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 anti-angiogenic agents has been proposed as a means to improve radiation therapy by enhancing tumor oxygenation, but only short-lived effects for this strategy have reported so far. Here we show that NVP-BEZ235, a dual inhibitor of phosphoinoside-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 indicate 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.

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 tumour 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 vascular endothelial growth factor (VEGF) network playing an important role in tumor angiogenesis. While the main goal in using anti-angiogenic 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). Cerniglia et al. observed similar effects using the EGFR inhibitor erlotinib that resulted in improved delivery of cisplatin and synergistic inhibition of tumor growth (15). Importantly, inhibition of this pathway appeared to result in more durable induction of vascular normalization than with agents targeting VEGF signaling (14).

In the present study, we investigated the potential of the dual PI3K/mTOR inhibitor NVP-BEZ235 (BEZ235) and the single PI3K inhibitor NVP-BKM120 (BKM120) to improve radiotherapy by inducing vascular normalization. Here we have asked whether vascular normalization induced in this way 1) would be durable and 2) 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 demonstrate 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 (SABiosciences), 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 cells (1 x 10^6 in 100 μL serum-free medium) were inoculated s.c. in athymic nude mice, in front of the right hind leg. In HT-1080 study, two 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, based on the dose-dependent inhibition of PI3K signaling.

For the first series of experiments (non-radiation) 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 mm^3 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 performed (radiation) using FaDu-HRE-luc xenografts, BEZ235 (20 mg/Kg) administration was started once tumors reached a volume of 100 mm^3 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 \( V = \frac{a \times b^2}{2} \), where \( a \) and \( b \) are the largest and the smallest perpendicular diameters, respectively.

In vivo real-time optical and ultrasound imaging

Mice were anesthetized (2% isofluorane in oxygen) and injected i.p. with 150 mg/kg luciferin (Xenogen). Imaging was performed 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 Micro-Ultrasound platform with the RMV 704 probe by image enhancement using contrast microbubbles (Vevo 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 nitromidazole (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 DMRBE microscope with a Hamamatsu camera. Immunohistochemistry for phosphorylated Akt (Ser473) and mTOR (Ser2448) (Ab from Cell Signaling; dilution 1:50) was as described (16). Images were acquired using a Nikon Eclipse E800 microscope with a Nikon DMX1200 digital camera (x10 and x40 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 two-tailed t-test or one-way ANOVA using the
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 seven 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-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 demonstrated 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. Fig 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-C). A moderate but significant decrease in VEGF levels was found in FaDu tumours (Suppl. Fig. 1). 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-B and Suppl. Fig. 2A). Thus the pathophysiological changes only occurred at drug doses giving substantial inhibition of pAKT. The improvement in perfusion was confirmed in FaDu tumors (Fig. 3C-D and Suppl. Fig. 2B-C).

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

Vascular remodelling consistent with vascular normalization underlies the increased perfusion. *Ex vivo* confocal/multiphoton images from sections of the tumors were reconstructed to reveal the vascular structure and Trace 3D software was applied to quantify the vascular characteristics (14). Increased average vascular diameter, decreased branching (increased length between branch points) and decreased tortuosity were induced only after treatment that had reduced pAKT immunostaining (Fig. 4A-B). The vascular remodelling effect of BKM120 and BEZ235 was confirmed in FaDu tumors (Suppl. Fig. 3A-B). Vasculature in the treated tumors had significantly increased pericyte coverage, as assessed by NG2 staining, characteristic of a more mature vessel morphology (Suppl. Fig. 4A-B). Thus changes in vascular morphology, decreased hypoxia and increased perfusion appeared to be dependent on PI3K inhibition.

**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 (BEZ2350-22), respectively. The median time to reach this volume was increased by 12 days by radiation alone (XRT7). In contrast, radiation combined with BEZ235 delayed tumor growth by 58 days (BEZ2350-58XRT7), a strongly supra-additive growth delay.

Because inhibition of PI3K or PI3K/mTOR can sensitise 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 to 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 and was continued throughout the experiment (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-B). Tumors in the XRT\textsubscript{7} and BEZ235\textsubscript{7}XRT\textsubscript{7} group did not show changes in hypoxia when compared to control and remained hypoxic until they reached their maximum size (day 30 and 40, respectively). Mice in the BEZ235\textsubscript{0-22} 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\textsubscript{0-58}XRT\textsubscript{7} 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\textsubscript{10-34}XRT\textsubscript{7} tumors also showed a persistent decrease in hypoxia. EF5 staining at the time of sacrifice confirmed that the luciferase measurements correlated with hypoxia (Suppl. Fig. 5A). Thus the decrease in hypoxia persisted throughout the time of the experiment regardless of radiation. VEGF levels were decreased in tumours treated with BEZ235 for long time (Suppl. Fig. 5B).

