Dual Inhibition of the PI3K/mTOR Pathway Increases Tumor Radiosensitivity by Normalizing Tumor Vasculature

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Abstract

The aberrant vascular architecture of solid tumors results in hypoxia that limits the efficacy of radiotherapy. Vascular normalization using antiangiogenic agents has been proposed as a means to improve radiation therapy by enhancing tumor oxygenation, but only short-lived effects for this strategy have been reported so far. Here, we show that NVP-BEZ235, a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mTOR, can improve tumor oxygenation and vascular structure over a prolonged period that achieves the aim of effective vascular normalization. Because PI3K inhibition can radiosensitize tumor cells themselves, our experimental design explicitly distinguished effects on the blood vasculature versus tumor cells. Drug administration coincident with radiation enhanced the delay in tumor growth without changing tumor oxygenation, establishing that radiosensitization is a component of the response. However, the enhanced growth delay was substantially greater after induction of vascular normalization, meaning that this treatment enhanced the tumoral radioresponse. Importantly, changes in vascular morphology persisted throughout the entire course of the experiment. Our findings indicated that targeting the PI3K/mTOR pathway can modulate the tumor microenvironment to induce a prolonged normalization of blood vessels. The substantial therapeutic gain observed after combination of NVP-BEZ235 with irradiation has conceptual implications for cancer therapy and could be of broad translational importance.

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Introduction

Radiotherapy is an important tool in cancer therapy. Both intrinsic and extrinsic factors can influence the sensitivity of tumor cells to radiation (1). The sensitivity of the cancer cell itself (intrinsic component) is an important determinant of the efficacy of radiotherapy (2) and is adversely affected by the activation of oncogenic signal transduction cascades such as the PI3K/Akt pathway (3, 4). We have previously shown that inhibition of this cascade can enhance radiosensitivity of tumor cells leaving the normal cells unaffected, providing an attractive concept for improving therapeutic outcome (5).

Tumor hypoxia and extrinsic factors reduce radiosensitivity. This occurs both because oxygen contributes to fixation of DNA damage and also through less well-understood mechanisms, by which hypoxia signaling leads to activation of cytokine signaling and alteration in DNA damage repair mechanisms that promote tumor cell survival after DNA damage (6). As a consequence, reduction in hypoxia has been sought as a means to improve the efficacy of radiation therapy. Manipulation of tumor angiogenesis may be one way to achieve this goal (7, 8).

The vasculature of solid tumors is morphologically aberrant leading to leaky immature vessels (8, 9). This, in conjunction with impaired lymphatic drainage, creates an abnormal tumor microenvironment (TME) characterized by elevated interstitial fluid pressure, poor perfusion, and hypoxia (10). These factors can lead to decreased delivery of chemotherapy and a reduced response to radiation and, ultimately, treatment failure (6).

Angiogenesis is regulated by molecular pathways with the VEGF network playing an important role in tumor angiogenesis. Although the main goal in using antiangiogenic agents in cancer therapy was to impair tumor angiogenesis, treatment with some of these agents such as VEGFR2 inhibitors resulted in the transient formation of a more regularized vascular system (11, 12) termed "vascular normalization" that increased radiation efficacy due to reduced hypoxia (7). The difficulty in clinical application of this strategy, however, is that the induction of vascular normalization is transient and with greater exposure to the anti-VEGFR2 agents, the vasculature becomes diminished and hypoxia increases, potentially counteracting the previously beneficial effect (13).
We and others have previously investigated the possibility of inhibiting oncogenic signaling in cancer cells as an alternative strategy to modulate tumor vasculature. Inhibition of the Ras-EGFR-Pi3K-Akt signaling at multiple points in this pathway led to vascular normalization accompanied by improved tumor oxygenation and perfusion (14). Cerigilia and colleagues observed similar effects using the epidermal growth factor receptor (EGFR) inhibitor erlotinib that resulted in improved delivery of cisplatin and synergistic inhibition of tumor growth (15). Importantly, inhibition of this pathway seemed to result in more durable induction of vascular normalization than with agents targeting VEGF signaling (14).

