Gastric Cancer Growth Control by BEZ235 *In Vivo* Does Not Correlate with PI3K/mTOR Target Inhibition but with [*18F*]FLT Uptake

Thorsten Fuereder¹, Thomas Wanek⁴, Pamina Pfleger², Agnes Jaeger-Lansky¹, Doris Hoeflmayer¹, Sabine Strommer¹, Claudia Kunten², Friedrich Wrba², Johannes Werzowa³, Michael Hejna³, Markus Müller¹, Oliver Langer¹,⁴, and Volker Wacheck¹

**Abstract**

**Purpose:** In this study, we tested the antitumor activity of the dual phosphoinositide 3-kinase (PI3K)/mTOR inhibitor BEZ235 against gastric cancer *in vitro* and *in vivo*.

**Experimental Design:** Gastric cancer cell lines (N87, MKN45, and MKN28) were incubated with BEZ235 and assessed for cell viability, cell cycle, and PI3K/mTOR target inhibition. *In vivo*, athymic nude mice were inoculated with N87, MKN28, or MKN45 cells and treated daily with BEZ235. *18F*-Deoxy-3-*F*fluorothymidine (*[^18F]FLT*) uptake was measured via small animal positron emission tomography (PET).

**Results:** *In vitro*, BEZ235 dose dependently decreased the cell viability of gastric cancer cell lines. The antiproliferative activity of BEZ235 was limited to a G1 cell-cycle arrest. *In vivo*, BEZ235 treatment resulted in PI3K/mTOR target inhibition as shown by dephosphorylation of AKT and S6 protein in all xenograft models. However, BEZ235 treatment only inhibited tumor growth of N87 xenografts, whereas no antitumor effect was observed in the MKN28 and MKN45 xenograft models. Sensitivity to BEZ235 *in vivo* correlated with downregulation of the proliferation marker thymidine kinase 1. Accordingly, *[^18F]FLT* uptake was only significantly reduced in the BEZ235-sensitive N87 xenograft model as measured by PET.

**Conclusion:** *In conclusion, in vivo* sensitivity of gastric cancer xenografts to BEZ235 did not correlate with *in vitro* antiproliferative activity or *in vivo* PI3K/mTOR target inhibition by BEZ235. In contrast, *[^18F]FLT* uptake was linked to BEZ235 *in vivo* sensitivity. Noninvasive *[^18F]FLT* PET imaging might qualify as a novel marker for optimizing future clinical testing of dual PI3K/mTOR inhibitors. *Clin Cancer Res; 17(16): 5322–32. ©2011 AACR.*

**Introduction**

Gastric cancer, although declining in incidence, is still a leading cause of cancer-related deaths worldwide (1, 2). Advanced gastric cancer is resistant to most of the conventional chemotherapeutic strategies and has a poor prognosis (2, 3). Activation of tumor growth promoting phosphoinositide 3-kinases (PI3K) results in AKT phosphorylation, which in turn plays a crucial role for tumor cell survival, invasion, growth, metabolism, and cancer cell motility (4–6). In gastric cancer, the PI3K pathway is frequently activated constitutively because of mutations of phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), PI3K3C, and AKT (7–9). The biological relevance of this signal transduction pathway is highlighted by the fact that the loss of *PTEN* heterozygosity serves as an independent prognostic factor in gastric cancer (10, 11). Furthermore, the PI3K/AKT downstream target mTOR is a relevant molecular target for antitumor therapy including gastric cancer (12–14). Our group has recently showed that mTOR inhibition by the mTOR complex (mTORC) 1 inhibitor everolimus impairs gastric cancer growth *in vitro* and leads to sustained tumor growth control *in vivo* (12).

mTOR inhibition by rapalog leads to mTORC2-mediated hyperphosphorylation of AKT via a P70S6K/IRS1/PI3K-negative feedback loop, which might attenuate the benefit of mTORC1 inhibition by rapalog (15–17). To overcome the feedback activation of AKT induced by mTORC1 inhibition, dual PI3K/mTOR inhibitors have been developed (6). The novel dual pan-class 1 PI3K/mTOR inhibitor BEZ235 has shown antitumor activity in various preclinical tumor models such as pancreatic cancer, glioblastoma, and renal cell cancer (18–20). BEZ235 is currently being tested in clinical trials for the treatment of solid tumors.
Translational Relevance

