Lack of Microvessels in Well-Differentiated Regions of Human Head and Neck Squamous Cell Carcinoma A253 Associated with Functional Magnetic Resonance Imaging Detectable Hypoxia, Limited Drug Delivery, and Resistance to Irinotecan Therapy

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ABSTRACT

Purpose: Combination chemotherapy with irinotecan (CPT-11; 50 mg/kg/week × 4 intravenously), followed 24 hour later by 5-fluorouracil (50 mg/kg/week × 4 intravenously), results in 10 and 100% cure rates of animals bearing human head and neck squamous cell carcinoma xenografts A253 and FaDu, respectively. A253 consists of 30% well-differentiated and avascular and 70% poorly differentiated regions with low microvessel density (10/×400), whereas FaDu is uniformly poorly differentiated with higher microvessel density (19/×400). Studies were carried out for determining the role of well-differentiated and avascular regions in drug resistance in A253 and detection of such regions with noninvasive functional magnetic resonance (fMR) imaging.

Experimental Design: Tumors were harvested for histopathologic evaluation and immunohistochemistry (CD31, CD34; differentiation marker: involucrin; hypoxia markers: carbonic anhydrase IX, pimonidazole; vascular endothelial factor (VEGF) and Ki67) immediately after fMR imaging following the 3rd dose of chemotherapy. High-performance liquid chromatography determination of intratumoral drug concentration of 7-ethyl-10-hydroxy-9-14C-labeled CPT-11 concentrations compared with poorly differentiated regions (positive for involucrin) lacking microvessels with a hypoxic rim (positive for carbonic anhydrase IX and pimonidazole) containing few proliferating (Ki67 positive) poorly differentiated cells. Autoradiography revealed that well-differentiated A253 tumor regions showed 5-fold lower 14C-labeled CPT-11 concentrations compared with poorly differentiated areas (P < 0.001). Blood oxygen level dependent fMR imaging was able to noninvasively distinguish the hypoxic and well-vascularized regions within the tumors.

Conclusion: Avascular-differentiated regions in squamous cell carcinoma offer sanctuary to some hypoxic but viable tumor cells (carbonic anhydrase IX and Ki67 positive) that escape therapy because of limited drug delivery. This study provides direct evidence that because of a specific histologic structure, avascular, well-differentiated hypoxic regions in tumors exhibit low drug uptake and represent a unique form of drug resistance.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the fifth leading cause of cancer incidence and the sixth leading cause of cancer-related death in the United States (1). These tumors frequently respond to chemotherapeutic agents primarily used for palliation or as adjuvant therapy in conjunction with surgery and radiation (2). Irinotecan (CPT-11), a top I inhibitor in clinical development, is enzymatically converted in vivo by carboxylesterase 2 in liver and intratumorally to its most active metabolite, 7-ethyl-10-hydroxy-9-camptothecin (SN-38; ref. 3–5). The lactone form is active, with SN-38 lactone being 100- to 1,000-fold more cytotoxic than the prodrug in its lactone form (6). The antitumor activity and toxicity of combining CPT-11 with 5-fluorouracil (FUra) is highly sequence dependent, with a sequence of CPT-11 preceding FUra that results in the most significant increase in therapeutic index (7). Earlier studies from this laboratory in which clinically relevant combination of CPT-11 (50 mg/kg/week × 4 intravenously) was used followed 24 hours later by FUra (50 mg/kg/week × 4 intravenously) on FaDu and A253 tumors growing subcutaneously in nude mice achieved cure rates of 100 and 10%, respectively (8).

Moreover, our preliminary neoangiogenesis studies in these tumors yielded a surprising and interesting observation about the morphological distribution of the apparently chaotic microvessels that has not been reported previously to the best of our knowledge. Using CD31 immunostaining specific for microvessels, we observed that well-differentiated regions in A253...
Differentiated, Avascular Regions and Drug Resistance

Tumors (with or without keratin pearl formation) had no microvessels. This finding is consistent with the fact that normal squamous epithelium also lacks microvessels. Because A253 originates from the squamous epithelium, it is not surprising that well-differentiated tumor regions share this aspect of the normal differentiation process. In these regions, multilayered cells are bound tightly together with no space for connective tissue containing microvessels. A253 tumors consist of about 30% well-differentiated regions without microvessels and 70% poorly differentiated regions with a low microvessel density (MVD; 10/400 high power fields). FaDu tumors are uniformly, poorly differentiated with a higher MVD (19/400 high power fields). Because of the potential therapeutic impact of these observations in relation to clinical outcomes, this study was undertaken to determine the role and significance of differentiation-linked absence of MVD, and the possible consequence this might have for drug distribution and therapy resistance. We hypothesized that avascular and, as a consequence, hypoxic regions within tumors would have limited drug distribution and uptake, and thus these regions could confer resistance to therapy. Indeed, poorly differentiated proliferating tumor cells located in the outer rims of such areas were found to escape therapy, supporting this premise.

Tumor vasculature is characterized by the following: (a) increased permeability to macromolecules because of large endothelial cell gaps or fenestrae (9); (b) incomplete basement membrane and relative lack of pericyte or smooth muscle associated with endothelial cells (10); (c) increased vascular tortuosity (11); (d) vasodilation (12); (e) intermittent or unstable flow (12); and (f) spontaneous hemorrhage and low vascular density mixed with regions of high angiogenic activity (11). Tumor hypoxia is thus a potential therapeutic hurdle resulting from this aberrant vasculature. In fact, hypoxia has also been recently considered a morphogen, inducing terminal differentiation of cells in well-differentiated squamous cell carcinoma (13). For the immunohistochemical detection of hypoxia, carbonic anhydrase IX (CAIX) has been proposed as an intrinsic, innate constitutive marker and was found to correlate with lowered oxygen tension (≤1%) in tumors (14–16). Pimonidazole hydrochloride stains hypoxic regions with a partial pressure of oxygen < 10 mm Hg. Hypoxia-regulated CAIX has been found to relate to poor vascularization and resistance of HNSCC to chemoradiotherapy in patients (17).

