invasion.** Endothelial cell migration and tube formation are important steps in the angiogenesis process. HUVEC exposure to monotherapies of 4 Gy, Pemetrexed, or SU11657 exhibited reduced migration by 20 to 30% (mean ± SD) compared with the nontreated controls \((P < 0.02; \text{Fig. 2A and B})\). The combination of two modalities further significantly inhibited the cell migration (45-65% versus each single therapy \(P < 0.05\)). Triple combination resulted in a 90% inhibition of HUVEC migration \((P < 0.01\) versus dual combinations). These data elucidate the superior inhibitory effect of the triple combination on endothelial cell migration compared with each dual combination.

**Endothelial tube formation.** When endothelial cells such as HUVEC are plated on Matrigel, they organize into lumen-like structures with multicentric anastomoses, as depicted 6 hours after plating in \(\text{Fig. 2B}\). Exposure to monotherapies of irradiation or SU11657 alone resulted in fewer tubes and weaker anastomoses versus controls \((P < 0.05\), whereas Pemetrexed alone had no significant effects \((P > 0.5\) versus controls; \(\text{Fig. 2C}\)). Each tested dual combination led to a further reduction of tubular structures versus single therapy \((P < 0.05\). The triple combination (\(\text{Fig. 2C}\)) resulted in the greatest inhibition of tube

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>D60 (Gy) (\pm) SD</th>
<th>Enhancement ratios (\frac{D60_{Rx}}{D60_{Rx+drug}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>Rx</td>
<td>7.5 ± 0.5</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + SU</td>
<td>6.6 ± 0.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + Pem</td>
<td>5.5 ± 0.4*</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Triple combination</td>
<td>4.4 ± 0.3↑</td>
<td>1.7</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Rx</td>
<td>4.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx + SU</td>
<td>4.3 ± 0.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + Pem</td>
<td>3.6 ± 0.2*</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Triple combination</td>
<td>3.2 ± 0.2↑</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 1.** Radiation doses which reduce cell proliferation to 60% that of controls (D60 in Gy) alone, and in combination with the drugs Pemetrexed and/or SU11657.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>D5 (\pm) SD (Gy)</th>
<th>Enhancement ratios (\frac{D5_{Rx}}{D5_{Rx+drug}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>Rx</td>
<td>6.5 ± 0.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + SU</td>
<td>5.7 ± 0.3*</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + Pem</td>
<td>5.7 ± 0.3*</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Triple combination</td>
<td>5.0 ± 0.3↑</td>
<td>1.3</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Rx</td>
<td>5.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rx + SU</td>
<td>4.6 ± 0.2*</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Rx + Pem</td>
<td>4.7 ± 0.2*</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Triple combination</td>
<td>4.3 ± 0.2↑</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 2.** Similar to Table 1, but for clonogenic survival as described in Materials and Methods reduced to 5% by irradiation (D5 in Gy).

**Figure 2.** Endothelial cell migration and tube formation. HUVEC migration was determined by counting cells that had migrated to the lower side of an uncoated invasion chamber 18 hours after cells were treated with: PBS (control), SU11657 (1.0 μmol/L), 4 Gy Radiation (Rx), or the triple combination (SU11657 + 4 Gy + Pemetrexed). A, stained cells in representative fields. B, the number of HUVEC that had migrated are shown as a histogram: columns, mean; bars, ± SD; Rx, 4 Gy; Pem, Pemetrexed; Su, SU11657; *, \(P < 0.05\) versus control; **, \(P < 0.05\) versus control and each single treatment; ***, \(P < 0.05\) versus all other treatments. C, the ability of HUVEC to form tubule-like structures when plated on Matrigel was determined for cells treated at 6 hours after plating with: PBS (control), SU (SU11657, 1 μmol/L), 1.06 μmol/L Pemetrexed (Pem), 4 Gy Radiation (Rx), and combinations. Photographs of representative fields are shown.
formation with the least number of anastomoses ($P < 0.05$ versus dual combinations).

