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Molecular Cancer Therapeutics

Differential Effects of PI3K and Dual PI3K/mTOR Inhibition in Rat Prolactin-Secreting Pituitary Tumors

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Abstract

Aggressive pituitary tumors are rare but difficult to manage, as there is no effective chemotherapy to restrict their growth and cause their shrinkage. Within these tumors, growth-promoting cascades, like the PI3K/mTOR pathway, appear to be activated. We tested the efficacy of two inhibitors of this pathway, NVP-BKM120 (Buparlisib; pan-PI3K) and NVP-BEZ235 (dual PI3K/mTOR), both *in vitro* on immortalized pituitary tumor cells (GH3) and on primary cell cultures of human pituitary tumors and *in vivo* on a rat model of prolactin (PRL) tumors (SMtTW3). *In vitro*, NVP-BEZ235 had a potent apoptotic and cytostatic effect that was characterized by decreased cyclin D/E and Cdk4/2 protein levels and subsequent accumulation of cells in G_1. *In vivo*, the effect was transient, with a decrease in mitotic index and increase in apoptosis; long-term treatment had no significant inhibitory effect on tumor growth. In contrast, while NVP-BKM120 had little effect *in vitro*, it dramatically limited tumor growth *in vivo*. Increased Akt phosphorylation observed only in the NVP-BEZ235–treated tumors may explain the differential response to the two inhibitors. Primary cell cultures of human PRL pituitary tumors responded to NVP-BEZ235 with reduced cell viability and decreased hormone secretion, whereas NVP-BKM120 had little effect. Altogether, these results show a potential for PI3K inhibitors in the management of aggressive pituitary tumors.
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Introduction

Aggressive pituitary tumors and carcinomas have to date shown persistent resistance to hormonal therapy with dopamine agonists or somatostatin analogs and as such represent a therapeutic challenge (1). Although conventional chemotherapies are largely ineffective (2, 3), recent case reports using temozolomide, an oral alkylating agent used in the management of glioblastoma, have given some hope, especially at early stages (4–6). However, about 60% of the published cases demonstrated only an initial response to temozolomide therapy (5, 7) with up to 25% of these patients becoming resistant to temozolomide during follow-up (5, 7–9). Because temozolomide treatment is not effective for all pituitary carcinomas or aggressive tumors, the development of new therapeutic options is necessary.

The PI3K/Akt/mTOR pathway is constitutively activated in human tumors (10) and is a key regulator of tumor cell growth, proliferation, and apoptosis. This pathway is overexpressed and/or activated in pituitary tumors (11, 12), suggesting that pituitary adenomas would be sensitive to treatment with mTOR inhibitors. Treatment with the allosteric mTOR inhibitor everolimus decreased viability among pituitary tumor cell lines (13) and primary cell cultures of human nonfunctioning pituitary tumors (14), indicating mTOR inhibition as a promising antiproliferative therapeutic option for aggressive pituitary tumors. However, mTOR inhibitors fail to induce a response in most human pituitary tumors *in vitro* (15) and to date have had no successful application in clinical practice (16).

Resistance to the mTOR inhibitor rapamycin is in part attributed to elimination of the negative feedback loop of the mTOR target p70 S6K onto the PI3K pathway. In an attempt to bypass this resistance by an upstream blockade of the PI3K pathway (17), PI3K inhibitors and dual PI3K/mTOR inhibitors were developed with favorable safety profiles (18, 19). The dual PI3K/mTOR inhibitor NVP-BEZ235 has induced G_1–S cell-cycle arrest and apoptosis in neuroendocrine tumor cell lines of various origins (20), reduced viability and activated apoptosis among human bronchial carcinoid tumor cells *in vitro* (21), and inhibited cell viability of a rat model of pituitary adenomas *in vitro* (22). In xenograft models, the pan-Class I PI3K inhibitor NVP-BKM120

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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(Buparlisib) has demonstrated dose-dependent inhibition of tumor growth *in vivo* (19).

The aim of our present study was to investigate the potential use of PI3K/Akt/mTOR pathway inhibitors in the treatment of aggressive pituitary tumors. We chose prolactin (PRL)-secreting pituitary tumors as our test model, because while most of these tumors are easily managed with dopamine agonists, those that acquire resistance constitute a large portion of all aggressive pituitary tumors and carcinomas with a poor prognosis (5, 23). We compared the effects of the pure PI3K inhibitor NVP-BKM120 with those of the dual PI3K/mTOR inhibitor NVP-BEZ235 both *in vitro* on somatolactotroph pituitary tumor GH3 cells and *in vivo* on a rat model of PRL pituitary tumor, SMtTW-3 (24, 25).