We also monitored tumor perfusion throughout the experiment (Suppl. Fig. 6A-D). There was no significant alteration in the level of perfusion in the control, XRT\textsubscript{7} or BEZ235\textsubscript{7}XRT\textsubscript{7} groups during the course of the experiment. As expected, both the BEZ235\textsubscript{0-22} tumors and the BEZ235\textsubscript{0-58}XRT\textsubscript{7} 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 regarding hypoxia, no significant improvement in perfusion was observed in the BEZ235\textsubscript{10-34}XRT\textsubscript{7} tumors.

Vascular morphology was determined in the tumors after sacrifice. Mice in the control group had typically chaotic tumor vasculature (day 18). Mice from the XRT\textsubscript{7} and
BEZ235XRT7 (day 30 and 40, respectively) treatment groups also had typical tumor vasculature composed of irregular networks. Importantly, the BEZ2350-22 group displayed normalized tumor vasculature at the end of the treatment (day 22). The BEZ2350-58XRT7 tumors also showed morphology (day 58) consistent with vascular normalization, but to an extent lower than the BEZ235-only group. Indeed, although the vessels retained a larger diameter, increased tortuosity and vessel density were observed compared to BEZ235-only group (Suppl. Fig. 7A-B). Thus irradiation appears to have a distinctive modifying effect on tumor vasculature in the presence of the drug. In line with the perfusion findings, BEZ23510-34XRT7 tumors showed no improvement in vascular morphology.

Pericyte analysis revealed improved coverage in the BEZ2350-22 group while a less prominent but significant increase was also observed in the BEZ2350-58XRT7 tumors. Immunohistochemical analysis of Akt and mTOR phosphorylation confirmed blockade of the pathway targets after prolonged treatment with BEZ235 (Suppl. Fig. 9), at the time of sacrifice.

DISCUSSION

Vascular remodeling plays an important role in controlling tumor microenvironment and its regulation evolves as a promising approach for cancer therapy (18). In this study, we report that inhibition of the PI3K/mTOR pathway markedly normalizes the tumor vasculature. BKM120 and, more potently, BEZ235 significantly remodelled vascular morphology towards 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 remodelling 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 tumor microenvironment in a similar way (15).

We hypothesized that the improvement in tumor blood flow and oxygenation as observed in treated tumors at day 7 should 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 (BEZ235XRT7) resulted in an additional 10 days growth delay over XRT7 alone which is significantly lower the 28 days growth delay observed in the BEZ2350.58XRT7 (p<0.001). Moreover, BEZ235 administration initiated 3 days post-irradiation (BEZ23510-34XRT7) had essentially no effect on growth delay. These results indicate that (a) PI3K/mTOR inhibitors function by increasing both the intrinsic and the extrinsic sensitivity of cancer cells to irradiation (b) alteration of TME can substantially improve response to radiotherapy, underlying the importance of tumor oxygenation for radiotherapy (4, 14). These findings demonstrate 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-epidermal growth factor receptor (EGFR) antibody cetuximab after radiation but the tyrosine kinase inhibitors BIBIX1382BS and erlotinib failed to improve local tumour 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 tumour 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 BEZ230-58XRT7 group, as compared to 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 et al. showed that radiotherapy was most effective when administered after treatment with bevacizumab in ovarian, breast and melanoma models (25). Bevacizumab and continuous delivery of interferon-β presented changes consistent with normalization and increased radiation response in an orthotopic glioma model (26).

However, the vascular remodeling that occurs after antiangiogenic 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 to partial vessel collapse and rebound hypoxia (27-29). Interestingly, FaDu tumours 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 tumours grown in preirradiated tumour 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 counterbalance 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 from Qayum et al. and Cerniglia et al. 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 demonstrated 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 remodelling (43). Transient expression of myrAkt was capable of altering the normal response to oxygen-induced remodeling without causing vascular malformations Therefore, physiological levels of Akt signaling modulate microvascular patterning (43). Phung et al. demonstrated 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 demonstrate PI3K inhibition in tumors.

