In Vivo Assessment of Antiangiogenic Activity of SU6668 in an Experimental Colon Carcinoma Model

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ABSTRACT

Purpose: The purpose of this research was to assess in vivo by dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) the antiangiogenic effect of SU6668, an oral, small molecule inhibitor of the angiogenic receptor tyrosine kinases vascular endothelial growth factor receptor 2 (Flk-1/KDR), platelet-derived growth factor receptor, and fibroblast growth factor receptor 1.

Experimental Design: A s.c. tumor model of HT29 human colon carcinoma in athymic mice was used. DCE-MRI with a macromolecular contrast agent was used to measure transendothelial permeability and fractional plasma volume, accepted surrogate markers of tumor angiogenesis. CD31 immunohistochemical staining was used for assessing microvessels density and vessels area. Experiments were performed after 24 h, and 3, 7, and 14 days of treatment.

Results: DCE-MRI clearly detected the early effect (after 24 h of treatment) of SU6668 on tumor vasculature as a 51% and 26% decrease in the average vessel permeability measured in the tumor rim and core (respectively). A substantial decrease was also observed in average fractional plasma volume in the rim (59%) and core (35%) of the tumor. Histological results confirmed magnetic resonance imaging findings. After 3, 7, and 14 days of treatment, postcontrast magnetic resonant images presented a thin strip of strongly enhanced tissue at the tumor periphery; histology examination showed that this hyperenhanced ring corresponded to strongly vascularized tissue adjacent but external to the tumor. Histology also revealed a strong decrease in the thickness of peripheral viable tissue, with a greatly reduced vessel count. SU6668 greatly inhibited tumor growth, with 60% inhibition at 14 days of treatment.

Conclusions: DCE-MRI detected in vivo the antiangiogenic efficacy of SU6668.

INTRODUCTION

Angiogenesis plays an essential role in cancer biology. For a tumor to grow beyond a certain size, it must develop a network of blood vessels to supply nutrients and oxygen, and to remove waste products.

Angiogenesis is regulated through the production of several proangiogenic and antiangiogenic factors. It is well established that tyrosine kinases receptors of vascular endothelial growth factor (VEGF), fibroblast growth factor, and platelet-derived growth factor are strongly implicated in angiogenesis associated with solid tumors. In particular, VEGF, also known as vascular permeability factor, is up-regulated during hypoxia and is considered one of the most potent proangiogenic molecules: it induces intracellular signaling in endothelial cells by activating the mitogen-activated protein kinase/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/AKT pathways, and is also a survival factor for normal and tumor endothelium (1). VEGF-stimulated neovascularization is highly permeable, allowing for leakage of large plasma proteins into extravascular stroma, which, in turn, facilitates neovascularization, tumor expansion, and metastasis. Receptor tyrosine kinases participate in the transmission of proliferation and survival signals to endothelial cells, and inhibiting cell signaling through these receptors represents a good strategy for antiangiogenic therapeutic intervention (2, 3).

SU6668 is a novel small molecule oxindole compound, which is a selective inhibitor of the angiogenic receptor tyrosine kinases Flk-1/KDR (VEGF receptor-2), platelet-derived growth factor receptor β, and fibroblast growth factor receptor (4). Previous studies reported that SU6668 has competitive inhibitory properties with respect to ATP, inhibits phosphotyrosine levels in VEGF receptor-2 and platelet-derived growth factor receptor β in vivo, and rapidly induces tumor microvessel apoptosis, followed by apoptosis of tumor cells (5). In cellular systems, it inhibits receptor tyrosine phosphorylation and mitogenesis in a dose-dependent manner after stimulation of cells by appropriate ligands (4). Furthermore, intravital multiluorescence videomicroscopy of C6 glioma xenografts in the dorsal skinfold chamber model revealed that SU6668 suppressed angiogenesis (4). Oral administration of SU6668 has been shown previously to result in regression, stasis, or marked growth inhibition of many large established s.c. tumor xenografts in mice (4). In a preclinical model of colon cancer metastasis, SU6668 inhibited metastases (55%), microvessel formation (36%), and cell proliferation (27%; Ref. 6), and in A/J mice bearing SCK tumors, SU6668 decreased the number and size of...
metastatic nodules in the lung and significantly prolonged the survival time (7).