**Apoptosis in endothelial cells.** Induction of apoptosis is one possible mechanism by which the various treatments manifest their antiproliferative effects. To explore this, the sub-G$_1$ (apoptotic) fractions of HUVEC (Fig. 3D) were determined by propidium iodide fluorescence-activated cell sorting after exposure to various combinations of agents. Irradiation alone, Pemetrexed, or SU11657 treatment alone induced 5 to 10 $\pm$ 2% apoptotic cells (mean $\pm$ SD, $P < 0.05$ versus control) at 24 hours. Exposure to each dual combination increased the sub-G$_1$ fractions to 10 to 20 $\pm$ 3%, which was significant versus single treatments ($P < 0.05$). The triple combination substantially enhanced the apoptosis rate to 25 $\pm$ 4% versus all other combination ($P < 0.02$).

To further analyze apoptosis induction, a flow cytometric analysis of apoptotic and nonapoptotic populations of HUVEC using the anti-active caspase-3 monoclonal antibody was done. Figure 3C shows representative results at 6 hours after treatment, demonstrating that untreated control endothelial cells were primarily negative for caspase-3; a slight shift of the curves was observed after radiation and Pemetrexed single therapy, indicating some caspase-3 activity. SU11657 (1 $\mu$mol/L) induced more caspase-3 activity than radiation (4 Gy) or Pemetrexed (1.06 $\mu$mol/L) at the concentrations chosen. All combinations resulted in more caspase-3 activity than monotherapies, and triple combination markedly shifted the curve further to the right indicating the most caspase-3 activity. Thus, triple combination markedly induced endothelial cell apoptosis.

**Akt phosphorylation in endothelial cells in vitro.** Akt is thought to contribute to survival signaling mediated by receptor tyrosine kinases such as VEGFR2 and PDGFR. Therefore, the extent of Akt activation was examined after exposure to various treatments (Fig. 3C and D). Although irradiation or Pemetrexed increased apoptosis, as single agents or in combination, these treatments were also observed to increase Akt phosphorylation suggesting the induction of a cell survival mechanism in response to toxicity. In contrast, SU11657 attenuated Akt activity and suppressed the enhancement of Akt phosphorylation observed after irradiation or exposure to the chemotherapeutic agent. We also found that the combination of radiation and chemotherapy resulted in the highest Akt phosphorylation. Interestingly,
SU11657 down-regulated radiation-, chemotherapy-, and radio/chemotherapy-induced Akt phosphorylation significantly in human endothelial cells (P < 0.05; Fig. 3D).

**Tumor growth of A431 xenografts in nude mice.** We then investigated the combined effects of irradiation, chemotherapy with Pemetrexed, and antiangiogenic therapy with SU11657 in vivo in A431 tumor xenografts growing s.c. on hind limbs of BALB/c nude mice. Because radiotherapy (7.5 Gy) was to be given only once, and the optimal schedule of combining antiangiogenic therapy and radiotherapy was in particular unknown, we first asked whether the order of administration of radiotherapy and angiogenesis inhibition influenced tumor growth. We found that monotherapies with either radiotherapy or SU11657 significantly attenuated tumor growth versus vehicle control (P < 0.05, from day 7 onwards; Fig. 4A). Furthermore, both schedules of combined antiangiogenic and radiotherapy were more efficacious than monotherapies from day 12 onwards (P < 0.05). Importantly, we found that when radiotherapy was given 1 day after starting SU11657 therapy (SU + Rx), tumor growth delay was greater than when radiotherapy preceded SU11657 (Rx + SU; P < 0.05, from day 12 onwards).

Therefore, for the following trimodal tumor growth experiments, we chose the more efficacious schedule with SU11657 administration starting prior to radiotherapy. Here, mice with established tumors were stratified into nine therapy arms (Fig. 4B). We observed reduced tumor growth in each treatment arm versus control (Fig. 4C) from day 7 onwards (P < 0.05 versus controls from day 7 onward, by day comparison). Tumor growth delay was significantly increased in each dual combination compared with monotherapies (P < 0.05, from day 9 onward). The triple combination significantly further reduced tumor growth compared with dual therapies (from day 11 onwards).