Tumor vascular function (i.e., the ability of vessels to transport oxygen and nutrients) has been noninvasively studied by mapping changes in the blood oxygen level dependant (BOLD), functional magnetic resonance (fMR) signal intensity in functional vessels in response to a controlled modulation of blood oxygenation by inhalation of carbogen or other injectibles systemically administered (19). Initial reports indicate BOLD fMR signal intensity maps correlate with temporal and spatial fluctuations in the measured partial pressure of oxygen values in tumor and other tissues (20–22). This study uses the BOLD fMR method as a noninvasive tool for assessing the tumor vascular status: i.e., the net effect of vascular functionality, vessel density, and maturity in individual tumors.

Such an approach is important for several reasons. First, there is a broad spatial and temporal variation in tumor oxygenation and perfusion over many tumors as well as within individual tumors depending on their stage of development and environmental variables (23). And, because noninvasive methods for routine assessment of these factors do not currently exist, optimization of therapeutic efficacy in relation to these variables is not possible. Second, regional in vivo variability of angiogenic activity cannot be sufficiently assessed by a static, purely morphological analysis such as MVD measurements of histology samples. A more robust, easily implemented in vivo assay is needed. To this end, the feasibility of using BOLD fMR imaging methods for noninvasive monitoring of hypoxia and blood flow has been examined herein and validated with detailed histopathologic evaluation and immunohistologic methods. Spin echo, used for our study, is spatially more precise than gradient-echo, because large vessels downstream from tumors do not contribute to signal changes (24). Spin echo with a lower magnitude of response (a factor of ≥3) can be a limitation for brain activation studies but is not a drawback for tumor studies at the higher field strength of 4.7T (26).

**MATERIALS AND METHODS**

**Animals.** Female athymic nude mice (nu/nu, body weight, 20–25 g), 12 weeks of age, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and were kept three to five mice per cage with water and food ad libitum according to a protocol approved by the Institute Animal Care and Use Committee at Roswell Park Cancer Institute. Non-necrotic tumor pieces were transplanted subcutaneously and bilaterally on the back flanks. Treatments were started when tumor weights reached ~150 to 200 mg.

**Tumors.** Human HNSCC cell lines A253 (less sensitive to CPT-11/FUra therapy) and FaDu (sensitive to CPT-11/FUra therapy) were purchased from American Type Culture Collection (Manassas, VA). The cell lines were maintained as a monolayer in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), and were tested for *Mycoplasma* every two months with the *Mycoplasma* T.C. Rapid Detection System (Gen-Probe Inc., San Diego, CA). The xenografts were established initially by implanting subcutaneously and bilaterally on the back flanks. Treatments were started when tumor weights reached ~150 to 200 mg.

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tumor capsule facing the interior of the animal was stained with black ink (Bradley Products Inc., Bloomington, MN).

**Patient Samples of HNSCC.** We also studied formalin/paraffin sections of six human surgical samples of HNSCC (4 well-differentiated and 2 poorly differentiated tumors like A253 and FaDu xenografts, respectively) for intratumoral microvessel distribution using factor VIII (Von Willebrand factor) to confirm the preclinical results in corresponding human tumors.

**Drugs.** CPT-11 (Pharmacia, Kalamazoo, MI), FUra (Hoffmann-La Roche, Inc. Nutley, NJ), and 14C-labeled CPT-11 (Pharmacia Italia S.p.A, Nerviano, MI, Italy) were dissolved in sterile saline before use at a final concentration of 20 and 10 mg/mL, respectively. 14C Radiolabel is part of the quinoline diethyl active moiety of CPT-11 that does not get removed with the cleaved piperidino side chain during conversion to the biological active form by carboxylesterase intratumorally and in liver.

**Drug Doses and Schedules.** When tumor weights reached ~150 to 200 mg, the mice were treated with CPT-11 (100 mg/kg/week × 3 intravenously) followed 24 hours later by FUra (50 mg/kg/week × 3 intravenously). The maximum-tolerated dose for CPT-11 and FUra administered individually is 100 mg/kg for the weekly schedule. The combination therapy did not result in any substantial weight loss or adverse reaction in the mice.

**Immunohistochemistry.** Because detection of mouse endothelial cells by antmouse CD31 monoclonal antibody requires the use of zinc fixatives, we developed and optimized immunohistochemical methods for involucrin, CAIX, Hypoxyprobe, and Ki67 in zinc fixatives. This also ensured identical experimental conditions for all of the markers. The staining characteristics of the four markers after zinc fixation, and after the traditional 10% neutral buffered formalin fixation, were very similar. Before immunostaining, conventional H&E-stained sections were prepared for general histopathologic evaluation and for comparison with BOLD fMR images. Harvested tumors were fixed in zinc fixative (BD Biosciences, PharMingen, CA) overnight and then processed for paraffin sections. Immunohistochemical staining was carried out on 5-μm sequential sections on charged slides after conventional deparaffinization and quenching of endogenous peroxidase with 3% H2O2. Casein (0.03%) in PBS with 500 μL Tween for 30 minutes at room temperature was used to block unspecific binding in each immunoperoxidase reaction. We carried out all immunohistochemical procedures using the automated immunostainer (DAKO, Carpinteria, CA). Antigen retrieval was done with citrate buffer (pH 6) in microwave for 4 minutes (Hypoxyprobe and CAIX) or 10 minutes (Ki-67, factor VIII), allowed to cool for 15 minutes and then washed with PBS/500 μL/L Tween. Nuclear counterstaining was done with hematoxylin before being dehydrated, cleared, and coverslipped. All washes were done with PBS/500 μL/L Tween, and chromogen 3,3′-diaminobenzidine (DAKO) was used for all of the immunostainings for 5 minutes. An isotype-matched negative control was used on a duplicate slide in place of the primary antibody in the same concentration as the primary antibody. All histopathologic and immunohistologic analysis and interpretation was done directly by a board certified and experienced pathologist (Dr. Károly Tóth MD, Ph.D.) or under his supervision.