The number of antiangiogenic compounds entering clinical trials is rapidly increasing, and functional characterization of tumor vasculature will be important for the treatment in patients receiving antiangiogenic therapies, because the effect on tumor vasculature may precede by some considerable time the effect on tumor size reduction. Objective tumor size reduction has been widely used as a standard end point in clinical trials, but traditional methods of testing cytotoxic compounds might not be adequate for this class of drugs, which are in fact mainly cytostatic, slowing or stopping tumor growth. Magnetic resonance imaging (MRI) technique allows morphological and functional evaluation of the microvascular bed in human and living animals at high spatial and temporal resolution. A unique approach is dynamic contrast-enhanced (DCE) -MRI, which detects properties of tumor vessels by virtue of differential distribution of contrast media in normal and tumoral tissues. MRI enhanced with small molecular weight (M<1,000) contrast agents is currently used in the clinic to evaluate tumor microvascular characteristics; on the other hand, the advantage of using macromolecular contrast media (blood-pool contrast agents) has been demonstrated in several preclinical studies (8–10). Good correlations have been obtained in several studies between DCE-MRI data and histological microvessel density (MVD), whereas others have shown controversial results. MRI characterization of microvasculartiy would offer advantages over the histological analysis in that MRI is noninvasive, is able to evaluate the entire tumor, and can be repeated frequently to monitor the effect of therapy.

In this study, DCE-MRI with a macromolecular contrast agent has been used to measure characteristics of tumor microvessels, such as transendothelial permeability (kPS) and fractional plasma volume (fPV) in an experimental colon carcinoma model. The aim of this study was to evaluate whether a time course of noninvasive DCE-MRI was able to assess antiangiogenic effects of SU6668 in vivo. An automated method for generation of quantitative anatomical maps of kPS and fPV has been developed (11), and this provides the unique opportunity to explore tumor vasculature heterogeneity in vivo. The effect of SU6668 on tumor vascularity was assessed for the first time quantitatively and noninvasively by MRI at different time points after treatment to better understand the sequence of vascular events that precede tumor growth inhibition. Changes in functional data from MRI have been correlated with changes in histological and immunocytochemical features of vessels in different tumor areas.

MATERIALS AND METHODS

Animals and Experimental Model. Athymic nu/nu mice (Harlan, San Pietro, Italy) were housed in a ventilated, temperature-controlled room, with a 12 h light/dark cycle. HT-29 human colon carcinoma fragments were implanted s.c. in the flank of 30 nude mice weighing ~25 g. SU6668 and vehicle (Cremophor-based vehicle) were obtained by Sugen Inc. (San Francisco, CA). Animals were inserted in the study when the tumors reached a weight of ~500 mg and were subdivided into three groups according to the duration of treatment. Animals in the first group received a single dose of SU6668 (200 mg/kg, p.o.; n = 5) or vehicle (n = 5) and were observed by MRI before and 24 h after treatment. Animals in the second group received daily administration of SU6668 (200 mg/kg/day, p.o.; n = 5) or vehicle (n = 5) for 3 days and were observed by MRI before and 72 h after beginning of treatment. Animals in the third group underwent daily administration of SU6668 (200 mg/kg/day, p.o.; n = 5) or vehicle (n = 5) for a period of 14 days. MRI for this group of animals was performed at day 0, day 7, and day 14. The drug was well tolerated, with no weight loss or any other adverse side effects observed. Tumor volumes were obtained by caliper measurement of tumor diameters (d and D), according to the formula d2 × D/2. The investigation complied with the national legislation about the care and use of laboratory animals.

Contrast Agent. Gd-DTPA-albumin, synthesized according to Ogan (12), was obtained from Robert Brasch (Constrast Media Laboratory, University of California, San Francisco, San Francisco, CA) and is characterized by an average molecular weight of M, 94,000, corresponding to ~45 molecules of Gd-DTPA covalently bound to each albumin molecule. A bolus of Gd-DTPA albumin was injected into the tail vein at a dose of 30 μmol of Gd/kg; the total injected volume was 26 ml/kg (typically 60 μl for mouse).

