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Response of HT29 Colorectal Xenograft Model to Cediranib Assessed with ¹⁸F-FMISO PET, Dynamic Contrast-Enhanced and Diffusion-Weighted MRI

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Abstract

Cediranib (AZD2171, AstraZeneca, UK) is a small-molecule pan-VEGFR inhibitor. The tumor response to short-term cediranib treatment was studied using dynamic contrast-enhanced (DCE) and diffusion-weighted (DW) MRI at 7 T as well as ¹⁸F-fluoromisonidazole (¹⁸F-FMISO) PET and histological markers. Rats bearing subcutaneous HT29 human colorectal tumors were imaged at baseline, then received three doses of cediranib (3 mg/kg per dose daily) or vehicle (dosed daily), with follow up imaging performed 2 hours after the final cediranib or vehicle dose. Tumors were excised and evaluated for the perfusion marker Hoechst 33342, endothelial cell marker CD31, smooth muscle actin (SMA), intercapillary distance (ICD) and tumor necrosis. DCE-MRI-derived parameters decreased significantly in cediranib-treated tumors relative to pre-treatment values: the muscle-normalized initial area under the gadolinium concentration curve (nIAUC90) by 48% ($p = 0.002$), the enhancing fraction (EnF) by 43% ($p = 0.003$) and K^{trans} by 57% ($p = 0.003$), but remained unchanged in controls. No change between pre- and post-treatment tumor apparent diffusion coefficient (ADC) in either cediranib- or vehicle-treated group was observed over the course of this study. ¹⁸F-FMISO SUVmean decreased by 33% ($p = 0.008$) in the cediranib group, but showed no significant change in the control group. Histological analysis showed that the number of CD31-positive vessels (59 per mm²), the fraction of SMA-positive vessels (80 to 87%) and ICD (0.17 mm) were similar in cediranib- and vehicle-treated groups. The fraction of perfused blood vessels in cediranib-treated tumors (81±7%) was lower than in vehicle controls (91±3%, $p = 0.02$). The necrotic fraction was slightly higher in cediranib-treated rats (34±12%) than in controls (26±10%, $p = 0.23$). These findings suggest that short-term treatment with cediranib causes a decrease of tumor perfusion/permeability across the tumor cross-section, but changes in vascular morphology, vessel density or tumor cellularity do not manifest at this early time point.

Keywords

Cediranib; HT29 Tumors; Dynamic Contrast-Enhanced Magnetic Resonance Imaging; Diffusion Magnetic Resonance Imaging; Histological Analysis

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Introduction

Antiangiogenic therapy delays tumor growth by inhibiting the formation of the new tumor blood vessels (1). Although significant increases in progression-free survival have been achieved, some patients never respond and others relapse after a transient response to antiangiogenic therapy without an overall survival benefit (2,3). Prediction and early identification of response to treatment as well as noninvasive treatment monitoring is therefore required for selecting an optimal therapy for an individual patient (4). Imaging biomarkers of tumor perfusion, hypoxia and metabolism are increasingly used for this purpose and can be combined when multiparametric approach is used to create a comprehensive picture of the tumor microenvironment (5). Dynamic contrast-enhanced (DCE) MRI and CT provide measures of tumor blood flow and/or permeability and blood volume (6,7). The reductions in these parameters after antiangiogenic treatment were found to be predictive of progression-free survival (8). Diffusion-weighted (DW) MRI provides estimates of the apparent diffusion coefficient (ADC), which is sensitive to tissue cellularity, edema and necrosis. Changes in tumor ADC after chemotherapy, radiotherapy or administration of vascular disruptive agents were shown to be markers of response in many studies (9,10), but the behavior of ADC after antiangiogenic therapy has been studied less extensively (11,12). Tumor hypoxia is predictive of poor outcome and can be probed by using hypoxia tracer ^{18}F -FMISO PET imaging (13). Although perfusion and hypoxia tend to be complementary (14), their relationship on a local level can be complex (15). Several studies reported decreases in ^{18}F -FMISO uptake after antiangiogenic treatment (16,17). A multifunctional MRI/PET study in rat gliomas has demonstrated that treatment with sunitinib resulted in increased cerebral blood volume, decreased vascular permeability and decreased ^{18}F -FMISO uptake (18). The reduction of the PET tracer uptake may be interpreted as a decrease in hypoxia due to the vascular normalization (19); however, decreased tracer delivery after treatment may also play a role and needs to be further investigated (20).

In this study we explored the early changes of the tumor parameters after administration of cediranib (AstraZeneca, UK), a potent small-molecule inhibitor of VEGF receptors (21-23). In a variety of tumor models, cediranib was shown to cause vascular regression, reduce vessel density and inhibit tumor growth (24-27). DCE MRI studies in mouse gliomas and subcutaneous C6 and LoVo tumors models have found approximately 30% decreases in tumor plasma-to-interstitium transfer constant K^{trans} after two days of treatment with cediranib (12,28,29). These changes were corroborated by the histologically measured vascular permeability in brain tumors (29) and by the large decreases in vessel permeability in SW620 tumors studied with intravascular contrast agents (30). The decrease of perfusion/permeability appears to occur before significant changes in microvascular density can be detected (29). The fractional volume of extracellular-extravascular space (EES) v_e decreased after treatment by 32% in mouse gliomas (12), but was not found to change significantly in C6 and LoVo tumors (28). Cediranib was shown to decrease edema in mouse brain; however, the ADC, which is often considered to be a marker of edema, decreased only by about 6% (12). Similarly, in human gliomas, ADC did not change within one day after cediranib administration (31).

The purpose of this study was (i) to determine the early changes in the tumor perfusion/permeability parameters and ADC derived from DCE and DW MRI after cediranib administration, (ii) to measure changes in ^{18}F -FMISO uptake in PET imaging over the same course of treatment, and (iii) to corroborate these changes with the histological measures of vascular function and morphology.