**Involucrin Staining.** Involucrin, a marker for keratinocyte terminal differentiation (like cytokeratin), is expressed in the suprabasal layers while being absent in the undifferentiated basal layer of stratified squamous epithelium. It is a marker for differentiation in human squamous cell carcinomas present in the differentiated cells and absent in the poorly differentiated tumor cells (27). Epidermis of a human skin biopsy was used as a known positive (suprabasal layers) and negative (basal layers) control to optimize the method. No antigen retrieval was necessary. Ten-minute fixation in 10% neutral buffered formalin was done after deparaffinization and before immunostaining.

**CD31 and CD34.** No antigen retrieval was necessary. The rat antmouse primary antibody CD31 (BD Biosciences PharMingen) and 10 μg/mL CD34 (clone RAM34, Research Diagnostics Inc., Flanders, NJ) was applied for 1 hour at room temperature. Biotinylated secondary antibody (antirat, BD Biosciences PharMingen) was used for 30 minutes followed by the streptavidin complex (Zymed Lab Inc., San Francisco, CA) for 30 minutes.

**Von Willebrand Factor (Factor VIII).** Rabbit antihuman polyclonal antibody (DAKO) in 11.4 μg/mL concentration was applied for 1 hour at room temperature. Biotinylated goat antirabbit (Vector Labs, Burlingame, CA) was used for 30 minutes, followed by streptavidin complex (Zymed Lab Inc.) for 30 minutes.

**Carbonic Anhydrase IX (M75).** The primary antibody M75 (20 μg/mL, a gift from Dr. Pastorek, Institute of Virology, Slovak Academy of Sciences, Slovak Republic) was applied for 90 minutes at room temperature. Incubation with Elite biotinylated secondary antibody (Vector Labs) for 30 minutes was followed by Elite ABC reagent (Vector Labs) for 30 minutes.

**Hypoxyprobe.** Mice received injection intraperitoneally with 60 mg/kg of Hypoxyprobe (pimonidazole hydrochloride, NPI, Belmont, MA) 45 minutes before BOLD fMR scanning. Sections were incubated with the primary antibody Hypoxyprobe-monoclonal antibody (1.4 μg/mL, NPI, Belmont, MA) for 1 hour at room temperature. Incubation with biotinylated IgG fragment [F(ab′)2, Accurate Chemical & Scientific Corporation, Westbury, NY] for 10 minutes was followed by the streptavidin complex (Zymed Lab Inc.) for 30 minutes.

**Vascular Endothelial Growth Factor.** Formalin/paraffin sections of untreated A253 xenografts were used for vascular endothelial growth factor (VEGF) detection. Polyclonal rabbit antihuman VEGF (0.25 μg/mL, A-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied overnight at 4°C as primary antibody. Incubation with Elite biotinylated secondary antibody for 30 minutes was followed by Elite ABC reagent (Vector Labs) for 30 minutes.

**Ki-67.** The mouse primary antibody against human Ki-67 (2 μg/μL, clone MB67, Diagnostic Biosystems, Pleasanton, CA) was incubated for 1 hour at room temperature. Incubation with biotinylated secondary antibody for 30 minutes was followed by the streptavidin-peroxidase complex for 30 minutes.
were euthanized by cervical dislocation, and the tumor was metabolite (see Drugs section above). After 2 hours, the mice according to the manufacturer’s instructions (Thermo Shandon, Pittsburg, PA). HNSCCs were implanted bilaterally in nude mice, so that both A253 and FaDu tumors would have the same drug exposure from circulation after drug treatment. Three separate mice with established tumors (approx. 200 mg) were treated with CPT-11 (100 mg/kg), tumors were excised, and blood was collected 2 hours after the treatment. Immediately after the collection, 5 mL of ice-cold methanol and acetonitrile (1:1) were added to tumor samples and homogenized with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). After centrifugation of the homogenate, the supernatant was evaporated to dryness, reconstituted to mobile phase, and subjected to high-performance liquid chromatography. Protein measurement was done on the pellet with Bradford protein assay (28). Plasma was obtained at 4°C immediately after the blood collection and was subjected to the same solvent extraction procedure and to high-performance liquid chromatography. Three replicates for these experiments were done. CPT-11 and SN-38 Measurements. We measured the lactone forms of CPT-11 and its active metabolite, SN-38, using a validated high-performance liquid chromatography method with fluorescence detection described by Warner and Burke (29). The separation method was carried out on a Waters Nova-Pak C18 column equipped with μBondapak C18 guard column, with mobile phase consisting of 20% acetonitrile and 80% triethylamine acetate. The detection was by fluorescence, with excitation at 370 nm and emission at 510 nm. The limits of quantitation were 2.5 ng/mL for both. Quality assurance was maintained by simultaneously assaying the quality control samples prepared in bulk before assay validation.