**Figure 4.** In vivo growth of A431 tumor xenografts. Mice with A431 human epidermoid cell carcinoma growing s.c. on BALB/c nu/nu mice were treated with irradiation (Rx, 7.5 Gy), with the chemotherapeutic agent Pemetrexed (150 mg/kg for 4 consecutive days starting at day 0, 600 mg total dose) and/or with SU11657 (100 mg/kg thrice weekly until end of observation). A second triple therapy regimen (Triple metron) with a metronomic Pemetrexed administration over six injections until day 10 was introduced (100 mg/kg, 600 mg total dose). Abbreviations: Rx, 7.5 Gy; Pem, Pemetrexed (150 mg/kg); SU, SU11657 (100 mg/kg); Triple, SU + Pem + Rx (150 mg/kg x 4); Triple metron, SU + Pem + Rx (100 mg/kg x 6). The tumor growth of s.c. A431 tumors in BALB/c nu/nu mice is shown, with each treatment group containing 10 to 12 mice, and the data points representing the mean ± SE of tumor volumes. A, tumor volumes after radiotherapy and angiogenesis inhibition to investigate the role of the schedule of administration. Irradiation 1 day after start of SU11657 therapy (SU + Rx) was more effective than irradiation 1 day prior to SU11657 start (Rx + SU; #, P < 0.05 from day 12 onwards). Monotherapy significantly attenuates tumor growth from day 7 onwards (*P < 0.05). Both schedules of dual irradiation and SU11657 also result in more tumor growth delay than each respective single therapy from day 12 onwards (**P < 0.05 for each comparison). B, schedule diagram for the nine groups in the trimodal therapy experiment. The more effective strategy from (A) is employed with SU11657 starting prior to irradiation. Two chemotherapy arms are tested, one conventional arm and one metronomic arm. C, tumor growth for the eight groups in the trimodal therapy experiment. The by day comparison shows significant differences for all treatment groups versus control from day 7 onwards (*P < 0.05). Each combination of two therapies resulted in more tumor growth delay than each respective monotherapy from day 9 onwards (**P < 0.05 for each comparison). Both triple schedules resulted in more tumor growth delay than all other groups, which was significant from day 11 on (***P < 0.05). The triple metronomic chemotherapy was significantly more effective than conventional chemotherapy therapy (##P < 0.05, from day 21 onwards). D, the tumor growth delay was also derived from T5, which represents the time required to reach five times the initial tumor volume, as an overall measure of tumor growth dynamics. Columns, difference in T5 of the respective treatment group and T5 in control group ± SD. Statistical significance is designated as: *, significant (P < 0.05) versus control; **, significant (P < 0.05) versus control and each single treatment group; ***, significant (P < 0.05) versus control, each single and double treatment group.
Figure 5. Immunohistochemistry of A431 tumors. Tumor cell proliferation and microvessel density were assessed by Ki-67 and CD31 immunostaining in A431 tumors excised on day 11 after start of therapy with: no treatment (control tumor), radiation (Rx, 7.5 Gy), Pem (Pemetrexed), SU (SU11657), SU + Rx and the reverse schedule Rx + SU as shown in Fig. 4. Rx + Pem, SU + Pem + Rx (conventional triple therapy and metronomic chemotherapy dosing as shown in Fig. 4). A, representative fields of Ki-67 staining are shown. B, quantitative analysis of Ki-67-positive cells determined by counting immunostained cells in tumors treated with: *, significant ($P < 0.05$) versus control; **, significant ($P < 0.05$) versus control and each monotherapy; ***, significant ($P < 0.05$) versus all other groups. C, representative fields of CD31 immunostaining in A431 tumor. D, quantitative analysis of CD31-positive vessels in the center of A431 tumors: *, significant ($P < 0.05$) versus control; **, significant ($P < 0.05$) versus control and respective monotherapies; ***, significant ($P < 0.05$) versus all other groups, #, significant ($P < 0.05$) versus conventional triple therapy. 

E, representative figures of phosphorylated Akt (p-Akt) immunostaining in A431 tumors after indicated treatments. Paraffin-embedded tissue sections of control (Ctrl), SU11657 (SU), Pemetrexed (Pem), radiation (Rx), radiation + Pemetrexed (Rx + Pem) and triple combination (radiation + Pemetrexed + SU11657, Triple) were stained using rabbit anti phospho-Akt (Ser473) antibody. Left column, ×100 view; right column, insets of the ×100 view at ×400. The black arrows point to endothelial cells. *, erythrocytes within the vessels.
Interestingly, the metronomic triple chemotherapy dosing was slightly but significantly more effective than the conventional triple chemotherapy schedule (*P < 0.05*, from day 21 onwards).

In addition to the by day comparison, the tumor growth delay was assessed on the basis of the time, *T5*, required to reach five times the initial tumor volume (Fig. 4D). In principle, agreement with the above by day comparison, single treatments induced a

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**Figure 6.** *In vivo* functional analysis by MRI and IFP measurements.