Materials and Methods

Reagents

NVP-BEZ235 and NVP-BKM120 (Buparlisib) were kindly provided by Novartis Pharma. Compounds were dissolved in DMSO (Sigma-Aldrich) for *in vitro* studies. NVP-BEZ235 was prepared in a 5% (w/v) methylcellulose solution (Colorcon), and NVP-BKM120 in the same solution with 0.5% (v/v) tween80 as vehicle for *in vivo* studies. The cell counting kit assay (CCK-8) was purchased from Sigma. The Cycle Test Plus DNA reagent Kit and FITC Annexin V Apoptosis detection kit were purchased from BD Biosciences. Primary antibodies against mTOR and p-mTOR (Ser2448; Millipore); Akt, p-Akt-Ser473, S6, p-S6-Ser235/236, p-Rb-Ser780, PARP, cleaved PARP (cl-PARP); cleaved caspase-3 (cl-caspase-3); p-p44/42 MAPK (Thr202/Tyr204); p44/42 MAPK, PTEN; and β-actin were made in rabbit (Cell Signaling Technology). Primary antibodies against cyclin D3, Cdk4 (Cell Signaling Technology), cyclin E (Santa Cruz Biotechnology), and Cdk2 (BD Transduction laboratories) were made in mouse. Anti-mouse and anti-rabbit horseradish peroxidase–conjugated secondary antibodies were used (purchased from Cell Signaling Technology.

Cell line and culture conditions

GH3 cell lines obtained from the ATCC were a kind gift from Fabienne Rajas (INSERM U855) in 2009.The cells were authenticated by measuring PRL and GH levels in culture media just before our experiments (February 2014). Regular PCR tests were performed to ensure the GH3 cell lines remained mycoplasma free. GH3 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine 200 mmol/L, and 2% (v/v) penicillin (10.000 U/mL)–streptomycin (10.000 μg/mL; Life Technologies) at 37°C and 5% CO_2.

SMtTW tumor model

The rats used in the experiments were 2-month-old female Wistar/Furth WF/Ico inbred strain (Charles River Laboratories). All rats were treated according to guidelines meeting French Ethics Committee approval (agreement n°BH2011-37).

The SMtTW lineage used was one of four generated in our laboratory since 1985 from spontaneous pituitary tumors of Wistar/Furth rats. The main characteristics of the strains produced and the grafting procedure for their generation have previously been described in detail (25). Briefly, from each spontaneous tumor, a thin piece (2 × 2 mm) was slipped under the kidney capsule of female consanguineous rats (heterotopic and allogenic graft) and produced a tumor. Each lineage was maintained by serial grafts provided from these tumors growing under the kidney capsule. In this study, we used the SMtTW3 tumor lineage that presented a PRL phenotype with high plasma PRL levels (1–150 μg/mL) and a low secretion of GH (0.4 μg/mL) that grows rapidly, is invasive and sometimes necrotic and metastatic, and shares common characteristics and gene expression profile with the human aggressive prolactinomas (23, 25).

Four weeks after grafting, tumor-bearing rats were divided into two groups of comparable tumor size distribution before being administered via oral gavage 5 days a week, a vehicle (control), or one of either the PI3K inhibitor NVP-BKM120 or the dual PI3K/mTOR NVP-BEZ235. NVP-BEZ235 was administered at 20 mg/kg/d for 3 (control, $n = 10$; NVP-BEZ235, $n = 13$) or 6 weeks (control, $n = 9$; NVP-BEZ235, $n = 13$). NVP-BKM120 was administered at a reduced dose of 5 mg/kg/d ($n = 12$) for 4 weeks only and compared with control ($n = 8$). This reduced dose and duration were due to poor tolerance, and hyperglycemia induced by high doses initially tested. Animal weight, blood glucose (Freestyle blood glucose monitor; Abbott Diabetes Care), and plasma PRL levels were regularly measured during treatment and at autopsy. Tumors were removed, separated from kidney tissue, measured, weighed, and prepared immediately according to different analytical techniques.