Activation of the phosphoinositide 3-kinase (PI3K)/mTOR pathway is a common event in gastric cancer and correlates with poor prognosis. PI3K/mTOR inhibition by the dual kinase inhibitor BEZ235 provides a novel and promising strategy for more effective treatment of gastric cancer patients. BEZ235 was recently reported to be well tolerated in a phase I clinical study and inhibited S6 phosphorylation in tumor specimens.

In this study, we show that PI3K/mTOR target inhibition by BEZ235 does not correlate with antitumor sensitivity in gastric cancer xenograft models. In contrast, \[^{18}F\]FLT PET uptake is linked to BEZ235 sensitivity in vivo. Our data suggest that the role of PI3K/mTOR target inhibition as a marker for dose finding and sensitivity to PI3K/mTOR inhibitors in translational studies should be reviewed. \[^{18}F\]FLT PET imaging might be considered for monitoring the activity of PI3K/mTOR inhibitors in future clinical trials.

It was the aim of this study to evaluate the antitumor activity of the PI3K/mTOR inhibitor BEZ235 in gastric cancer cells and in vivo. As secondary objectives, we studied whether dephosphorylation of mTOR target proteins and uptake of 3’-deoxy-3’-[\(^{18}\)F]fluorothymidine (\[^{18}F\]FLT) measured by small animal positron emission tomography (PET) were correlated with sensitivity to dual PI3K/mTOR inhibition in vivo.

Materials and Methods

Cell lines

NCI-N87 human gastric cancer cells (intestinal type) were purchased from American Type Culture Collection. MKN28 and MKN45 human gastric cancer cells (diffuse type) were kindly provided by Prof. H. Yokozaki, First Department of Pathology, Hiroshima University School of Medicine. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and an antibiotic mixture containing penicillin, streptomycin, and amphotericin B (all Gibco Invitrogen). Cell lines were cultured in a fully humidified 5% CO\(_2\), 95% ambient air atmosphere at 37°C.

Compounds

BEZ235 was kindly provided by Novartis Pharma. For in vitro studies, a 10 mmol/L BEZ235 stock solution was prepared in dimethylsulfoxide (DMSO) and further diluted to the desired concentrations. Compounds were treated with appropriate concentrations of DMSO. For in vivo experiments, BEZ235 (20 mg/kg or 40 mg/kg) was suspended in 1 volume of 1-methyl-2-pyrrolidone (Sigma-Aldrich) and 9 volumes of PEG300 (Sigma-Aldrich).

Cell viability assay

Cell Titer Blue cell viability assay (Promega) was used to quantify the fraction of viable cells in in vitro experiments. Tumor cells in exponential growth were seeded at 1 × 10\(^3\) cells per well in 50-µL medium in 96-well plates and incubated overnight at 37°C. The cells were then incubated for 24, 48, and 72 hours with BEZ235. After treatment, 20 µL of Cell Titer Blue reagent were added, and the color reaction was measured using a fluorescence detection system (Victor 1620 Multilabel Detector; Wallac/Perkin Elmer).

Western blot

For in vitro studies, 3 × 10\(^5\) cells were seeded in 6-well plates in 3 mL of culture medium. Cells were incubated at the indicated concentrations with BEZ235 for 6 hours, and protein was subsequently extracted as previously described (21). For in vivo studies, snap frozen tumor xenografts were pulverized with a MM 200 mixer mill (Retsch). Protein was extracted as previously described (22), loaded on a 10% SDS-polyacrylamide gel and electrophoretically separated, followed by blotting on polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies diluted in blocking solution at 4°C overnight. Antibodies were directed against phospho-AKT Ser473, phospho-AKT Thr308 (both dilution 1:1,000), phospho-S6 ribosomal protein Ser235/236, and Ser240/244 (both dilution 1:1,000), phospho-4E-BP1 at Thr37/46 and Thr70 (both dilution 1:1,000), cyclin D1 (dilution 1:1,000; all Cell Signaling Technology), thymidine kinase 1 (TK1; dilution 1:1,000; Abcam), and actin (purified from rabbit serum, dilution 1:10,000; Sigma). Primary antibodies were detected by horseradish peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz; 1:4,000) and visualized by chemiluminescence using enhanced chemo luminescence (Thermo Fisher Scientific) substrate.