MRI. Mice were anesthetized by inhalation of a mixture of air and O2 containing 0.5–1% halothane and placed in prone position into a 3.5-cm i.d. transmitter-receiver birdcage coil. Images were acquired using a Biospec tomograph (Bruker, Karlsruhe, Germany) equipped with a 4.7 T, 33-cm bore horizontal magnet (Oxford Ltd., Oxford, United Kingdom). Coronal Spin Echo (SE) and transversal multislice, fast Spin Echo T2-weighted (RARE, T2eff = 70 ms) were acquired for tumor localization and good visualization of extratumoral tissues. Afterward, a dynamic series of three-dimensional, transversal Spoiled-Gradient Echo images were acquired with the following parameters: Repetition-Time (TR)/Echo-Time (TE) = 50/3.5 ms, Flip Angle (α) = 90°, matrix size 128 × 64 × 32, Field-of-View (FOV) 5 × 2.5 × 3 cm3 (corresponding to 0.39 × 0.39 mm2 in-plane resolution and 0.94 mm slice thickness), Number of Acquisitions (NEX) = 1. The acquisition time for a single three-dimensional image was 104 s; a dynamic scan of 24 images was acquired with 30-s time intervals between each image (total acquisition time 53 min). The contrast agent was injected in bolus during the time interval between the first and the second scan. A phantom containing 1 ml Gd-DTPA in saline was inserted in the field of view and used as an external reference standard. The experimental protocol closely followed the one reported in reference (9), with the modifications already described in reference (11). Briefly, precontrast T1 values were measured using the IR-SnapShot Flash technique (13). Under the present experimental conditions it was not possible to measure the signal directly from the blood vessels, because the signal of the blood was hyperintense due to flow effects. Consequently, the plasma kinetics of the contrast medium was determined ex vivo (11). This procedure is widely adopted for such kinds of investigations (Ref. 14 and references therein).

Theoretical Equations and Data Analysis. Data analysis was adapted from a method described previously (9) for the special case of macromolecular contrast agents (11). Briefly, the
time dependence of longitudinal relaxation time in different tissues, after injection of the contrast agent, is obtained from the experimentally measured signal intensity (SI) values according to the theoretical dependence of SI for a GRE sequence (once $T_1$ has been independently measured):

$$\frac{SI(t)_{\text{post}}}{SI_{\text{pre}}} = \frac{1 - \exp \left[ -\frac{1}{T_R} T_1(t)_{\text{post}} \right]}{1 - \exp \left[ -\frac{1}{T_R} T_1(t)_{\text{pre}} \right]}$$  (A)

$T_1$ values of a tissue can be correlated to the tissutal Gd content by the relationship:

$$\Delta R_1(t) = \frac{1}{T_1(t)_{\text{post}}} - \frac{1}{T_1(t)_{\text{pre}}} \times [\text{Gd}(t)] r_1$$  (B)

where $T_1_{\text{pre}}$ and $T_1(t)_{\text{post}}$ represent, respectively, the longitudinal relaxation times of the tissue before and at different time points after contrast agent injection; $[\text{Gd}(t)]$ indicates the molar concentration and $r_1$ (expressed in mm$^{-1}$ s$^{-1}$), the relaxivity of Gd ion in the tissue. Assuming that $r_1$ is constant in the different tissues examined, the parameter $\Delta R_1(t)$ is directly proportional to the Gd concentration in the tissue. The time dependence of $\Delta R_1(t)$ was analyzed in terms of a two-compartment tissue model composed of plasma and interstitial water equilibrating pools (9, 11). In this model it is assumed that the exchange is due to passive diffusion of the contrast agent. As explained in detail previously (11), the time dependence of $\Delta R_1(t)$ can be expressed by:

$$\Delta R_1(t) \propto CT(t) = \int_0^t Coexp(-\delta A) d\delta + f_{PV} Coexp(-At)$$  (C)

where CT(t) is the total concentration of Gd in both interstitial water and plasma space, $Coexp(-At)$ is the experimentally measured concentration of Gd in plasma, $f_{PV}$ is the fractional plasma volume of the tissue, and kPS is the extravascular permeability. This expression was fitted to the $\Delta R_1(t)$ values extracted from the experimental signal intensity using a two-parameter best fit nonlinear algorithm. The analysis was performed on a pixel-by-pixel basis, and parametric maps of kPS and fPV were produced.