Cell viability

The effect of NVP-BEZ235 and NVP-BKM120 on cell viability was established using the CCK-8 assay according to the procedure recommended by the supplier. Cells were plated in 96-well plates at a concentration of 5×10^4 cells per 100 μL of medium/well, incubated for 24 hours with each drug at 1, 10, 100, and 250 nmol/L. Controls were performed in DMSO with the same dilutions. Absorbance was measured at 450 nm using a multiplate reader (Multiskan Ex; ThermoFisher). Three replicate wells were used for each analysis, and at least three independent experiments were conducted.

Flow cytometry

After 24 hours of treatment, all cells were collected and assigned to different analytical procedures. For cell viability analysis, 1 μL propidium iodide (BD Biosciences) was added to cells just before data acquisition on the flow cytometer (Canto II; BD Biosciences). For cell-cycle analysis, the Test Plus DNA reagent Kit was used according to the recommended procedure. For apoptosis analysis, the FITC Annexin V Apoptosis detection Kit was used according to the recommended procedures, and immunolabeling was performed on 4% (v/v) paraformaldehyde-fixed GH3 cells with anticleaved caspase-3 rabbit antibody and Alexa488-labeled goat anti-rabbit antibody. All experiments were repeated three times to ensure reproducibility. All data concerning viability and apoptosis were analyzed using the DIVA software, and those relating to the cell cycle were evaluated using the ModFit software.

Western blot

Pelleted GH3 cells or tumor fragments were extracted by sonication in lysis buffer complemented with phosphatase and protease inhibitors (Roche Diagnostics; ref. 26). Protein concentrations were assessed by fluorometry using the Quant-iT Assay Kit (Life Technologies). SDS-PAGE electrophoresis (Criterion XT Precast Gel, 4%–12% Bis-Tris; Bio-Rad Laboratories) was then performed using 15 μg of total proteins for GH3 cells and 12 μg for tumors before transfer to a nitrocellulose membrane (Protan 0.45, Whatman; ThermoFisher). After blocking with TBS-T

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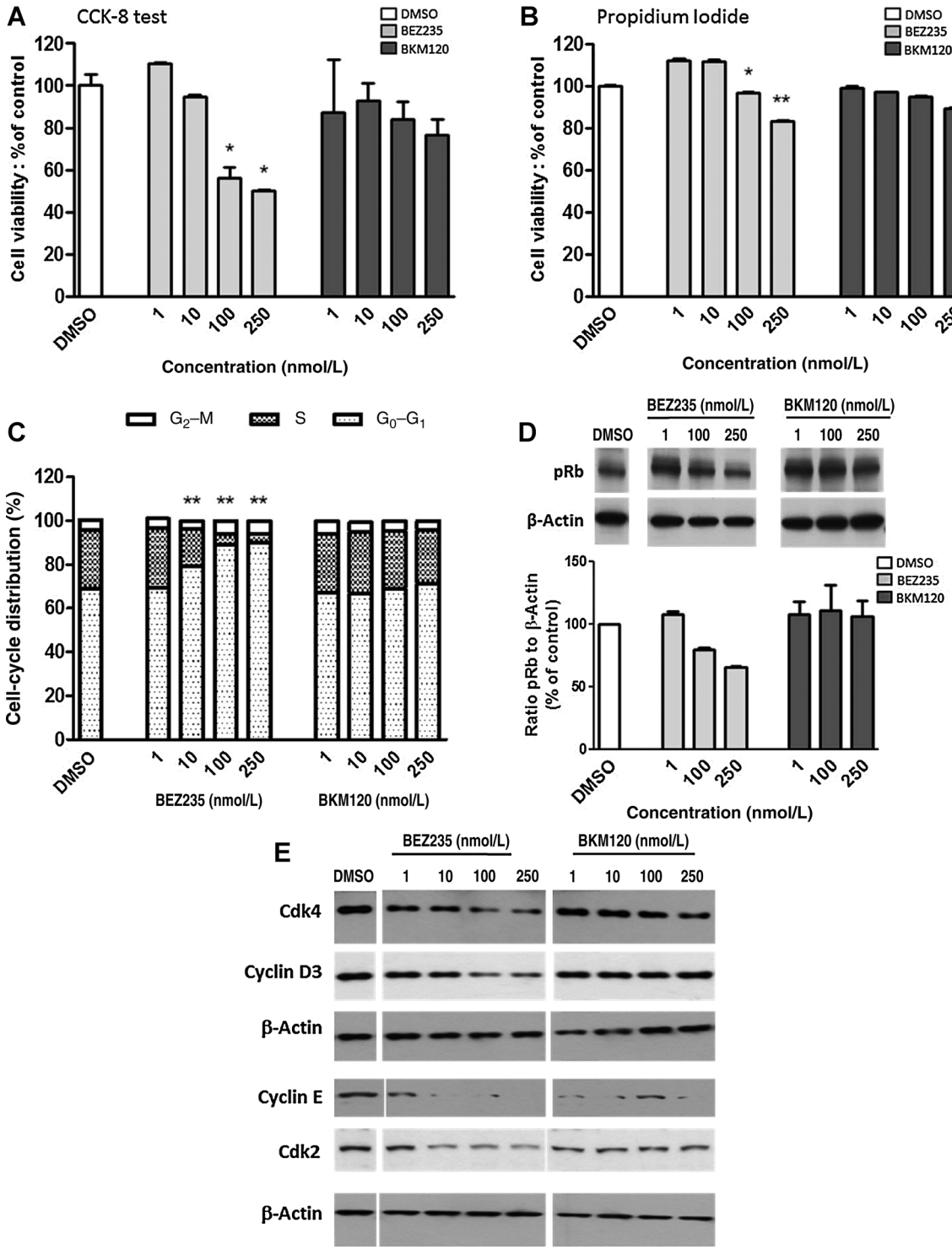

