Combination of Vascular Endothelial Growth Factor Receptor/Platelet-Derived Growth Factor Receptor Inhibition Markedly Improves Radiation Tumor Therapy

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Abstract

Purpose: Investigations on the combination of radiotherapy with vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) antiangiogenic agents, which has the potential to improve the clinical outcome in cancer patients.

Experimental Design: Here, we analyze the combined VEGF (SU5416) and PDGF (SU6668) receptor tyrosine kinase inhibition with irradiation in human endothelium (HUVEC), prostate cancer (PC3), and glioblastoma (U87) in vitro and in vivo.

Results: Combined inhibition of VEGF and PDGF signaling resulted in enhanced apoptosis, reduced cell proliferation, and clonogenic survival as well as reduced endothelial cell migration and tube formation compared with single pathway inhibition. These effects were further enhanced by additional irradiation. Likewise, in PC3 and U87 tumors growing s.c. on BALB/c nu/nu mice, dual inhibition of VEGF and PDGF signaling significantly increased tumor growth delay versus each monotherapy. Interestingly, radiation at ~20% of the dose necessary to induce local tumor control exerts similar tumor growth-inhibitory effects as the antiangiogenic drugs given at their maximum effective dose. Addition of radiotherapy to both mono- as well as dual-antiangiogenic treatment markedly increased tumor growth delay. With respect to tumor angiogenesis, radiation further decreased microvessel density (CD31 count) and tumor cell proliferation (Ki-67 index) in all drug-treated groups. Of note, the slowly growing PC3 tumor responded better to the antiangiogenic drug treatments than the faster-growing U87 tumor. In addition to the beneficial effect of abrogating VEGF survival signaling when combined with radiation, we identified here a novel mechanism for the tumor escape from radiation damage. We found that radiation induced up-regulation of all four isoforms of PDGF (A-D) in endothelial cells supporting adjacent smooth muscle cells resulting in a prosurvival effect of radiation. The addition of SU6668 attenuated this undesirable paracrine radiation effect, which may rationalize the combined application of radiation with PDGF signaling inhibition to increase antitumor effects.

Conclusion: A low relative low radiation dose markedly enhances local antitumor effects of combined VEGF and PDGF signaling inhibition, suggesting a promising combination regimen for local tumor treatment with radiotherapy remaining an essential element.

The combination of radiotherapy with targeted drugs ("biologicals") with antiangiogenic or antivascular effects may become an interesting anticancer strategy (1–5). The concept of combining antivascular compounds or angiogenesis inhibitors has been extensively investigated for the combination of vascular endothelial growth factor (VEGF) signaling inhibitors concurrently or sequentially together with radiotherapy (6–15). Moreover, trimodal combinations of VEGF targeting angiogenesis inhibitors, radiation, and chemotherapy have been investigated in preclinical models and have entered clinical cancer trials (16–18). Other potential radiotherapy combinations include inhibition of integrin signaling (19) and platelet-derived growth factor (PDGF) signaling (20–22). To improve cancer therapy, combinations of drugs targeting both VEGF and PDGF signaling have been suggested (23–26). Hereby, both endothelial cells and the vessel-supporting pericytes are interrupted with the potential to treat the intractable late-stage solid tumor (23, 24). Whereas SU5416,
an inhibitor of the VEGF receptor (VEGFR) 2 receptor tyrosine kinase (RTK; refs. 27, 28), was found to be effective against early-stage angiogenic lesions, SU16668, an inhibitor with high activity against PDGF receptor β (PDGFRβ; refs. 29, 30), was active in end-stage tumors, eliciting detachment of pericytes and disruption of tumor vascularity (23). The therapeutic regimens consisting of dual VEGF + PDGF RTK inhibition (RTKI) were more efficient than each single treatment and showed broad-spectrum activity against both early- and late-stage tumors (23, 24).

The present article investigates the therapeutic potential of dual VEGF and PDGF RTKI in combination with radiotherapy as the present mainstay of nonsurgical local tumor therapy in vitro and in vivo. Although dual inhibition of VEGF and PDGF signaling was more efficient than single pathway inhibition, we found that addition of radiation markedly enhanced the therapeutic efficacy in all groups investigated in vitro and in human glioblastoma (U87) and human prostate carcinoma (PC3) tumors growing s.c. on BALB/c nu/nu mice. This study shows that radiation is an integral component of local tumor therapy and encourages the use of radiotherapy with combined VEGF and PDGF RTKI.