Protein Binding Measurements. CPT-11 analogs bind extensively to proteins. The lactone form seems to bind to proteins to a greater extent than the carbonate form (30, 31). To estimate the actual free (nonprotein bound) drug concentrations in the samples, control experiments were analyzed to evaluate the protein binding as described by Stewart et al. (31). This constitutes spiking mouse plasma with known concentrations of CPT-11 and SN-38 (100 to 1,000 ng/mL), equilibration at 25°C and centrifugal ultrafiltration with the Amicon Centrifuge ultrafiltration system. The ultrafiltrate was processed as described earlier to calculate the nonprotein bound fraction. CPT-11 metabolism to SN-38 lactone was measured in plasma and tumor tissue samples were taken 2 hours after drug administration. Samples were processed as outlined earlier.

High Resolution Autoradiography. To preserve and maintain the original tumor in vivo drug distribution, we did autoradiography using frozen sections; thereby, we avoided the normal embedding procedures that are known to lead to changes in the drug location during processing. 14C-labeled CPT-11 (specific activity, 59 mCi/mmol) was mixed with cold CPT-11 and given intravenously to achieve a dose of 100 mg/kg, with an activity of 10 μCi (specific activity of 0.29 μCi/mmol/L) to mice bearing HNSCC xenografts. On the basis of the location of the 14C label, the radiolabel remains a part of the drug’s active metabolite (see Drugs section above). After 2 hours, the mice were euthanized by cervical dislocation, and the tumor was excised and frozen with cryogel (Instrumedics Inc, Hackensack, NJ) in liquid nitrogen before being stored in −80°C freezer until sectioning. Frozen sections (6–8 μ) were cut by cryostat (−30°C) and put on amino-acyl silane-treated slides (charged slides) and stored in −80°C freezer until further processing. They were brought to room temperature in a dark room with only a red safety light where remaining processing was carried out. For negative controls, mice with tumors were treated similarly, with nonlabeled CPT-11, and the same procedure was carried out. Kodak-NB2 emulsion was placed in a water bath at 42°C for 15 minutes. The slides were dipped into a small amount of emulsion and dried before storing in a black, light-free box at 4°C with desiccant (silica gel). Exposure for duration required (7 weeks) was done at 4°C under low humidity conditions. For developing, slides at room temperature were immersed for 5 minutes in Kodak D19 developer at 22°C. After a 30 second wash with distilled water, they were immersed in Kodak fixer (1:1) for 4 minutes before washing for 15 minutes and fixing in 10% neutral buffered formalin for 15 minutes. The conventional H&E counterstaining followed a final wash of 30 minutes. Slides were dehydrated, cleared, and coverslipped (32).

Image Analysis of Autoradiographic Slides. For quantitation of the grains, color microphotographs were taken at ×1,000 magnification. Slides were digitized at 1350 dots per inch, and the digital images were converted to gray-scale and filtered with an unsharp mask (amount 150%, radius 4 pixels, Adobe Photoshop, Adobe Systems Inc., San Jose, CA). We then analyzed the resultant images by commercial image analysis and visualization software (AnalyzePC Version 5, Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) for grain counting using the object counter module.

BOLD fMR Imaging. Mice were anesthetized with 100 mg/kg ketamine HCl/10 mg/kg xylazine (intraperitoneally) and imaged with T2*-weighted spin echo-based rapid acquisition with refocused echoes (RARE) imaging sequence (Echo time = 76.9 ms, Repitition time = 4622.5 ms, number of excitations = 2, field of view = 3 cm, 1-mm thick axial slices perpendicular to the spinal axis, with an in-plane spatial resolution of 234 μm). RARE imaging delivers images practically free from artifacts with an appropriate phase-encoding scheme. Biological structures including tumor, with long relaxation times are the ones most sought after in clinical magnetic resonance imaging, and thus use of a long TR time with a relatively short scan time is possible with RARE sequences. It can be used to acquire images with a very high signal:noise ratio in a reasonable time by averaging (33). We acquired sequential BOLD fMR images first while the mouse was breathing room air followed later by carbogen (93% O2 + 7% CO2, for 9 minutes) using a GE CSI 4.7T/33 cm imaging spectrometer (GE NMR Instruments, Fremont, CA) incorporating AVANCE digital electronics (Biospec platform with Paravision 2.1 operating system, Bruker Medical, Billerica, MA). For processing fMR data, a customized and interactive fMR module was developed and interfaced to a commercially available three-dimensional image analysis package (AnalyzePC Version 5, Biomedical Imaging Resource, Mayo Clinic). A functional map was subsequently calculated pixel-by-pixel for regions visually identified as containing tumor. fMR image intensity changes from pre- and post-carbogen−paired T2*-weighted magnetic resonance images were calculated with the following equation: % signal intensity
change = \frac{(\text{post} - \text{pre})}{(\text{pre})} \times 100\), where “pre” refers to the T2-weighted images obtained with the mice breathing room air, and “post” refers to images acquired while breathing carbogen. To serve as a control, functional maps were obtained from back-to-back scans while the animal breathed room air to ensure pre/post image changes were nonrandom. To aid in visualization of regions of change, a color lookup table was applied to the functional map and an anisotropic diffusion filter (iterations = 6, time interval = 0.25) and low-pass filter (kernel $3 \times 3 \times 1$) were applied to reduce systemic noise while preserving gross areas of change from baseline. The colorized functional map was then overlaid on a T2-weighted scan to localize areas of signal intensity change. In resultant BOLD fMR maps, minimum or no change was depicted as “green” whereas regions with net-positive change from baseline values were depicted as “blue-purple” (50 being the highest positive change), and regions with net-negative change are depicted as “yellow-red” (−50 of lookup table being the largest negative change) as illustrated. Regions of different fMR image signal intensity changes were compared for correlation with MVD count for that region.

**Statistical Analysis.** We analyzed differences between the mean values of the autoradiographic grains and tumor CPT-11/SN-38 lactone levels for significance using unpaired two-tailed $t$ test for independent samples, with Welch correction applied. Linear regression and correlation analysis were carried out for fMR mean signal intensity and MVD with runs test. GraphPad InStat (Version 3.01 for Windows 95, GraphPad Software, San Diego, CA) was used to do all analysis. A $P$ value of $<0.05$ was considered statistically significant.