**Magnetic Resonance Quantitative Evaluation and Statistics.** After acquisition, data were transferred on a PC for analysis. The fitting routine was written in Matlab 5.2 (The MathWorks, Inc, Natick, MA). Images were analyzed on a pixel-by-pixel basis, to obtain parametric maps of kPS and fPV, or on a region-of-interest (ROI) basis to obtain the average value of kPS and fPV in the selected ROI; in each animal, the central five slices of the three-dimensional data set were analyzed. Two different kind of analysis were performed: first, for each considered slice, ROIs were manually tracked to cover the tumor rim and the tumor core, as described previously (11). A band ~2 mm wide at the periphery, on the external side of the tumor was considered as the rim. The signal in the rim (and in the core) was averaged and analyzed to obtain the mean kPS and fPV values in the rim (or in the core) of the selected slice. To minimize the influence of ROI selection, a histogram-based approach was also used: the analysis was performed pixel-by-pixel obtaining parametric maps of kPS and fPV; the whole tumor was manually selected, and histograms showing kPS and fPV value distribution across the whole areas were obtained. Statistical significance of the differences between pre- and post-treatment values was assessed using paired t test at the 95% confidence intervals.

**Histological and Immunocytochemical Methods.** After the last MRI examination, mice were sacrificed. Before removal of tumor a mark was made on the overlying skin with an ink pen, perpendicular to and in the midpoint of cranial caudal axis of tumor. After fixation in zinc fixative for 6 h, the tumor was cut in half exactly along the marking line and embedded in paraffin. From each of the two halves, five slices (5-μm thick) were taken. The sections obtained were in planes corresponding to those used for the magnetic resonance images. Three of the five sections were stained with H&E; the remaining two were used for CD31 immunohistochemical staining of endothelial cells using a 1:800 dilution of the CD31 Pharmingen (Rat A-mouse #557355) primary antibody incubated overnight. The resultant products were visualized with the 3,3'-diaminobenzidine chromogen. The primary antibody was replaced by rat nonimmune serum (DAKO - X0912) at the same protein concentration for negative controls.

**Histological Image Analysis.** Sections stained with H&E showed a central necrotic area and a peripheral area with viable tumoral cells. For measuring the number of vessels, four fields for each slide were examined using a ×10 objective (each field measuring 647 × 483 μm), selected in the viable area of the tumor (see scheme reported in Fig. 1). The image analysis program Image ProPlus 4.0 was used. Vessels were selected on color basis and, for each field, the number of positive objects was counted. Vessels located in the capsule and objects with area <10 μm$^2$ were excluded. The average area of vessels was

![Fig. 1 Schematic representation of a tumor.](https://clincancerres.aacrjournals.org)
measured within the same fields as before, using a ×20 objective (each field measuring 323.7 × 241.8 μm). Each vessel was manually lined, and area values were collected.

RESULTS

Early Effect on Tumor Vasculature, 24-h Study. In Fig. 2 we show representative images obtained for one animal belonging to the treated group before treatment: a T2w image (Fig. 2A) as well as T1w images acquired before (Fig. 2B) and 50 min after (Fig. 2C) contrast agent administration are shown. T1w and T2w images were acquired approximately at the same position in the center of the tumor, although slice thickness in the T2w image was higher than in T1w images (2 mm against 0.94 mm). T2w images allowed a better distinction of tumor from extratumoral tissues (highest signal of peripheral rim). Signal intensity enhancement in T1w images was more marked at the tumor periphery (Fig. 2C, white arrows), in agreement with the higher vascularization observed histologically in this region (see below). In frames (Fig. 2D) and (Fig. 2E), we report fPV and kPS maps obtained for the same slice.

Fig. 3 shows corresponding images obtained for the same animal as in Fig. 2, 24 h after treatment with a single dose of SU6668. No relevant differences were observed after treatment in standard T2w and T1w-precontrast images. However, the comparison between Fig. 2 (B and C) and Fig. 3 (B and C) shows that signal enhancement induced by contrast agent on T1w images is strongly decreased after drug treatment (see lack of enhancement in Fig. 3C). Comparison between kPS and fPV maps (Fig. 3, D and E) also indicates a strong decrease in both parameters after treatment.