Figure 1.

In vitro effects of NVP-BEZ235 and NVP-BKM120 treatments on cell viability and cell cycle in GH3 cells. Treatments consisted of DMSO or of the inhibitors NVP-BEZ235 or NVP-BKM120 at 1, 10, 100, and 250 nmol/L (A, B, C, and E) or all except 10 nmol/L (D) for 24 hours. Cell viability was measured by colorimetric assay with the CCK-8 test (A) and by flow cytometry with propidium iodide (B). Data are expressed as a percentage of control (mean ± SEM). Data were analyzed statistically by Mann–Whitney test: *, $P < 0.05$; **, $P < 0.01$ compared with DMSO. Cell cycle was assessed by flow cytometry (C), and S-phase data were analyzed statistically by Mann–Whitney *U* test: **, $P < 0.01$ compared with DMSO. Protein expression levels of phosphorylated Rb (D) were determined by Western blot analysis. The bar graphs represent the mean and SEM. Equal protein loading was examined by detection of β-actin, and data are expressed as a percentage of control. One representative experiment out of three independent experiments at least is shown. The expression level of G_1–S cell-cycle proteins was established by Western blot (E) using anti-cyclin D3, -Cdk4, -cyclin E, and -Cdk2; respective levels of β-actin are shown. Representatives of two experiments are shown.