**RESULTS**

**Antitumor Response in Xenograft.** We reported previously the efficacy of the therapeutic synergy of CPT-11 and FUra (8). Briefly, Fig. 1 summarizes the key findings of the use of CPT-11 (50 mg/kg/week ×4 intravenously) followed 24 hours later by FUra (50 mg/kg/week ×4 intravenously) on FaDu and A253 tumors growing subcutaneously in nude mice, giving a cure rate of 100% and 10%, respectively. The sequential combination with CPT-11 at 100 mg/kg/week, used in this study, gave a lower median tumor volume in A253 as compared with the combination using CPT-11 at 50 mg/kg/week. The higher dose of CPT-11 in the sequential combination was used to highlight and confirm the impact of intratumoral tissue architecture in hampering uniform drug distribution in the less sensitive xenograft A253.

**Detection of Avascular Hypoxia by Immunohistochemical Methods in Xenografts.** To understand the role of differentiation and avascular hypoxia in drug resistance in HNSCC, the poorly differentiated FaDu ($n = 6$) and the well-differentiated A253 ($n = 6$) xenografts were analyzed histologically for differentiation status (H&E, involucrin), MVD with monoclonal antibodies CD31 and CD34, hypoxia with two markers (CAIX and pimonidazole HCl), proliferation marker Ki67, and angiogenesis marker VEGF.

Figure 2 summarizes the histologic and immunohistochemical characterization of untreated A253 (left) and FaDu (right) xenografts. The representative photomicrographs show the fundamental differences between the two tumors. Panel A shows the histologic structure of the well-differentiated HNSCC, A253, containing well-differentiated part with keratin formation (left part) and the characteristic localization of poorly differentiated cells at its rim (arrow), whereas the poorly differentiated tumor cells are present (right side of the panel). Panel B shows the uniformly poorly differentiated HNSCC, FaDu. Panel C clearly shows the complete absence of microvessels in a large microscopic field of the well-differentiated part of A253 (arrow), whereas panel D shows a relatively homogeneous distribution of numerous microvessels in FaDu. FaDu has twice as many microvessels (19/×400 high power fields) as A253 (10/×400 high power fields), whereas a higher proportion of the total vessels are large in A253. Panels E (brown regions) and G (left, arrow) confirm that the well-differentiated regions in A253 show positive immunostaining for both hypoxia markers whereas panel G (right, arrow) indicates that such regions are strongly positive for VEGF. Panels F and H indicate the lack of hypoxia immunostaining almost in the entire FaDu tumor and only the narrow edge of necrosis at the typical localization (arrows) exhibits hypoxia.

Figure 3 summarizes the remarkable change in intratumoral tissue architecture over the course of therapy when the differentiation marker, involucrin, was used. Panel A shows A253 before therapy with about 30% differentiated regions. Panel B depicts remnant A253 tumor 24 hours after the 3rd sequential treatment with CPT-11 followed 24 hours later by FUra and shows that the remaining small A253 tumor characteristically consists of about 80% well-differentiated areas. Panel C shows the presence of poorly differentiated cells (arrow) in a rim surrounding the differentiated region. Tumor cells in these locations and nearby are the major source for tumor survival and regrowth.

Figure 4 summarizes the histologic and immunohistologic features of A253 (left) and FaDu (right) 24 hours after the 3rd sequential treatment with CPT-11 followed 24 hours later by...
Fura. Panel A shows that the remaining small A253 tumor characteristically consists of about 80% well-differentiated areas, with poorly differentiated cells at their rim (arrow). The untreated A253 has only about 30% well-differentiated parts. This comparison indicates a remarkable increase in the fraction of the well-differentiated part of A253. Panel B shows the remaining tumor cell nests (arrows) of FaDu in a connective tissue stroma. Panel C shows again that there are no vessels in the large well-differentiated areas of A253, as opposed to the highly vascularized poorly differentiated FaDu (panel D). Panel E shows strong CAIX immunostaining in the poorly differentiated areas at the rim of the well-differentiated area, proving that the tumor cells within this location remained hypoxic, whereas the surviving FaDu (panel F) tumor cell nests show no immunostaining (arrows), indicating that they are not hypoxic (surrounding mouse stroma shows some unspecific brown staining). Panel G shows positive immunostaining for the other hypoxia marker in the typical localization of the well-differentiated areas of A253, whereas panel H shows lack of immunostaining in the FaDu tumor cells, indicating lack of hypoxia. Panel I visualizes the presence of Ki67-positive cells among the tumor cells located within the rim region, indicating that they are surviving and proliferating cells, whereas in FaDu (panel J) the surviving tumor cells (arrows) are Ki67 negative, indicating that they are not in cell cycle.

We confirmed lack of microvessels in the well-differentiated parts of HNSCC xenografts before and after therapy using CD34, an additional marker for endothelial cells. Microvessels within the poorly differentiated regions were positive for CD34 although weaker when compared with CD31 immunostaining.
Levels of CPT-11 and SN-38 in Xenografts. Table 1 summarizes the plasma and tumor tissue levels of CPT-11 and SN-38. The SN-38 levels in plasma were ~9% that of CPT-11. The SN-38 levels were much lower relative to CPT-11 in the tumor. SN-38 in A253 tumor was 1.8% that of CPT-11, and in FaDu it was <0.4%. Although the drug and metabolite levels in plasma were the same for the two tumors, the intratumoral drug accumulation and conversion, especially for SN-38, differed considerably. The CPT-11 levels in A253 and FaDu tumors were 1.9 and 2.4%, respectively, of the circulating plasma levels at this 2 hour sampling time, but the SN-38 levels were 0.4 and 0.1% of the circulating plasma levels, respectively. The intratumoral level of the SN-38 lactone was four times higher in A253 (3.64 ng/mg protein) compared with FaDu (0.93 ng/mg protein; \( P < 0.049 \)). Despite a three times higher concentration of the active metabolite SN-38 in A253, FaDu is more responsive to CPT-11 treatment. The in vitro \( IC_{50} \) for SN-38 in A253 and FaDu is 0.35 and 0.1 \( \mu \)mol/L, respectively (29). Intratumoral drug concentration, therefore, does not correspond to treatment response in A253 and, to better understand the role of intratumoral drug distribution, \( ^{14} \)C-labeled CPT-11 autoradiography was carried out.