In this study, we investigated the potential of the dual Pi3K/mTOR inhibitor NVP-BEZ235 (BEZ235) and the single phosphoinositide-3-kinase (Pi3K) inhibitor NVP-BKM120 (BKM120) to improve radiotherapy by inducing vascular normalization. Here, we have asked whether vascular normalization induced in this way (i) would be durable and (ii) would act in combination with radiation in delaying tumor regrowth. We distinguished between the contributions of the vascular normalization and hypoxia and the intrinsic cell radiosensitivity to show the contribution of vascular normalization to the improved response to radiation.

Materials and Methods

Cell culture and transfection

Tumor cells were cultured as reported (14). The HT1080-HRE-luc was constructed and cultured as reported (14). FaDu cells were transfected using the Cignal Lenti HIF ReporterCL luciferase (SA Biosciences), according to the manufacturer’s instructions.

Xenograft studies

All animal experiments were carried out in accordance with U.K. Home Office regulations. FaDu-HRE-luc and HT1080-HRE-luc (1 × 10⁶ in 100 μL serum-free medium) were inoculated subcutaneously in athymic nude mice, in front of the right hind leg. In HT-1080 study, 2 different doses were tested for BKM120 (30 and 15 mg/kg) and BEZ235 (20 and 10 mg/kg) p.o., via oral gavage. Only the high dose was applied in FaDu-HRE-luc experiment, on the basis of the dose-dependent inhibition of Pi3K signaling.

For the first series of experiments (nonradiation) testing both cell lines, mice were divided into cohorts of 4 mice per drug per dose. A placebo cohort that received vehicle (NMP/PEG300) was included as well. Drug administration was commenced once tumors reached a volume of 100 mm³ and were given daily for 1 week. At the end of the experiments, the mice were euthanized and the tumors were fixed in 3% neutral-buffered formalin. For the second series of experiment done (radiation) using FaDu-HRE-luc xenografts, BEZ235 (20 mg/kg) administration was started once tumors reached a volume of 100 mm³ and was given daily, throughout the experiment. A single dose (6 Gy) was applied at day 7 with a 250-kV orthovoltage irradiator (Philips RT 250) at a dose rate of 2.63 Gy/min, by copper shielding. The source-to-target distance was 30 cm. Tumor size was measured with calipers using the formula V = (a × b²)/2, in which a and b are the largest and the smallest perpendicular diameters, respectively.

In vivo real-time optical and ultrasound imaging

Mice were anesthetized (2% isoflurane in oxygen) and injected intraperitoneally with 150 mg/kg luciferin (Xenogen). Imaging was done with the IVIS 200 system (Xenogen). Bioluminescence was calculated as previously described (14). Tumor hypoxia, represented by photon flux, was normalized to the untreated (control) group.

Evaluation of tumor perfusion was done with the VisualSonics VEVO770 Mirco-Ultrasound platform with the RMV 704 probe by image enhancement using contrast microbubbles (Veo MicroMarker Contrast Agent Kit) and Power Doppler, as described (14).

Microscopy, immunostaining, and VEGF ELISA

The vascular network was visualized using anti-CD31-RPE–conjugated antibody (Biolegend) at the end of each treatment, and tumor hypoxia using EF5 nitroimidazole (14). Immunofluorescence staining for NG2 (1:100; Chemicon) and CD31 (1:50; BD Pharmingen) was completed using the TSA biotin system (Pelkin-Elmer) and streptavidin-conjugated fluorophores Alexa Fluor 488 and 546 (Invitrogen). Images were acquired using the Leica DMIRBE microscope with a Hamamatsu camera. Immunohistochemistry for phosphorylated Akt (Ser473) and mTOR (Ser2448, dilution 1:50; Ab from Cell Signaling) was as described (16). Images were acquired using a Nikon Eclipse E800 microscope with a Nikon DXM1200 digital camera (%10 and ×40 magnification). VEGF levels were measured by the Quantikine VEGF ELISA kit (R&D Systems).

Statistical analyses

Quantitative data were expressed as means ± SD. The significance of differences between the means was assessed by 2-tailed t test or 1-way ANOVA using the GraphPad Prism program version 4.0 (GraphPad Software). Statistically significant difference was considered as P < 0.05.

