Effects of the Dual Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Inhibitor NVP-BEZ235 on the Tumor Vasculature: Implications for Clinical Imaging

Christian R. Schnell, Frédéric Stauffer, Peter R. Allegrini, Terence O'Reilly, Paul M.J. McSheehy, Celine Dartois, Michael Stumm, Robert Cozens, Amanda Littlewood-Evans, Carlos García-Echeverría, and Sauveur-Michel Maira

Abstract

Dysregulated angiogenesis and high tumor vasculature permeability, two vascular endothelial growth factor (VEGF)-mediated processes and hallmarks of human tumors, are in part phosphatidylinositol 3-kinase (PI3K) dependent. NVP-BEZ235, a dual PI3K/mammalian target of rapamycin (mTOR) inhibitor, was found to potently inhibit VEGF-induced cell proliferation and survival in vitro and VEGF-induced angiogenesis in vivo as shown with s.c. VEGF-impregnated agar chambers. Moreover, the compound strongly inhibited microvessel permeability both in normal tissue and in BN472 mammary carcinoma grown orthotopically in syngeneic rats. Similarly, tumor interstitial fluid pressure, a phenomenon that is also dependent of tumor permeability, was significantly reduced by NVP-BEZ235 in a dose-dependent manner on p.o. administration. Because RAD001, a specific mTOR allosteric inhibitor, was ineffective in the preceding experiments, we concluded that the effects observed for NVP-BEZ235 are in part driven by PI3K target modulation. Hence, tumor vasculature reduction was correlated with full blockade of endothelial nitric oxide (NO) synthase, a PI3K/Akt-dependent but mTORC1-independent effector involved in tumor permeability through NO production. In the BN472 tumor model, early reduction of permeability, as detected by \( K_{\text{trans}} \) quantification using the dynamic contrast-enhanced magnetic resonance imaging contrasting agent P792 (Vistarem), was found to be a predictive marker for late-stage antitumor activity by NVP-BEZ235. [Cancer Res 2008;68(16):6598–607]

Introduction

The formation of new microvessels from the existing tumor vasculature is an essential initial step in the mass expansion process of solid tumors. This phenomenon ensures proper delivery of growth factors, nutrients, and oxygen indispensable to tumor cell proliferation and tumorigenic phenotype maintenance. Moreover, in contrast to normal vasculature, tumor blood vessels are distinctly abnormal in structure and function: they are enlarged, tortuous, and poorly invested by pericytes and exhibit arteriovenous shunts and blind ends (1). As a consequence, tumor blood flow is not regular, leading to localized hypoxia. When tumors reach a certain size, or when located to a certain distance from existing blood vessels, an angiogenic phenotype (the angiogenic “switch”) is initiated. This is in part due to local overexpression of vascular endothelial growth factors (VEGF; ref. 2). In addition, VEGFs are responsible for the hyperpermeable state of tumor blood microvessels. This increase in tumor vessel permeability is thought to contribute to the deposition of plasma proteins, which may provide a provisional matrix for the inward migration of fibroblasts and endothelial cells into tumors and amplify the signals important for tumor growth. Moreover, leakage of tumor vessels may facilitate the traffic of tumor cells into the bloodstream and the formation of metastases (3).

Inhibition of VEGF expression or activity was shown to prevent the vascularization and growth of experimental tumors. Hence, antiangiogenic therapies aiming at affecting tumor vessel formation and function through endothelial cell targeting are attractive approaches for the treatment of cancer. This includes antibodies or small molecular mass inhibitors (4, 5) directed against key regulators, including the VEGFs or their cognate high-affinity receptor tyrosine kinases Flt-1 (VEGFR1) and Flk-1 (VEGFR2/KDR).

The phosphatidylinositol 3-kinase (PI3K) pathway is of relevance for VEGF-induced endothelial signaling (6). Indeed, PI3K/Akt pathway activation in endothelial cells promotes their survival when cultured in vitro (7, 8) and in the tumor vasculature in vivo (9, 10). Tumor cells actively participate in this phenotype by secreting VEGF in the stromal space. This promotes endothelial cell proliferation owing to increased sensitivity to VEGF because of constitutive Akt activation. In that respect, Akt is the immediate upstream activator of nitric oxide (NO) synthase (11) specifically expressed in endothelial cells (eNOS; refs. 12, 13). On VEGF challenge, production and release of free radical gas NO in the vascular endothelium lead to increased angiogenesis and vascular permeability (14).