Materials and Methods

Cell cultures and treatment conditions. Primary isolated human umbilical vascular endothelial cells (HUVEC, Promocell) were cultured up to passage 4. 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 basic fibroblast growth factor. Human prostate (PC3) and glioma (U87) tumor cells (Tumorbank, German Cancer Research Center, Heidelberg, Germany) were cultured in DMEM with 10% FCS. SU5416 and SU6668 were provided by SUGEN, Inc. SU5416 is an ATP-competitive inhibitor of the Flk-1/KDR/VEGFR2 RTK, with a Ki value of 0.04 μM/L and only minimal activity against PDGFR or fibroblast growth factor receptor (27, 28). SU6668 is a potent inhibitor of PDGFRβ with a Ki value of 8 μM/L. It also inhibits the kinases of VEGFR2 (Flk-1/KDR) and fibroblast growth factor receptor 1, with Ki values of 2.1 and 1.2 μM/L, respectively (29). Cell exposures with the drugs were done for a period of 1 h before irradiation with 6 MV X-rays (Mevatron, Siemens) at a dose rate of 2.5 Gy/min.

Proliferation, clonogenic survival, and tube formation assay. Proliferation/cell viability, clonogenic, and tube formation assays were done as previously described (7, 16). Briefly, to measure effects on cell proliferation/viability, 50,000 endothelial cells were seeded on 25 cm² collagen-coated flasks overnight at standard conditions. Drugs were added to the cells, and cells were incubated for 72 h and counted. In radiation combination experiments, cells were treated with/without drugs for 1 h, then irradiated and immediately incubated for another 72 h, and counted. For the clonogenic assay, increasing numbers of cells (10² to 5 × 10⁵) were plated in 25 cm² flasks, incubated with drugs for 1 h, irradiated, and returned to the incubator for 14 to 17 d, after which they were stained with crystal violet (Sigma). For the tube formation assays, 24-well plates were coated with 300 μL Matrigel (Becton Dickinson). Cells were plated on Matrigel, inhibitors were added, and cells were irradiated, incubated for 6 h, fixed, and stained with Diff-Quik II reagents (Dade Behring AG).

Matrigel invasion/migration and coculture assays. Invasion of endothelial cells in vitro was measured on Matrigel-coated (0.78 mg/mL) Transwell inserts with 8-μm pore size (Becton Dickinson). Cells were trypsinized and 200 μL of cell suspension (3 × 10⁵ cells/mL) and 500 μL medium per condition were added in triplicate Transwells. After 18 h of incubation, cells that had invaded to the opposite of the membrane were fixed and stained using Diff-Quik II solution (Dade Behring AG), sealed on slides, and counted by microscopy. For smooth muscle/endothelial cell coculture studies, human smooth muscle cells (SMC) were seeded, and after selective irradiation of the human SMCs, Matrigel-coated Transwells with nonirradiated endothelial cells (HUVEC) were added to the upper compartment and counted after 18 h of coculture incubation as described (7, 16, 17, 22).

Apoptosis and flow cytometry. Up to 72 h after treatments, endothelial cells (HUVEC) were prepared for fluorescence-activated cell sorting analysis (FACSscan, Becton Dickinson) as described previously (7, 16, 17). Cells were fixed in Hank’s solution and 70% ethanol, centrifuged, and washed in PBS, and the supernatant was removed. Cells were resuspended in the staining solution of PBS, RNase, and propidium iodide and analyzed for cells with sub-G₁ DNA content involving forward/sideward scatter gating to eliminate debris. To measure caspase-3 activity, HUVECs were exposed to single and combination modalities (irradiation, SU5416, and SU6668). At 6 h after incubation, cells were washed twice with cold PBS, resuspended using the Cytofix/Cytoperm solution (BD PharMingen), and incubated for 20 min on ice. Cells were pelleted, washed with washing buffer, and resuspended in washing buffer plus phycoerythrin-conjugated monoclonal active caspase-3 (BD PharMingen) using 20 μL/1 × 10⁹ cells and incubated for 30 min at room temperature. Following incubation with the antibody, cells were washed in buffer and resuspended in PBS and analyzed by flow cytometry.

