ABSTRACT

N-[4-(3-amino-1H-indazol-4-yl)phenyl]-N1-(2-fluoro-5-methyl phenyl)-urea (ABT-869) is a novel multitargeted receptor tyrosine kinase inhibitor that demonstrates single-agent activity in preclinical studies and has undergone phase I and II clinical trials. We characterized the mechanism of action of ABT-869 by examining vascular changes after treatment (25 mg/kg per day) in HT1080 fibrosarcoma and SW620 colon carcinoma cells, using immunohistochemistry, dynamic contrast enhanced-magnetic resonance imaging (DCE-MRI), and hypoxic protein detection. We observed the inhibition of vascular endothelial growth factor receptor 2 and platelet-derived growth factor receptor β phosphorylation in both tumors and changes in tumor vasculature. Reductions in microvessel density and diameter were observed. Vascular-wall integrity was assessed by colocalization of pericytes and basement membrane. Although both microvessel density and total number of pericytes decreased with treatment, the percentage of pericyte coverage on remaining vessels significantly increased. These data suggest the selective ablation of microvessels lacking pericyte coverage. Functional vascular measures DCE-MRI and hypoxia formation were also tested. After 2 days of treatment on the HT1080 model, vascular permeability, Ktrans, was reduced by >60% and hypoxic tumor fraction was significantly decreased, which was also seen in the SW620 tumors after 4 days of treatment. Taken together, decreases in vascular permeability and changes in vascular integrity observed in these studies define the mode of action of ABT-869 and may aid in optimizing the timing of therapeutic window for combination therapies.

Introduction

Angiogenesis and vascular maturation are highly complex processes that require the sequential activation of a series of growth factor receptors (Folkman, 1971; Ferrara, 1999; Carmeliet, 2000; Yancopoulos et al., 2000). A subset of receptor tyrosine kinases (RTKs) contributes to tumor progression by mediating tumor angiogenesis and lymphangiogenesis and enhancing vascular permeability. These angiogenic RTKs include members of the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptor families (Blume-Jensen and Hunter, 2001). Participation of these two families in the angiogenic process has been demonstrated in several studies, with VEGF functioning in the initiation of new blood vessel formation and PDGF functioning in the maintenance of the vessels, especially in tumor vasculature (Ferrara, 1999; Dvorak, 2002). Tumor vasculature is structurally and functionally abnormal, characterized by impaired endothelial cell organization and pericyte and basement membrane coverage, with chaotic and inefficient vessels resulting in vessel leakage (edema), hypoxia, and interstitial fluid hypertension (Benjamin et al., 1999; Jain, 2005; Batchelor et al., 2007). N-[4-(3-amino-1H-indazol-4-yl)phenyl]-N1-(2-fluoro-5-methylphenyl)-urea (ABT-869) is a structurally novel RTK inhibitor that is a potent inhibitor of members of the VEGF and PDGF families (Albert et al., 2006) and has demonstrated activity in a phase I clinical trial, including compound-mediated decreases in DCE-MRI (Wong et al., 2009; Zhou et al., 2009).