Methods

Cell line and tumor implantation

Human colorectal carcinoma HT29 cells (ATCC Number HTB-38) were maintained in Dulbecco's Modified Eagle Medium (MediaTech Inc., Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. Xenografts were initiated by subcutaneous injection of 5.0×10^6 cells in 0.2 mL of PBS into right hind limb of 6–8 week old female athymic *nu/nu* rats (weight, 200–280 g). Animal studies were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Administration of cediranib

Cediranib was provided by AstraZeneca (Alderley Park, UK). Initially, the dose of cediranib that inhibited the tumor growth was established by treating the rats with HT29 tumors of approximately 300 mm³ volume (n = 24) with 0.75, 1.5 and 3 mg/kg cediranib (n = 6 for each dose) or vehicle solution (0.5% methylcellulose in water, n = 6) daily by oral gavage for 26 days. Tumor dimensions were measured with calipers and tumor volume was calculated as $V = (\pi/6) \times l \times w^2$, where *l* was the largest diameter of the tumor and *w* was the corresponding perpendicular diameter. The slopes of the growth curves (tumor volumes versus time) for different treatment protocols were compared using the Student's t-test. The dose of 3 mg/kg/day was selected for subsequent imaging studies.

MRI experiments

MRI studies were performed on rats (n = 12) bearing tumors of approximately 700 mm³ volume. Rats were imaged before treatment and were then randomly assigned to the cediranib treatment group (n = 6) or vehicle control group (n = 6). Animals then received three doses of cediranib (3 mg/kg per dose daily) or vehicle (dosed daily), with follow up imaging performed 2 hours after the final cediranib or vehicle dose.

MRI data were acquired on a 7-Tesla, horizontal 30-cm-wide bore BioSpec magnet (Bruker BioSpin, Billerica, MA) equipped with 55 G/cm gradients. Rats were anesthetized by inhalation of 1–2% isoflurane in air and the tail vein was catheterized with a 24-gauge catheter for contrast agent administration. Animals were covered with towels to maintain body temperature and placed prone on a custom built holder with the tumor-bearing leg inside a 4-cm diameter Helmholtz coil (14). Breathing was monitored with a respiration monitor pad placed under the rat's abdomen. After tuning and matching of the coil and shimming of the magnet, pulse calibration was performed for each animal. To localize the tumor, T₂-weighted coronal images were acquired using a rapid acquisition with refocused echoes (RARE) sequence with the following parameters: repetition time, TR = 2–6 s depending on the number of slices required to cover the entire tumor; echo time, TE = 60 ms; slice thickness 0.8 mm; field of view, FOV = 3.0 × 3.0 cm²; matrix, 128 × 128. Using the T₂-weighted image as a reference, four coronal slices were selected in the central part of the tumor and DW and DCE MRI data were acquired in the same location and with the following shared settings: FOV = 3.0 × 3.0 cm²; slice thickness, 0.8 mm; matrix, 128 × 128; voxel size, 0.234 × 0.234 × 0.8 mm³. DW was performed using a spin echo sequence with TR = 2 s, TE = 34 ms, and diffusion-weighting factors *b* = 0, 300, 500, 700 and 900 s/mm². Pre-contrast tumor T₁ was determined using inversion recovery T₁-weighted RARE sequence (TR = 8 s; TE = 7.5 ms; FOV = 3.0 × 3.0 cm²; matrix, 128 × 128; single slice placed through the center of the tumor; inversion times TI = 64, 100, 200, 400, 600, 800, 1000 and 1200 ms). This was followed by the T₁-weighted DCE MRI performed using a fast low-angle shot (FLASH) sequence with TR = 34.2 ms; TE = 3.2 ms; flip angle, 30°; 200 dynamic images acquired with the temporal resolution of 4.38 s per image. After 2 min of

baseline signal acquisition (27 images), a bolus of gadopentetate dimeglumine (0.2 mmol/kg, Magnevist; Berlex Laboratories, Inc., Wayne, NJ) was manually injected and dynamic images were acquired continuously for additional 12 min.

DCE and DW MRI data analysis—Voxel-wise DCE and DW MRI data analyses were performed using software written in Matlab (Mathworks, Natick, MA). The post-contrast signal enhancement was converted into the change of the longitudinal relaxation rate, $\Delta R_1(t) = 1/T_1(t) - 1/T_1(t=0)$ using the gradient echo signal equation (32) and a fixed pre-contrast T_1 value, found to be the same in the tumor and muscle ($T_1(t=0) = 1750$ ms). Gadolinium concentration versus time curves were obtained by dividing $\Delta R_1(t)$ by the longitudinal relaxivity of Gd-DTPA, $r_1 = 3.3 \text{ mM}^{-1} \text{ s}^{-1}$ (33).

The initial area under the curve (IAUC) was calculated by integrating the concentration-time curve between the bolus arrival time and 90 s afterwards (IAUC90). To minimize the variability of the vascular input among animals, the tumor IAUC90 was normalized by the median IAUC90 value within an ROI placed in the adjacent muscle and the ratio was denoted nIAUC90 (34). The highly enhancing portion of the tumor, EnF, was defined as the fraction of the tumor voxels with nIAUC90 > 1 (35).

The reference region (RR) model was used to determine the plasma-to-EES transfer constant K^{trans} and the fractional volume of EES v_e (36). An empirical model (37) was first fitted to the muscle $\Delta R_1(t)$ data and the resulting smooth fitting curve was used as the RR curve with fixed parameters, $K^{\text{trans}} = 0.021 \text{ min}^{-1}$ and $v_e = 0.07$ (38,39). The $\Delta R_1(t)$ data in every tumor voxel were fitted with the RR model with K^{trans} constrained within an interval of 0.001–1.0 min^{-1} and v_e within 0.01–1.0.

Maps of the apparent diffusion coefficient (ADC) were calculated from DW images by fitting the signal intensity S versus the b -value with the monoexponential equation: $S(b) = S_0 \exp(-b \cdot \text{ADC})$, where S_0 is the signal intensity at $b = 0 \text{ s/mm}^2$. In DCE and DW MRI analyses, the goodness of curve fit was assessed with $R^2 = 1 - \text{SSE}/\text{SS}$, where SSE is the sum of squared distances between the data and fitting curve and SS is the sum of squared distances between the data and the mean of all data values.