**Distribution of \( ^{14} \)C-labeled CPT-11 in Xenografts by Autoradiography.** Figure 5 illustrates the characteristic autoradiographic distribution of \( ^{14} \)C-labeled CPT-11 (black silver grains) in the xenografts 2 hours after intravenous administration of the drug. Panels A and B show the same A253 tumor, demonstrating that the well-differentiated region (A), including the poorly differentiated tumor cells at its rim (arrow), contain many less silver grains, indicating that less \( ^{14} \)C-labeled CPT-11 is present than in the region with the poorly differentiated cells (B). Panel C, as a control, shows few grains because of baseline background radiation (system noise) independent of tissue whereas panel D shows the grain distribution in the uniformly poorly differentiated FaDu.

Table 2 summarizes the mean (n ≥ 4) intratumoral grain distribution for both xenografts, after we adjust for background using image analysis of autoradiographic slides. The rim of cells surrounding the well-differentiated regions in A253 had a much lower (22 ± 14 grains/mm²) grain density after the 1st chemotherapy treatment compared with the poorly differentiated regions (103 ± 16 grains/mm²), indicating a significantly lower drug distribution (\( P < 0.001 \)) and, therefore, a lower drug exposure of the cells located within this rim region. The difference was even more significant (rim, 28 ± 15 compared with 193 ± 36 grains/mm² in poorly differentiated regions) after the 4th chemotherapy treatment (\( P < 0.001 \)). The grain distribution in the poorly differentiated regions of A253 after the 4th cycle of chemotherapy showed a significant increase (\( P = 0.004 \)) when compared with the grain density in a similar region after the 1st chemotherapy cycle. This is indicative of effective vasculature in the remaining poorly differentiated regions of the tumor in A253 during therapy. In FaDu there was no significant (\( P = 0.0759 \)) increase in the grains during therapy between the 1st and 4th cycle. The poorly differentiated well-vascularized regions in A253 had three times more grains than the poorly differentiated regions of FaDu (\( P < 0.001 \)), consistent with the differences seen with total intratumoral SN-38 lactone measurements by high-performance liquid chromatography (Table 1).

**Noninvasive Characterization of Tumor Hypoxia by BOLD fMR Imaging.** BOLD fMR imaging results were compared with histopathology and immunohistochemical tumor findings. Figure 6 summarizes the correlation between well-vascularized, nonhypoxic tumor regions with areas of negative BOLD fMR signal intensity change (Pearson coefficient \( r = -0.89, r^2 = 0.79, P < 0.001 \)). As an example, these regions are
illustrated in Fig. 6 (right column) as yellow/red in the resultant BOLD fMR image maps from a FaDu tumor obtained after therapy. The higher the number and/or the greater the vessel size, the greater was the decrease in BOLD fMR signal intensity observed. Hypoxic regions with poor, little, or no vasculature were associated with regions of positive BOLD fMR signal intensity change. Figure 7 (left column) depicts representative BOLD fMR results obtained from a A253 tumor after therapy. The lookup table used for better visualization shows the color range for positive and negative signal intensity change with yellow and red being toward negative change corresponding to MVD whereas blue and purple denotes positive intensity change. As shown, a significant area within the tumor contains blue/purple pixels as regions with increased BOLD fMR signal intensity.
intensity change associated with hypoxia. The figure legend below each fMR image lists MVD results from outlined individual tumor regions. CD31 results confirm the excellent correlation between immunohistochemical data and BOLD fMR image results. Moreover, Fig. 7 shows the ability and sensitivity of BOLD fMR imaging techniques to be used as a noninvasive "research probe" to identify well-differentiated hypoxic tumor regions and their surrounding rims in our tumor model.

**Patient Samples of HNSCC.** We detected microvessel distribution by immunohistochemical method using antihuman factor VIII to detect intratumoral endothelial cells in formalin/paraffin sections of the surgical specimens (n/H110056) of HNSCC. Smaller and bigger microscopic areas of the well-differentiated parts (with or without keratin pearl formation) of tumors did not show presence of microvessels. Only the poorly differentiated parts of the tumors showed the typical intratumoral chaotic distribution of microvessels. These findings, which use another endothelial cell marker (factor VIII), confirm a microvessel distribution pattern in human surgical samples similar to that seen in HNSCC xenografts, which use CD31 and CD34 endothelial cell markers.

### Discussion

We have used the human HNSCC xenografts A253 and FaDu in this study to understand intratumoral architectural changes during therapy that are linked to resistance of the FDA approved combination therapy of CPT-11/FUra. Our focus has been on the role of avascular differentiation-linked areas, a unique feature of well-differentiated squamous cell carcinoma such as A253 and its possible role in facilitating nonuniform intratumoral drug distribution, thereby leading to therapy resistance. This study does not focus on hypoxia in any other way except thus far as it is related to such avascular regions and its detection using fMR imaging techniques. *In vitro* IC$_{50}$ for SN-38 in A253 and FaDu is 0.35 and 0.1 H9262 mol/L, respectively (34). Despite a three times higher concentration of the active metabolite of CPT-11, SN-38, in A253, FaDu is more responsive to CPT-11 treatment *in vivo*. Intratumoral drug concentration therefore does not correspond to treatment response in A253. FaDu and A253 have been used as a representative model of poorly and well-differentiated HNSCC, respectively, in this study. Any conclusion and extrapolation from these two models onto others should be taken with adequate caution because of inherent differences between the two tumor types.