Real-time quantitative reverse transcription-PCR. Expression levels of PDGF RNA transcripts were quantitated by real-time PCR. Total RNA from HUVEC was isolated using RNeasy kit (Qiagen). RNA with a 28S/18S rRNA ratio of 2.0 ± 0.3 was used. The quality was insured by lab-on-chip technology (Agilent 2100 Bioanalyzer in combination with the RNA 6000 Lab Chip kit, Agilent Technologies). SYBR Green assay and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) were used for the real-time quantitation of the RNA. A consensus sequence was derived from Locus Link/RefSeq database (National Center for Biotechnology Information), 6 with the following accession numbers: mPDGF-A, NM_008808; mPDGF-B, NM_011057; mPDGF-C, NM_019971; mPDGF-D, NM_027924; mPDGF-E, NM_002607; hPDGF-B, NM_002608; hPDGF-C, NM_016205; and hPDGF-D, NM_025208. The primers were as follows: mPDGF-A, 5'-GGCTGGCTCGAAGTCAGATC-3' (forward) and 5'-GCTGCCACGCCTGCTCCTG-3' (reverse); mPDGF-B, 5'-AAGCTCGGTAGTACCGCTC-3' (forward) and 5'-ACTTGGGTTCTGGCGCAGT-3' (reverse); mPDGF-C, 5'-CTGGCGGCTGCTGCTGTCTG-3' (forward) and 5'-TGGCAATTGATGGTCAAAGG-3' (reverse); mPDGF-D, 5'-CCAGCCACCGCTGCTGCTT-3' (forward) and 5'-TGGCAATTGATGGTCAAAGG-3' (reverse); hPDGF-A, 5'-CGGCGGCGCTGCTGCTGCTG-3' (forward) and 5'-CCAGCTCCAAATGCCTCCTTA-3' (reverse); hPDGF-B, 5'-AGATGCAATGTTGCGGCG-3' (forward) and 5'-TGGCAATTGATGGTCAAAGG-3' (reverse); hPDGF-C, 5'-CGGCGGCGCTGCTGCTGCTG-3' (forward) and 5'-TGCTGTCTGCTGCTGTCTG-3' (reverse); hPDGF-D, 5'-CCAGCTCCAAATGCCTCCTTA-3' (reverse). After RNA isolation, genomic DNA was removed by DNase 1 treatment (DNA-free; Ambion). First-strand cDNA was reverse transcribed from total RNA using the cDNA Archive kit (MultiScribe Reverse Transcriptase, Applied Biosystems) and stored at -20°C until use. Complementary DNAs were mixed with SYBR Green PCR master mix (Applied Biosystems) and primers, and real-time PCR was done. A threshold cycle (Cₚ) was observed in the exponential phase of amplification, and the quantitation of relative expression levels was achieved using standard curves for both the target and endogenous controls. All assays were done in triplicates.

Animal studies. All experiments were approved by in-house and governmental animal protection committees. For tumor growth

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6 http://www.ncbi.nlm.nih.gov/LocusLink
experiments with s.c. growing human xenotransplants, athymic mice (BALB/c, nu/nu, 8 wk, 20 g) were obtained from Charles River Laboratories. Human prostate carcinoma cells (PC3) and glioblastoma (U87) cells were injected s.c. into the right hind limb (1 \times 10^7 to 5 \times 10^8 in 100 \mu L PBS). Animals were randomized (n = 6-12; Tables 1 and 2) for therapy starting on day 0 when tumors were established and tumors reached a volume of \(-150 \text{ mm}^3\). Tumor volume was determined thrice weekly by direct measurement with calipers (volume = length \times width \times depth \times 0.5). SU6668 was given s.c. in carboxymethylcellulose every other day at 250 mg/kg starting on day 0 until the end of observation. SU416 was given s.c. in carboxymethylcellulose twice weekly at 100 mg/kg starting on day 0 until the end of observation. Drug doses were chosen to induce maximum tumor growth inhibition at tolerable toxicity (9, 26–30). To induce tumor growth delay in the same range with clinically relevant fractionated radiation, a dosing schedule of 5 \times 2.5 Gy was chosen, which had been determined by previous dose-finding studies (data not shown). This relative low radiation dosage is \(~20\%\) of the dose necessary to induce local control. Radiation was delivered on 5 consecutive days to the tumor starting on day 0 while shielding the other body parts using a cobalt-60 source (Gammamatron, Siemens).

**Histology and immunohistochemistry.** For histologic analysis, tumors were harvested from animals (PC3: at days 24 and 50; U87: day 10) and fixed in buffered formalin and embedded in paraffin. Tissue slices (5 \mu m) were stained with H&E. General tissue morphology was visualized by H&E and photographed using a camera (Nikon Super Coolscan 4000 ED) mounted on a Zeiss microscope (Carl Zeiss). To assess the tumor cellular proliferation (% Ki-67 positivity), immunohistochemical staining was done using the MIB-1 monoclonal mouse anti-human Ki-67 antigen (Dako). Sections were counterstained with hematoxylin. Ki-67 staining was quantified by counting the number of positively stained cells of 200 to 250 nuclei in 10 randomly chosen fields at \(\times 400\) magnification. To quantify tumor vessel counts, frozen sections were fixed and stained with primary antibody to CD31 (Becton Dickinson) and 10 random fields at \(\times 400\) magnification were chosen for counting.