¹⁸F-FMISO PET imaging

A separate cohort of rats ($n = 10$) with HT29 tumors of approximately 700–800 mm^3 volume was studied with ¹⁸F-FMISO PET. Rats were imaged at baseline, randomized into two groups and treated with $3 \times 3 \text{ mg/kg/day}$ cediranib ($n = 6$) or vehicle ($n = 4$). Post-treatment PET imaging was carried out 2 h after the last treatment. Prior to imaging, rats were anesthetized with isoflurane and administered 1 mCi of ¹⁸F-FMISO via tail vein injection. PET images were acquired between 90 and 95 minutes post-injection using a microPET scanner (R4 or Focus 120, Concorde Microsystems, Knoxville, TN) and reconstructed as described previously (20). ROIs were manually drawn on pre- and post-treatment images around the whole tumor and in the muscle using ASIPro VM software (Concorde Microsystems, Knoxville, TN). The mean and maximum standardized uptake values, SUV_{mean} and SUV_{max}, respectively, were calculated from the mean and maximum tissue activity expressed as percent injected dose per gram (%ID/g) within the ROI: $\text{SUV} = [\% \text{ID/g} \cdot \text{body mass (g)}]/100\%$.

Histology and immunohistochemistry

After the last imaging session, vascular perfusion marker Hoechst 33342 (15 mg/kg in 0.2 mL of sterile saline) was administered via the tail vein catheter 5 min before sacrifice. Animals were euthanized and tumors were quickly excised. Series of contiguous fresh-

frozen tumor sections were obtained (40) and dual immunofluorescence staining for the endothelial cell marker CD31 and pericyte marker smooth muscle-actin (SMA) was performed on the same sections as previously described (20).

Stained sections were imaged using an Olympus BX-40 fluorescence microscope equipped with a computer-driven motorized stage, a CC-12 digital camera and Olympus 5 acquisition software (Olympus America, Center Valley, PA). Whole tumor sections were imaged at $\times 40$ magnification by montage assembly of a series of individual image frames. For fluorescence microscopy of CD31/SMA expression and Hoechst 33342 marker, each tumor section was imaged three times using appropriate filters for each fluorophore. The tumor sections adjacent to the ones imaged with fluorescence microscopy were stained with hematoxylin and eosin (H&E) and bright field images of H&E-stained sections were acquired using the same setup. For each section, sets of immunohistochemistry and histology images were superimposed in Olympus 5 software.

Tumor vasculature was evaluated on a single middle section from each tumor using sets of digital images of CD31, SMA and Hoechst 33342, which were coded and randomized before scoring. Using Photoshop software (Adobe Systems, San Jose, CA), six 1 mm^2 areas were selected in non-necrotic areas of each section and manually evaluated for the numbers of (i) CD31-positive structures, (ii) CD31-positive structures co-localizing with SMA, (iii) CD31-positive structures co-localizing with Hoechst 33342. The intercapillary distance (ICD) was determined on combined CD31/SMA/Hoechst images as the mean distance between a given vessel and its nearest neighbors in four quadrants (41).

Necrotic tumor areas were subjectively delineated on the images of H&E stained sections by two observers (S.C. and M.R.) using Photoshop software. Each observer independently measured the area of necrotic regions and the area of the whole section on at least two sections from each tumor and averaged the results. These measurements were then averaged between the observers, and the necrotic fraction was calculated for each tumor as the ratio of the necrotic area to the total tumor area. The perfused fraction was determined from the Hoechst images as the fractional area with the staining signal intensity greater than the intensity in the necrotic tumor regions.

Statistical analysis

Statistical analyses were performed in Matlab's Statistics Toolbox. For each tumor, the mean parameters were determined from ROIs manually drawn around the whole tumor on all tumor-containing slices. The parameter means, standard deviations and the fractional post-treatment parameter changes $\Delta P = (P_{\text{post}} - P_{\text{pre}})/P_{\text{pre}}$ were calculated for each animal and averaged within each treatment group. The parameter distributions (fractions of tumor voxels versus the parameter value) were examined using the normalized histograms of voxel data pooled within each treatment group. The correlations between imaging-derived and histological parameters were assessed using Pearson's correlation coefficient. Within each treatment group, the MRI- and PET-derived parameters before and after treatment were compared using paired Student's t-test. The comparison between the cediranib-treated and control groups was done with an unpaired t-test. All statistical tests were two-sided and results were deemed statistically significant at $p < 0.05$.

Results

Effect of cediranib on tumor DCE and DW MRI parameters

Cediranib was well tolerated by the animals at all doses used in the dose selection study (0.75, 1.5 and 3 mg/kg/day). No significant differences in weight were observed between the animals in the drug treatment and control groups over the course of treatment. Administered

daily over 26 days, cediranib at 1.5 mg/kg and 3 mg/kg inhibited the growth of HT29 tumors significantly better than cediranib at 0.75 mg/kg or vehicle ($p < 0.001$ for all t-tests) (Fig. 1). The dose 3 mg/kg/day was selected for subsequent MR and PET imaging studies.

In cediranib-treated tumors, the initial upslopes of the mean gadolinium concentration versus time curves decreased on average by $60 \pm 26\%$ relative to their respective pre-treatment values ($p = 0.006$) (Fig. 2A). The maximum tumor concentration showed a tendency to decrease after treatment, but this trend did not reach significance ($p = 0.08$). The enhancement of the vehicle-treated tumors (Fig. 2B) or muscle in either group (Fig. 2C,D) did not show significant changes after treatment.

Maps of MRI-derived parameters $nIAUC_{90}$, K^{trans} , v_e and ADC for a representative tumor before and after treatment with cediranib are shown in Figure 3. The maps of $nIAUC_{90}$ and K^{trans} are similar. In the outer regions of the tumor, both $nIAUC_{90}$ and K^{trans} are several times higher than their respective values in the muscle, but in the tumor center these parameters are comparable to their muscle values. The values of v_e are high ($v_e > 0.5$) around the tumor and v_e in the tumor periphery is about two to three times higher than in the muscle.