The hypoxia markers CAIX and pimonidazole HCl were used in our study to correlate and compare the BOLD fMR intensity changes. We carried out BOLD fMR imaging to study the feasibility of using a noninvasive imaging modality to monitor intratumoral vessel functionality and tissue oxygenation status during therapy in a preclinical mouse model. The ultimate goal is to evaluate the imaging modality for its role as a prognostic predictor of cancer chemotherapy. Our results indicate that resultant BOLD fMR signal intensity changes pinpointed areas of chronic hypoxia as areas of positive intensity change (blue-purple range in the color look-up table) in both

### Table 1

<table>
<thead>
<tr>
<th>Plasma/xenograft</th>
<th>CPT-11 lactone *</th>
<th>SN-38 lactone *</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse plasma</td>
<td>10,436.53 ± 2872.25</td>
<td>937.87 ± 212.44</td>
<td>0.029</td>
</tr>
<tr>
<td>A253</td>
<td>197.31 ± 18.56</td>
<td>3.64 ± 1.05</td>
<td>0.437</td>
</tr>
<tr>
<td>FaDu</td>
<td>251.83 ± 96.19</td>
<td>0.93 ± 0.24</td>
<td>0.049</td>
</tr>
</tbody>
</table>

NOTE. Data are the means of ± SD of three tumors per experiment, three replicates.

* Expressed as ng/ml for plasma and ng/mg protein for the tumor tissue.

† Unpaired two-tailed t test Welch corrected.
A253 and FaDu tumors, and this method shows promise for noninvasive longitudinal monitoring of tumor hypoxia and vascular function. The contributing factors to this positive signal intensity change is not well understood although the release of diamagnetic oxygen molecules diffusing to the hypoxic regions could be one contributing factor. These areas colocalized well with immunohistologically identified tumor hypoxic regions, whereas areas with negative intensity change (yellow-red regions) colocalized with areas rich in vasculature in the corresponding immunohistochemically stained sections. The observed BOLD fMR intensity changes were commensurate with the number and type (small versus large) of vasculature as visually confirmed with CD31 immunostaining for the corresponding regions (Fig. 6). One has to be cautious about extrapolating the results to other tumor models, because variations in response to both carbogen breathing and the magnetic resonance scan protocols have been reported. The potential limitation of BOLD fMR imaging of tumor is also attributable to confounding factors like vessel vasoreactivity, perfusion, and flow. Significant efforts have been made recently to elicit effectively the presence and the extent of tumor hypoxia (35, 36). However, in vivo assessment of human tumors has been difficult because of the lack of consistent and reproducible methods to measure intratumoral oxygen tension. To this end, several single photon emission computed tomography and positron emission tomography imaging-based hypoxia agents have been developed and are under evaluation (37). These include iodinated azomycin arabinoside for single photon emission computed tomography and 60Cu(II)-diacetyl-bis(N4-methylthiosemicarbazone and flumoromisonidazole for positron emission tomography imaging. However, limitations in spatial resolution and imaging characteristics have restricted their clinical utility. BOLD fMR imaging techniques do not suffer from these limitations and therefore may have future clinical implications and relevance.

CAIX and Hypoxyprobe immunohistochemical staining are accepted as reliable markers for chronic hypoxia. Involucrin, a specific marker of differentiation, is oxygen regulated in human squamous cell carcinoma and colocalizes with Hy-

**Table 2**

<table>
<thead>
<tr>
<th>Microscopic fields of samples</th>
<th>Number of average grains†/mm² ± SD</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A253 rim region after 1st treatment</td>
<td>22 ± 14</td>
<td></td>
</tr>
<tr>
<td>A253 poorly differentiated region after 1st treatment</td>
<td>103 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A253 rim region after 4th treatment</td>
<td>28 ± 15</td>
<td></td>
</tr>
<tr>
<td>A253 poorly differentiated region after 4th treatment</td>
<td>193 ± 36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FaDu after 1st treatment</td>
<td>28 ± 15</td>
<td></td>
</tr>
<tr>
<td>FaDu after 4th treatment</td>
<td>59 ± 13</td>
<td>&lt;0.08</td>
</tr>
</tbody>
</table>

* 2 hours after intravenous administration.
† Average number of grains determined by scoring 4 to 7 individual microscopic fields and accounting for background radiation (24 ± 16 grains/mm²).
‡ Unpaired two-tailed t test.

![Fig. 6](image.png)

**Fig. 6** Correlation of MVD in well-vascularized, non-hypoxic tumor regions with areas of negative BOLD fMR signal intensity change. Pearson coefficient $r = -0.89$, $r^2 = 0.79$, $P < 0.001$. **Table 2** Quantitation of the Distribution of 14C-labeled CPT-11* using image analysis of the autoradiographic slides of A253 and FaDu xenografts.
CAIX staining more accurately reflects tissue hypoxia than does hypoxia-inducible factor-1α alone, although it depends on hypoxia-inducible factor-1α activity and oxygen level (38). CAIX is regulated by cell density and is triggered by an intermediate decrease of oxygen tension (5% and 1%, mild hypoxia), related to increased oxygen consumption (39). Some CAIX-stained regions in A253 colocalize with Hypoxyprobe (the indicator for true hypoxia with partial pressure of oxygen < 10 mm Hg; refs. 40 and 41), whereas all Hypoxyprobe regions were positive for CAIX. Expression of CAIX, as in A253, is associated with a tighter intratumoral cellular arrangement