Genetic alterations in \( PIK3CA \) and \( PTEN \) genes are often observed in human tumors. \( PIK3CA \) encodes the catalytic subunit responsible for the conversion of phosphatidylinositol-3,4-bisphosphate in phosphatidylinositol-3,4,5-trisphosphate, whereas \( PTEN \) is responsible for the expression of the lipid phosphatase catalyzing the opposite reaction. Consequently, \( PIK3 \) pathway constitutive activation can be seen as a hallmark of tumorigenesis (15), and \( PI3 \) is considered as a well-validated cancer target. Multiple small-molecule inhibitors aiming at intercepting PI3K signaling in the tumor cells are currently being tested for efficacy in clinical trials (16). Given the importance of the PI3K pathway for endothelial cell signaling, it is also anticipated that PI3K inhibitors might also display significant effects on the tumor vasculature.
NVP-BEZ235 is a novel dual pan-PI3K/mammalian target of rapamycin (mTOR) inhibitor that exhibits significant antitumor activities in a broad range of experimental tumors (17). In the present study, we investigated the effect of NVP-BEZ235 on VEGF-induced angiogenesis and on tumor vasculature permeability using the BN472 orthotopic and syngeneic rat mammary carcinoma model. Results suggesting that the reduction of tumor leakage caused by NVP-BEZ235 can be monitored noninvasively and used as a surrogate marker for efficacy for this compound are presented and discussed.

Materials and Methods

Compound preparation for in vitro and in vivo studies. For in vitro studies, stock solutions of PTK787/ZK222584 (PTK/ZK), NVP-BEZ235, or RAD001 (Novartis) were prepared in 100% DMSO and then diluted in medium at the desired concentrations. For in vivo studies, NVP-BEZ235 was administered p.o. daily either in NMP/PEG300 (10/90, v/v) for mice treatment (10 mL/kg) or in 0.5% methylcellulose for rat treatment (5 mL/kg); PTK/ZK was administered p.o. (5 mL/kg) daily in a vehicle of 100% PEG200; RAD001 was formulated as a microemulsion containing 2% active substance and diluted in 5% glucose and applied p.o. (10 mL/kg).

Human umbilical vascular endothelial cell proliferation, in vivo chamber implant angiogenesis, and vascular permeability assays. The effects on VEGF-induced proliferation of human umbilical vascular endothelial cell (HUVEC) were tested using a bromodeoxyuridine (BrdUrd) incorporation kit (Biotrak Cell Proliferation ELISA System v.2, Amersham). The assay was run as recommended by the vendor and as described (18). The chamber implant assay has been described previously (19). To assess vascular permeability in normal tissue, a modified version of the Miles assay was used. Two hundred microliters of Evans blue (0.5%) were injected into the tail vein of female FVB mice. Thirty minutes later, the mice were anesthetized (3% isoflurane in O2, Forene, Abbott AG) and then injected into the mammary fat pad of BN rats as previously described (20). When the tumors were established by orthotopic implantation of tumor fragments into the mammary fat pad of BN rats as previously described (20), rats were randomized into different treatment groups for efficacy studies. Tumor progression is shown in Supplementary Fig. S3. Magnetic resonance imaging. All experiments were performed on a Biospec DBX 47/30 spectrometer (Bruker BioSpin) at 4.7 T equipped with a self-shielded 12-cm bore gradient system capable of switching 200 mT/m in 170 μs, Paravision version 4.0 software for data acquisition, and a 1H resonator with a diameter of 70 mm (Bruker BioSpin). Animals were anesthetized outside the magnet with isoflurane (initial, 4%; after several minutes, 2.5–3.5%) in an O2/N2O 1:2 mixture applied with a face mask at a rate of 0.7 L/min. The contrast agent Vistarem (Ref P792, Guerbet) was injected as bolus (0.028 mmol/kg) via catheterized tail vein, automatically by a pulse program controlled infusion pump, after seven baseline acquisitions (injection rate set to 3 mL/min). The temporal resolution for dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) was 8 s and overall experimental duration was 12 min. Vistarem extravasation and the procedure for ktrans measurement in this tumor model used an adapted TrueFISP method, as described (21). For data analysis, the entire visible tumor on the MR image was included into the region of interest. Outliers were determined statistically using Grubb’s test (22). The significance of changes between the mean values before and after treatment and between the groups was assessed using one-way ANOVA with Dunnett’s or Tukey post hoc test, for which the significance value was set at P < 0.05.