T2-weighed MRI of A431 tumors growing s.c. on hind limbs of BALB/c *nu/nu* mice was done to assess tumor size, necrosis, and edema on day 10 after start of therapy. Tumor borders are outlined to indicate the region of interest. Closed arrows, regions of necrosis; open arrows, regions of edema. A, representative T2-weighed transversal scans of the tumors. B, representative parameter images of the distribution of the T2 relaxation time. The T2-map scans enable calculation of the T2-relaxation times of tumor tissue, tumor necrosis, and edema. C, quantitative analysis of T2-map scans is shown: T2-relaxation times of 140 to 180 milliseconds correspond to regions of edema and those >180 milliseconds represent regions of necrosis. D, IFP was measured in A431 tumor xenografts on day 1, at 24 hours after start of the respective therapy, as shown in Fig. 5. IFP was significantly decreased after treatment with SU11657. Columns, mean; bars, ± SD; *, significant (*P < 0.05*) versus control, irradiation alone and Pemetrexed alone.
significant tumor growth delay versus control of 4.1 ± 1 days (radiation), 3.5 ± 1 days (Pemetrexed), or 1.8 ± 0.5 days (SU11657), respectively (P < 0.05 for all three). Each of the dual combinations produced a further significant tumor growth delay compared with single treatments (P < 0.05, each). Triple combination markedly enhanced tumor growth delay versus all other groups (9.7 ± 1.5 days, P < 0.02 versus dual combinations).

All therapies were well-tolerated by the animals. No differences in animal weight or clinical behavior between groups were detected. All animals survived until the end of the observation period unless sacrificed for scheduled histology. Furthermore, histology in HE-stained sections from organs including liver, spleen, kidney, skin, and muscle did not reveal therapy-related toxicity.

Histopathology of A431 tumors. In order to assess tumor cell proliferation under different treatment regimens, the Ki-67 index was determined (Fig. 5A). The total number of Ki-67-positive A431 cells was reduced in all treated groups versus control. Dual combinations had lower Ki-67 indices than single therapies. Significantly, the triple combination resulted in a marked further decrease of the Ki-67 index versus all dual combinations (P < 0.05, Fig. 5A). In addition, the tumor cells exhibited phenotypic and morphologic alterations, such as swollen cytoplasm, which was most pronounced after triple-therapy. When comparing the schedule of radiation and antiangiogenic treatment from the tumor growth experiments shown in Fig. 4A, antiangiogenic prior to radiation (SU + Rx versus Rx + SU) had less Ki-67-positive cells, but without statistical significance.

Vessel density in the tumors was determined by CD31 staining (Fig. 5C). A significant decrease in vessel count was observed after single treatment with SU11657 or irradiation (P < 0.05), whereas treatment with Pemetrexed alone had no significant influence on vessel count (P > 0.8). Combining SU11657 with irradiation further reduced vessel count compared with single treatments (P < 0.05). Combining Pemetrexed with irradiation reduced vessel density to a comparable amount as irradiation alone, as expected based on the lack of activity of Pemetrexed alone. However, when Pemetrexed was combined with SU11657, fewer vessels were observed than with SU11657 alone. The triple combination caused the greatest antiangiogenic effects in vivo, with the lowest vessel number versus all other groups (P < 0.05; Fig. 5D). When comparing the schedule of radiation and antiangiogenic treatment, antiangiogenic treatment prior to radiation caused significantly less vessel density than (P < 0.05, SU + Rx versus Rx + SU).

In vitro endothelial cells, we had shown (Fig. 3C and D) that Akt was phosphorylated after radio/chemotherapy and SU11657 attenuated this process. To transfer the in vitro results immunohistochemistry was done in treated A431 tumors. We found that radiotherapy, chemotherapy, and their combination induced Akt phosphorylation which was attenuated by SU11657, particularly visible after combined radio/chemotherapy. This is highlighted in Fig. 5E, showing phosphorylated Akt (p-Akt) immunostaining in control (Ctrl), SU11657 (SU), Pemetrexed (Pem), radiation (Rx), radiation + Pemetrexed (Rx + Pem) and triple combination (radiation + Pemetrexed + SU11657, Triple) treated A431 tumors (Fig. 5E).