After treatment with cediranib, both $nIAUC_{90}$ and K^{trans} decrease to the muscle level or below throughout most of the tumor except for a narrow region around the tumor (Fig. 3, middle row). The inner regions with slow pre-treatment enhancement show nearly no enhancement after treatment. The parameter v_e also decreases after treatment, though not uniformly throughout the tumor. The largest drop in v_e occurs in areas around the tumor and in the tumor center, but is not as dramatic in the tumor rim. The post-treatment tumor ADC does not show marked changes and appears to be only slightly higher than its pre-treatment value, which may be due to a difference in slice location between the pre- and post-treatment imaging sessions.

The differences in enhancement across the tumor before and after treatment are illustrated by the concentration versus time curves and the corresponding RR model fits and parameters from four voxels selected in (1) the tumor rim, (2) just outside the tumor, (3) the slowly enhancing central region and (4) non-enhancing central region (Fig. 3, bottom row). The peripheral voxels 1 and 2 show the highest initial upslope and amplitude of enhancement and yield the highest K^{trans} and $R^2 > 0.9$. Voxels with low overall enhancement, such as voxel 4, show lower goodness of fit ($R^2 = 0.7$) and model parameters that are close to their lower bounds. Voxels in which the concentration curve reaches a plateau (1 pre and post, 2 post and 3 post) provide lower v_e values ($v_e = 0.3$) than voxels in which the concentration increases continuously throughout the experiment (2 pre, 3 pre; $v_e > 0.5$). The pre-treatment curve of voxel 3 has a low initial upslope, but eventually reaches a level comparable to the concentration in the tumor rim, and therefore yields low K^{trans} and a high v_e value ($v_e = 0.88$).

ADC is low and relatively uniform in the periphery of the tumor (below $0.7 \cdot 10^{-3} \text{ mm}^2/\text{s}$) (Fig. 3). Central areas of slightly elevated ADC ($(0.8\text{--}1.2) \cdot 10^{-3} \text{ mm}^2/\text{s}$) correspond to the slowly enhancing regions on DCE MRI that have low K^{trans} values. Areas with the highest ADC values ($(1.2\text{--}2.5) \cdot 10^{-3} \text{ mm}^2/\text{s}$) correspond to the non-enhancing regions with the lowest values of K^{trans} and v_e .

The histological sections (H&E and Hoechst perfusion marker, Fig. 3, bottom row) show that the tumor rim contains perfused, viable tissue, which corresponds to the areas of high $nIAUC_{90}$ and K^{trans} . The inner regions of the tumor are mostly necrotic. Within the necrotic areas, the acellular and cystic regions correspond to the regions with the lowest perfusion/permeability parameters and the highest ADC values, whereas regions containing islands of

viable tissue correspond to the areas of decreased nIAUC90 and K^{trans} , higher v_e and intermediate ADC.

The mean pre- and post-treatment parameters nIAUC90, EnF, K^{trans} , v_e and ADC for each tumor are shown in Figure 4A–E and the mean values within each group are summarized in Table 1. The fractional post-treatment changes in each parameter are illustrated in Figure 4F. Before treatment, none of the parameters were significantly different between the cediranib group and control group. After treatment with cediranib, both nIAUC90 and EnF decreased in all six animals (Fig. 4A,B) and the mean tumor nIAUC90 decreased on average by $48 \pm 18\%$ ($p = 0.002$, paired t-test between pre- and post-treatment values), while the EnF dropped by $43 \pm 20\%$ ($p = 0.003$) (Fig. 4F). In the control group, nIAUC90 and EnF did not change significantly (nIAUC90, $p = 0.99$; EnF, $p = 0.32$). The mean post-treatment nIAUC90 values were lower in the drug-treated group than in the control group, although the difference did not reach significance ($p = 0.077$). The post-treatment EnF was significantly lower in the cediranib group than in the vehicle group (0.45 ± 0.21 versus 0.72 ± 0.07 , $p = 0.012$, unpaired t-test).

The post-treatment changes were also evident in the RR model parameters (Fig. 4C,D,F). In the cediranib group, K^{trans} decreased in all six animals and dropped on average by $57 \pm 11\%$ ($p = 0.003$). The EES volume v_e also showed a tendency to decrease by $24 \pm 25\%$, which was just short of significance ($p = 0.059$). In the control group, neither K^{trans} nor v_e showed significant differences (K^{trans} , $p = 0.55$; v_e , $p = 0.90$). The values of K^{trans} correlated well with nIAUC90 in all twelve tumors before treatment ($R = 0.85$, $p = 0.0004$) and after treatment ($R = 0.84$, $p = 0.0005$), as expected from simulations (34,42).

The mean tumor ADC values were concentrated in the range of $(0.6\text{--}0.9) \cdot 10^{-3} \text{ mm}^2/\text{s}$ except for one outlier with a higher ADC in the cediranib-treatment group (Fig. 4E). No significant changes between the mean pre- and post-treatment ADC values were observed in animals treated with cediranib ($p = 0.33$) or vehicle ($p = 0.58$) (Fig. 4F). There was a weak and not statistically significant correlation between the mean tumor ADC and v_e values for all tumors before treatment ($R = 0.50$, $p = 0.13$) or after treatment ($R = 0.33$, $p = 0.32$).

The treatment-induced changes in the distributions of MRI-derived voxel parameters are illustrated by the histograms in Figure 5. The pre-treatment distributions of nIAUC90 values in the cediranib and control groups have similar means (2.57 ± 0.97 and 2.31 ± 0.86 , respectively, Table 1) and long right-hand-side tails. After treatment with cediranib, the number of voxels with $1.6 < \text{nIAUC90} < 9$ was considerably reduced with a concomitant increase in the number of voxels with $\text{nIAUC90} < 1.6$ (Fig. 5A). This indicates that the drug has an effect on voxels within a wide range of intermediate nIAUC90 values instead of a handful of highly enhancing voxels. In control animals, a small fraction of voxels is shifted towards higher nIAUC90 values after treatment (Fig. 5B). The distributions of K^{trans} reflect similar behavior: in the cediranib-treated group, voxels with $K^{\text{trans}} > 0.03 \text{ min}^{-1}$ migrate towards lower values (Fig. 5C). Within the control group, the distribution of K^{trans} changes little (Fig. 5D). A slight shift towards lower v_e values can be seen in the cediranib-treated group (Fig. 6E), and in the control group the distribution of v_e stays unchanged (Fig. 5F). The distributions of ADC are similar between the drug-treated animals (Fig. 5G) and control animals (Fig. 5H) and do not change after treatment.