Immunoblot analyses and immunohistochemistry. For immunoblot analyses, cells, tumors, ears, or lungs were lysed [50 mmol/L Tris-HCl, 120 mmol/L NaCl, 1 mmol/L EDTA, 6 mmol/L EGTA, 1% NP40, 20 mmol/L NaF, 1 mmol/L Pefabloc SC, 2 mL/L Na2VO4, 0.1%, 10 mmol/L SDS, Protease tablets (1 for 50 mL)]; insoluble proteins were removed by centrifugation (3,000 rpm for 30 min at 4°C); and supernatants were analyzed by SDS-PAGE followed by Western blotting. Antibodies were either from Cell Signaling (Akt, S473P-Akt, S1177P-eNOS, and S235/236P-RPS6) or from Spring Bioscience (tubulin). Quantiﬁcation of S240P-RPS6 levels was done on croiedbeded dissected tumor tissue (Tissue-Tek, Medite) after immersion in 4% phosphate buffered formaldehyde (pH 7.4; J.T. Baker, Medite, Nunningen, Switzerland) for 24 h at 4°C followed by parafﬁn embedding. Tissue sections (3 μm) were mounted on poly-lysine–coated microscope slides, dried, and stained with clone DAK-S6-240 antibody (Dako). Detection was ensured by incubation of a biotinylated horse anti-rat (mouse adsorbed) antibody (Vector Laboratories, Medite) and standard Vectorstain Elite ABC (Medite) and was followed by visualization with brown color substrate (Liquid DAB+ Substrate Chromogen System, Dako). Quantiﬁcation was done using the H-score, which combines percentage of stained tumor cells with obtained strength of staining intensity multiplied by weighting factors. The following formula was applied: H-score = (low %) × 1 + (medium %) × 2 + (high %) × 3. Statistical analysis was performed with one-way ANOVA.

Results

NVP-BEZ235 inhibits VEGF-induced endothelial cell proliferation in vitro and in vivo. The angiogenic factor VEGF promotes vascular endothelial cell proliferation (23), a prerequisite for the formation of new blood vessels from preexisting ones, in physiologic or pathologic conditions. To test whether NVP-BEZ235 could influence endothelial cell proliferation, HUVECs were plated in growth factor–depleted medium (Fig. 1) and exposed to VEGF in the presence of increasing concentrations of either NVP-BEZ235 (left) or the potent VEGFR1/2 inhibitor PTK/ZK (right). As expected, VEGF (10 ng/mL) increased BrdUrd uptake (compare lane 0 with lane +V, in the absence of inhibitors). Consistent with previously described studies (18), PTK/ZK reduced VEGF-induced proliferation with an IC50 of 15 mmol/L (n = 4) and cytostasis was.
observed at 100 nmol/L. NVP-BEZ235 provoked a more profound effect with an IC50 value of 1.8 nmol/L (n = 4) and cytostasis was obtained at 10 nmol/L. Moreover, at a concentration of 100 nmol/L, the BrdUrd uptake was less than the one observed in starved, VEGF-untreated cells, indicative of cell death induction. Biochemical analyses of HUVECs (Fig. 1B) revealed that NVP-BEZ235 efficiently abolished VEGF-induced S473P-Akt and S235/236P-RPS6 levels, showing efficient PI3K pathway inhibition.