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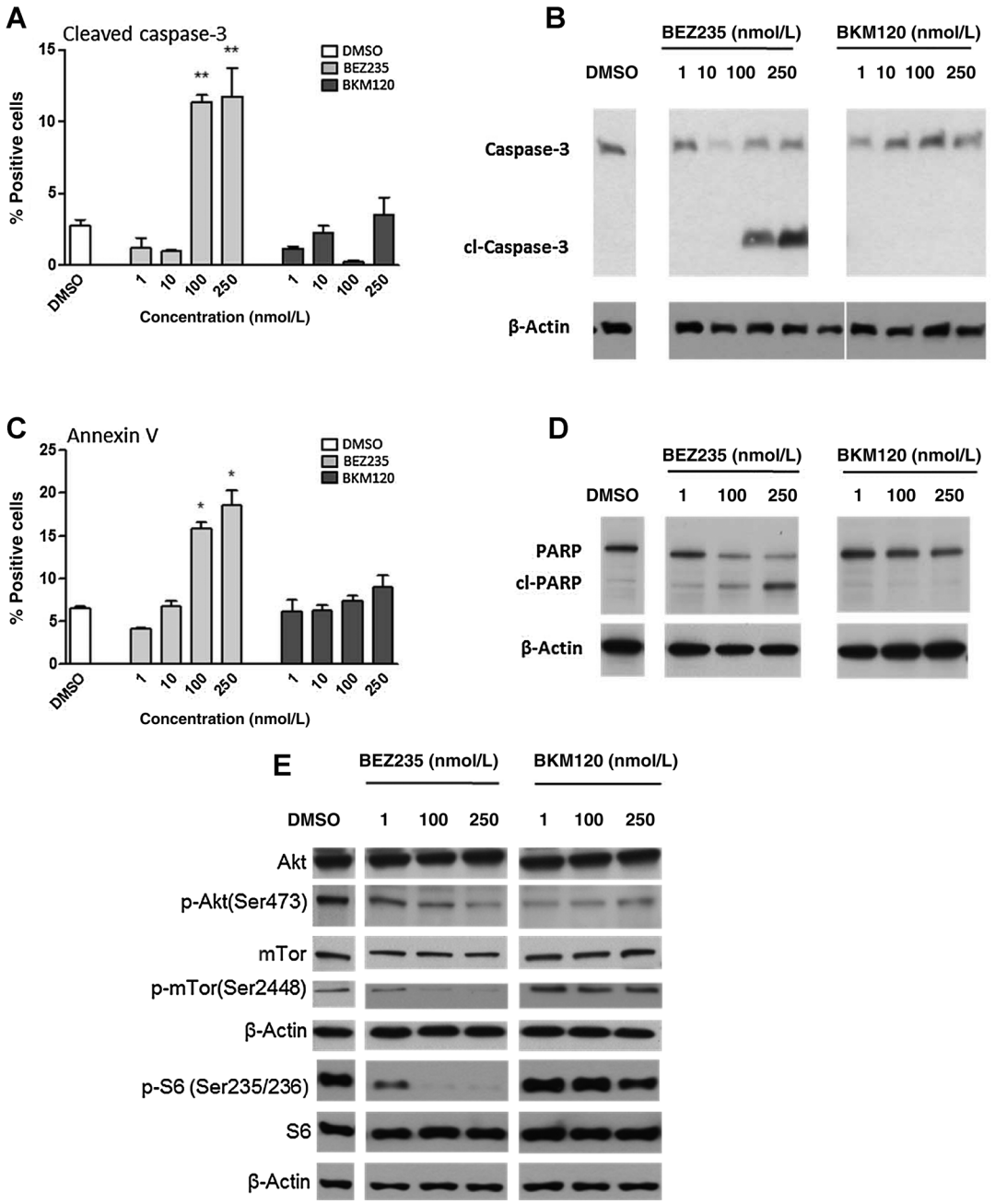

Figure 2.
In vitro effects of NVP-BEZ235 and NVP-BKM120 treatments on apoptosis and on the PI3K/Akt/mTOR pathway in GH3 cells. Treatments consisted of DMSO or of the inhibitors NVP-BEZ235 or NVP-BKM120 at 1, 10, 100, and 250 nmol/L (A–C) or all except 10 nmol/L (D and E) for 24 hours. Staining of cleaved caspase-3 (A) and Annexin V (C) was measured by flow cytometry. Data were analyzed statistically by Mann–Whitney *U* test, and values shown are the mean ± SEM: *, $P < 0.05$; **, $P < 0.01$ compared with DMSO. One representative experiment out of three independent experiments at least is shown. Protein expression level of total caspase-3 and cl-caspase-3 (B) and PARP and cl-PARP (D) was observed by Western blot analysis; respective levels of β-actin are shown. Representatives of two experiments are shown. Expression levels and phosphorylation status of Akt, mTOR, and p-S6 were examined by Western blot analysis (E). One representative blot out of three performed is shown for NVP-BEZ235 and out of two for NVP-BKM120.

BSA5% (w/v), membranes were incubated with specific primary antibodies (overnight), before their incubation for 1 hour with secondary antibodies. Detection was achieved using an enhanced chemiluminescence system (Immobilon Western; Millipore). Densitometric quantification of the immunoblot bands was performed using ImageJ software.

Mitosis

For light microscopy, tumor fragments were fixed in Bouin–Hollande, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin–eosin. For mitotic index, mitotic cells were counted at 400× magnification in 10 representative fields per tumor, with an average count of 5,000 nuclei.

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Human prolactinomas in primary cell culture

This study was approved by the Max-Planck-Institute ethics committee, and informed consent was received from each patient or from their relatives. Seven prolactinomas were included. The tumors were removed by transphenoidal surgery, and a diagnosis was reached according to clinical, biochemical, radiological, and surgical findings. Postsurgical specimens were prepared as previously described (15). Cell viability, as determined by acridine orange and ethidium bromide staining, was considered acceptable above 80%. Cells were seeded in 96-well plates (10,000 cells per well) and were left for 48 hours before the 24-hour treatment. Cell proliferation was assessed using the nonradioactive colorimetric WST-1 assay (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells treated with DMSO alone (in which NVP-BEZ235 and NVP-BKM120 were dissolved) were used as a control. All treatments were carried out in quadruplicate.