Results

Dose–response of BEZ235 and BKM120 in HT1080 and FaDu xenografts

We first established the biologically effective doses for HT1080 xenografts using immunostaining for pAKT as an indicator of Pi3K activity and pmTOR for mTOR activity. Tumors were examined after the mice had received 7 daily oral doses (Fig. 1A). BEZ235 at 20 mg/kg completely abrogated both pAKT and pmTOR staining. At 10 mg/kg pmTOR staining was still apparent. pAKT staining was reduced but also still apparent. BKM120 at the higher dose resulted in the absence of pAKT staining with partial reduction at the lower dose. pmTOR was not affected by BKM120 at either dose. We then tested the effective doses on mice bearing FaDu xenografts with equivalent results (Fig. 1B). Despite the high degree of signaling inhibition, the growth of the treated tumors was equivalent to that of controls, regardless of the drugs or doses used (Fig. 1C and D).
PI3K/mTOR inhibition reduces tumor hypoxia and increase perfusion in a dose-dependent fashion

We previously showed that inhibition of the RAS–PI3K pathway led to reductions in tumor hypoxia and increased perfusion using a different series of drugs (14). The effect was evaluated in mice bearing tumors derived from cell lines bearing the hypoxia reporter HRE-luciferase, FaDu-HRE-Luc, and HT1080-HRE-Luc. We previously showed that...
the level of luciferase expression correlated with hypoxia as measured by EF5 staining (14). After 7 days of treatment with the indicated drugs luciferase was again assessed using bioluminescence. Figure 2A shows that hypoxia was reduced in the HT1080 tumors only at the higher doses that led to substantial pAKT reduction. Treatment of FaDu tumors also led to decreased hypoxia, independently confirmed by EF5 staining (Fig. 2B and C). A moderate but significant decrease in VEGF levels was found in FaDu tumors (Supplementary Fig. S1). Similarly, perfusion measured by Doppler ultrasound using microbubbles was increased in the HT1080 tumors treated by doses sufficient to cause a substantial decrease in pAKT levels, but not at the lower doses (Fig. 3A and B and Supplementary Fig. S2A). Thus the pathophysiologic changes only occurred at drug doses giving substantial inhibition of pAKT. The improvement in perfusion was confirmed in FaDu tumors (Fig. 3C and D and Supplementary Fig. S2B and S2C).

**BEZ235 and BKM120 promote vascular normalization and remodeling**

Vascular remodeling consistent with vascular normalization underlies the increased perfusion. *Ex vivo* confocal/
The effect of BEZ235 on radiation response

Because hypoxic tumors are substantially less responsive to radiation therapy, we hypothesized that treatment with PI3K inhibition should lead to better radiation response. As the drug with dual PI3K and mTOR inhibition led to the greatest magnitude of vascular modification, we tested the treatment with the dual inhibitor for the ability to enhance the efficacy of radiation therapy using a tumor regrowth delay assay (Fig. 5A). Mice bearing FaDu tumors were treated with BEZ235 (20 mg/kg) for 7 days prior to irradiation with a single dose of 6 Gy. We continued to administer BEZ235 daily throughout the course of this experiment. In unirradiated mice treated with vehicle or BEZ235, tumors reached the maximum permitted volume (~750 mm³) at days 18 (control) and 22 (BEZ235), respectively. The median time to reach this volume was increased by 12 days by radiation alone (XRT). In contrast, radiation combined with BEZ235 delayed tumor growth by 58 days (BEZ235-XRT), a strongly supra-additive growth delay.

Because inhibition of PI3K or PI3K/mTOR can sensitize tumor cells to radiation, independently of changes in oxygenation, we asked whether some component of the radiosensitization could be attributed to this effect by treating mice with a single dose of BEZ235 only on the day of irradiation (Fig. 5B). In addition, BEZ235 has been shown to inhibit ATR, ATM, and DNA-PK, all of which would be expected to lead to radiosensitization (17). This schedule did not alter tumor oxygenation or perfusion. It did however lead to a superadditive growth delay of 40 days compared with 30 of radiation alone. Thus, the drug must lead to alteration in the intrinsic radiosensitivity of the tumor independent of oxygenation changes.