NVP-BEZ235 was then tested for its ability to affect blood vessel formation in mice carrying VEGF-loaded agar chambers (19). In the absence of inhibitors, the weight of the chamber (Fig. 1C, left; Supplementary Fig. S1) as well as levels of the endothelial cell marker Tie-2 (Fig. 1C, right; ref. 24) were significantly increased in the presence of VEGF. Both effects were significantly inhibited (P < 0.05) in a dose-dependent manner when the mice were treated p.o. with either NVP-BEZ235 (p.o., 30 mg/kg), PTK/ZK (p.o., 100 mg/kg), or L-NAME (i.v., 20 mg/kg) and subjected to a modified Miles assay (Fig. 2A, left). PTK/ZK as well as NVP-BEZ235 treatment almost completely abrogated VEGF-induced Evans blue dye extravasation. Analysis of surrogate, highly vascularized, lung tissues (right) revealed that NVP-BEZ235 treatment led to strong reduction of S473P-Akt and S235/236P-RPS6 levels, consistent with the dual PI3K and mTOR inhibitory activity of the compound. Interestingly, S1177P-eNOS levels were similarly reduced, suggesting that the permeability blockade by NVP-BEZ235 might be the result of Akt inactivation, as a consequence of PI3K inhibition. Acute PTK/ZK treatment significantly reduced S473P-Akt but not S473P-RPS6 levels, suggesting that only PI3K and not mTOR is required for VEGF-induced permeability. NO production by eNOS plays a critical role in NVP-BEZ235 inhibits VEGF-induced permeability in normal tissue. As VEGF-induced vasculature leakage is controlled in part by activation of the PI3K/Akt pathway, we anticipated that NVP-BEZ235 might affect vascular permeability. To test this hypothesis, FVB mice were treated with either NVP-BEZ235 (p.o., 30 mg/kg), PTK/ZK (p.o., 100 mg/kg), or L-NAME (i.v., 20 mg/kg) and subjected to a modified Miles assay (Fig. 2A, left). PTK/ZK as well as NVP-BEZ235 treatment almost completely abrogated VEGF-induced Evans blue dye extravasation. Analysis of surrogate, highly vascularized, lung tissues (right) revealed that NVP-BEZ235 treatment led to strong reduction of S473P-Akt and S235/236P-RPS6 levels, consistent with the dual PI3K and mTOR inhibitory activity of the compound. Interestingly, S1177P-eNOS levels were similarly reduced, suggesting that the permeability blockade by NVP-BEZ235 might be the result of Akt inactivation, as a consequence of PI3K inhibition. Acute PTK/ZK treatment significantly reduced S473P-Akt but not S473P-RPS6 levels, suggesting that only PI3K and not mTOR is required for VEGF-induced permeability. NO production by eNOS plays a critical role in...
VEGF-induced permeability (25) and is directly regulated by Akt independently of mTOR activity (11, 12). Treatment with the eNOS inhibitor L-NAME also significantly reduced dye leakage (left), underlining the importance of eNOS in this model, as described by others (26). Time course analysis on NVP-BEZ235 treatment (Fig. 2B) revealed that VEGF-induced permeability is maximally inhibited for a period of 6 h, with complete recovery observed 48 h after treatment, consistent with the mouse pharmacokinetic of the compound (17), and shows the reversibility of the inhibition on permeability (14). To further confirm the importance of PI3K modulation, mice were treated with the mTOR allosteric inhibitor RAD001, and local VEGF challenge was performed either 1, 6, or 24 h after administration (Fig. 2C, left). In contrast to the effect observed with the dual PI3K/mTOR inhibitor, a single administration of RAD001 was unable to reduce the dye extravasation even at the 1-h time point (plasma $T_{\text{max}}$ of RAD001). Moreover, ex vivo analyses of lung tissues showed that S473P-Akt levels were unaffected (right).

NVP-BEZ235 inhibits vasculature leakage in the tumor tissue environment. The highly angiogenic nature of the

![Figure 2. NVP-BEZ235 blocks VEGF-induced neovascularization in vivo. A and B, FVB mice pretreated with either NVP-BEZ235 (30 mg/kg, p.o., n = 8, for 6 h), L-NAME (20 mg/kg, i.v., n = 5, for 1 h), or PTK/ZK (100 mg/kg, p.o., n = 6, for 2 h) or the vehicle control (n = 10) were injected i.v. with Evans blue and challenged 30 min later with VEGF injection in the ear. Mice were then sacrificed, the dye extravasation area was measured (left), and extracts from lungs were analyzed by Western blotting for the mentioned proteins (right), as described in Materials and Methods (modified Miles assay). B and C, FVB mice were pretreated p.o. with either NVP-BEZ235 (30 mg/kg; B) or RAD001 (5 mg/kg; C, left) for the indicated time, and VEGF-induced leakage in the ear was then performed as described in A. C, right, lungs from RAD001-treated mice (t = 6 h) were analyzed by Western blotting for S473P-Akt levels. *, $P < 0.05$.](image)
orthotopic rat mammary BN472 model (20) was confirmed by the fact that p.o., once per day treatment of tumor-bearing animals with PTK/ZK led to statistically significant antitumor activity (T/C of 27%) at a dose level of 100 mg/kg (Fig. 3A, left). NVP-BEZ235 was able to produce antitumor activity similar to PTK/ZK when given p.o., once per day. The efficacy was dose dependent, with T/C ratio of 90%, 51%, and 17% obtained at 3, 10, and 20 mg/kg dose levels, respectively (right). Ex vivo analyses of tumor tissues 6 h after the last administration (Fig. 3B) revealed that NVP-BEZ235 had, and in contrast to PTK/ZK, a profound effect on S473P-Akt, S235/236P-RPS6, and S1177P-eNOS levels.