Multiple studies describing the relationship of antiangiogenic therapy and tumor vasculature normalization have
been published (Benjamin et al., 1998; Winkler et al., 2004; Franco et al., 2006; Batchelor et al., 2007; Fenton and Paoni, 2007). The tumor vasculature normalization hypothesis proposed by Jain (2005) describes a role for antiangiogenic therapy to potentially normalize both the structure and function of abnormal tumor vasculature by selectively pruning the chaotic and inefficient vessels and improving the vascular wall integrity of remaining vessels, thus providing more efficient delivery of drugs and oxygen. A study of VEGFR2 blockade by treatment with DC101, a monoclonal antibody against VEGFR2 (Flk-1), demonstrated an increase in pericyte coverage of glioma model vessels through up-regulation of angiopoietin-1 as well as degradation of their pathologically thick basement membrane through matrix metalloproteinase activation during the “normalization window” (Winkler et al., 2004). Another study with DC101 demonstrated that treatment of an orthotopically transplanted human breast carcinoma (MDA-MB-231) reduced microvessels (MV) density and blood flow, resulting in an increase in the hypoxic tumor fraction within 5 days that remained throughout the entire course of treatment up to 21 days (Franco et al., 2006). Vascular improvement and normalization have also been observed in clinical studies with small-molecule inhibitors of VEGF signaling (Batchelor et al., 2007). These studies consistently observed that the antiangiogenic therapy normalized structurally and functionally abnormal tumor vasculature and improved blood flow in the tumor microenvironment, although the mechanistic details associated with these functional outcomes remain undetermined. The current study was designed to interrogate the effect of ABT-869 on tumor vasculature, specifically the response of vascular wall integrity to the compound during tumor growth inhibition in ectopic flank xenograft models. Because ABT-869 targets both the VEGF and PDGF kinase families, it simultaneously inhibits two signaling pathways thought to be essential to tumor angiogenesis (Albert et al., 2006). We explored changes in vascular density, vessel permeability, vascular wall integrity, and hypoxic status between ABT-869 and vehicle control-treated mice during tumor growth inhibition and regression studies. The results from the present study provide insights into the mode of action of ABT-869 and the role of VEGF and PDGF in regulating tumor angiogenesis.

Materials and Methods

In Vivo Tumor Growth and ABT-869 Treatment. Cell lines were obtained from the American Type Culture Collection (Manassas, VA). A total of $5 \times 10^5$ tumor cells were suspended in 0.5 ml of PBS, mixed with 0.25 ml of Matrigel (phenol red free; BD Biosciences, San Jose, CA), and inoculated into the flank of the mice. At the designated time after inoculation, tumor-bearing animals were divided into groups ($n = 5$/group), and administration of vehicle (2% ethanol, 5% Tween 80, 20% polyethylene glycol 400, 73% saline) or ABT-869 at 25 mg/kg/day b.i.d. was initiated. The tumor size was assessed with calipers and calculated using the formula (length $\times$ width$^2$ $\times$ 0.5). For the morphological study, the HT1080 tumor was allowed to grow 7 days before treatment, and the tumors were collected at baseline, 2 and 5 days after treatment; the SW620 tumor was allowed to grow 21 days before treatment, and the tumors were collected at baseline and 4 days after treatment.

Tumor Processing/Preparation for Hypoxia and Vasculature Assessment. For the hypoxia assessment, tumor-bearing mice received an intraperitoneal injection of pimonidazole hydrochloride (60 mg/kg; Millipore Bioscience Research Reagents, Temecula, CA) 90 min before euthanasia. Subsequently, the mouse received an intravenous injection of 100 $\mu$g of Isoleotin GS-IB/Alexa 594 (Invitrogen, Carlsbad, CA), and the dye was allowed to circulate for 5 to 10 min for the assessment of individual tumor vessels. A subset of these mice was injected only with fluorescein isothiocyanate (FITC)-labeled Lycopersicon esculentum (tomato) lectin (Vector Laboratories, Burlingame, CA) before tumor collection. All tumors were removed, snap-frozen in liquid nitrogen, and kept at $-80^\circ$C until used. The sections were fixed in 4% paraformaldehyde for 30 min, PBS-washed, air-dried, and stored at $4^\circ$C. Cryosections (90 $\mu$m) were cut for the observation of global vasculature. Cryosections (20 $\mu$m) were cut and further stained by hematoxylin and eosin, immunohistochemistry (IHC), or hypoxic protein.

Immunohistochemistry. Two micrograms of each primary antibody was used to identify the cancer cells, endothelial cells, pericytes, and receptors in tumor tissues: von Willebrand factor (vWF; DK-2600; Dako Denmark A/S, Glostrup, Denmark), $\alpha$-smooth muscle actin antibody (a-SMA; Abcam Inc., Cambridge, MA), phospho-PDGFR $\beta$ (Tyr1009; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phospho-VEGFR 2 (Tyr1214; Spring Bioscience, Pleasanton, CA). The specificity of the antibody signal was determined by using matched isotype control antibodies and blocking the binding of the pPDGFR $\beta$ and pVEGFR 2 antibodies by preincubation with the synthetic peptides used to generate the antibodies (data not shown), synthetic PDGFR $\beta$ peptide (Tyr1009; Santa Cruz Biotechnology, Inc.), and synthetic VEGFR 2 peptide (Tyr1214; ChemGenes, San Francisco, CA).