Morphologic and functional magnetic resonance imaging of A431 tumors. To obtain additional functional insights into the tumor physiology, MRI T2-weighed images were captured on day 10 after therapy began. The first column in Fig. 6A illustrates the tumor growth delay with dual therapies being more effective than single therapies, and triple therapies showing the greatest tumor growth inhibition. In addition, a reduction of tumor infiltration into the adjacent muscle tissue is shown, in particular, after dual and triple combination. MRI also enabled a quantitative assessment of tumor necrosis and edema using the pixelwise analysis, as depicted in T2-maps (Fig. 6B). The histogram analysis (Fig. 6C) represents quantification of the relative amount of tumor edema (T2-relaxation time, 140-180 milliseconds) and necrosis (T2 >180 milliseconds) within the area depicted as tumor. Of note, tumors treated with SU11657 in dual combinations contained a lower percentage of pixels assigned as edema than respective monotherapies (Table 3). This edema-reductive effect of SU11657 was particularly visible in combination with Pemetrexed, radiotherapy and in triple combination as shown in Table 3. The table also shows that SU11657 + Pem + Rx triple therapy had the highest percentage of tumor pixels, and at the same time, the least percentage of edema and necrosis.

Interstitial fluid pressure in A431 tumors. Because IFP has been suggested to be a critical factor in tumor perfusion, edema formation, and in other variables relevant to the tumor

### Table 3. Quantitative histogram analysis of MRI T2-maps as the number of pixels indicating tumor, edema, and necrosis in two animals at day 10

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>% (mean ± SE) of all pixels in tumor volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor tissue</td>
</tr>
<tr>
<td>Control</td>
<td>93 ± 2.5</td>
</tr>
<tr>
<td>SU</td>
<td>91 ± 0</td>
</tr>
<tr>
<td>Pem</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>Rx</td>
<td>87 ± 4.5</td>
</tr>
<tr>
<td>SU + Rx</td>
<td>94 ± 2*</td>
</tr>
<tr>
<td>Pem + Rx</td>
<td>93 ± 1.5</td>
</tr>
<tr>
<td>Pem + SU</td>
<td>97 ± 1*</td>
</tr>
<tr>
<td>SU + Pem + Rx</td>
<td>99 ± 0</td>
</tr>
</tbody>
</table>

Abbreviations: Rx, irradiation; SU, SU11657; Pem, Pemetrexed.
*P < 0.05 for dual combinations containing SU versus respective monotherapies.
**P < 0.05 for triple versus all other except for triple versus Pem + SU.
pathophysiology, we determined the IFP in A431 tumors in vivo. IFP was measured 1 day after the beginning of each monotherapy to determine the condition for the subsequent therapies which were scheduled for that day. SU11657 markedly decreased the IFP in tumors 1 day after institution. The IFP decreased significantly from 8.8 ± 2.6 for controls to 4.2 ± 1.5 mm Hg (mean ± SD, P < 0.02) with SU11657. In contrast, radiotherapy and chemotherapy alone had no significant effects on IFP (P > 0.5; Fig. 6D). Tumor size also influenced IFP. Figure 6D illustrates that for control tumors with increasing tumor size, IFP increased from 8.8 ± 2.6 mm Hg on day 1 to 12.7 ± 2.8 on day 3 (P < 0.01). SU11657-treated tumors, alone or in combination with other therapies, had significantly lower IFP values than controls (P < 0.05), and triple therapy resulted in smaller IFP than monotherapies (P < 0.05). Figure 6D also shows the IFP results from tumors of the radiotherapy/SU11657 scheduling experiments (Fig. 4A). The regimen with radiotherapy given after institution of SU11657 (SU + Rx) had lower IFP than the schedule with radiotherapy prior to SU11657 (Rx + SU), although this difference did not reach statistical significance (P > 0.1).

Discussion

Here we report on the beneficial combination of a trimodal anticancer strategy consisting of radiotherapy, chemotherapy, and angiogenesis inhibition in vitro and in vivo. We present molecular, cellular, and physiologic evidence for the beneficial use of this multimodal combination. Due to the clinical availability of the three modalities, including angiogenesis inhibitors such as VEGF antibodies, it is possible that this or a similar trimodal therapy regimen can be investigated in a variety of clinical entities.