**Statistical analysis.** The tumor volume \(V\) at a given time after treatment was normalized to the initial volume \(V_0\) at the onset of treatment (day 0). Statistical evaluation of tumor growth was undertaken by comparing the volumes per given day. In addition, the general response to treatment was assessed based on the time \(T_n\) required to reach \(n\) times the initial tumor volume \(V_0\). For multiple comparisons, the Kruskal-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. Significance level was \(P < 0.05\).

### Table 1. U87 tumor response in vivo

<table>
<thead>
<tr>
<th>U87 human glioma</th>
<th>Days required for tumors to grow five times the initial volume (V_0) ± SE</th>
<th>Growth delay (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 12)</td>
<td>7.0 ± 1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>SU5416 (n = 10)</td>
<td>8.1 ± 1.4*</td>
<td>1.1</td>
</tr>
<tr>
<td>SU5416 + radiation (n = 6)</td>
<td>15.5 ± 1.7*†</td>
<td>8.5</td>
</tr>
<tr>
<td>SU6668 (n = 10)</td>
<td>7.9 ± 1.8*</td>
<td>0.9</td>
</tr>
<tr>
<td>SU6668 + radiation (n = 6)</td>
<td>15.5 ± 2.0*†</td>
<td>8.5</td>
</tr>
<tr>
<td>SU416 + SU6668 (n = 10)</td>
<td>13.2 ± 1.5*†</td>
<td>6.2</td>
</tr>
<tr>
<td>Radiation (n = 6)</td>
<td>9.9 ± 1.8*</td>
<td>2.9</td>
</tr>
<tr>
<td>SU416 + SU6668 + radiation (n = 6)</td>
<td>17.6 ± 1.9*†</td>
<td>10.6</td>
</tr>
</tbody>
</table>

* \(P < 0.05\) versus control.
† \(P < 0.05\) versus compound monotherapy.
‡ \(P < 0.05\) versus dual-compound therapy.

### Table 2. PC3 tumor response in vivo

<table>
<thead>
<tr>
<th>PC3 human prostate carcinoma</th>
<th>Days required for tumors to grow two times the original volume (V_0) ± SE</th>
<th>Growth delay (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>17 ± 1.5/37.0 ± 3</td>
<td>14/11</td>
</tr>
<tr>
<td>SU5416 (n = 8)</td>
<td>31 ± 3.5*/48.0 ± 5*†</td>
<td>46/45</td>
</tr>
<tr>
<td>SU5416 + radiation (n = 6)</td>
<td>63 ± 6*/82.0 ± 7*†</td>
<td>15/42</td>
</tr>
<tr>
<td>SU6668 (n = 8)</td>
<td>32 ± 3*/79.0 ± 7*†</td>
<td>74/58</td>
</tr>
<tr>
<td>SU6668 + radiation (n = 6)</td>
<td>91 ± 8*/95*††</td>
<td>28/—</td>
</tr>
<tr>
<td>SU416 + SU6668 (n = 8)</td>
<td>45 ± 4*/1—†</td>
<td>17/26</td>
</tr>
<tr>
<td>Radiation (n = 6)</td>
<td>34 ± 3*/63.0 ± 6*†</td>
<td>75/58</td>
</tr>
</tbody>
</table>

* \(P < 0.05\) versus control.
† \(P < 0.05\) versus drug monotherapy.
‡ \(P < 0.05\) versus dual-drug therapy.

### Results

**Endothelial and tumor cell viability/proliferation.** We first did dose-effect experiments with SU6668 and SU5416 to determine the ability of the compounds to inhibit endothelial cell proliferation/viability in the 72-h cell number assay (Fig. 1A and B). For both compounds, we found a dose-dependent inhibitory effect with maximal inhibition observed at the highest doses tested: 10 \(\mu\text{mol/L for SU6668 (} \sim 60\% \text{ inhibition versus control) and 2 } \mu\text{mol/L for SU5416 (} \sim 60\% \text{ inhibition versus control). The IC}_{50} values for SU5416 and SU6668 were estimated to be approximately 1.5 and 8 \(\mu\text{mol/L, respectively. Therefore, concentrations of 1 and 5 } \mu\text{mol/L, which were slightly below the IC}_{50} values for SU5416 and SU6668, respectively, were selected for the doses used in the in vitro combination experiments. Radiation alone and the combination radiation + compound(s) reduced HUVEC cell number in a dose-dependent manner between 0 and 10 Gy. The maximum effect reaching a plateau at \(\sim 90\%\) endothelial cell number reduction versus controls was achieved with the triple combination 10 Gy radiation + SU5416 + SU6668, whereas 10 Gy + the drug monotherapy (both SU5416 and SU6668) reduced HUVECs by \(\sim 80\%\). This difference was significant for the entire radiation range between 0 and 10 Gy. Radiation combined with both dual SU5416 and SU6668 drug combination significantly reduced HUVEC viability versus all other groups (\(P < 0.05\); example in Fig. 1D).