The figure illustrates the demonstration of microscopic hypoxic regions by BOLD fMR imaging. Red marked regions in the T1-weighted magnetic resonance images (A) and (B) shows the anatomic tumor locations. A magnetic resonance reference standard (5 mmol/L CuSO4) is clearly seen as a hyperintense circle used for orientation and signal intensity calibration. BOLD fMR signal intensity changes after 3 weeks of therapy from a small (102 mg, 82 mm³) A253 tumor (C) indicate the absence of blood vessels (purple) for a significant portion of the tumor as confirmed by CD31 (×200) results (E). These regions consist mainly of multiple well-differentiated tumor cell nests. In contrast, BOLD fMR signal intensity changes after 3 weeks of therapy from a small (49 mg, 33 mm³) FaDu tumor (D) indicate the presence of blood vessels (red) for a significant portion of the tumor as confirmed by CD31 (×200) results (F). HPF, high power fields.
nonuniform and apparently chaotic arrangement of tumor neovascularature becomes more unfavorable for drug delivery to individual tumor cells in A253 because of the presence of avascular islands of well-differentiated cells. The outer rims surrounding these well-differentiated regions have a low partial pressure of oxygen (Hypoxprobe positive) because of the vessel distribution, and they are hypoxic but contain many proliferating Ki67-positive tumor cells (Fig. 4, panel I) that do not get sufficient drug delivery, leading to a tumor regrowth. Some poorly differentiated cells in these regions are nonproliferating Ki67 negative (i.e., in G0 stage of cell cycle that is less sensitive to CPT-11). Nonuniform drug distribution has been confirmed by high resolution autoradiography (Fig. 5), demonstrating 5- to 7-fold less drug in these “rim” regions (Table 2) compared with the poorly differentiated, well-vascularized regions in the same tumor ($P < 0.0002$). Studies with simulated percolation processes to mimic the situation in vivo have produced a highly variable intervascular distance that does not allow free diffusion of oxygen (a freely diffusing molecule). Macromolecular drug, such as CPT-11 and FUra with lower diffusivities than oxygen, have little chance of uniform distribution in such a heterogeneous intratumoral microenvironment, especially in the rim region. Highly heterogeneous and chaotic networks akin to tumor vasculature have areas of higher resistance to blood flow (42). This is particularly the case for A253, where the differentiated regions are devoid of blood vessels, with their tighter cellular architecture as evidenced by CAIX staining. In these regions, the tumor cells are very close to each other with no stroma and no microvessels between them. The well-differentiated part of the tumor mimics a histologic structure seen in normal squamous cell epithelium of the oral mucosa and epidermis of the skin, in which connective tissue and microvessels are absent. A recent study (43) with human basal cell skin carcinoma has confirmed the absence of intratumoral blood vessels, and may explain why basal cell skin carcinoma does not metastasize, despite its invasive character. Our studies with human surgical samples of HNSCC confirms the presence of similar avascular well-differentiated histologic regions that can result in lower drug delivery to these specific areas of the tumors.

Immunohistochemical staining for VEGF in A253 showed a relatively stronger staining in the well-differentiated tumor cells (Fig. 2, panel G, right) than in the poorly differentiated cells. The increased proportion of hypoxic well-differentiated regions during treatment provides for higher proangiogenic factors, but the tighter cellular architecture of these regions does not facilitate penetration by the endothelial cells and thus precludes blood vessel formation. Instead it can lead to higher vessel numbers in the poorly differentiated nests of A253 during therapy. After 3 weeks of therapy, the well-vascularized poorly differentiated regions in A253 had a significantly ($P < 0.004$) higher drug delivery when compared with similar regions after the 1st week of therapy. Such an effect was not seen in FaDu during therapy. Therefore, the poorly differentiated regions are not a problem for tumor cell kill during therapy in the well-differentiated tumor A253.

The well-vascularized poorly differentiated compartments of A253 during therapy showed a uniform and 3- to 4-fold higher uptake of the drug compared with FaDu, as represented by the autoradiographic grain distribution. This is consistent with the results of the $^{14}$C-labeled CPT-11 uptake study. Our studies indicate that a changing tumor architecture over the course of therapy, because of an increasing proportion of differentiated areas, is linked with unequal drug distribution and resistance in A253. Because of the low drug delivery, avascular-differentiated regions offer sanctuary to some poorly differentiated tumor cells within the surrounding outer rim where the cells escape therapy and regrow. Recent data from our laboratory (44) with another sequential combination, which uses methyl seleno cysteine as a chemo modulator with CPT-11, has yielded the same intratumoral architectural changes in resistant A253 xenografts, indicating the likelihood of this as one of the possible mechanism for well-differentiated squamous cell carcinoma tumors and tumor cells evading the effects of anticancer agents. Such tumors are also unlikely to respond favorably to antiangiogenic therapy by themselves. In the same study, use of CPT-11 at double the maximum-tolerated dose (200 mg/kg/week $\times$4 intravenously) was possible with organic selenium compounds (decreasing the toxicity of CPT-11; ref. 45) and improved cure rate from 60 to 80%. This improvement could partially be attributable to a better drug distribution to this rim region through diffusion because of a better drug availability at the higher drug concentration used, in turn allowing for a better tumor cell kill.

**ACKNOWLEDGMENTS**

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Lack of Microvessels in Well-Differentiated Regions of Human Head and Neck Squamous Cell Carcinoma A253 Associated with Functional Magnetic Resonance Imaging Detectable Hypoxia, Limited Drug Delivery, and Resistance to Irinotecan Therapy

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