One reason for the beneficial trimodal combination could be that SU11657 interferes with the radio/chemotherapy-associated paracrine tumor cell-endothelial cell interaction. For example, it has been shown that ionizing radiation exerts both proangiogenic and antiangiogenic effects (9). Whereas irradiation of endothelial cells has antiproliferative and proapoptotic effects, irradiation of the tumor cell compartment can increase the expression of key proangiogenic cytokines such as VEGF and basic fibroblast growth factor (9, 11). Irradiation of endothelial cells may also induce expression of PDGF (48). Thus, radiation may induce paracrine proangiogenic effects which have been reported to ultimately reduce the radiosensitivity of tumor endothelium (9, 11). Because SU11657 inhibits VEGF and PDGF signaling, SU11657 may be able to attenuate this escape mechanism and thereby enhance the antiendothelial radiation effects.

Another reason for the efficacious trimodal combination might be their opposite effects on Akt signaling. Akt might be important here, because Akt is a cytoplasmic serine/threonine kinase that, among other things, mediates survival signaling from receptor tyrosine kinases such as VEGFR and PDGFR (36). It is activated in many cancers and may promote drug resistance (34, 35). Our data show that irradiation and/or exposure to Pemetrexed induces Akt phosphorylation, a surrogate for activation in endothelial cells in vitro and in vivo, and we also show that SU11657 attenuates this Akt activation. It is likely that this occurs through interruption of autocrine signaling. We found in functional angiogenesis assays that SU11657 enhances the sensitivity of endothelial cells to radiation or radio/chemotherapy, presumably by attenuating Akt activation. It has been suggested that microvascular damage mediates the sensitivity of tissues to radiotherapy in general (49). On a molecular and cellular level, it is thus plausible that endothelial cells are directly resensitized by SU11657 to radio/chemotherapy.

Cell cycle and caspase-3 activity assessment further showed that apoptosis played an important role in the reaction of endothelial cells to triple combination.

Aside from the effects on the endothelial cell compartment, we also found that triple therapy was remarkably effective directly against A431 cells. In vitro proliferation or clonogenic survival assays showed that A431 tumor cells were sensitive to SU11657 alone and in combination with irradiation or Pemetrexed. This might suggest that SU11657 interferes with autocrine proliferation mediated by split kinases, but it is more likely to inhibit another kinase that contributes to A431 proliferation and survival. The identity of this kinase is not currently known. EGFR, which is known to be overexpressed and activated in A431 cells, is not inhibited by SU11657 (IC50 > 20 μmol/L), rendering it an unlikely candidate.

An unresolved issue in multimodal therapy, both experimentally and clinically, is the issue of the optimal treatment scheduling. For many cancer types, concurrent or sequential, and continuous or bolus type applications are pursued. Although combining antiangiogenic therapy and radiotherapy has been shown empirically to be beneficial in experimental tumor systems, their optimal scheduling is in particular unclear, due to issues of a potential shutdown of blood vessels, inducing hypoxia and thus reducing radiosensitivity. In our A431 tumor system in mice, we found that when single-dose radiotherapy was given after starting the SU11657 therapy, tumor growth delay was greater, and microvessel density was reduced versus the schedule when radiotherapy preceded SU11657 therapy. Our data thus suggest that radiotherapy is more effective when tumors are pretreated with antiangiogenic therapy. However, both schedules of combined dual antiangiogenic and radiotherapy reduced tumor growth more efficacious than each respective monotherapy. Most important to us, trimodal therapy with additional chemotherapy clearly resulted in the greatest tumor growth delay in the A431 xenograft model. Remarkably, of the two trimodal arms tested, metronomic chemotherapy (lower daily, but the same total dosage) was more efficacious in tumor growth delay than the conventional arm, in particular towards the end of observation.

This therapeutic enhancement by the trimodal therapy was accompanied by a significantly decreased Ki-67 proliferation index and reduced vascular density versus all other groups. Of note, at the histology assay time, tumors in some groups had different sizes, and thus their differences in histology may not be exclusively treatment-related, but are additionally size-dependent. However, when tumors were excised at day 11, no significant differences in tumor size between conventional and metronomic chemotherapy dosing were apparent yet, whereas histology was different, suggesting an inherent treatment-related effect: metronomic administration of Pemetrexed caused reduced tumor microvessel density compared with the conventional Pemetrexed regimen. Later in the course of the observation, this histologic difference in CD31 count translated into a tumor size difference. Together with published antiangiogenic effects of metronomic chemotherapy dosing (1, 50), we conclude from our data that the antitumor effects after trimodal therapy partially result from the combined enhancement of antiangiogenic effects.