Hypoxic protein was detected by anti-mouse IgG/FITC conjugates following the manufacturer’s directions. For IHC fluorescence detection of individual antibodies, including pPDGFR $\beta$, pVEGFR 2, and vWF antibodies, the tissue sections were permeabilized with 0.3% Triton X-100 in PBS then incubated with the antibody that was conjugated with an Alexa dye (Zenon labeling technology; Invitrogen) for 2 h at room temperature. Stained slides were then washed with PBS, rinsed with double-distilled H$_2$O, and air-dried, then the slides were covered with Prolong gold antifade mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen). Dual labeling of pericyte and pPDGFR $\beta$ was used to evaluate colocalization of pPDGFR $\beta$ and pericytes. The samples were permeabilized with 0.3% Triton in PBS, then incubated with a-SMA antibody/FITC overnight at 4°C followed by labeling with pPDGFR $\beta$ antibody labeled with Alexa 594 overnight at 4°C as described above. For chromogenic detection, the horseradish peroxidase visualization polymer system (Biocare Medical, Concord, CA) was used in combination with diaminobenzidine and counterstained with hematoxylin.

Fluorescent or chromogenic images were captured with a Zeiss AxiosPhot 2 fluorescent microscope (Carl Zeiss Inc., Thornwood, NY).

DCE-MRI Experimental Design and Measurement. The HT1080 tumor-bearing animals were divided into vehicle-treated (2% ethanol + 5% Tween 80 + 20% polyethylene glycol 400 + 73% hydroxypropyl methyl cellulose) and ABT-869-treated (25 mg/kg per day) groups ($n = 4–6$/group). Oral administration of vehicle and ABT-869 began on day 9 after the inoculation and continued until the end of the study. DCE-MRI was performed 1 day before treatment and 2 days after treatment. All MRI experiments were conducted on a 4.7 T/40-cm magnet (Magnex Scientific, Kidlington, Oxfordshire, UK) with a 12-cm bore gradient insert operated via a Varian INOVA imaging console (Varian Inc., Palo Alto, CA). The mouse tail vein was catheterized with catheters preloaded with gadopentetate dimeglumine-diethylenetriamine pentaacetic acid (Gd-DTPA; Bayer HealthCare, Wayne, NJ) before the mouse was positioned in a 4-cm volume coil. Body temperature was maintained at 37°C during the imaging experiment with warm air. DCE-MRI was acquired from four 1.5-mm-thick slices covering the whole or partial tumor using a $T_1$-weighted gradient echo imaging sequence. After eight baseline images, a bolus injection of Gd-DTPA (0.2
mmol/kg i.v.) was administered via tail vein, and the acquisition was
continued for 8 min at a time resolution of 19 s/image using the
imaging parameters time recycle/time echo of 150 ms/3 ms and field
of view of 3 × 3 cm². All calculations were performed using custom-
built software developed in IDL (ITT Visual Information Solutions,
Boulder, CO). DCE-MRI data were analyzed based on the two-com-
partment tracer kinetic modeling described by Tofts (1997). First,
the recorded time course DCE-MRI signal was converted into time
course contrast agent concentration with the aid of precontrast T₁
measurement. The plasma contrast agent concentration was derived
from the arterial input function measured from large vessels present
in the imaging slices. The Tofts-Kermode equations associating tis-
sue and plasma contrast agent concentrations were solved via curve
fitting to calculate pharmacokinetic parameter Kᵣ. The volume
derived from blood pool to extravascular extracellular
space per unit tissue volume (Tofts, 1997). The calculation was

carried out pixel by pixel from manually outlined tumor regions of all
prescribed slices. Because of the inhomogenous nature of tumor
tissue, a log transform was applied to pixel-wised Kᵣ data to
achieve normal distribution, and thereafter their geometric means
were calculated to provide an average Kᵣ for each lesion.