An additional schedule was tested whereby daily administration of BEZ235 was initiated 3 days after irradiation (Fig. 5C). This treatment was not significantly different from XRT alone. Thus, we could conclude that radiosensitization by BEZ235 was partially due to enhanced oxygenation when administered prior to radiation but also affected by direct radiosensitization of the tumor cells themselves (Fig. 5D).

Hypoxia, perfusion, and vascular morphology in irradiated tumors

We monitored tumor hypoxia using luciferase during the radiation-induced growth delay experiments described above (Fig. 6A and B). Tumors in the XRT and BEZ235-XRT group did not show changes in hypoxia when compared with control and remained hypoxic until they reached their maximum size...
(day 30 and 40, respectively). Mice in the BEZ235_{20 mg/kg} group had a dramatic decrease in hypoxia after 7 days of treatment that persisted until the tumors reached their maximum size. A similar decrease in hypoxia was observed in the treated, radiated BEZ235_{5 mg/kg/XRT7} group. With time, as these tumors regrew hypoxia increased, but did not reach the initial pretreatment level by the time of sacrifice (day 58). BEZ235_{5 mg/kg/XRT7} tumors also showed a persistent decrease in hypoxia.

Perfusion increased significantly above the control. Some tumors regrew hypoxia after 7 days of treatment that persisted until the tumors reached their maximum size. A similar decrease in hypoxia was observed in the treated, radiated BEZ235_{5 mg/kg/XRT7} group. With time, as these tumors regrew hypoxia increased, but did not reach the initial pretreatment level by the time of sacrifice (day 58). BEZ235_{5 mg/kg/XRT7} tumors also showed a persistent decrease in hypoxia. EF5 staining at the time of sacrifice confirmed that the luciferase measurements correlated with hypoxia (Supplementary Fig. S5A). Thus, the decrease in hypoxia persisted throughout the time of the experiment regardless of radiation. VEGF levels were decreased in tumors treated with BEZ235 for long time (Supplementary Fig. S5B).

We also monitored tumor perfusion throughout the experiment (Supplementary Fig. S6A and S6D). There was no significant alteration in the level of perfusion in the control, XRT7, or BEZ235_{20 mg/kg/XRT7} groups during the course of the experiment. As expected, both the BEZ235_{20 mg/kg} tumors and the BEZ235_{5 mg/kg/XRT7} tumors had increased perfusion that persisted until sacrifice. As with hypoxia, the level of perfusion began to decrease but remained significantly above the control. Some of the decrease may be due to areas of necrosis observed by ultrasound monitoring at this time. In contrast to the finding about hypoxia, no significant improvement in perfusion was observed in the BEZ235_{10 mg/kg} group.

Vascular morphology was determined in the tumors after sacrifice. Mice in the control group had typically chaotic tumor vasculature (day 18). Mice from the XRT7 and BEZ235_{5 mg/kg/XRT7} groups during the course of the experiment.

Figure 4. BEZ235- and BKM120-induced vascular remodeling and normalization. HT1080-bearing mice were treated as indicated and CD31-PE was injected intravenously 1 minute before sacrifice (day 7). A, representative images from a single 1-μm optical section of tumor vasculature (top: ×10 magnification). AMIRA software 3D reconstructions (middle), and Trace3D software vascular tree tracings (bottom) in HT1080 tumors. B, Trace3D software enabled calculation of vessel density, vessel diameter, length, and tortuosity in HT1080 model. Columns, mean; bars, SD. *P < 0.05 over vehicle-treated control.
Vascular Normalization by PI3K/mTOR Inhibitors