Quantitative evaluation of kPS and fPV values was performed separately in the tumor rim and in the core. ROIs were selected as described in “Materials and Methods.” The results were obtained separately for the rim and the core of the tumor, as summarized in Fig. 4. The early effect of SU6668 on tumor vasculature was a significant decrease in transendothelial permeability (kPS) to Gd-DTPA-albumin (by 51% and 26% in the rim and in the core, respectively). A significant decrease was also observed in plasma volume (fPV) both in the rim (by 59%) and in the core (35%) of the tumor. The effect on both permeability and blood volume was more pronounced in the rim than in the core of the tumor. No significant alteration in fPV and kPS values was observed in the controls. Due to scarce penetration of the contrast agent in central necrotic area, data referred to the core are more prone to experimental errors and, therefore, more difficult to interpret; for this reason histology findings and their correlation with DCE-MRI data were always and only referred to the rim of viable tissue.

In Fig. 5, kPS histograms at 0 and 24 h for two control animals are compared with correspondent histograms for two drug-treated mice. At 24 h, a clear shift to lower values of kPS (shift to the left) is appreciated in both treated animals, whereas no significant shift is seen in the controls. KPS histograms were quantitatively evaluated by determining the percentage of pixels having a kPS value above a certain threshold. For each animal the threshold was fixed at the average value of the permeability as determined from histograms before the administration of SU6668 (or vehicle). The percentage number of pixels in which the permeability was above the threshold decreased in the treated group (from 43.8 ± 1.9% to 22.5 ± 9.8%; P = 0.006), whereas it did not decrease significantly in the control group (from 41.9 ± 2.5% to 30.5 ± 11.1%; P = 0.118).
Histological results confirmed the MRI data. Sections stained with H&E showed the presence of a central necrotic area and a peripheral zone with viable tumor cells. Image analysis was used to count vessels in the viable areas of CD31 stained slides. Table 1 reports mean number (MVD) and area of vessels ± SD for control and treated animals in selected regions of tumor rim (see “Materials and Methods”). A highly significant decrease in both vessel count (40%) and

![Image](image_url)

**Fig. 3** T2w, T1w-pre, and T1w-postcontrast images acquired approximately at the tumor center for the same animal reported in Fig. 1 but acquired 24 h after treatment. A, axial T2w RARE image; B and C, axial T1w GRE images acquired before (B) and 50 min after the injection of Gd-DTPA-albumin (C); D and E, fPV and kPS maps obtained for the same slice. Comparison between B and C shows that the enhancement of signal intensity in the tumor rim is small if compared with the one observed before treatment (see Fig. 2, B and C).

**Fig. 4** Mean values of transendothelial permeability (kPS; in ml/min/cm² of tissue) and fractional plasma volume (fPV; in ml/cm³ of tissue) in the tumor rim and core, before and 24 h after administration of SU6668 or vehicle. Data are averaged over five central slices for each animal and are shown as mean; bars, ±SD. Statistical significance is indicated by asterisks: * P < 0.05, ** P < 0.001.
vessel area (18%) was found in treated tumors versus controls.

3-, 7-, and 14-Day Studies. Standard T1w and T2w magnetic resonance images acquired 3 days after treatment resemble those obtained at 24 h. However, after administration of contrast agent, three animals of five showed a strong enhancement in a thin strip at the tumor periphery (Fig. 6). Such pronounced and localized enhancement was not observed in the control group or in the 24 h study. Quantitative kPS and iPV data for the 3-day study are reported in Fig. 7. MRI results indicate that a relatively small (20%) decrease in transendothelial permeability occurs in the tumor rim of treated animals after 3 days of treatment. However, a similar decrease was also observed in the control group. We can hypothesize that the decrease in kPS observed in the control group is due to the normal evolution of tumor vasculature. In contrast, a decrease (20%) in the vascular permeability was observed in the tumor core of treated animals, whereas this parameter tended to increase in tumors from control animals. No significant effect was detected in plasma volumes.

After 3 days of treatment, histological examination revealed a strong decrease in thickness of the peripheral viable tissue and corresponding enlargement of the necrotic core. Vessel counts in the viable rim of treated tumors were strongly decreased (70%) when compared with vessel counts in control tumors (see Table 1). Fig. 8A shows that in treated animals a well-vascularized capsule is contiguous to a thin area of scarcely vascularized viable tissue. Fig. 8B shows an analogous tumor section from the control group, where the vascularization in the viable area is clearly preserved.