Serum PRL assay

Before and after 3- and 6-week treatments with control or NVP-BEZ235 (20 mg/kg), or after a 4-week treatment with control or NVP-BKM120 (5 mg/kg), blood was drawn from the retro-orbital sinus or collected at autopsy (24). Serum was collected for the hormone assay, and PRL concentrations were measured by double antibody radioimmunoassay with the reagents purchased at the National Institute of Diabetes and Digestive and Kidney Diseases (PRL rat reagents NIDDK). The intra-assay variability was less than 10%, and the inter-assay variability was less than 12%. Results are expressed as the mean ± SD.

Human PRL was determined using a radioimmunoassay from DPC Biermann, according to the manufacturer's instructions. PRL values were divided by the cell viability values, and data are given in (ng/mL)/OD450 nm.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software. The nonparametric Mann–Whitney *U* test was used to compare two groups (DMSO or control vs. treated). *In vitro* data were assessed at least three times in triplicate. A $P \leq 0.05$ was considered to be statistically significant.

Results

Effects of NVP-BEZ235 and NVP-BKM120 on GH3 cells *in vitro*

In GH3 cells, treatment with either NVP-BEZ235 or NVP-BKM120 (1, 10, 100, and 250 nmol/L) for 24 hours dose-dependently reduced cell viability, reaching statistical significance for NVP-BEZ235 only. NVP-BEZ235 had a stronger inhibitory effect on cell viability compared with NVP-BKM120 (50% vs. 23% decrease, respectively, compared with control; $P < 0.05$; Fig. 1A),