DNA extraction and mutation analysis

Gastric cancer genomic cell DNA was extracted employing a wizard genomic DNA purification kit (Promega), according to the manufacturer’s recommendation. Briefly, cells were centrifuged, washed, lysed, and protein was precipitated. Supernatant was mixed with isopropanole and centrifuged followed by ethanol addition. Genomic DNA pellets were then rehydrated and used for sequence analysis. Mutation analysis of PIK3CA (exon 9 and 20), RAS (codon 12, 13, and 61), and BRAF (V600E) was carried out as previously described (23).

Fluorescence-activated cell sorting

For cell-cycle analysis, cells were tryspinized 24 hours after treatment, resuspended in Dulbecco’s phosphate-buffered saline (DPBS), fixed with ice-cold ethanol-DPBS (70:30) solution, and stored at 4°C. Following centrifugation, cells were incubated with RNase A (Sigma-Aldrich; 125 µg/mL) and propidium iodide (Sigma-Aldrich; 10 µg/mL) at 37°C for 30 minutes and analyzed immediately by flow cytometry.
flow cytometry. Cell-cycle distribution was assessed by using Modfit software (Verity Software House).

**Tumor xenograft model**

Pathogen-free, 4- to 6-week old, female athymic nude mice (Harlan Winkelmann) were housed under sterile conditions. Mice (n = 5/group) were inoculated with 8 × 10^6 NCI-N87, MKN45, or MKN28 cells on both flanks. Animals were randomly assigned to one of the following treatment groups: (i) carrier control (Ctrl), BEZ235 solvent; (ii) BEZ235 20 mg/kg; and (iii) BEZ235 40 mg/kg.

Tumor volume was assessed twice per week by calliper measurements and calculated according to the approximation formula: volume (mm^3) = 4/3 π × (long diameter × short diameter)/2. Treatment was initiated when tumor volumes reached approximately 150 mm^3 and continued until tumor volume reached 1,000 mm^3 as the predefined surrogate endpoint for terminal disease. Animals were treated daily by oral administration of 0.2 mL BEZ235 (20 mg/kg or 40 mg/kg) or Ctrl. BEZ235 was well tolerated as determined by clinical assessment. The study was approved by the local animal welfare committee and was carried out in accordance with the local regulations.

**ELISA**

For detection of VEGF in *in vivo* experiments, blood was drawn via cardiac puncture before sacrificing the animals and then centrifuged to obtain murine serum. Human and murine VEGF serum levels were quantified by Quantikine human VEGF immunoassay and Quantikine murine VEGF Immunoassay (both from R&D Systems), respectively. Absorption was measured using a Victor II multilabel plate reader (Wallac). Secreted VEGF levels were calculated after subtraction of blanks.

**Immunohistochemistry**

Ki-67 (anti–Ki-67 monoclonal mouse antibody, clone MIB-1; Dako; dilution 1:50) and CD31 (anti–CD31 antibody, clone MEC 13.3; BD-Pharmingen; dilution 1:50) staining was carried out as previously described (24). For quantitative assessment of Ki-67 immunolabeling, a total of 500 tumor cells were evaluated in each specimen in fields showing the highest density of immunopositive cells. For assessment of microvessel density (MVD), anti-CD31 immunostained tissue sections with the highest density of distinctly highlighted microvessels ("hot spot") were manually counted at 200-fold magnification within an examination area of 0.25 mm^2. All immunohistochemistry slides were interpreted by a trained pathologist blinded regarding treatment groups.