Histological Image Acquisition and Analysis. Tumor sections
were visualized using bright filter or fluorescence filters: DAPI for
nuclei, FITC or Alexa 594 for lectin, α-SMA, hypoxia protein, and
receptor staining. Images were captured with a Zeiss Axiocam cam-
era connected to the microscope using Zeiss AxioVision 4.6 software.
In general, two to four sections in each of three to five tumors from
each treatment group were examined, and four to six highly vascular
areas (0.4 mm²/each) from each section were randomly selected for
image acquisition and analysis. MV were identified by the staining of
endothelial cells using wVFN antibody or fluorescence labeled-lectin
vascular infusion (Minamikawa et al., 1987; Gee et al., 2003) and
quantified at 200× magnification.

Pathological scores were determined for pPDGFR β and pVEGFR
2 immunostaining. The stain intensity for each antibody was as-
seased using the scale of 0 to 3: 0, no staining; 1, weak staining; 2,
medium staining; 3, strong staining. The scores were integrated into
two groups (0–1 and 2–3) before statistical analysis. Colocalization of
pPDGFR β (or pVEGFR 2) and pericytes was detected by merging of
Alexa 594 and FITC fluorescence to yield the third yellow color.
Isotype antibody and peptide competitive staining were used as
negative controls for each antibody.

Leaky vessels, which were defined by perivascular tumor cell
labeling with infused lectin/FITC, and fluorescence intensity of spe-
cific hypoxia protein or pericyte immunostains were quantified on
to four to six randomly selected vascular areas (0.4 mm²/each) per
section using Zeiss AxioVision 4.6 software. The threshold setting
was determined by the intensity of the background fluorescence that
was measured on images stained with FITC secondary antibody
without a primary antibody and used throughout the image acqui-
sition procedure. The fluorescence intensity of hypoxia protein or
pericyte stains represented the average brightness of all cell-related
pixels. The mean value was calculated for the tissue section stained
with a particular antibody.

To assess vascular maturity further analysis of pericyte coverage
on individual tumor vessels was performed by measuring the per-
centage of α-SMA+ cells surrounding lectin-stained vessels at 200×
magnification. The pericyte covered-vessels were divided into three
groups of 0 to 30, 50 to 60, and 80 to 100% pericyte coverage. A
pericyte coverage index was obtained by calculation of the average
pericyte coverage per section, using the following equation (number
of % coverage vessels/total number of vessels in a section × 0% +
number of 50% coverage vessels/total number of vessels in a sec-
tion × 50% + number of 80% coverage vessels/total number of vessels
in a section × 80%). These section-based values were further
averaged for each tumor and compared between vehicle- and ABT-
869-treated groups.

Statistics. A mixed-effect model was fitted to estimate the MV
diameter and density for each group. MV density values (number of
vessels in the area of 0.4 mm²) and Kᵣ values from DCE-MRI
measurement were log-transformed to achieve normality. The two-
sample t test was performed for the assessment of the average per-
cyte coverage per section to identify differences between the
treatment groups, as well as vessel leakage, hypoxic protein, and
Kᵣ quantification. For IHC staining intensity assessment,
Fisher’s exact test was used for the number of tumors with the
scale ≥2 or <2. Values are expressed as mean ± S.E. Statistical
analysis was carried out using the SAS version 9.1 software (SAS
Institute, Cary, NC). p < 0.05 was considered a statistically
significant difference.