Figure 5. Therapeutic efficacy of BEZ235 (20 mg/kg) and radiation in FaDu-HRE-luc xenograft model. A–C, when tumors reached a volume of approximately 100 mm³ cm⁻³, mice were treated with either vehicle (control), BEZ235 (on days 0–22; BEZ2350–22), 6 Gy (on day 7; XRT7), BEZ235 single dose at the day of irradiation (BEZ235×XRT7), BEZ235 pretreatment followed by 6 Gy and continued throughout the course of experiment (BEZ235 on days 0–58 and 6 Gy on day 7; BEZ2350–58×XRT7) and 6 Gy followed by BEZ235 (6 Gy on day 7 and BEZ235 on days 10–34). Points, mean of tumor volume (mm³) of each treatment group (n = 4–5); bars, SD. D, Pretreatment with BEZ235 results in supra-additive tumor growth delay effect. Columns, mean time of each treatment group to reach 700 mm³; bars, SD. **, P < 0.01; ***, P < 0.001.

remodeled vascular morphology toward well-organized, longer vessels with large diameter that were enriched with pericytes, resembling those in normal tissues. These changes were accompanied by improved tumor perfusion and oxygenation and improved response to radiotherapy but only at drug doses that showed substantial inhibition of pAKT, highlighting the potential of signaling in this pathway in modulating vascular remodeling and altering the TME. These data are in line with our previous work on oncogenic signaling inhibitors of the Ras/PI3K/Akt pathway (14, 19) and those of using the EGFR inhibitor erlotinib-modulated TME in a similar way (15).

We hypothesized that the improvement in tumor blood flow and oxygenation as observed in treated tumors at day 7 result in enhanced response to radiation in vivo. BEZ235 leads to radiosensitization of tumors derived from cell lines with Kras mutations. However, this work did not distinguish between the effects on radiosensitization of the tumor cells themselves and effects on the TME (20). Administration of BEZ235 only on the day of irradiation (BEZ235×XRT7) resulted in an additional 10 days growth delay over XRT7 alone which is significantly lower than the 28 days growth delay observed in the BEZ2350–58×XRT7 (P < 0.001). Moreover, BEZ235 administration initiated 3 days postirradiation (BEZ2350–34×XRT7) had essentially no effect on growth delay. These results indicate that PI3K/mTOR inhibitors function by increasing both the intrinsic and the extrinsic sensitivity of cancer cells to irradiation (ii) alteration of TME can substantially improve response to radiotherapy, underlying the importance of tumor oxygenation for radiotherapy (4, 14). These findings show that the benefit of PI3K/mTOR inhibition might be enhanced by precise treatment scheduling. These (our) experiments utilized the tumor growth delay assay which can, under some circumstances, yield different results from tumor control assays. Relevant to this study, Baumann and colleagues found a growth delay similar to the anti-EGFR antibody cetuximab after radiation, but the tyrosine kinase inhibitors BIBX1382BS and erlotinib failed to improve local tumor control in the FaDu model (21–23). However in these experiments, the drugs were first administered on the same day as radiation was initiated, a dosing schedule that would not have maximized the effect of decreased hypoxia. Undoubtedly, the local tumor control remains an important and relevant parameter in determining the therapeutic effect of irradiation when combined with different drugs (24). Nevertheless, the impressive growth delay of 38 days observed in the BEZ2350–58×XRT7 group, as compared with XRT7, indicates a substantial response to radiotherapy.

The effects of PI3K/Akt/mTOR inhibition on the cancer vasculature and radiation outcome resemble the transient changes reported from Jain and colleagues after anti-VEGFR treatment (11, 12). Blockade of the VEGF pathway normalized blood vessel and improved oxygenation, providing a window for combination with radiotherapy (7). Dings and colleagues showed that radiotherapy was most effective when administered after treatment with bevacinuzumab in ovarian, breast, and melanoma models (25). Bevacizumab and continuous delivery of IFN-β presented changes consistent with normalization and increased radiation response in an orthotopic glioma model (26).

However, the vascular remodeling that occurs after anti-angiogenic therapy is only transient, lasting approximately 5
...days (7). In contrast, our data suggest that PI3K/mTOR could further extend this normalization window. Even though irradiation did not alter vascular density in our study, we observed areas of leakage in previously irradiated vessels. Importantly, no improvement in vascular morphology was observed in the BEZ23510–34XRT7 arm. We are tempted to speculate that this can be attributed to the effect of irradiation on endothelial cells, at least in part, or to compensatory mechanisms of the tumors cells. Radiation can lead to endothelial cell damage which could result in partial vessel collapse and rebound hypoxia (27–29). Interestingly, FaDu tumors grown in preirradiated tissues presented a better response to the VEGFR inhibitors, indicating that irradiated blood vessels are more susceptible to these drugs than unirradiated vessels (30).
Whether PI3K/mTOR inhibitors could potentially have similar effects on tumors grown in preirradiated tumor bed remains to be investigated.