**Small animal PET**

For PET studies, mice (n = 4/group) were inoculated with NCI-N87, MKN45, or MKN28 cells bilaterally. Animals were randomly assigned to one of the following treatment groups: (i) Ctrl, BEZ235 solvent; and (ii) BEZ235 40 mg/kg. [18F]FLT was synthesized by radiofluorination of the 5'-O-benzyol-2,3'-anhydrothymidine precursor (ABX) by using an automated synthesis module (Tracerlab FX, GE Healthcare), as published in the literature (25). PET scans were carried out by using the microPET Focus 220 system (Siemens Medical Solutions; ref. 26). For PET, mice were placed in an induction box and anesthetized with 2% isoflurane in oxygen. Under anesthesia, [18F]FLT at a dose of 8.3 ± 1.6 MBq in 100 μL saline was injected in the lateral tail vein, and the mice were placed back into the induction box where they were kept anesthetized for the whole uptake time. Body warming was achieved by placing the induction box on a heating pad kept at 38°C. Warming was started approximately 30 minutes before tracer injection and continued throughout the uptake and imaging period. One hour after [18F]FLT injection a 10-minute static image (250–750 keV energy window, 6 ns timing window) was acquired. Baseline imaging was carried out prior to the initiation of treatment (day 0). Imaging was repeated on day 2 after treatment with identical acquisition parameters. Images were reconstructed by using FORE rebinning followed by filtered back projection algorithm resulting in a voxel size of 0.4 × 0.4 × 0.796 mm^3. The standard data correction protocol (normalization, decay correction, and injection decay correction) was applied to the data.

For image analysis, a calibration factor for converting units of PET images into absolute radioactivity concentration was first determined by imaging a phantom with a known activity concentration. Radioactivity concentration in tumors was quantified by manually drawing a volume of interest (VOI) around the whole tumor on the reconstructed image using the image analysis software Amide (27). Tracer uptake by the tumors was expressed as standardized uptake value where only the pixels exhibiting more than 75% of maximum radioactivity in the tumor VOI were included (SUV_max). Data are presented as mean ± SD. Image analysis was carried out in a blinded fashion, where the image analysts were unaware of whether the PET images were from treated or Ctrl group.

**Statistical analysis**

For multiple comparisons, ANOVA was followed by Tukey’s HSD post hoc test. The statistical significance between treatment groups for PET studies was tested using a 2-sided unpaired Student’s t test. For changes within treatment groups in the PET studies, data were analyzed using the paired Student’s t test. P < 0.05 was considered statistically significant. Data are shown as percentage of control values unless indicated otherwise. Statistical analysis was conducted using SPSS 16.0 software (SPSS Inc.).

**Results**

**BEZ235 reduces gastric cancer cell growth and induces G1 cell cycle arrest in vitro**

The antitumor activity of BEZ235 was studied in 3 gastric cancer cell lines (NCI-N87, MKN28, and MKN45) *in vitro*. Exposure of gastric cancer cell lines to BEZ235 (1–10 μmol/L) resulted in dose-dependent growth...
inhibition (Fig. 1A). Concentrations of BEZ235 known to result in specific kinase inhibition (28) led to approximately 50% growth inhibition in all gastric cancer cell lines tested (Fig. 1B; NCI-N87: 45%; MKN45: 65%; MKN28: 53%).

Next, we investigated whether the growth inhibitory effect of BEZ235 on gastric cancer cells was mediated by effects on the cell cycle (Fig. 1C–E). Fluorescence-activated cell sorting analysis revealed a significant dose-dependent increase of cells in the G1 fraction in all 3 gastric cancer cell lines (\(P < 0.05\) for all). At 80 nmol/L BEZ235, we observed approximately 20% increase in G1 phase for N87 and MKN45, and 10% increase for MKN28 (Fig. 1C–E).