Results

ABT-869 Treatment Inhibited Tumor Growth. To study the effects of ABT-869 on tumor vasculature we se-
lected two models, the highly vascular HT1080 fibrosarcoma and the SW620 colon carcinoma that demonstrated effective
targeting of tumor vasculature by angiogenesis inhibitors (Mukhopadhyay et al., 1998; Yao et al., 2000). Both models
are also characterized by leaky vasculature, high levels of VEGR, and robust angiogenesis, and they exhibit effective
response to ABT-869 (Albert et al., 2006). The current study of tumor growth inhibition with these two models used the
recommended dose of 25 mg/kg per day that was consistent with our previous studies (Albert et al., 2006). In the HT1080
model, treatment began 7 days after inoculation, and vehicle- and ABT-869-treated groups were harvested on day 7 before
therapy (day 0), day 9 (2-day treatment), and day 12 (5-day treatment). In the SW620 model, treatment began 21 days
after inoculation, and vehicle- and ABT-869-treated groups were harvested on day 21 before therapy (day 0) and day 25
(4-day treatment). The treatment with ABT-869 resulted in >50% tumor growth inhibition within 4 days in both models
(Fig. 1). There were no differences in animal weights between the treatment groups.

ABT-869 Treatment Inhibited Phosphorylation of
PDGR β and VEGFR 2. Our previous studies demon-
strated that ABT-869 inhibited phosphorylation of PDGR β
and VEGFR 2 in cellular assays (Albert et al., 2006; Guo et al.,
2006; Shankar et al., 2007). The current IHC study demon-
strated that ABT-869 inhibited target receptors in vivo for
both tumor models. The antibodies used in this study specif-
cally recognized pPDGR β expression primarily in per-
cytes and tumor cells and pVEGFR 2 expression predomi-
nantly in endothelial cells and tumor cells. Representative
images of the antibody staining are shown in Fig. 2A, dem-
strating the inhibition of the phosphorylation of the recep-
tors. The quantitative pPDGR β staining was globally mea-
sured on tumor cells and pericytes (Fig. 2B) by measuring
immunostaining intensity using the traditional 0 to 3+ scale.
Untreated tumors demonstrated high expression of both
phosphorylated receptors with a median expression of 3+
(Fig. 2B). After treatment with ABT-869 all groups had a
median expression of 0, and the differences were statistically
significant by Fisher’s exact test. In the HT-1080 model, after
2 days of treatment the mean score was 0.08+ (ABT-869)
differs 2.94+ (vehicle) for the staining intensity of pPDGR
β and 0.08+ (ABT-869) versus 2.93+ (vehicle) for pVEGFR 2
(both receptors p < 0.01). After 5 days of treatment the
average score was 0.17+ (ABT-869) versus 2.92+ (vehicle)
for the staining intensity of pPDGFR β and 0.33+ (ABT-869) versus 3+ (vehicle) for pVEGFR 2 (both receptors p < 0.05). Likewise, in the SW620 model, after 4 days of treatment the average score was 0.38+ (ABT-869) versus 3+ (vehicle) for the staining intensity of pPDGFR β and 0.08+ (ABT-869) versus 2.7+ (vehicle) for pVEGFR 2 (both receptors p < 0.01) (Fig. 2B). Then the tissues were costained with α-SMA antibody to identify the pericytes, which were defined by their physical proximity to the vessels.

**ABT-869 Treatment Changed Global Vasculature and Reduced MV Diameter and Density.** Fluorescence-labeled lectin injected into the bloodstream binds rapidly and uniformly to the luminal surface of the vasculature that maintains blood flow, thus actively perfused blood vessels can be identified (Minamikawa et al., 1987; Debbage et al., 1998; Hashizume et al., 2000; Morikawa et al., 2002). Using this technique, tumor global vasculature was examined on thick tissue sections (90 μm) by fluorescent microscopy. In vehicle-treated HT1080 tumors, necrotic areas were observed in the tumor center, and only the rim of the tumor displayed clearly identified vasculature, characterized by a haphazard pattern of interconnection (Fig. 3A). Perivascular tumor cells stained with lectin/FITC indicated the presence of vessel leakage (Fig. 3C). After 2 and 5 days of treatment with ABT-869 on HT1080 tumors, the vessels seemed straight and well organized (Fig. 3B) in contrast to the chaotic and leaky MV in untreated tumors, and the leakage of the lectin was significantly reduced from 84.09% ± 3.61 (2 days) and 93.44% ± 5.82 (5 days) to 17.86% ± 4.17 and 49.44% ± 5.82, respectively (mean ± S.E.) (Fig. 3, D and G). Similar results
were observed in the SW620 model. The vessels in vehicle-
treated tumors were also tortuous and leaky (Fig. 3E),
whereas after ABT-869 treatment the MV density was re-
duced and the vessels were more straight and organized with
less leakage (Fig. 3F).