Another important issue for multimodal cancer therapy is treatment-related toxicity. Despite the better antitumor effects in the trimodal groups, we did not observe an increase of the overall...
toxicity as determined by clinical parameters or in histology in a variety of organs. However, it is obvious that adverse effects cannot be completely excluded, particularly in case of the translation in the clinical setting.

Apart from direct cellular effects, combination treatments also influence general tumor physiology such as IFP. Elevated IFP is a hallmark of solid tumors that emerges due to the expansive growth characteristic of the tumors and their abnormal vascular architecture (3, 12, 19, 39). We found that the IFP in A431 tumors was significantly reduced by ~50% at 24 hours after SU11657 administration. One explanation would be that SU11657 decreases vascular permeability via inhibition of VEGFR or PDGFR, which in turn may lower IFP. In contrast, both radiation and chemotherapy did not substantially alter the IFP. Likewise, quantitative T2-weighed MRI showed that the IFP reduction was accompanied by reduced intratumoral edema. MRI parameters may thus be used as surrogate markers to predict the efficacy of antiangiogenic therapy early in the treatment course.

From these physiologic data, we found that in our trilobal therapy study, radiotherapy was delivered at reduced IFP 1 day after the start of SU11657 treatment. Thus, the reduction of tumor IFP and edema offers one physiologic indication for the improved efficacy of radiotherapy or chemotherapy when given after SU11657. This view is supported by our data showing that changing the order of therapy and giving SU11657 after radiotherapy was less effective in reducing tumor growth than vice versa.

This concept of increased radio/chemotherapy efficacy through normalization of tumor vasculature by SU11657 inhibition is consistent with preclinical and clinical observations from other strategies targeting the VEGF pathway (3, 11, 12, 19, 24, 38–41). In agreement with our data using SU11657, a previous study has shown that SU11248 (a chemical analogue of SU11657) inhibited VEGF signaling and led to reduced vascular permeability in mice (22). On the other hand, it has been shown that treatment with a PDGFR receptor tyrosine kinase inhibitor, STI571 (Gleevec), also decreased the interstitial hypertension and increased capillary-to-interstitium transport of 5-fluorouracil in an experimental colon carcinoma model (51, 52). Therefore, it is conceivable that the combined VEGF and PDG signaling interruption by the receptor tyrosine kinase inhibitor SU11657 is a powerful tool to normalize the tumor vasculature (53, 54). Likewise, the inhibition of both VEGF and PDG signaling using SU11248, an analogue to SU11657, has recently been reported to produce survival benefits in combination with metronomic chemotherapy schedule as a beneficial anticancer strategy in pancreatic islet tumors of mice (55).

On the underlying signaling level, we found that SU11657 inhibited Akt-mediated survival signals in vitro in HUVEC and in vivo in the A431 tumor model. Akt is considered a major escape mechanism from tumor therapy (33–37, 44). In the context of the proposed trilobal therapy, our results suggest a dual role for Akt, which is downstream from VEGF/PDG, and thus also represents a link to decreased IFP after VEGF/PDGF inhibition (56). First, the inhibition of Akt signaling can contribute to the beneficial physiologic effects for the subsequent radio/chemotherapy (and may indirectly affect other “normal” and tumor cells). Second, Akt inhibition by SU11657 also represents a mechanism by which the individual endothelial cell is directly resensitized to radio/chemotherapy, which is otherwise disturbed by the up-regulation of Akt as a self defense to cell toxicity.

It should be noted that similar experiments have not yet been done with spontaneously arising tumors. Because the vasculature of s.c. tumor xenografts may not accurately reflect the vasculature of tumors growing in other organs or tissues, it has yet to be shown that all of the effects of triple therapy reported here will also be observed in more natural tumors.

Current approaches in clinical cancer therapy favor multimodal strategies (3–6). One rationale is the concept that the side effects of different therapies do not overlap. The combination of irradiation and chemotherapy has become a standard treatment and is associated with improved survival rates in many tumors (2). The simultaneous combination of chemotherapy and antiangiogenesis has shown clinical promise (4–6). We suggest that, given the disparate modes of action, the proposed triple combination of irradiation, chemotherapy, and antiangiogenesis using a VEGF/ PDG receptor antagonist has clinical potential as an anticancer strategy.

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References


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