**Downregulation of the PI3K/mTOR pathway in vitro**

To confirm the activity of BEZ235 treatment on the PI3K/mTOR pathway, we evaluated the phosphorylation status...
of the key proteins of the PI3K/mTOR signaling cascade (Fig. 2A–C). Gastric cancer cell lines exposed to 20 nmol/L BEZ235 showed almost complete dephosphorylation of the mTOR downstream targets ribosomal pS6RP (phosphorylation sites Ser240/244 and Ser235/236) and p4EBP-1 at Thr70, the mTOR inhibitor sensitive phosphorylation site (29). Downregulation of p4EBP-1 Thr37/46 by BEZ235 led to inhibition of cap-dependent translation as shown by dose-dependent downregulation of cyclin D1. Incubation of cells with BEZ235 at concentrations of 20 nmol/L and above resulted in dose-dependent dephosphorylation of AKT at phosphorylation sites Ser473 and Thr308 in all cell lines tested (Fig. 2A–C). PTEN protein was detectable in all cell lines and did not change upon incubation with BEZ235. PCR analysis revealed no hot spot mutation of PIK3CA (exon 9 and 20), RAS (codon 12, 13, and 61), or BRAF (V600E) in the 3 gastric cancer cell lines.

**BEZ235 reduces growth of NCI-N87, but not MKN45 and MKN28 gastric cancer xenografts**

To evaluate the effects of dual PI3K/mTOR blockage by BEZ235 in vivo, xenograft models for NCI-N87, MKN45, and MKN28 were established. After formation of palpable tumor disease, animals were treated with 20 or 40 mg/kg/d BEZ235 for 2 weeks. Therapy was well tolerated in all treatment groups with no obvious signs of toxicity as judged by mouse weight and daily clinical assessment. Treatment with BEZ235 resulted in significant dose-dependent control of tumor growth of NCI-N87 xenografts ($P < 0.01$; Fig. 3A). After 2 weeks of treatment, 20 and 40 mg/kg of BEZ235 therapy led to a 53% and 70% tumor growth suppression compared with vehicle control, respectively (20 mg/kg: 315 mm$^3$; 40 mg/kg: 203 mm$^3$; Ctrl: 666 mm$^3$). In contrast to our in vitro data, MKN45 and MKN28 xenografts turned out to be resistant to treatment with
BEZ235 treatment of MKN45 xenografts did not result in a significant antitumor effect (Fig. 3B; 20 mg/kg: 791 mm³; 40 mg/kg: 613 mm³; Ctrl: 773 mm³). Likewise, treatment of MKN28 xenografts did not inhibit significantly tumor growth (Fig. 3C; 20 mg/kg: 687 mm³; 40 mg/kg: 314 mm³; Ctrl: 445 mm³).

To assess long-term effects of BEZ235 treatment on NCI-N87 gastric cancer xenografts, we extended BEZ235 treatment in a further animal study (Fig. 3D). Daily treatment with BEZ235 for 6 weeks resulted in significant tumor growth inhibition relative to the control animals. When animals in the control group had to be sacrificed due to terminal disease after 6 weeks, BEZ235 treatment was stopped, and tumor volume was assessed twice per week until the predefined abortion criteria were reached. Tumor volumes presented as means ± SD.

Modulation of PI3K/mTOR pathway proteins does not correlate with BEZ235 in vivo sensitivity

Downregulation of PI3K/mTOR pathway proteins has been studied as a pharmacodynamic marker for mTOR inhibitor activity (30, 31). To assess target regulation following BEZ235 therapy in vivo, we carried out immunoblot analyses of tumor xenografts. BEZ235 treatment led to a dose-dependent dephosphorylation of AKT at phosphorylation sites Ser473 and Thr308 in all xenograft models (Fig. 4A–C). S6 protein phosphorylation at Ser240/244 was completely abrogated by BEZ235 treatment (40 mg/kg) in the NCI-N87 and MKN45 xenograft models and markedly reduced in the MKN28 xenografts. p4EBP1 at Thr70 and cyclin D1 protein levels were downregulated in NCI-N87 and MKN45 cells, whereas in MKN28 cells no significant effects on these proteins were observed.