Without radiation, the combination of SU5416 and SU6668 enhanced the antiproliferative effects of the drug monotherapy by \(\sim 10\%\) (\(P < 0.05\)). When the samples were additionally irradiated with 2 Gy, the clinically important typical fractionation dose, all groups showed reduced endothelial cell number by approximately 30% to 40% versus the nonirradiated samples.

Importantly, the analogous experiments with PC3 and U87 tumor cells showed that (a) the drugs had only modest (\(< 10\%\) versus controls) but significant antiproliferative effect on tumor cells if given alone (\(P < 0.05\)) and (b) radiation at 2 Gy had
Fig. 1. Cell response \textit{in vitro} to SU5416, SU6668, radiation, and combinations in the 72-h proliferation/cell number assay. Cells were exposed to the indicated treatment for 72 h and the cells were counted. Cell numbers normalized to their cognate untreated control are shown as a function of treatment. A, SU6668 dose response in endothelial cells (HUVEC). Points, mean (n = 5); bars, SD. B, SU5416 dose response in endothelial cells (HUVEC). Points, mean (n = 5); bars, SD. C, radiation dose response alone and in combination with compounds (1 \(\mu\)mol/L SU5416 and 5 \(\mu\)mol/L SU6668) in endothelial cells (HUVEC). *, \(P < 0.05\) versus control; **, \(P < 0.05\) versus the dual combination of SU5416 + SU6668. D, detailed analysis for 2 Gy \(\times\) SU5416 \(\times\) SU6668 in HUVEC. Columns, mean (n = 5); bars, SD. *, \(P < 0.05\) versus controls; **, \(P < 0.05\) versus the respective monotherapy; ***, \(P < 0.05\) versus the respective dual drug combinations. E, analysis for 2 Gy \(\times\) SU5416 \(\times\) SU6668 in PC3 and U87 tumor cells. Columns, mean (n = 5); bars, SD. *, \(P < 0.05\) versus controls; **, \(P < 0.05\) versus the dual combination of SU5416 + SU6668. RT, radiation.
strong effects (~40% inhibition versus controls) but radiation drastically enhanced the antiproliferative drug effect in both prostate cancer (~70% inhibition) and glioblastoma cells (~60% inhibition) versus each monotherapy (P < 0.01; Fig. 1E).

**Endothelial and tumor cell clonogenic survival.** Both SU5416 (1 μmol/L) and SU6668 (5 μmol/L), alone and their combination, resulted in a modest but statistically significant (P < 0.05) reduction of endothelial cell and tumor cell (PC3 and U87) clonogenic cell survival (Fig. 2A and B). The combination of both agents showed a nonsignificant trend to be more efficacious than the single agents. In contrast, radiation exposure with 1, 2, or 4 Gy resulted in a clear dose-dependent marked reduction in clonogenic survival of the endothelial cells and PC3 and U87 tumor cells. The radiation-induced anticolonogenic effect was drastically enhanced when the endothelial cells were additionally treated with single drugs (data not shown) and was more pronounced with combined drug treatment (Fig. 2A). Interestingly, the dual VEGF + PDGF inhibition also modestly further reduced clonogenic survival in irradiated U87 and PC3 tumor cells but much less compared with the endothelial cells (Fig. 2B).

**Endothelial cell migration and tube formation.** Endothelial cell migration is an important step in tumor angiogenesis. Figure 3A shows that SU5416 (1 μmol/L), SU6668 (5 μmol/L), as well as 2 Gy radiation markedly reduced endothelial cell migration (P < 0.05). The combination of SU5416 and SU6668 was more effective than the respective single exposures (P < 0.05), and the additional 2 Gy irradiation further reduced the antimigratory effects significantly (P < 0.05). Sprouting of endothelial cells and the formation of tubes are also crucial steps during angiogenesis. When assessing endothelial cell tube formation, we found that SU5416, SU6668, and their combination inhibited the tube formation process, whereas 2 Gy alone induced only a modest tube formation inhibition (Fig. 3B). Triple combination of both drugs and radiation inhibited tube formation most effectively versus all other combinations.