These global vasculature studies demonstrated the changes in tumor vasculature after ABT-869 treatment. To
further interrogate this observation, we quantified MV den-
sity and diameter at higher magnification levels (Fig. 4). For
the HT1080 tumors (Fig. 4A), mean vessel diameter was
reduced significantly with ABT-869 treatment for 2 days ($p < 0.01$) and 5 days ($p < 0.01$). MV density was also reduced
significantly after treatment for 2 days ($p < 0.01$) and 5 days
($p < 0.01$). For the SW620 tumors, similar differences were
observed with reductions in vessel density and diameter ($p < 0.01$) (Fig. 4B).

**ABT-869 Treatment Improved Vascular Wall Integrity.** Vessel maturation or later stages of vascularization
includes recruitment of mural cells (pericytes or smooth
muscle cells) to the vessels, production of basement membrane,
and induction of vessel bed specializations. As an important
compontent of the vessel wall, pericyte coverage has been
used broadly as a vessel maturation marker (Inai et al., 2004;
Bagley et al., 2005; Jain, 2005). In this study, the pericyte
coverage on individual vessels was also measured (as de-
scribed under Materials and Methods) to test the vessel wall
integrity after treatment with ABT-869. For the HT1080
tumors, the pericyte coverage index was 0.17 ± 0.1 and
0.09 ± 0.1 on day 2 and day 5 vehicle-treated tumors, respec-
tively. After ABT-869 treatment for 2 and 5 days, the index
increased to 0.52 ± 0.1 ($p < 0.05$) and 0.46 ± 0.14 ($p < 0.01$),
respectively (Fig. 4, A and C). For the SW620 tumors, the
index increased to 0.59 ± 0.03 (ABT-869) compared with
0.36 ± 0.03 (vehicle; $p < 0.01$) (Fig. 4, B and C). To further
assess the effect of ABT-869 treatment on the vascular wall
integrity, we evaluated the effect of treatment on the base-
ment membrane of the vasculature in a limited subset of the
HT1080 tumors by visualizing the main structural protein,
collagen IV, using IHC. The results similar to what was
observed with the pericytes demonstrated that the basement
membrane continuation was interrupted in vehicle control
tumor vessels, and that after ABT-869 treatment the base-
ment membrane coverage was continuous and tightly asso-
ciated with the MV. These results implied that ABT-869
inhibited total pericytes by pruning chaotic MV but improved
vascular wall integrity of the remaining vessels.

**ABT-869 Treatment Induced Modification of Func-
tional Vascularity.** The functional impact of vascular nor-
malization was assessed using DCE-MRI in the HT1080
model. The $K^{trans}$ values that are a function of both vessel
permeability and surface area were generated by DCE-MRI
and subjected to longitudinal analysis. Consistent with the
histological studies, rapid reduction of vascular permeability
to Gd-DTPA and vessel size was observed upon treatment
with ABT-869. Figure 5A provides examples of DCE-MRI
images showing signal enhancement within tumors by 8 min
after contrast agent injection. Before ABT-869 treatment,
significant contrast agent uptake was seen in the tumor. The
uptake was highly variable because of the necrotic nature of
this fast-growing tumor type, with higher uptake in tumor
rim and in some areas within the tumor. Longitudinal mea-
surement via DCE-MRI demonstrated that $K^{trans}$ in vehicle-
treated animals increased as tumors grew and that treat-
ment with ABT-869 for 2 days significantly reduced $K^{trans}$
in tumors (Fig. 5B). These DCE-MRI data significantly
strengthen the use of this technique in the study of drug
action, for example, in detecting differential regional changes
in the tumor core and enhancing rim (Galbraith et al., 2003).