Figure 3. Growth inhibition of gastric cancer NCI-N87 xenografts but not in MKN45 and MKN28 xenografts. Gastric cancer xenografts of NCI-N87 cells (A), MKN45 cells (B), and MKN28 cells (C; confirmed by 2 independent animal experiments) were treated with 20 or 40 mg/kg BEZ235 by oral gavage for 2 weeks. Tumor volume was assessed twice per week by calliper measurement. D, long-term treatment of NCI-N87 gastric cancer xenografts with 20 or 40 mg/kg BEZ235 for 6 weeks (black bar). When animals in Ctrl group reached the predefined abortion criteria after 6 weeks, BEZ235 treatment was stopped, and tumor volume was assessed twice per week until the abortion criteria were reached. Tumor volumes presented as means ± SD.
Given the lack of correlation between PI3K/mTOR pathway proteins and BEZ235 sensitivity in vivo, further markers of mTOR activity were assessed. BEZ235 has been reported to inhibit angiogenesis (32, 33). Analysis of MVD displayed no differential effects of BEZ235 treatment in sensitive versus resistant xenograft models (data not shown). In the BEZ235-sensitive NCI-N87 xenograft model, human (tumor derived) serum VEGF levels were approximately 8-fold lower in BEZ235 treatment groups compared with Ctrl group (Supplementary Fig. S1). In the BEZ235-resistant MKN45 and MKN28 animal models, no effect on human VEGF serum level was observed. Murine (host derived) VEGF serum levels were not altered in any of the xenograft models upon BEZ235 treatment (data not shown).

**BEZ235 reduces tumor proliferation measured by thymidine kinase protein expression in NCI-N87, but not in MKN45 and MKN28 xenografts**

To assess whether the observed tumor growth inhibition of NCI-N87 xenografts was because of inhibition of tumor cell proliferation, Ki67 immunohistochemical staining of BEZ235-treated xenografts was carried out. We noted no decrease of Ki67 staining in the BEZ235 treatment groups relative to Ctrl-treated animals (data not shown). As an alternative marker for tumor cell proliferation, TK1 activity has been reported to be linked to cell proliferation (34). Additionally, TK1 is detected in serum of tumor patients and correlates with clinical stage and tumor prognosis (34). We observed a clear decrease in TK1 expression in the BEZ235-sensitive NCI-N87 xenografts, but no change in TK1 protein expression in the BEZ235-resistant MKN45 and MKN28 xenografts (Fig. 5A).

**Decrease in [18F]FLT uptake correlates with BEZ235 sensitivity of NCI-N87 xenografts**

TK1 has been reported as the most important determinant of [18F]FLT PET uptake (35, 36). The uptake of [18F]FLT is believed to be dependent on cell proliferation (37). Thus, we studied whether BEZ235 efficacy correlates with [18F]FLT uptake quantified with small animal PET. [18F]FLT uptake increased in control groups from baseline to day 2 in all xenograft models (Fig. 5B and C). In the sensitive NCI-N87 xenograft model, [18F]FLT uptake was decreased after 2 days of BEZ235 treatment by approximately 20% relative to control (P < 0.05). In contrast, in the MKN45 and MKN28 xenograft model no statistically significant decrease was observed (Fig. 5B and C).

**Discussion**

In this study, we show that tumor growth inhibition by BEZ235 does not correlate with in vitro sensitivity or PI3K/mTOR target modulation in gastric cancer xenografts. Despite inhibition of AKT and S6 protein phosphorylation, only TK1 expression correlated with BEZ235 sensitivity in vivo as quantified by Western blotting and [18F]FLT uptake via small animal PET.

In line with previous reports for other malignancies (18, 28, 38), BEZ235 administration resulted in a significant dose- and time-dependent decrease of cell proliferation in vitro in gastric cancer cell lines. mTOR-dependent phosphorylation of S6 ribosomal protein (Ser240/244 and Ser235/236) and 4EBP1 (Thr70) was inhibited at much lower concentrations than phosphorylation of AKT at Ser473 and Thr308. A similar result was published for PI3K/mTOR blockage by BEZ235 in sarcoma and breast...
cancer cells (38, 39). These data suggest that BEZ235 is more effectively in inhibiting mTOR activity than PI3K activity. At higher concentrations, the additional inhibition of PI3K as indicated by abrogation of AKT phosphorylation (Fig. 2A–C) further enhanced the antiproliferative effects of BEZ235, supporting the dual targeting concept of PI3K/mTOR. The antiproliferative activity of BEZ235 in vitro correlated with a dose-dependent increase of gastric cancer cells in the G1 phase of the cell cycle and cyclin D1 downregulation. There are controversial reports about the impact of cell-cycle arrest on the antiproliferative activity of BEZ235 (40, 41). Depending on the tumor model assessed, PI3K/mTOR blockade by BEZ235 was reported to result in cytostatic or cytotoxic effects (44, 45). In our gastric cancer cell lines, G1 arrest seems to be the predominant mode of action and not cell death induction (data not shown).