Histological evaluation of vascular parameters and necrosis

The histological parameters of tumor vasculature and necrosis measured in the tumors studied by MRI are summarized in Table 2. The cediranib-treated and control tumors had the same number of CD31-positive vessels (59 per mm^2) and mean ICD (0.17 mm). The fractions of pericyte-associated (CD31/SMA-positive) vessels were also similar between the

two groups (80% versus 87%, $p = 0.12$). However, a significantly lower fraction of perfused (Hoechst-positive) vessels was observed in the cediranib treatment group than in the control group (cediranib, $81 \pm 7\%$; control, $91 \pm 3\%$, $p = 0.02$).

The perfused tumor fraction was slightly lower in the cediranib treatment group than in the control group (37% vs 44%, $p = 0.20$) and the necrotic fraction was slightly higher than in controls (34% vs 26%, $p = 0.23$), but neither of these differences was significant. There was a significant negative correlation between the histological necrotic fraction and EnF ($R = -0.74$, $p = 0.006$) (supplementary material, Figure S1). A negative correlation was also found between the necrotic fraction and the mean post-treatment nIAUC90 ($R = -0.61$, $p = 0.035$) or K^{trans} ($R = -0.58$, $p = 0.046$) values from all twelve animals. There was no significant association between the histological perfusion fraction and any of the MR-derived parameters.

Effect of cediranib treatment on ^{18}F -FMISO SUV

The pre-treatment SUVmean values were similar between the cediranib-treated and control animals (cediranib, 0.53 ± 0.04 ; control, 0.53 ± 0.06 , Table 3). Treatment with cediranib decreased the tumor ^{18}F -FMISO uptake (Fig. 6A,B) and reduced the post-treatment tumor SUVmean values by $33 \pm 19\%$ ($p = 0.008$) and SUVmax by $22 \pm 20\%$ ($p = 0.04$) (Figs. 6C-E). In the control group, the changes in the ^{18}F -FMISO uptake measures were not statistically significant: SUVmean changed by $-4 \pm 9\%$ ($p = 0.16$) and SUVmax by $-6 \pm 12\%$ ($p = 0.18$) (Figs. 6C-E).

The histological necrotic fraction in tumors studied with PET was similar to the necrotic fraction in tumors studied by MRI and was slightly higher in cediranib-treated tumors than in controls (cediranib, $31 \pm 5\%$, controls, $22 \pm 9\%$, $p = 0.078$). The perfusion fraction was slightly, but not significantly higher in the drug-treated tumors than in controls (cediranib, $40 \pm 4\%$; controls, $33 \pm 14\%$, $p = 0.30$). There was a significant negative correlation between the pooled drug-treated and control post-treatment SUVmax values and perfusion fraction ($R = -0.74$, $p = 0.016$) and a non-significant correlation between SUVmean and perfusion fraction ($R = -0.52$, $p = 0.13$).

Discussion

We used DCE and DW MRI and ^{18}F -FMISO PET to examine the acute effects of cediranib on HT29 colorectal tumors in nude rats. Treatment with cediranib (3×3 mg/kg daily) resulted in approximately 60% reduction in the initial upslope of the mean tumor gadolinium concentration and a trend towards decreased maximum concentration (Fig. 2). Comparable reductions of the upslope and the maximum gadolinium concentrations were observed by Bradley *et al.* in C6 and LoVo tumors treated with cediranib (3×3 mg/kg over 52 h) (28). The muscle-normalized initial uptake integral over 90 s, nIAUC90, decreased significantly by 48%, which was greater than the reductions of IAUC over 60 s observed in C6 and LoVo tumors (23% and 33%, respectively) (28). The post-treatment reduction of the contrast uptake affected a large part of the tumor, as shown by the decrease of the enhancing fraction EnF from 78% at baseline to 45% after cediranib treatment (Figs. 3 and 5). This finding is also illustrated by the voxel histograms of nIAUC90, which show that after treatment a considerable fraction of voxels shifted towards the lower values (Fig. 5). The K^{trans} values derived using RR model were in the range of 0.015 – 0.1 min^{-1} (Fig. 4) and were similar to the K^{trans} obtained by Bradley *et al.* using the Tofts model and an average arterial input function (28). In our study, K^{trans} decreased on average by 57% in cediranib-treated tumors, which was a larger effect than the 30% reduction in K^{trans} reported by Bradley *et al.* in subcutaneous tumors (28) and a 34% drop observed by Farrar *et al.* in mouse brain tumors (12). In contrast, in the control group, nIAUC90, EnF or K^{trans} showed no significant

changes after treatment. The mean tumor values of v_e (<0.35) obtained in this study were within the range reported in animal tumors and decreased after cediranib treatment by 24%, which was non-significant ($p = 0.059$). In comparison, Farrar *et al.* observed a significant post-treatment drop in v_e by 32% (12), whereas Bradley *et al.* reported very low baseline v_e values (2–3%) that remained unchanged after treatment (28).