**ABT-869 Treatment Decreased Tumor Hypoxia.** Pi-
monidazole, a substituted 2-nitroimidazole, which is prefer-
entially reduced in viable hypoxic cells, forms irreversible
protein adducts at $pO_2$ levels ≤10 mm Hg (Varia et al., 1998;
Raleigh et al., 1999) and has been optimized for detection
with a FITC-conjugated monoclonal antibody against the
protein adducts for use as a marker to detect hypoxia (Ra-
leigh et al., 2000; Dings et al., 2007). Nuclei were stained
with DAPI to ensure that the hypoxia was measured in cells
rather than nonspecific binding to necrotic regions. Overall
tumor hypoxia in both tumor models was quantified. Statistical analysis indicated that there was a transient decrease in the amount of hypoxic protein detected in HT1080 tumors ($p < 0.05$) after 2 days of treatment that increased to an intermediate value after 5 days of treatment (Fig. 6, A and C). Hypoxic protein decreased in the SW620 tumors with 4 days of treatment ($p < 0.01$; Fig. 6, B and D). The distribution pattern of hypoxic areas changed, such that hypoxic areas in the ABT-869-treated tumors were both smaller and located at a greater distance from the tumor vasculature than was observed in the vehicle control-treated tumors. This phenomenon implied that the remaining vessels were normalized.

**Discussion**

We have demonstrated previously that ABT-869 has robust inhibition in a broad spectrum of xenograft tumor growth models in a dose-dependent manner, including human fibrosarcoma and breast, colon, and small-cell lung carcinomas (Albert et al., 2006) that can presumably be attributed to the antiangiogenic effects of the compound. As an ATP-competitive inhibitor of VEGF and PDGF RTKs, the simultaneous inhibition by ABT-869 may result in greater antitumor efficacy and provide the potential to treat a broader range of human cancers than more selective agents. In a phase I study of patients with refractory solid malignancies ABT-869 was found to be tolerable and demonstrated partial response and prolonged tumor stabilization in a broad range of tumor types (Wong et al., 2009; Zhou et al., 2009). To define the mode of action of ABT-869, the current study focused on effects of ABT-869 on vascular wall integrity. The results demonstrated that ABT-869 therapy in the HT1080 fibrosarcoma and SW620 colon carcinoma xenografts resulted in a significantly reduced tumor growth rate within 2 days from the start of treatment, which is consistent with previous studies and reflects early action of ABT-869 on tumor growth in both models. Subsequent analysis was performed to interrogate multiple vasculature parameters to enhance our understanding of the antiangiogenic actions of ABT-869.

We performed a series of analyses to address the mechanism by which ABT-869 exhibits its activity in tumor growth inhibition. The first level of analysis addressed the presence of the target proteins in the tumor models studied on both tumor cells and the tumor vasculature. Through colocalization experiments we determined that phosphorylated PDGFR colocalized with pericytes (α-SMA stain) and phosphorylated VEGFR 2 colocalized with tumor vessels. Treatment with ABT-869 strongly inhibited staining with both phosphorylation-specific antibodies, indicating that the primary angiogenic targets were inhibited in vivo.

Our next level of analysis focused on the vasculature itself, examining global parameters and MV density and diameter. In both tumor models, responses to ABT-869 treatment were observed; after 2 days of treatment in the HT1080 model and after 4 days in the SW620 model, the global structure of vessels became better organized, and the vessels were smaller in size and less dense as tumor growth was significantly inhibited. Similar results have been reported with
The study demonstrated that ABT-869, as a single agent, had significant impact on the integrity of vascular wall. Tumors treated with ABT-869 demonstrated improved vascular wall integrity characterized by better pericyte coverage on the remaining vessels, which may reflect the role of VEGF as a negative regulator of pericyte function (Greenberg et al., 2008). These studies are consistent with reports in the literature with other antiangiogenic agents (Inai et al., 2004; Dings et al., 2007).