Interestingly, in vitro sensitivity as well as pAKT and mTOR target regulation in vivo did not reflect efficacy of BEZ235 in vivo. BEZ235 showed a significant dose-dependent antitumor response in NCI-N87 xenografts only. This antitumor activity was maintained just as long as daily treatment with BEZ235 was continued. Upon termination of BEZ235 administration, we observed a significant progression of tumor xenograft growth indicating that there is no sustained effect of BEZ235 treatment. In the other two xenograft models, BEZ235 treatment did not impair tumor growth despite of PI3K/mTOR target regulation. The underlying molecular mechanisms for this finding are still elusive. To elucidate, we investigated pharmacodynamic biomarkers typically assessed in proof of concept studies for PI3K and mTORC1 inhibitors. In several studies, the (pre)clinical activity of mTORC1 inhibitors was correlated with PTEN loss or mTOR inhibition measured by dephosphorylation of S6 protein (30, 31, 42, 43). However, these markers were not predicting BEZ235 sensitivity in our models. In all xenograft models, PTEN protein was expressed and BEZ235 modulated PI3K/mTOR pathway phosphorylation status. The phenomenon that in vitro sensitivity to mTORC1 inhibitors does not correlate with in vivo sensitivity was also reported by others (44). On
a cautionary note, the relevance of these markers as predictive biomarkers for mTORC1 inhibitors is discussed controversially. PTEN loss was reported not to serve as a predictive biomarker for response to the mTOR inhibitor everolimus (45). Previous publications of our group showed that the phosphorylation status of the mTOR downstream target S6 does not necessarily predict efficacy of mTORC1 inhibitors in tumor models (44, 46).

Despite the variability intrinsic to any in vivo model, we observed a clear trend of p4EBP1 Thr70 downregulation in all tumor samples in the NCI-N87 and MKN45 xenograft models, whereas no downregulation was observed in the MKN28 xenografts. Because both the MKN45 and the MKN28 xenograft models do not respond to BEZ235 treatment, this finding corroborates our notion that the in vivo efficacy of BEZ235 does not correlate with PI3K/mTOR target regulation such as in the gastric tumor models studied.

Aberrant activation of the PI3K/mTOR and RAS/MAP kinase pathway because of mutations in PTEN, PIK3CA or RAS, or Braf are common in gastric cancer and correlate with poor prognosis (7, 9–11, 47). Mutational activation of the PI3K/mTOR pathway was reported to influence the efficacy of PI3K inhibitors in vivo (48, 49). BEZ235 is highly effective in PIK3CA mutated cells (38, 50). The cell lines employed for the xenograft models in our study do not harbor any hot spot mutations in PIK3CA, RAS, or Braf. Thus, mutational activation of the PI3K/mTOR pathway is not likely to account for the observed different BEZ235 in vivo sensitivity.

Because BEZ235 as well as specific mTOR inhibitors are known for their antiangiogenic properties (32, 33), we assessed whether markers of angiogenesis are related to BEZ235 sensitivity in vivo. In contrast to previous reports, no evidence for an antiangiogenic activity of BEZ235 was observed in our models. Murine VEGF levels (as marker for the angiogenic host response) and tumor MVD observed in our models. Murine VEGF levels (as marker for the angiogenic host response) and tumor MVD observed in our models. Murine VEGF levels (as marker for the angiogenic host response) and tumor MVD observed in our models.

References

5. Marone R, Cmiljanovic V, Giese B, Wymann MP. Targeting phospho-


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