The reduction in perfusion/permeability measures $nIAUC90$ and K^{trans} after cediranib treatment did not appear to be associated with the decrease in the histologically measured microvessel density or pericyte coverage, which were found to be similar in the cediranib treatment and control groups (21,29). However, we observed a slightly, but significantly lower number of *perfused* blood vessels in the cediranib-treated group than in the control group (80% versus 91%, respectively, Table 2). It has been noted that the loss of VEGF signaling can result in local vasoconstriction via reduction of nitric oxide production (43), which may be partially responsible for the observed effect. Previous studies have shown that cediranib causes rapid drops in the vascular permeability (12,25,29). In SW620 colorectal xenografts in rats treated with cediranib (2×3 mg/kg over 24 h), Bradley *et al.* (30) observed small decreases in the initial upslope of the concentration of an intravascular contrast agent (gadomelitol) and dramatic reductions in the amplitude of enhancement curves. These changes corresponded to a 20% reduction in the tumor blood flow, an 80% drop in the permeability surface area product and a 70% drop in the blood volume. In the current study performed with low molecular weight gadolinium chelate, a large reduction in the initial concentration upslope and a corresponding decrease in K^{trans} likely reflect combined post-treatment reductions in both the blood flow and permeability.

We chose the RR model with the muscle as the reference tissue, because measuring the arterial input function in individual animals was not feasible in our experiments and using an average (population) input function from literature (44) was problematic, because the effect of cediranib on the rat systemic circulation has not been established. The tumor parameters calculated using the RR model may be affected by the variations in the reference tissue curve and the accuracy of the reference tissue parameters, especially K^{trans} (36,45). We found that the muscle concentration curves and their $IAUC90$ values in the cediranib treatment and control groups were similar and did not change significantly after treatment in either group (Fig. 2). We used the reference tissue parameters obtained in the rat flank muscle using measured AIF ($K^{trans} = 0.021 \text{ min}^{-1}$ and $v_e = 0.07$) by Yankeelov *et al.* (38). Similar values were obtained by Beaumont *et al.* in rat temporal muscles ($K^{trans} = 0.019 \text{ min}^{-1}$, $v_e = 0.074$) (39) and by Kershaw and Buckley in human periprostatic muscle ($K^{trans} = 0.015 \text{ min}^{-1}$ and $v_e = 0.08$) (46).

The spatial distributions and mean values of ADC (Figs. 3 and 5) obtained in this study are similar to the ADC results reported in HT29 tumors by Jordan *et al.* (47). In our study, neither the mean ADC values (Fig. 4) nor the voxel ADC distributions (Fig. 5) showed significant changes after treatment with either cediranib or vehicle, which suggests that treatment with cediranib over our study period did not result in abrupt changes of tumor necrosis. This observation is supported by the non-significant difference of the histological necrotic fraction between the drug-treated and control tumors and is also consistent with published studies that have found no significant ADC changes after short-term antiangiogenic treatment (11) or small reductions in ADC (6%) (12). In contrast, vascular disruptive agents, which induce rapid onset of necrosis, often cause a significant increase of ADC within few days after treatment (10). It should also be noted that the ability of ADC to reflect tumor necrosis may depend on the pattern of necrosis: as shown by Lyng *et al.* (48), ADC correlates with histological necrotic fraction in tumors with larger contiguous necrotic areas, but not in tumors that contain multiple small islands of necrosis.

In a recent study of human breast tumors, Arlinghaus *et al.* (49) reported an absence of correlation between the mean or voxel values of the tumor v_e and ADC, although both these parameters are thought to reflect the properties of the EES and therefore are expected to be directly related. We too found that v_e and ADC were not significantly correlated. We believe that the lack of correlation is caused by the tumor heterogeneity and various extrinsic factors. In particular, the reliability of determining v_e depends on the SNR of the experiment, magnitude of initial contrast uptake, temporal resolution and total acquisition time. Simulations by Jaspers *et al.* (50) have shown that v_e approximately equal to its value in the muscle ($v_e = 0.09$) can be reliably estimated for true K^{trans} values ranging between 0.01 min^{-1} and 0.1 min^{-1} from data with 3% noise acquired for 15 min. For K^{trans} values to the left and right of this interval, v_e tends to become increasingly overestimated with an error up to 50%. Our observations are consistent with this prediction. Voxels in the tumor rim have intermediate K^{trans} ($0.02\text{--}0.05 \text{ min}^{-1}$) and v_e values ($0.1\text{--}0.3$). Voxels with enhancement that increases throughout the experiment and shows no wash-out or plateau tend to yield high v_e values ($v_e > 0.5$). Such voxels may be located in the tumor center, where the contrast wash-in is slow and K^{trans} is low ($K^{trans} \sim 0.01 \text{ min}^{-1}$, e.g., voxel 3 pre in Fig. 3) or in the tumor periphery, where K^{trans} is high ($K^{trans} > 0.1 \text{ min}^{-1}$, voxel 2 pre). In non-enhancing voxels, fitting converges to the lower parameter bounds.

On ADC maps, three tissue types can be roughly identified: viable tumor ($\text{ADC } 0.8\cdot 10^{-3} \text{ mm}^2/\text{s}$), early necrosis or mixed viable and necrotic tissue ($0.8\cdot 10^{-3} < \text{ADC} < 1.2\cdot 10^{-3} \text{ mm}^2/\text{s}$) and mostly necrotic tissue ($1.2\cdot 10^{-3} < \text{ADC} < 2.5\cdot 10^{-3} \text{ mm}^2/\text{s}$). Viable tissue shows low and relatively uniform ADC values and corresponds to the similarly uniform areas of $v_e < 0.3$. Mixed tissue corresponds to the heterogeneous central regions that include areas with high v_e values ($v_e > 0.5$). Necrotic areas have high ADCs and correspond to the regions with the lowest v_e ($v_e < 0.05$). Thus a tumor containing viable tissue and early necrosis might produce a correlation between the voxel values of v_e and ADC. For a tumor comprising all three tissue types (Fig. 3), a scatter plot of v_e versus ADC contains three overlapping clouds of points and shows no correlation between parameters. In such tumors, segmenting different tissue subpopulations using various clustering approaches may be required to correctly assess the effect of treatment (51).