To check whether the antitumor activity observed in this tumor model is linked to tumor vasculature permeability reduction, BN472 tumor-bearing animals were treated acutely, p.o., with either NVP-BEZ235 or PTK/ZK at efficacious doses (20 and 100 mg/kg, respectively), and 4 to 6 h after being subjected to an i.v. administration of FITC-dextran (vascular compartment labeling) and Evans blue dye (labeling of plasma proteins in the tumor extracellular matrix). Analyses of tumor sections (Fig. 3C, left) revealed that, at comparable vasculature density, the leakiness observed in the vehicle-treated animals was strongly reduced by PTK/ZK. This result supports the assumption that the tumor permeability is VEGFR dependent in this tumor model. NVP-BEZ235 showed a similar inhibitory profile as PTK/ZK. Evaluation of the Evans blue to FITC-dextran area ratios (right) confirmed a statistically significant 4-fold decrease in tumor vasculature permeability.

Figure 3. NVP-BEZ235 is efficacious against highly angiogenic BN472 mammary carcinoma tumors and reduces BN472 vasculature permeability. A, orthotopic BN472 tumor-bearing BN rats (n = 6–8) were treated p.o., once per day, with either PTK/ZK (100 mg/kg, left) or NVP-BEZ235 (3, 10, and 20 mg/kg, right). Tumor volume evolutions were recorded throughout the course of the study and plotted as fraction of the initial volume at day 0 of treatment. B, same as in A (treatment with NVP-BEZ235 was at a dose of 20 mg/kg), except that the animals were sacrificed 4 h after the last dose, 6 d after initiation of the treatment. Tumors were then recovered and analyzed by Western blotting for the mentioned proteins. C, BN rats bearing 2,500 mm³ orthotopic BN472 tumors were pretreated once with either NVP-BEZ235 (20 mg/kg, p.o.), PTK/ZK (100 mg/kg, p.o.), or the vehicle control. Four hours (NVP-BEZ235) or 6 h (PTK/ZK) after, i.v. administrations of Evans blue followed by FITC-dextran were performed. Animals were then sacrificed, and tumors were excised and snap frozen. Tumor sections were then analyzed (left, representative images) for vasculature content (FITC-dextran) and permeability (Evans blue), and Evans blue to FITC-dextran area ratio was calculated (right). *, P ≤ 0.05 versus vehicle-treated rats.
permeability. Similar observations were made when the A15 syngeneic rat glioma model was used (Supplementary Fig. S2).

**NVP-BEZ235 reduces tumor IFP.** Solid malignancies contain structurally abnormal and hyperpermeable vessels. This is often associated with a reduction of lymphatic vessels, resulting in high stromal fluid pressure. Reduction of vasculature permeability is therefore predicted to produce a decrease in tumor intrafluid pressure. To determine whether NVP-BEZ235 would affect IFP in tumors, a pressure-sensing catheter linked to a radio transmitter was introduced in BN472 tumor tissues grown orthotopically in BN rats (Supplementary Fig. S3). IFP values were then measured continuously in freely moving animals over a period of 24 h. PTK/ZK treatment produced a dose-dependent effect on tumor IFP, as reflected by the decrease of the area under the curve (AUC; Fig. 4A, top and bottom). The IFP was recorded for 24 h and variations (Δ) were plotted (top, dark lines, untreated animals; gray line, applied treatment as indicated above the graph). Bottom, the effects were quantified by integration of the AUC compared with untreated controls. B, same as in A, except that animals were treated p.o. twice with 20 mg/kg NVP-BEZ235 for a total duration of IFP recording of 48 h. Arrows, administration schedule. *, P < 0.05 (ANOVA-Dunnett’s).