**Endothelial cell apoptosis.** The role of endothelial cell apoptosis, although potentially important for the angiogenic process, is yet unclear in radiotherapy of tumors (3, 22). Figure 4A shows that the incubation of endothelial cells with SU5416 and SU6668 induced an increase of the sub-G1 DNA content at 12 h after therapy compared with controls, which was further enhanced when both compounds were combined. Radiation with 2 Gy also produced a statistically significant enhancement of apoptosis in endothelial cells, but it was much less than that observed with either of the compounds. Triple combination of 2 Gy with both compounds significantly (P < 0.05) increased the sub-G1 rate in HUVECs. To further analyze apoptosis induction, a flow cytometric analysis using the anti-active caspase-3 monoclonal antibody was done. We found that untreated control endothelial cells were primarily negative for caspase-3. Figure 4B further shows that 12 h after treatment that the monotherapies radiation, SU5416, and SU6668 resulted in little caspase-3 activity, which was slightly increased when SU5416 and SU6668 were combined. Triple combination consisting of radiation, SU5416, and SU6668 markedly shifted the curve further to the right, indicating the most caspase-3 activity. Thus, triple combination of radiation with the combined VEGF/PDGF RTKI induced the most endothelial cell apoptosis.

![Fig. 2](clincancerres.aacrjournals.org) Clonogenic survival in response to SU5416, SU6668, radiation, and combinations. A. HUVEC. B. U87 and PC3. Columns, mean (n = 6); bars, SD. *, P < 0.05 versus control; **, P < 0.05 versus the dual combination of SU5416 + SU6668.
Radiation up-regulates PDGF isoforms in endothelial cells. On a signaling level, we next sought to investigate a potential rationale for combining radiation with PDGF inhibitors. One possible rationale would be up-regulation of PDGF survival signaling induced by radiation. To analyze if endothelial cell up-regulates PDGF after irradiation and to determine which isoforms are released, the expression levels of all four PDGF isoforms (A-D) were measured using real-time quantitative reverse transcription-PCR (Fig. 5A). All four PDGF isoforms exhibited some induction at 12 h after 2 Gy radiation in HUVEC ($P < 0.05$). PDGF-B (>3-fold) and PDGF-C (>3-fold) were the predominant radiation-induced PDGF isoforms, whereas PDGF-D ($\approx 2$-fold) and PDGF-A ($\approx 1.3$-fold) were less strongly up-regulated. Because endothelial cell activation can be a critical event in autocrine and paracrine cell survival, these results suggested that concurrent inhibition of PDGF signaling during or after radiation might be a way to attenuate this process, which would ultimately help to prevent tumor growth or regrowth.

**VEGF and PDGF inhibition in SMCs.** Next, we sought to dissect the respective effects of SU6668 and SU5416 on PDGF and VEGF signaling using a coculture model of endothelial cells and SMCs as models for pericytes. SU6668 treatment of SMCs led to a significant inhibition of endothelial cell proliferation, whereas treatment with SU5416 had almost no effect, suggesting that SMCs were primarily stimulated by PDGF but not by VEGF. Further, the selective irradiation of SMCs markedly enhanced the proliferation of endothelial cell (35% increase compared with nonirradiated SMCs in the coculture), which had not been irradiated. The treatment of SMCs with SU6668, but not SU5416, resulted in a significant reduction of radiation-induced paracrine promitotic effects ($P < 0.05$). These results suggest that, within the context of radiation-inducible paracrine effects and angiogenesis, VEGF signaling (inhibited by SU5416) may be less important than PDGF signaling (inhibited by SU6668).

**PC3 prostate and U87 glioblastoma tumors in mice.** Next, we sought to analyze the effects of treatment combinations in vivo in s.c. growing PC3 and U87 tumors on BALB/c nude mice (Fig. 6A and B). Both the drug treatment and fractionated radiotherapy started when tumors were established at a volume of $\approx 150$ mm$^3$ on day 0. The administration and drug doses were chosen from previous experiments (29) and our own pilot studies (data not shown) to induce maximum tumor growth inhibition. Radiation dose was chosen to induce similar growth-inhibitory effects as the drugs. This dose is $\approx 20\%$ of...
SU5416 and SU6668 was more effective than the monotherapy induced significant tumor growth delay. The combination of with SU5416 and SU6668, as well as 5 times the initial size. In both PC3 and U87 tumor models, monotherapy effective that tumors did not reach the 5-fold volume of their volume was chosen because several treatment regimens were so given factor were taken as a general overall measure for tumor the days required to increase the initial volume on day 0 by a treatment was the primary experimental end point. In addition, fig. 6A and B; Table 1). Concurrent radiation markedly increased tumor growth delay in single-drug groups (U87: 8-fold; PC3: 3-fold) and in the dual-drug combination groups (U87: 1.5-fold; PC3: 3-fold). In general, the maximum tumor growth delay was achieved when radiation was combined with SU5416 and SU6668. However, irradiation plus the dual-drug combination did not delay growth of PC3 tumors more than those treated with SU6668 plus irradiation. Interestingly, tumor regression and tumor control were seen in some animals bearing the PC3 prostate tumors, indicating high susceptibility of this tumor to antiangiogenic therapy involving VEGF/PDGF inhibitors. The additional day by day comparison of tumor growth curves showed that all treatments slowed tumor growth (P < 0.05) from day 10 and beyond for PC3 and from day 12 and beyond for U87. In the PC3 model, the combination of radiotherapy and the respective drug was more effective from day 20 (day 10 for U87) onward (P < 0.05) versus the respective monotherapies. The combination of SU5416 and SU6668 was more effective than the single compounds from day 32 onward (day 10 for U87). Finally, the triple combination of radiotherapy and the combined two VEGF/ PDGF signaling inhibitors were more effective than dual-drug therapies from day 60 and beyond. In U87 tumors, the combination of all three modalities was particularly effective. Interestingly, however, in general, the slowly growing PC3 tumor seemed to respond better to the antiangiogenic therapy than the faster-growing U87 tumor. With respect to side effects, SU5416 was relatively toxic to the animals as shown by reduced weight.