The pre-treatment values of ^{18}F -FMISO SUVmean and SUVmax obtained in this study are comparable to the results previously reported in HT29 tumors in mice (20). Both SUV measures decreased significantly after cediranib treatment (Fig. 6) and the post-treatment SUV values negatively correlated with the histological perfusion fraction. Valable *et al.* (18) have shown that in mouse gliomas treatment with sunitinib over six days increased the MRI-derived cerebral blood volume and reduced vessel permeability and decreased ^{18}F -FMISO uptake measured 3 h after tracer injection, which was attributed to the decrease in hypoxia following vascular normalization. However, in mouse mammary tumors treated with an anti-VEGF antibody, hypoxia, assessed by the hypoxia marker pimonidazole, increased after two days of treatment and longer treatment intervals (52). Therefore post-therapy decreases in uptake of hypoxia PET tracers should be interpreted with caution. In our study, the similarity in the magnitude of the post-cediranib reductions in ^{18}F -FMISO SUV (33%), and the MR-derived parameters nIAUC90 (48%) and K^{trans} (57%) suggest that the decrease in the SUVmean may be caused by the reduced delivery of the tracer rather than an increase in hypoxia. A similar reduction in ^{18}F -FMISO uptake was observed following treatment with the antivascular compound DMXAA and attributed to a reduction in the delivery of ^{18}F -FMISO to the tumor, rather than to decrease of the underlying tumor oxygenation (20).

Our study had several limitations. First, different small cohorts of animals were used for MRI and PET studies. This was done because performing long sequential imaging studies in the same animals twice over the short study period was impractical. Second, no co-

registration between pre- and post-treatment images or between the images and histological sections was performed. Third, to derive perfusion/permeability parameters we used the RR model, which is less precise than the models that include an arterial input function. The RR model may be inaccurate in the central tumor regions, where the contrast may be delivered by diffusion through EES rather than by vascular perfusion, or in the highly vascularized areas that may contain a large vascular fraction which was ignored in the RR model used here.

In conclusion, we observed that treatment with cediranib ($3 \times 3\text{mg/kg}$ dosed daily) results in a change in tumor blood vessel functionality that can be measured using DCE MRI and ^{18}F -FMISO PET. These changes are apparent before significant morphological changes in tumor vasculature are observed. The decrease in ^{18}F -FMISO mean SUV observed after cediranib treatment may represent changes in both tumor hypoxia and tracer delivery. Therefore, without untangling the effects of tracer delivery from the overall tracer uptake, ^{18}F -FMISO PET imaging may not be a reliable early biomarker of hypoxia after anti-angiogenic treatment. The interpretation of the imaging biomarkers remains nontrivial and needs to be studied further in comparison with the histological analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biography

Jane Halliday

On October 3rd, 2010, Dr. Jane Halliday sadly passed away following an accident. Dr. Halliday was a gifted scientist with great insight and enthusiasm, which she used to make many significant contributions to her chosen field. Her sense of true enjoyment was evident in all that she did and was a source of inspiration to all who had the good fortune to know and work with her. Jane was a great friend and colleague to many and her loss is keenly felt.

Abbreviations

ADC	apparent diffusion coefficient
DCE MRI	dynamic contrast enhanced magnetic resonance imaging
DW MRI	diffusion weighted MRI
EES	extravascular extracellular space
EnF	enhancing fraction

^{18}F-FMISO	^{18}F -fluoromisonidazole
H&E	hematoxylin and eosin
IAUC	initial area under the curve
nIAUC	normalized IAUC
ICD	intercapillary distance
K^{trans}	volume transfer constant from the plasma to extracellular extravascular space
PET	positron emission tomography
v_e	fractional volume of extravascular extracellular space
RARE	rapid acquisition with refocused echoes
ROI	region of interest
SMA	smooth muscle actin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Figure 1.

Growth curves of HT29 human colorectal xenograft tumors grown subcutaneously in rats and treated for 26 days by daily administration of vehicle or cediranib at the dose of 0.75 mg/kg, 1.5 mg/kg and 3 mg/kg ($n = 6$ in each group). Symbols are mean volumes over six rats in each group; error bars are standard errors in one direction. At the beginning of the treatment, the mean $\pm$ SE control tumor volumes were $330 \pm 80 \text{ mm}^3$ and the mean drug-treated tumor volumes were $270 \pm 70 \text{ mm}^3$ (0.75 mg/kg), $230 \pm 80 \text{ mm}^3$ (1.5 mg/kg) and $260 \pm 60 \text{ mm}^3$ (3 mg/kg). Cediranib administered at the doses of 1.5 mg/kg and 3 mg/kg inhibited tumor growth significantly better than vehicle or cediranib at 0.75 mg/kg. At the end of the study, the mean volume of the control tumors was $1500 \pm 750 \text{ mm}^3$. The final tumor volumes in cediranib treatment group were $1300 \pm 1000 \text{ mm}^3$ (0.75 mg/kg), $90 \pm 30 \text{ mm}^3$ (1.5 mg/kg) and $100 \pm 30 \text{ mm}^3$ (3 mg/kg) in the three treatment groups. The dose of 3 mg/kg was adopted for the imaging studies.

Figure 2.

Gadolinium concentration versus time in the tumor (A, B) and muscle (C, D) before and after treatment with cediranib or vehicle. Data represent the mean tumor and muscle curves averaged within each treatment group (filled symbols, pre-treatment; open symbols, post-treatment) and the 95% confidence intervals (CI) within each group (shaded areas; darker gray, pre-treatment; lighter gray, post-treatment). Enhancement curves from individual ROIs were aligned so that bolus arrival time corresponds to $t = 0$. Treatment with cediranib significantly decreases the initial upslope of the tumor curve, as indicated by the non-overlapping CI between 0 s and 120 s. At later time points, the amplitudes of the pre- and post-treatment tumor curves are not significantly different (overlapping CI), mainly because in some tumors the enhancement continuously increases throughout the duration of experiment. Treatment with vehicle does not affect the contrast concentration in the tumor (B). The muscle curves show minimal changes after treatment with either cediranib or vehicle (C, D).