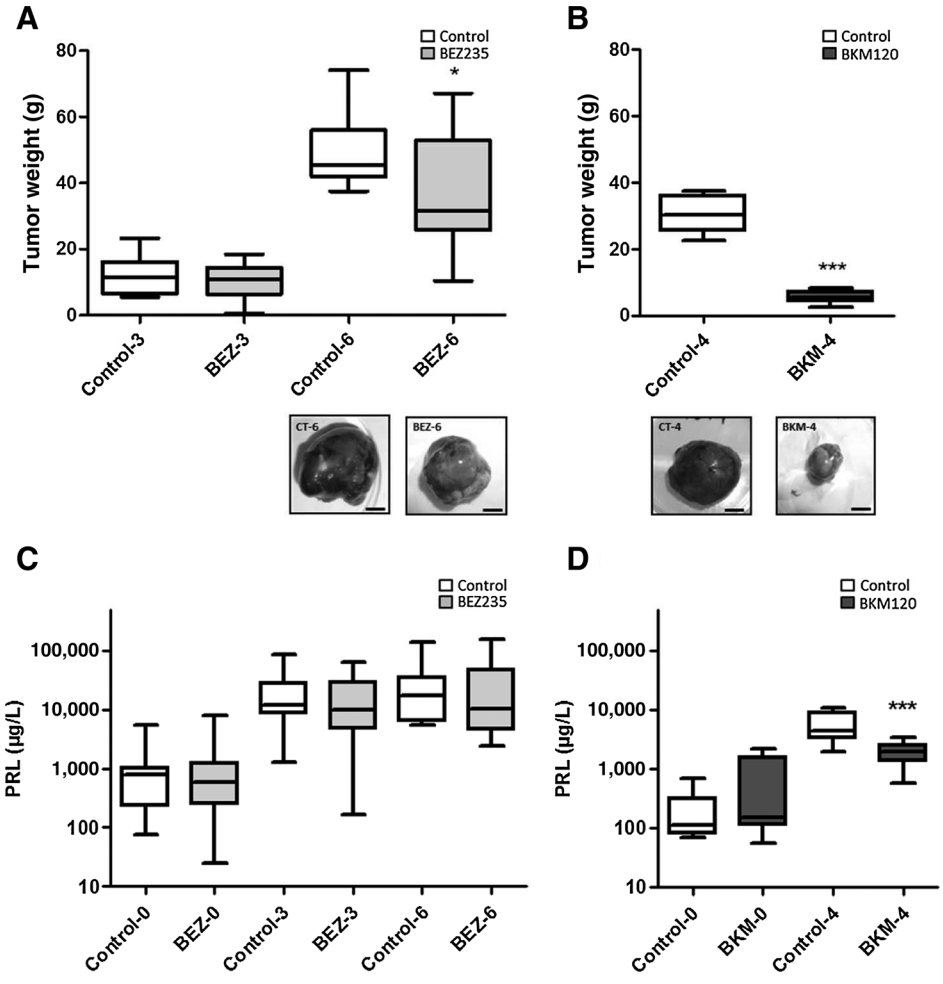

Figure 3.
In vivo effects of NVP-BEZ235 and NVP-BKM120 treatments on tumor growth and prolactin secretion in the SMtTW3 tumor model. Rats were treated 5 weeks after graft placement (A and C) with NVP-BEZ235 20 mg/kg/d, 5 days a week for 3 weeks (Control-3, $n = 10$; BEZ-3, $n = 13$) or 6 weeks (Control-6, $n = 9$ and BEZ-6, $n = 13$) and (B and D) with NVP-BKM120 5 mg/kg/d, 5 days a week for 4 weeks (Control-4, $n = 8$ and BKM-4, $n = 12$). Tumor weights (A and B) were noted, and different tumor sizes were illustrated by pictures: bar, 1 cm. Serum prolactin levels (logarithmic scale; C and D) were assessed by radioimunoassay before treatment (Control-0, BEZ-0, BKM-0) and at autopsy (Control-3, -4, -6, BEZ-3, -6, and BKM-4). Data were analyzed statistically by Mann–Whitney test, and values shown are mean ± SEM: *, $P < 0.05$; ***, $P < 0.001$ compared with the corresponding control.

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using the CCK8 assay. This difference was less prominent (17% and 11% reduction for 250 nmol/L NVP-BEZ235 and NVP-BKM120, respectively; $P < 0.01$ for NVP-BEZ235) using the propidium iodide staining test (Fig. 1B).

NVP-BEZ235 at concentrations of 10 to 250 nmol/L dose-dependently induced an accumulation of GH3 cells in the G_0–G_1 phase, alongside a 35% decrease of those in S-phase ($P < 0.01$ vs. DMSO). NVP-BKM120 treatment on the other hand had limited effect on the cell cycle (Fig. 1C). This differential effect on the cell cycle was reflected by a reduction in Rb phosphorylation observed only after NVP-BEZ235 treatment at concentrations of 1, 100, and 250 nmol/L; NVP-BKM120 had no effect (Fig. 1D).

Western blot for cell-cycle proteins involved in G_1–S revealed a strong reduction in Cdk2 and cyclin E protein levels in the NVP-BEZ235–treated GH3 cells (10–250 nmol/L; Fig. 1E). NVP-BEZ235 treatment also decreased Cdk4 and cyclin D3 protein levels, though only at the high nanomolar range (100–250 nmol/L) with no effect at lower doses. In contrast, NVP-BKM120 had no effect on Cdk4, cyclin D3, or Cdk2 levels, although it did suppress cyclin E (Fig. 1E).

NVP-BEZ235 at 100 to 250 nmol/L induced a significant and dose-dependent increase in apoptosis, as shown by an increase both in cleaved caspase-3 ($P < 0.01$; Fig. 2A and B) and in Annexin V ($P < 0.01$; Fig. 2C), both hallmarks of apoptosis. Furthermore, as shown in Fig. 2D, NVP-BEZ235 treatment increased cleaved PARP levels. NVP-BKM120 on the other hand had no significant effect on Annexin V, cleaved caspase-3, or cleaved PARP (Fig. 2).

Concerning the PI3K/Akt/mTOR pathway, NVP-BEZ235 treatment reduced levels of Akt and pS6 phosphorylation as well as mTOR phosphorylation at Ser2448 (mediated by p70 S6K; ref. 27) in a dose-dependent manner (Fig. 2E). Although NVP-BKM120 decreased Akt phosphorylation, it had no effect on pS6 phosphorylation and mTOR. Neither treatment affected total protein level at any dose.

Effect of NVP-BEZ235 and NVP-BKM120 on SMtTW3 tumors *in vivo*

At the doses used, neither inhibitor affected rat survival. However, although the growth curve of the NVP-BEZ235 group of rats was similar to that of the control group (243 g vs. 262.4 g at 6 weeks; Supplementary Fig. S1A and S1B), the NVP-BKM120 group showed a reduction in body weight after 4 weeks of treatment (183.7 g vs. 238.7 g, $P < 0.001$; Supplementary Fig. S2A). Moreover, blood glucose concentrations transiently increased after initiation of NVP-BKM120 treatment and then normalized for the remaining 4 weeks (132.833 mg/dl vs. 73.375 mg/dl, NS; Supplementary Fig. S2B).