**PC3 and U87 tumor histology.** Figure 6C and D shows that tumor cellular proliferation (% Ki-67 positivity) markedly decreased after SU5416 and SU6668 and was further reduced by additional radiation. Similarly, immunohistochemical staining for CD31 revealed a decrease in vessel count after SU5416 and SU6668. Quantitative analysis showed that the addition of radiotherapy to antiangiogenic monotherapy and combination therapy markedly reduced vessel count and Ki-67 – positive cells (P < 0.05), indicating a profound antiangiogenic effect of the triple combination regimen.

**Discussion**

The main goal of radiotherapy is local cancer therapy for patients. To improve the cure rates or reduce side effects, combinations of radiotherapy with biological or pharmaceutical agents are being investigated. In this regard, drugs carrying antiangiogenic or antivascular properties are of great interest. Large numbers of such compounds are already in clinical studies or approved for certain malignancies (18). However, it is still unclear which combinations of signaling inhibitors would be most effective as an anticancer regimen per se and which combinations would benefit from the addition of radiotherapy. In this context, we show here in two human tumor models (PC3 prostate cancer and U87 glioblastoma) that the combination of VEGF and PDGF signaling inhibition (SU5416 and SU6668 RTKI) enhances the effects of each monotherapy. We also show that radiotherapy in combination with both VEGF and PDGF signaling inhibition greatly enhances antiangiogenic and antitumor effects of the respective mono- and dual-drug therapies. In fact, it appears from our data that radiotherapy is more effective than drugs alone or in...
Fig. 6. Tumor growth curves of s.c. PC3 (A) and U87 (B) tumors in vivo on BALB/c nu/nu mice. Tumors were treated with SU5416, SU6668, radiation, and combinations on day 0 when tumors were established at ~150 mm³. Radiation was given on 5 consecutive days (days 0–4, 5 × 2.5 Gy) to the tumor region. Drugs were given concurrently with fractionated radiotherapy and then continuously until the end of observation. Points, mean of tumor volume normalized to the initial volume; bars, SE. *, P < 0.05 versus control; **, P < 0.05 versus the respective monotherapy; ***, P < 0.05 versus the respective dual compound combinations (details in Tables 1 and 2).

C, immunohistochemistry of PC3 and U87 tumors is given in representative photograph of CD31 and Ki-67 staining. D, quantitative analysis of CD31 staining in PC3 tumors given as vessel counts per high-power field (HPF) and evaluation of Ki-67 staining in PC3 and U87 tumors given as Ki-67 positive tumor cells. Columns, mean (n = 10); bars, SD. *, P < 0.05 versus controls; **, P < 0.05 versus the respective monotherapy; ***, P < 0.05 versus the respective dual compound combinations.
their optimal combination. This suggests that radiotherapy may remain a critical component for local therapy of most solid tumors even in multimodal regimens with newer targeted biologicals.

In these studies, we have used SU6668 as an inhibitor of PDGFR signaling and SU5416 as an inhibitor of VEGFR signaling. Neither of these compounds is considered as viable drug candidates any longer. However, they make useful tools for understanding the role that these receptor kinases may play in tumor pathophysiology. Although SU6668 has been reported to inhibit VEGFR, it does so with a Ki value that is >250-fold higher than the concentration at which it inhibits PDGFR (29). Similarly, SU5416 was reported to inhibit PDGFR kinase, but it was ~20-fold less potent against PDGFR as it was against VEGFR2 (27), so their relative selectivities can be used to examine contributions made by each RTK. Nevertheless, it should be kept in mind that ATP-competitive kinase inhibitors rarely exhibit complete selectivity (31), so the data should be interpreted with the understanding that there may be potential off-target effects. Such off-target effects may have contributed to the small but statistically significant effects that these compounds had on the tumor cells.