Figure 3. MR images and parametric maps (top row: pre-treatment; middle row: post-treatment with cediranib, 3 × 3mg/kg daily) and histology images (bottom row) for a representative slice from a cediranib-treated tumor: T2-weighted images ($b = 0 \text{ s/mm}^2$ DW image), nIAUC90, K^{trans} , v_e and ADC. The maps of nIAUC90 and K^{trans} show higher values in the tumor periphery and low values in the center of the tumor and both parameters decrease markedly after treatment. The fractional EES volume v_e is high around the tumor and some areas in the tumor center. After treatment, the v_e decreases dramatically in these areas, but remains relatively unchanged in the rest of the tumor. The post-treatment changes are not obvious on maps of ADC. Histological H&E-stained section shows the tumor consisting of a viable rim and a largely necrotic core. The distribution of Hoechst 33342 perfusion marker is also consistent with strongly perfused tumor rim and non-perfused core with islands of perfused tissue. The plots and table show voxel concentration curves, RR model fits and parameters in four voxels selected in different regions of the tumor: (1) tumor rim; (2) just outside the tumor; (3) slowly enhancing central region (4) non-enhancing central region. Voxels 1 and 2 (tumor rim and capsule) have high pre-treatment enhancement, which decreases considerably after treatment. Voxel 3 enhances slowly before treatment to the level of the tumor rim, but after treatment its enhancement drops. Voxel 4 shows very low enhancement throughout the experiment before or after treatment. The goodness of the model fits, as shown by R^2 , is higher for voxels 1–3 than for the barely enhancing voxel 4.

Figure 4. Mean tumor parameters before and after treatment: (A) nIAUC90, (B) EnF, (C) K^{trans} and (D) v_e , (E) ADC, and (F) fractional parameter changes after treatment. The thin horizontal lines indicate the mean values within each group and the vertical whiskers with short bars at the ends represent one standard deviation in each direction. In (F), the parameters that changed significantly after treatment with cediranib are indicated with asterisks: nIAUC ($p = 0.002$), EnF ($p = 0.003$), and K^{trans} ($p = 0.003$) (Table 1). The changes in v_e ($p = 0.059$) and ADC ($p = 0.33$) were not significant. In control group, none of the parameters changed significantly.

Figure 5.

Voxel histograms (fraction of tumor voxels versus parameter values) for pooled data from cediranib-treated animals (left column) and vehicle-treated animals (right column). In drug-treated animals, nIAUC90 (A) shows a shift of voxels to lower values after treatment, while nIAUC90 in controls (B) there is a slight redistribution of voxels to higher values. A shift to lower values is observed in K^{trans} in drug-treated group (C), but not in controls (D). The EES volume v_e shows a tendency to decrease after cediranib treatment (E), but remains unchanged in controls (F). The ADC does not change in either cediranib-treated animals (G) or in controls (H).

Figure 6. Hypoxia tracer ^{18}F -FMISO PET imaging of a cediranib-treated HT29 tumor. Representative PET images at 90 min after tracer administration acquired before treatment (A) and after treatment (B) with cediranib show decreased post-treatment tracer uptake. The values of SUVmean (C) and SUVmax (D) decrease in all cediranib-treated animals, whereas in control animals SUVmean and SUVmax do not show a clear trend. In panels (C) and (D), the thin horizontal lines indicate the mean values within each group and the vertical whiskers with short bars represent one standard deviation in each direction. Both parameters decrease significantly after treatment with cediranib (asterisks) (SUVmean, $p = 0.008$; SUVmax, $p = 0.04$), but did not change significantly in controls (E).

Table 1

MRI-derived parameters in rats with HT29 tumors treated with cediranib (3×3 mg/kg daily) and vehicle-treated controls.

Group	nIAUC90	EnF	K ^{trans} , min ⁻¹	v _e	ADC, 10 ⁻³ mm ² /s
Cediranib (n = 6)					
Pre-treatment	2.57±0.97	0.78±0.13	0.063±0.023	0.23±0.08	0.78±0.25
Post-treatment	1.40±0.97	0.45±0.21	0.027±0.012	0.17±0.08	0.74±0.18
p-value	0.002	0.003	0.003	0.059	0.33
Vehicle (n = 6)					
Pre-treatment	2.31±0.86	0.68±0.11	0.048±0.021	0.24±0.06	0.70±0.10
Post-treatment	2.31±0.58	0.72±0.07	0.051±0.021	0.24±0.03	0.73±0.10
p-value	0.99	0.32	0.55	0.90	0.58

Data are mean ± standard deviation within each group. The p-value is given for the paired t-test between the pre- and post-treatment data within each treatment groups.

Table 2

Histological vascular and necrosis markers measured in HT29 tumors after treatment with cediranib (3×3 mg/kg dosed daily, n = 6) or vehicle (n = 6).

	CD31 (number/mm ²)	ICD (mm)	SMA (%)	Hoechst (%)	Perfused fraction (%)	Necrotic fraction (%)
Cediranib	59±18	0.17±0.04	80±11	80±7	37±10	0.34±0.12
Vehicle	59±4	0.17±0.03	87±3	91±3	44±10	0.26±0.10
p-value	0.97	0.94	0.12	0.02	0.20	0.23

CD31, the number of vessels per square millimeter; SMA, the fraction of vessels positive for CD31 and SMA; Hoechst, the fraction of vessels positive for CD31 and Hoechst; ICD, intercapillary distance; perfused fraction, fractional area of the tumor with Hoechst staining intensity above the intensity of necrotic regions; necrotic fraction, fractional area of the necrotic regions outlined on H&E sections. The p-value corresponds to the two-tailed, unpaired Student's t-test between the cediranib-treated and control tumors.

Table 3

PET-derived parameters in rats with HT29 tumors treated with cediranib (3×3 mg/kg dosed daily) and vehicle-treated controls.

Group	Tumor SUV _{mean}	Tumor SUV _{max}
Cediranib (n = 6)		
Pre-treatment	0.53±0.04	0.97±0.09
Post-treatment	0.35±0.09	0.74±0.18
p-value	0.008	0.04
Vehicle (n = 4)		
Pre-treatment	0.53±0.06	1.09±0.15
Post-treatment	0.51±0.06	1.03±0.22
p-value	0.44	0.37

Data are mean ± standard deviation within each group. The p-value corresponds to the paired t-test between the pre- and post-treatment data within each treatment group.