**Figure 4.** NVP-BEZ235 reduces tumor IFP. A, orthotopic BN472 tumor-bearing rats containing a pressure-sensing catheter inserted in the tumor were treated once with either PTK/ZK at 12.5 mg/kg (n = 6), 25 mg/kg (n = 7), and 100 mg/kg (n = 6); NVP-BEZ235 at 3 mg/kg (n = 5; initial IFP: 17.6 mmHg ± 0.8), 10 mg/kg (n = 5; initial IFP: 14.7 mmHg ± 3.3), 20 mg/kg (n = 6; 14.5 mmHg ± 2.0), and 30 mg/kg (n = 6; 18.5 mmHg ± 2.5); or the vehicle control (n = 9; 18.2 mmHg ± 1.5). The IFP was recorded for 24 h and variations (Δ) were plotted (top, dark lines, untreated animals; gray line, applied treatment as indicated above the graph). Bottom, the effects were quantified by integration of the AUC compared with untreated controls. B, same as in A, except that animals were treated p.o. twice with 20 mg/kg NVP-BEZ235 for a total duration of IFP recording of 48 h. Arrows, administration schedule. *, P < 0.05 (ANOVA-Dunnett’s).

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To better refine the mechanism of NVP-BEZ235–induced IFP reduction, a similar study was performed with the rapamycin derivative mTORC1 allosteric inhibitor RAD001. RAD001 was previously found to be efficacious at a dose of 5 mg/kg, given once per day, against a rat pancreatic tumor model (27). At this dose, the IFP profile of BN472 tumors was unaffected by a single RAD001 treatment, being almost identical to the one obtained with the vehicle cohort (Fig. 5A). In the same experimental settings, RAD001 treatment did not affect tumor vasculature leak, as revealed by similar Evans blue dye extravasation in the tumor tissue and Evans blue to FITC-dextran area ratio to the one obtained with the vehicle-treated animals (Fig. 5B). To check whether RAD001 and NVP-BEZ235 would have a different antitumor activity profile, rats bearing orthotopic BN472 tumors were treated p.o. once per day, with either RAD001 (5 mg/kg; left) or NVP-BEZ235 (20 mg/kg; middle). C, tumor volume evolutions were recorded throughout the course of the study and plotted as fraction of the initial volume at day 0 of treatment. On day 6, animals were sacrificed, and tumors were excised and snap frozen. Tumor sections were then processed for p240/241-RPS6 level quantification (D) by immunohistochemistry (top, representative sections) and H-score evaluation (bottom), as described in Materials and Methods. *, P ≤ 0.05 versus vehicle-treated rats.

The effect on tumor vasculature can be monitored with noninvasive DCE-MRI. Tumor vasculature variables and characteristics can be visualized and quantified noninvasively by DCE-MRI. For instance, the use of high molecular weight contrasting such as Vistarem/P792 permits the estimation of the endothelial...
transfer coefficient ($K^{\text{trans}}$), a constant directly proportional to tumor vascular leakage (28). To this end, DCE-MRI imaging on Vistarem injection was carried out in the course of either PTK/ZK (100 mg/kg, p.o., once per day) or NVP-BEZ235 (20 mg/kg, p.o., once per day) treatment, 7 and 6 days, respectively, of orthotopic BN472 tumor-bearing animals. PTK/ZK treatment provoked a statistically significant ($P < 0.05$, Dunnett’s) $K^{\text{trans}}$ reduction already detectable on day 3 (62% reduction versus day 0, and 46% versus control) of the study (Fig. 6A). This effect was maintained up to day 7 (74% versus day 0, and 48% versus control), confirming, as expected from the tumor Evans blue leakage and IFP studies, that this process is VEGFR dependent. NVP-BEZ235 treatment led to a similar significant ($P < 0.05$) $K^{\text{trans}}$ reduction as early as day 2 (66% reduction versus day 0, and 50% versus control). This effect was even more pronounced on day 6 (72% reduction versus day 0, and 46% versus control). When $K^{\text{trans}}$ changes obtained on day 2 were plotted against the change in tumor size displayed by NVP-BEZ235 on day 6 (Fig. 6B), a statistically significant ($P < 0.01$) linear correlation was obtained ($r = 0.795$). A nonlinear mixed effect (NLME) approach (NONMEM, version V, double precision) was then used to analyze $K^{\text{trans}}$ measurements collected as a function of time and illustrate this last finding. This analysis was performed in rats treated by 20 mg/kg and provided a good fitting of the data with a structural model type "Imax," a log-normal interanimal variability and combined error model. As a final step, this model allowed to predict for each animal a value of $K^{\text{trans}}$ at days 1, 3, 4, and 5. These predictions obtained, where data were not available, were plotted versus observed change of tumor size at day 6 for each animal. This modeling analysis showed (see Fig. 6C) that the correlation observed before with values of $K^{\text{trans}}$ at day 2 is in fact very constant with time and could already be established at day 1 (see Fig. 6C).