Collectively, reduction of MV density/diameter and reduction of pericyte/pPDGFR β colocalization imply that active pericytes in the tumor region might be more susceptible to ABT-869, which results in selective pruning of MV through inhibition of PDGFR β phosphorylation. Tumor vessels that survived the inhibition of the receptors’ phosphorylation were more normal in global structure and at a cellular level, which may improve oxygen and drug delivery to adjacent tumor cells despite reduced tumor vascularity (Benjamin et al., 1999; Jain, 2005; Batchelor et al., 2007). The changes caused by ABT-869 to vascular wall components in the two tumor models demonstrate that this inhibition of RTK signaling does more than block growth of new tumor vessels; the agent has multiple effects that might prove useful in understanding the dependence of tumor vessels on VEGF and PDGF for survival, the process of blood vessel regression, and the mechanism of action of angiogenesis inhibitors.

Our last set of experiments explored the functional consequences of ABT-869-induced vascular changes. We observed, through Ktrans measurements using DCE-MRI, a reduction in vessel leakiness indicative of a more functional, normalized tumor vasculature. This observation was supported by our morphological findings and an observed reduction in hypoxia. Taken together, these results indicate that a reduction in vessel leakiness can lead to vascular normalization and improved tumor perfusion and are consistent with previous studies showing that tumor perfusion increased with antiangiogenic treatment, although vascular leakiness was reduced (Dvorak, 2002; Inai et al., 2004). It is noteworthy that although hypoxic areas in HT1080 tumors decreased after 2 days of ABT-869 treatment 3 additional days of treatment tended to increase areas of hypoxia, although the level was still lower than in the nontreated tumors. These results support previously reported transient changes in tumor oxygenation (Ansiaux et al., 2005; Franco et al., 2006) and the existence of a “tumor oxygenation window” similar to what has been observed in xenograft tumor models with anginex and avastin (Dings et al., 2007). The reversal of leakiness and transient decrease in hypoxia during single-agent therapy with ABT-869 suggests vascular normalization that may lead to improved delivery of chemotherapy that could be clinically beneficial.

In summary, we report an immediate and significant effect of ABT-869 on morphological and functional aspects of tumor vasculature, including the ability of ABT-869 to concomitantly reduce tumor growth, tumor vascular permeability, MV density, and diameter and to improve tumor vascular wall integrity. The data provide compelling evidence in support of ABT-869 effects on tumor vasculature that cause transient functional normalization and could indicate a therapeutic window for future combination therapy. These results will hopefully lead to a better understanding of the mechanism action of multiple tyrosine kinase receptor inhibitors on tumor vessels and stimulate the development of
innovative ways to assess their in vivo action and predict which tumors will be most responsive.

Acknowledgments

We thank David Reuter and Elizabeth Litvinovich for critical technical help with mouse tail vein injection, cryosection, and immunostaining.

Authorship Contributions

Participated in research design: Jiang, Albert, Luo, and McKeegan.
Conducted experiments: Jiang, Luo, and Tapang.
Performed data analysis: Jiang, Luo, and Zhang.
Wrote or contributed to the writing of the manuscript: Jiang, Albert, Luo, Davidsen, Fox, Lesniewski, and McKeegan.

References


**Fig. 6.** Assessment of tumor hypoxia after ABT-869 treatment. A and B, representative images of typical hypoxic protein staining (green) across each section. MV were identified by lectin/Alexa594 infusion (red). DAPI-stained nuclei are blue. A, HT1080. B, SW620. In both tumor treatment models, hypoxic areas distributed differently and were smaller compared with vehicle control. Bars represent 100 μm in A and B, top, and 50 μm in B, bottom. C and D, the graphs present the mean of fluorescence intensity of hypoxic area in each tumor (see Materials and Methods). *p* < 0.05, comparison at the same time point. **p** < 0.01, comparison at the same time point. Orange triangles indicate *p* < 0.05, comparison of treated versus baseline.


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