Table 1  CD31 immunohistochemical staining: microvessel density and mean area of vessels for the 24-h and 3-day study

<table>
<thead>
<tr>
<th></th>
<th>24-h study</th>
<th>3-day study</th>
</tr>
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<tbody>
<tr>
<td>Controls</td>
<td>Mean area ( \pm SD )</td>
<td>Mean area ( \pm SD )</td>
</tr>
<tr>
<td></td>
<td>41.7 ( \pm 24.2^a )</td>
<td>564.9 ( \pm 149.2^e )</td>
</tr>
<tr>
<td>Treated</td>
<td>25.0 ( \pm 19.9^b )</td>
<td>462.0 ( \pm 91.2^c )</td>
</tr>
<tr>
<td>Controls</td>
<td>73.8 ( \pm 28.1^b )</td>
<td>471.5 ( \pm 69.0^d )</td>
</tr>
<tr>
<td>Treated</td>
<td>19.8 ( \pm 12.0^d )</td>
<td>320.3 ( \pm 168.4^c )</td>
</tr>
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\( ^a P < 0.001 \)
\( ^b P < 0.001 \)
\( ^c P < 0.05 \)
\( ^d P < 0.05 \).
Histological examination of these tumors clarifies the origin of this hyperenhanced rim. Histological analysis performed on control and treated tumors at 14 days showed the presence of a prominent vascularization outside the viable tumor tissue in SU6668-treated animals (Fig. 10). The depth of viable tissue was strongly reduced compared with controls.

**Tumor Growth Inhibition.** The ability of SU6668 to inhibit s.c. growth of HT29 human colon carcinoma xenografts was evaluated. Fig. 11 shows that SU6668 orally administered at 200 mg/kg/day had significant *in vivo* activity against the HT29 tumor, resulting in 60% inhibition of growth on day 14 (*P* < 0.01). This result correlates with the early response on vessels detected by DCE-MRI at 24 h.

**DISCUSSION**

DCE-MRI techniques play an increasingly important role in the evaluation of tumor vasculature *in vivo* and specifically in the early detection of the therapeutic efficacy of antiangiogenic agents (15). By using DCE-MRI it has been demonstrated that different antiangiogenic treatments may result in different patterns of microcirculatory modification. Roberts et al. (16) have shown that anti-VEGF antibodies induce modification of both kPS and fPV in experimental tumors, as probed by MRI enhanced with a macromolecular contrast agent and a small molecular weight contrast agent in a model of glioblastoma multiforme (17). The same group also reported the effect of an anti-VEGF antibody on tumor kPS, with no effect on fPV (18). Turetschek et al. (19) showed that PTK787/ZK 222584, an inhibitor of VEGF receptor-2 kinase activity, induces a modification of kPS without significant changes in fPV. The effect of PTK787/ZK 222584 was also studied by Drevs et al. (20), who confirmed an effect on vessel permeability (to a low molecular weight contrast agent) in a murine renal cell carcinoma.

The data presented here provide additional evidence of microcirculatory modification induced by simultaneous inhibition of multiple angiogenic growth factor signaling pathways and confirm that administration of an inhibitor of angiogenic receptor tyrosine kinases results in a relevant reduction of kPS and fPV, as probed by DCE-MRI 24 h after treatment.

The effect of SU6668 on tumor vasculature at different time points of treatment has been studied *in vivo* by DCE-MRI using a macromolecular contrast agent. SU6668 induced a significant decrease in fractional plasma volume and transendothelial permeability, parameters strictly connected to tumor angiogenesis. Such effects were clearly detected by MRI 24 h after treatment, well before any effect on tumor size reduction was detectable. A significant reduction in MVD and vessel area was also detected by immunohistochemistry, in agreement with the