Thus, vasculature permeability reduction by NVP-BEZ235 could be considered as an early biomarker predictor of long-term efficacy, and this effect can be monitored noninvasively.

**Discussion**

Molecules such as Avastin (bevacizumab), sunitinib (SU11248), or sorafenib (BAY43-9006) have received marketing approval by health authorities based on their antitumor efficacy in the treatment of specific solid tumors. These compounds have provided clinical proof...
of concept for the use of antiangiogenic therapies that target the VEGF/VEGFR axis. The data obtained with NVP-BEZ235 suggest that modulation of PI3K biological activity can also exert an antiangiogenic activity in cellular and in vivo settings.

PI3K is recruited and activated by various mitogenic and survival signaling pathways. As occurs with other growth and survival factors (e.g., epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor-I), on VEGF treatment, VEGFR2 (KDR) has been shown to recruit p110/p85 complex, a phenomenon necessary for porcine aortic endothelial cells and HUVEC VEGF-stimulated cell proliferation. As previously shown with other PI3K inhibitors (29), NVP-BEZ235 completely abrogates VEGF-induced Akt and RPS6 phosphorylation in endothelial cells. Inhibition of VEGF-mediated proliferation of endothelial cells has also been reported for mTOR allosteric inhibitors such as rapamycin and derivatives thereof (30). It has been recently described that rapamycin influences Akt activation in HUVECs, suggesting that in these cells PI3K and mTOR pathways are tightly linked (31). NVP-BEZ235 also efficiently impaired VEGF-induced HUVEC proliferation but, in addition, displayed significant cell death induction at the highest concentration tested. These data agree with the survival function of the PI3K/Akt pathway (32) and with the notion that VEGF is a survival factor for endothelial cells, in part through activation of the PI3K pathway (33). Altogether, these data would tend to suggest that dual PI3K/mTOR inhibition has a negative effect on endothelial cell survival and proliferation, two critical phenomena in the angiogenic processes occurring in growing tumors. Evidence that this has an effect on neovascularization in vivo is revealed by the fact that NVP-BEZ235 significantly inhibited VEGF-induced formation of vascularized tissue around a s.c. growth factor–impregnated implant. It is unclear if this effect is entirely due to reduced endothelial cell proliferation and/or increased cell death. The possibility still exists that dual PI3K/mTOR inhibition might also negatively influence the migration of endothelial cells, which are normally attached to the extracellular matrix. Moreover, a broad effect on the overall vessel structure, or on the pericytes, cannot be excluded.

VEGF was originally discovered as a vascular permeability factor, a protein secreted by tumor cells that potently stimulates vascular leak (34). Vascular permeability (or leakiness) is a complex process that can be defined as the movement of fluids and molecules between the vascular and extravascular compartments and is dependent on several variables (35). Hence, VEGF is a secreted factor that regulates angiogenesis and vascular permeability, and although both phenomena seem to be independent, vascular leak is believed to augment the angiogenic response (36). NVP-BEZ235 potently inhibited VEGF-induced leakage both in an ear model and in the tumor environment. In both cases, the observed phenotype was associated with reduction in S1177P-eNOS and S473P-Akt levels, which is consistent with the observation that VEGF regulates eNOS activity in an Akt-dependent manner (11, 12) and that VEGF-induced leakiness in postcapillary venules can be readily blocked with PI3K inhibitors (37). RAD001 was not able to mimic this effect, although strong concomitant reduction in S240P-RPS6 levels was observed. These findings suggest that under the tested experimental conditions Akt-dependent eNOS activation is not mTORC1 dependent. Interestingly, PTK/ZK treatment did not significantly affect eNOS activation, although significant vascular leakage reduction was observed. The likely explanation for this result might come in part from the pleiotropic nature of the VEGF response on endothelial cells as several VEGF downstream signal transducers other than PI3K have been implicated in the regulation of the vasculature permeability (3). Additional biochemical analysis would therefore be required to decipher the mechanism of action of PTK/ZK.