Increased perfusion in normalized tumor vessels might have been expected to increase tumor growth rate. However, no alteration in tumor growth was seen with BEZ235 or BKM120. This could be attributed to the antiproliferative effect of the signaling inhibitors that counterbalanced increased tumor growth from the increased perfusion or, perhaps, improved oxygenation renders the tumor less aggressive.

How could inhibition of PI3K/mTOR pathway result in improvement of vascular functionality? One explanation could be that both BKM120 and BEZ235 caused a moderate down-regulation of VEGF expression, at the doses which normalized blood vessels. This is in accordance to the reports by Qayum and colleagues and Cerniglia and colleagues with the signaling inhibitors erlotinib and nelfinavir, respectively (14, 15). In contrast, antiangiogenic agents aim at inducing a complete inhibition of VEGF. Anti-VEGF therapy can induce tumor hypoxia, leading to increased invasion and metastasis in tumor models (31, 32). It should be mentioned that in preliminary studies that we conducted, BEZ235 and BKM120 resulted in extensive vascular damage with increased leakage, rather than normalization, when used at higher doses than were sufficient to inhibit pAKT (45 and 60 mg/kg, respectively; data not shown).

A second possible explanation could be the involvement of the PI3K/mTOR pathway in endothelial signaling (33–35). Activation of PI3K/Akt signaling upregulated VEGF and promoted vessel formation in chicken embryos (36). Promising preclinical activity has been shown for BEZ235, while blockade of neovascularization and downregulation of VEGF and HIF-1 have been reported (37–41). BEZ235 inhibited VEGF-induced permeability and vascular leakage and reduced interstitial fluid pressure (42). These data complement our findings. Moreover, Akt overexpression in endothelial cells resulted in embryonic lethality due to abnormal vascular remodeling (43). Transient expression of myrAkt was capable of altering the normal response to oxygen-induced remodeling without causing vascular malformations. Therefore, physiologic levels of Akt signaling modulate microvascular patterning (43). Phung and colleagues showed that Akt is chronically activated in tumor-associated endothelial cells but not in normal tissues. Sustained endothelial activation of Akt in otherwise healthy tissues, induced vascular malformations and recapitulated the abnormal structural and functional features of tumor blood vessels (44). These changes were reversible upon inactivation of Akt, providing a rationale for Akt inhibition to decrease angiogenesis and excessive vascular permeability in cancer (44). In our present work, vessel normalization was accompanied by global inhibition of Akt and mTOR phosphorylation, indicating a direct correlation between PI3K/Akt/mTOR signaling and vascular architecture. It is unclear whether the cancer cell, the host vasculature, or both are the target for these TME effects. If it is the cancer cell, then stratification for PI3K–AKT signaling would be warranted. However, if it is the host vessels, then the effect may be more universal. These data suggest that decreased hypoxia might be used as a surrogate marker to show PI3K inhibition in tumors.

Conclusion

The results of this study provide evidence that inhibition of the PI3K/mTOR signaling by BKM120 and BEZ235 displays significant effects on TME by remodeling blood vessels and enhancing tumor perfusion and oxygenation. BEZ235-induced changes promoted a substantial response to radiotherapy and showed a supra-additive effect in delaying tumor regrowth, as compared with other drug–radiation combinations. To the best of our knowledge, this is the first study to analyze in detail the effect of different scheduling of PI3K/mTOR inhibition on radiation efficacy. Modulation of oncogenic signaling could be potentially used as a therapeutic approach for cancer therapy.

Disclosure of Potential Conflicts of Interest

S.-M. Maira and W. Hackl are Novartis Pharma employees and shareholders. No potential conflicts of interest were disclosed by the other authors.

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