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*Fig. 6* Representative T1w images acquired for two SU6668 treated mice, after 3 days of treatment. Images are acquired before (A and C) and 50 min after administration of Gd-DTPA-albumin (B and D). A thin strip of signal enhancement is clearly visible in postcontrast images (white arrows).
MRI finding of decreased blood volume. Although potent antiangiogenic effects of SU6668 are dependent on chronic administration (21), an early effect on functional microcirculatory parameters exists in the first hour after treatment. This is in accordance with data showing numerous apoptotic endothelial cells by 6 h after initiation of treatment (5) and a significant decrease in tumor blood perfusion at 1 h after a single injection of SU6668 (7). In our experiments these data are confirmed, and the strict correlation between in vivo and histological findings demonstrates that the detected functional modifications have morphological bases. Several articles have recently assessed that microvessel density is not a good indicator of the therapeutic efficacy of antiangiogenic drugs (see Ref. 23 for a recent review) although widely used in preclinical and clinical investigations. Together with the invasiveness of the method that requires repeated biopsies during the therapy, this finding pushes toward a multimethods approach where radiological techniques, as DCE-MRI, play an important role. It has been observed that new microvessels formed in response to angiogenesis are hyperpermeable to macromolecules, and reduced permeability is potentially an excellent surrogate marker of biological activity of antiangiogenic compounds (16). The decrease in MVD observed in the present study was accompanied by a strong decrease in both fractional plasma volume and transendothelial permeability determined at MRI so providing a very robust demonstration of SU6668 efficacy.

Previous studies demonstrated the ability of SU6668 to inhibit VEGF-induced vascular permeability in mouse skin using the Miles assay (5). This assay is invasive and provides only a qualitative indication of permeability decrease. In the present study, we demonstrated an early effect of SU6668 on vascular leakage of albumin-Gd-DTPA and proved the ability of DCE-MRI to noninvasively and quantitatively detect and monitor vessel permeability in vivo. Such a quantitative approach is crucial for a direct comparison of data obtained in different experimental conditions.

Studies evaluating longer treatment periods (3, 7, and 14 days) showed other modifications in tumor vascularization: SU6668 induced a strong decrease in the number of vessels in the viable area of the tumor by 3 days, as determined by immunohistochemistry. In contrast, the capsule of the tumor remained well vascularized (as shown in Fig. 8). This effect was additionally enhanced with 14 days of treatment; histological sections taken at this time point clearly showed a region with prominent vasculature surrounding the tumor, but external to it. Our data seem to indicate that this region of prominent vasculature is the evolution of the well-vascularized capsule detected in some animal at 3 days. A greatly enhanced rim was detected by MRI in some treated tumors at 3 days (Fig. 6), matching the well-vascularized capsule observed histologically. To the best of our knowledge this time-dependent pattern of extratumoral vascularization after antiangiogenic therapy has not been described previously. The clinical relevance and meaning of the observed increase in vascularity of peritumoral tissue induced by the drug remain to be investigated. If detected in human tumors, it would have relevance in the radiological evaluation of the efficacy of
the therapy, because it appears in contrast-enhanced MRI as a very bright region not always easily discernable from the viable tumor tissue, as the present study demonstrates. Such a peripheral prominent vascularization could also affect the delivery of drugs to tumor tissue. Despite a strong decrease in vessel number in the viable area (70% at 3 days of treatment), MRI was not able to detect any decrease in plasma volume in the tumor rim. This is very likely due to the difficulty in defining a suitable region of interest in the thin viable remains of the tumor. The resolution used in this study (390 μm in-plane) was not high enough to distinguish the viable tissue rim from strongly vascularized extratumoral tissue. If a clear distinction of tumoral and extratumoral tissues has to be achieved, images will have to be acquired at a higher resolution to separate out the two compartments. This will be explored in future studies by using two-dimensional techniques. Several studies also reported a lack of correspondence between MRI-determined blood volume and histological MVD. This could be due to the inherent sensitivity of MRI measurement to the sole perfused and functional vessels, as compared with histological analysis, which takes into account all of the anatomically distinguishable vascular structures. Fractional plasma volume was not found to be different in tumors treated with an inhibitor of VEGF-receptor tyrosine kinase, despite a decreased MVD detected in treated tumors (19). After treatment with an inhibitor of the VEGF-receptor, Dreys et al. (20) detected an increase in blood volume by MRI. Despite a reduced number of vessels, this was explained by virtue of hemodynamic dilation of the remaining vessels. Griffin et al. (7) also showed that SU6668 induced a transient elevation in tumor blood perfusion before a clear reduction, due to a massive loss of endothelial cells.