In addition to its vascular hyperpermeability stage, the tumor is also unable to form its own lymphatic system so that solutes cannot be efficiently drained from its interstitial space. Furthermore, the blood vessels are compressed by proliferating cells and this increases the microvascular pressure (38). Together, these factors contribute to an elevated IFP in solid tumors compared with normal tissues. We describe here for the first time a fully implantable miniaturized radiotelemetry system that we used to monitor tumor IFP in nonanesthetized freely moving, unstressed rats maintained in their home cage, each animal being used as its own control. Confirming the data obtained with the direct assessment on permeability, NVP-BEZ235, similar to PTK/ZK but not RAD001, significantly reduced tumor IFP, already after a few hours after drug administration. In such a short period, neo-lymphangiogenic and tumor shrinkage processes could be considered as negligible entities in comparison with the more dynamic effect on the existing tumor vasculature.

To associate the effects observed on vessel permeability with antitumor activity, we examined the effects of NVP-BEZ235 and PTK/ZK administration on tumor progression in BN472 tumor-bearing rats. NVP-BEZ235 and PTK/ZK at their effective dose for vessel leakage inhibition significantly reduced tumor growth by 83% and 73%, respectively. To the best of our knowledge, this is the first time that a decrease in vessel permeability caused by a PI3K inhibitor has been linked to its antitumor activity. A similar link has been proposed for cavitratin, a selective inhibitor of NO-dependent vascular leakage, and tumor progression in mice (39). The relation between vessel permeability inhibition and tumor growth observed with PTK/ZK is similar to the one obtained with other angiogenesis inhibitors such as TNP-470, capostatrin, and angiotatin (40) or DC101, an anti-VEGFR2 antibody (41).

Distinguishing the effects of direct mTOR inhibition and inhibition of the PI3K/mTOR pathway in experimental tumors is difficult. Rapamycin and derivatives possess a broad antitumor activity, which comprises antitumor cell antiangiogenic and antivascular activities (42, 43), but the dosing regimen is critical to achieve an optimal antiangiogenic effect (44). RAD001 also possesses broad antitumor activity, including antiangiogenic activity, manifested by a reduction in tumor VEGF production and vascular density (45, 46). Some PTEN-negative tumors, which alleviate control of PI3K activity and facilitate PI3K/mTOR pathway dependency, show increased sensitivity to rapamycin-dependent mTOR block (47). Both mTORC1 and mTORC2 are required, acting at different times, for the proliferation of endothelial cells induced by hypoxia (48), but prolonged rapamycin treatment eventually reduces functional mTORC2.

Altogether, the results obtained with NVP-BEZ235 indicate that VEGF-induced eNOS activation and subsequent tumor-associated vascular leakiness are likely to be efficacy biomarkers for anti-VEGF/VEGFR and anti-PI3K therapies. DCE-MRI is a noninvasive imaging technology that measures vessel permeability, leakage space, and partial blood volume of tumors. These features could be exploited for measuring PI3K target inhibition in clinical settings. Such an approach has already been used for early assessment of PTK/ZK efficacy in phase I trials (49). In our preclinical studies with the BN472 tumor...
model, PTK/ZK and NVP-BEZ235 markedly decreased fractional $K'_{trans}$ of the observed changes in fractional $K'_{trans}$ preceded and were correlated with tumor response, suggesting that DCE-MRI measurements can be a suitable translational biomarker strategy for predicting antitumor activity in man. Our data suggest that NVP-BEZ235, a dual PTK/mTOR catalytic inhibitor, interferes with tumor growth likely by affecting tumor cells and their vasculature system. This latter effect might be evaluated in clinical settings by using a noninvasive technique.

Disclosures of Potential Conflicts of Interest

R. Cozens: Stockholder, Novartis. S-M. Main: Stockholder, Novartis. The other authors disclosed no potential conflicts of interest.

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References


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Christian R. Schnell, Frédéric Stauffer, Peter R. Allegrini, et al.


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