Previous reports had already suggested that SU6668 was more effective against a well-vascularized, established, end-stage pancreatic islet tumor, whereas SU5416 was only effective against early-stage angiogenic islet tumors (23–26), suggesting complementing antitumor activities through different target cell populations of the neovascularization. The data reported here show that in vitro endothelial cell proliferation was enhanced when the compounds were used in combination. This observation suggests that SU6668 was not a very effective inhibitor of VEGF signaling, such that SU5416 could complement it. At the same time, the increased efficacy of the combination over SU5416 suggests that SU6668 may inhibit another target in endothelial cells other than VEGFR2.

SU6668 had been shown to disturb blood vessel formation and maintenance by targeting pericytes through inhibition of the receptor of PDGF (25). Accordingly, using a coculture model of endothelial cells and SMCs, we found that treatment of SMCs with SU6668, but not with SU5416, inhibited endothelial cell growth, suggesting that SMCs were primarily stimulated by PDGF but not by VEGF. Further, the treatment of SMCs with SU6668, but not SU5416, resulted in a significant reduction of radiation-induced paracrine prosurvival effects in endothelial cells. This confirmed that SU5416 primarily inhibited VEGF signaling, whereas SU6668 acted primarily as a PDGF inhibitor relevant for SMCs and pericytes.

Considering all of the in vitro data together, and extrapolating to the in vivo setting where multiple cell types are involved in tumor formation, the increased antitumor activity of a combined SU5416 and SU6668 treatment is likely a reflection of several complementing mechanisms that involve direct effects on tumor cells as well as paracrine effects, the endothelial cell compartment, and endothelial cell interactions with pericytes. This signaling interaction between cell compartments is important for tumor irradiation because radiation induces growth factors such as VEGF in tumor cells as well as expression of VEGFR2 receptors in the tumor endothelium (7) and PDGF in endothelial cells (Fig. 5) and PDGFR in fibroblasts (22). Such autocrine and paracrine signaling cascades may contribute to the survival of tumors on irradiation by regulating endothelial cell survival through the antiapoptotic phosphatidylinositol 3-kinase/Akt signal transduction pathway (11, 13). Although this was shown with respect to Flk-1/KDR activation, other kinases (e.g., those downstream of the integrin family) may similarly contribute to prosurvival Akt phosphorylation (19). Similarly, protein kinase C has been implicated in fibroblast growth factor–mediated endothelial cell survival after irradiation (32). In addition, our data support the notion that endothelial cell apoptosis might be enhanced after combined radiation and VEGF/PDGFR inhibition. Because endothelial cell survival appears to be a regulatory modulator of tumor response to radiation (33), inhibition of such signaling pathways is expected to be beneficial for cancer treatment.

In this context, our data in PC3 and U87 tumors also show by reduced CD31 vascular counts as well as tumor proliferation (Ki-67 staining) that the triple combination of radiation with VEGF and PDGF signaling inhibitors had the highest antiangiogenic and antitumor activity among all tested schedules. Although this concept may constitute progress, combining inhibitors of VEGF and PDGF signaling with radiation will not completely shut down tumor angiogenesis because the genetic network architecture for tumor angiogenesis seems to be extremely complicated (34, 35). The organizing principles of a balanced process such as angiogenesis may require several (yet unknown) drug combinations, or a less selective inhibitor with the optimal spectrum of activity, to fully shift the balance of the system to an “antiangiogenic state” to finally reduce tumor angiogenesis and thus prevent further tumor growth (36, 37).

In conclusion, the present study reinforces a cancer therapy concept of PDGFR inhibitors synergizing with inhibitors of VEGF signaling (23–26, 38). However, our data also show that although the inhibitors were given at their maximum tumor growth inhibition efficacy, tumor control was not achieved. It is therefore conceivable that, in the most prevalent spontaneous human solid tumors, even more potent inhibitors of VEGFR/PDGFR signaling than the compounds used here will not be sufficient to induce sustained local control. In fact, our results suggest that ionizing radiation has superior tumor growth-inhibitory abilities in vivo compared with drug therapy. Moreover, radiation greatly enhances the antiangiogenic and antitumor effects of the drugs. Taken together, our data advocate the use of radiotherapy together with combined VEGF and PDGF signaling inhibition for local tumor therapy.

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References


Combination of Vascular Endothelial Growth Factor Receptor/Platelet-Derived Growth Factor Receptor Inhibition Markedly Improves Radiation Tumor Therapy

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