In the present study, together with microvessel density, we measured the average vessel area in the tumors to gain an indication of vessel size. We observed that a decrease in MVD was always accompanied by a significant but smaller decrease in vessel area (Table 1), indicating that a compensatory effect of the remaining vessels may occur in an attempt to counteract the loss of microvessels.

The strong decrease in permeability observed 24 h after treatment (Fig. 4) was partially lost at 3 days (Fig. 7). We hypothesize that this could be due to a compensatory mechanism, which leads to induction of VEGF transcription with consequent increases in vascular permeability and vasodilation (7). A similar early, but transient effect on microvascular permeability was also found recently after treatment of human breast cancer xenografts with a matrix metalloprotease inhibitor (24). However, the aforementioned partial volume effect, which prevented a clear distinction between capsule and viable tumor, could also play a role. A somewhat surprising result was the statistically significant decrease in vessels permeability in the rim accompanied by an increase in the core after 3 days of vehicle administration. We hypothesize that this is due to the decrease in treated area.
“normal” evolution of the tumor vasculature; it is well known that tumor vascularization strongly changes with tumor growth (23), and areas of high permeability to macromolecules have often been found in preferentially necrotic regions (11, 23). In this model, the rim and the core of the tumors appear strongly different by histology, the first being preferentially constituted by living tissue and the second mostly necrotic. It is possible that during tumor growth vessels in the two regions undergo different transformations. Concerning the pathogenetic mechanisms of the modification induced by inhibition of angiogenic tyrosine kinase activity, previous studies have mainly focused on tumor vasculature with scarce attention to the surrounding tissues. These aspects could be important, because the tumor vasculature is strictly dependent on the circulatory bed existing in the surrounding tissues. In our study we found a good conservation of the capsular and extratumoral vessels in treated animals. The specific CD31 staining of the vessels demonstrated that degenerative effects preferentially involved the vessels of the tumoral mass saving in part the microcircle of the peritumoral tissue. However, ectasic vessels and hemorrhages were detected in a limited area between the capsule and the tumoral mass. These data seem to suggest an elective action on the boundary between “normal” and tumoral vasculature that might cause a reduction of the hematic flow from the capsular to the intratumoral microcirculation. This aspect seems to suggest a particular sensitivity of the vessels in this area to simultaneous inhibition of multiple angiogenic growth factor signaling pathways. More work is needed to fully understand the mechanism of elective action on tumor vessels, but careful region selection is crucial if MRI data have to be referred to viable tumor tissue.

The early effect on vascularity detected by DCE-MRI after a single treatment with SU6668 lead to a time progressive damage and death of the tumor after continual treatment. After 14 days of treatment only a very thin layer of viable tumor cells...
remained at the tumor periphery, and tumor growth was inhibited by 60% (Fig. 11).

Angiogenesis inhibitors are a new class of drugs for which the general rules involving conventional chemotherapy might not apply. DCE-MRI is a very promising approach to noninvasively monitor the sequence of vascular events in a tumor, which characterize the activity of antiangiogenic and vascular targeting agents. Tumor microcirculation differs profoundly from that of normal organs with blood perfusion being spatially and temporally very heterogeneous. A major advantage of DCE-MRI is the opportunity to serially monitor over time the different regions in a tumor (11). Most of the studies published recently reported results concerning kPS and fPV values measured either very early (at 12 or 24 h after treatment) or very late (days or weeks of treatment), but a time-dependent assessment was not performed. Our time course study demonstrated a sequence of only partially understood vascular events characterized by mechanisms of compensatory and feedback regulation. Our findings could explain discrepant results of previous studies reported in the literature, which failed to detect antivascular efficacy of antiangiogenesis compounds due to time dependence of the effect. DCE-MRI time course studies in animal models are needed to carefully plan patient treatment and examination schedule in clinical trials. SU6668 has laid the groundwork for the development of a series of new compounds, which are under development or in clinical trials. None of the compounds belonging to the SU6668 class has been studied in vivo using DCE-MRI techniques. Our results will be of interest in the planning and evaluation of clinical trials for the whole class of compounds.

REFERENCES


Antiangiogenic Activity of SU6668

In Vivo Assessment of Antiangiogenic Activity of SU6668 in an Experimental Colon Carcinoma Model

Pasquina Marzola, Anna Degrassi, Laura Calderan, et al.


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