**CONCLUSION**

The results of the present study provide evidence that inhibition of the PI3K/mTOR signaling by BKM120 and BEZ235 displays significant effects on tumor microenvironment 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 to other drug-radiation combinations. To the best of our knowledge, this is the first study to analyse 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,

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REFERENCES


Figure 1. Immunohistochemical analysis of HT1080 and FaDu tumors from mice treated with signaling inhibitors. Evaluation of the effects of BKM120 and BEZ235 on p-Akt and p-mTOR in (A) HT1080 and (B) FaDu tumors. Sections were stained with the indicated antibodies. Magnification (X40). C, HT1080 and D, FaDu tumor growth after treatment with the signaling inhibitors. Columns, mean; bars, SD. *p<0.05 over vehicle-treated control.
Figure 2. BEZ235 and BKM120 reduce hypoxia in vivo. Hypoxia was monitored by bioluminescent imaging of the luciferase signal. Representative images and hypoxia measurements from (A) HT1080 and (B) FaDu tumors at day 0 and 7 treated with BEZ235 or BKM120, as indicated. C, immunostaining for EF5 in FaDu tumors confirmed reduction in hypoxia. Magnification (X40). *Columns, mean; bars, SD*, p<0.05 over vehicle-treated control.

Figure 3. Ultrasound analysis of the effects of PI3K/Akt/mTOR inhibition on tumor blood flow. A, representative images show perfusion as depicted by microbubbles (green signal), that is proportional to perfusion within the region of interest (ROI). B, perfusion was evaluated from the ROI (blue line) in HT1080 at day 0 and 7 after treatment with BEZ235 or BKM120, as indicated. C-D, representative images of blood perfusion and perfusion measurements in the FaDu model. *Columns, mean; bars, SD*, p<0.05 over vehicle-treated control.

Figure 4. BEZ235- and BKM120-induced vascular remodelling and normalization. HT1080-bearing mice were treated as indicated and CD31-PE was injected i.v. 1 min before sacrifice (day 7). A, representative images from a single 1-μm optical section of tumor vasculature (upper; X10 magnification), AMIRA software 3D reconstructions (middle) and Trace3D software vascular tree tracings (lower) 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.
**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 ~100 mm$^3$ cm, mice were treated with either vehicle (control), BEZ235 (on days 0-22; BEZ235$_{0-22}$), 6 Gy (on day 7; XRT$_7$), BEZ235 single dose at the day of irradiation (BEZ235$_7$XRT$_7$), BEZ235 pre-treatment followed by 6 Gy and continued throughout the course of experiment (BEZ235 on days 0-58 and 6 Gy on day 7; BEZ235$_{0-58}$XRT$_7$) and 6 Gy followed by BEZ235 (6 Gy on day 7 and BEZ235 on days 10-34). Points, mean of tumor volume (mm$^3$) 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 mm$^3$; bars, SD. *, p<0.05; **, p<0.01; ***, p<0.001.

**Figure 6.** Monitoring of hypoxia in irradiated FaDu-HRE-luc xenograft model. Tumors were treated using BEZ235, radiation or the different schedules (as described in “Results”). A, representative images of the luciferase signal and (B) their corresponding measurements are shown. Points, mean; bars, SD. *p<0.05 over vehicle-treated control.
1B

FaDu

pmTOR

BEZ235 (20 mg/Kg)

Control

BEZ235 (10 mg/Kg)

BKM120 (15 mg/kg)

BKM120 (30 mg/kg)

1C

HT1080

Tumor Volume (mm$^3$)

0 50 100 150 200 250 300

Control

BEZ235 (10 mg/kg)

BEZ235 (20 mg/kg)

BKM120 (15 mg/kg)

BKM120 (30 mg/kg)

Time (days)

0 3 7

1D

FaDu

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BEZ235 (20 mg/Kg)

BEZ235 (10 mg/Kg)

BKM120 (30 mg/Kg)

BKM120 (15 mg/Kg)

2B

Day: 0 7

Control

EZ235 (20 mg/Kg)

BKM120 (30 mg/Kg)

Tumor hypoxia (% control)

FaDu

HT1080

Hypoxia (% control)

2C
Day 7

3B

HT1080

Perfusion (% control)

- control
- BEZ235 (10 mg/kg)
- BEZ235 (20 mg/kg)
- BKM120 (15 mg/kg)
- BKM120 (30 mg/kg)

* day 0
* day 7

3C

Control
BEZ235 (20 mg/kg)
BKM120 (30 mg/kg)

Day 0

Day 7

3D

FaDu

*
4B

**Tortuosity (%)**

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**No. of vessels per field**

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*Significant difference compared to control.
6B

**Vehicle**
- **BEZ235_{0-22}
- XRT_{7}
- BEZ235_{7}XRT_{7}
- BEZ235_{0-58}XRT_{7}
- BEZ235_{10-34}XRT_{7}**

Hypoxia (% control)

Time (days)
Dual inhibition of the PI3K/mTOR pathway increases tumor radiosensitivity by normalizing tumor vasculature

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