In vivo, NVP-BEZ235 treatment showed a weak inhibitory effect on tumor growth that reached statistical significance after 6 weeks (10.1 ± 1.3 g vs. 11.8 ± 1.8 g at 3 weeks and 36.6 ± 5 g vs. 49.4 ± 3.9, at 6 weeks, $P < 0.05$; Fig. 3A). In contrast, NVP-BKM120 treatment induced a strong reduction of tumor weight relative to the control group (5.4 ± 0.5 g vs. 30.6 ± 1.9 g, $P < 0.001$) after 4 weeks of treatment, at which point the treatment was terminated in view of the endpoint

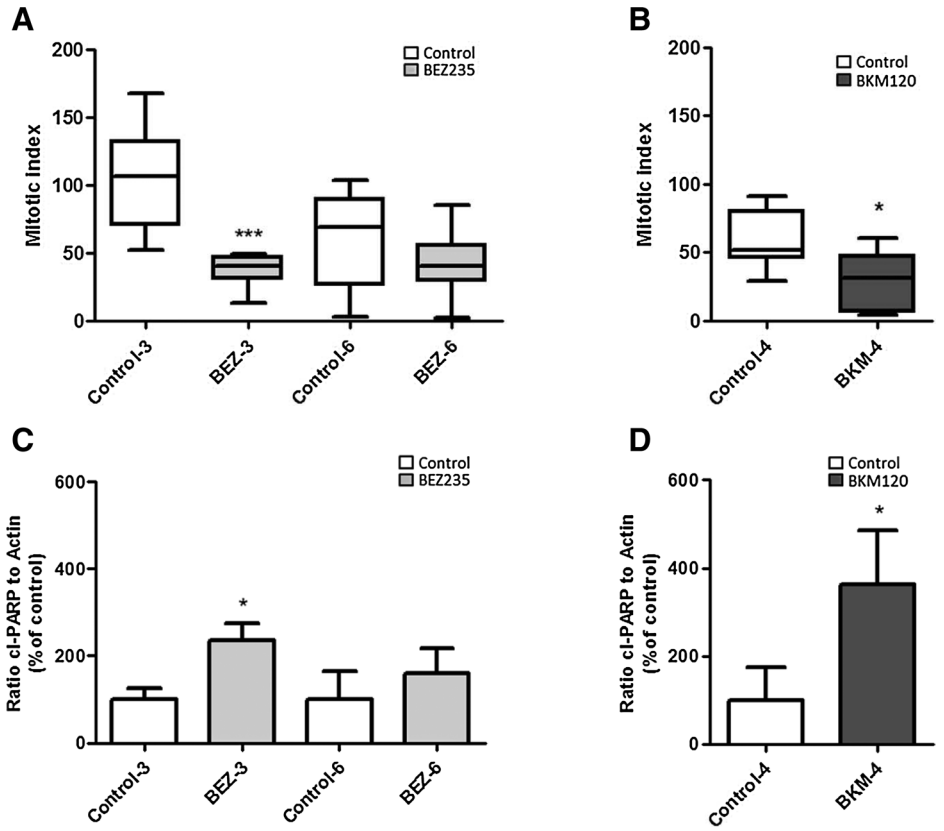

Figure 4.
In vivo effects of NVP-BEZ235 and NVP-BKM120 treatments on cell proliferation and apoptosis in the SMtTW3 tumor model. Rats were treated 5 weeks after graft placement, (A and C) with NVP-BEZ235 20 mg/kg/d, 5 days a week, for 3 weeks (Control-3, $n = 10$ and BEZ-3, $n = 13$) or 6 weeks (Control-6, $n = 9$ and BEZ-6, $n = 13$) and (B and D) with NVP-BKM120 5 mg/kg/d, 5 days a week, for 4 weeks (Control-4, $n = 8$ and BKM-4, $n = 12$). Mitoses (A and B) were assessed on hematoxylin–eosin staining and were counted at 400x magnification in 10 fields per tumor. Level of cl-PARP protein expression (C and D) was determined by Western blot analysis. The bar graphs represent the mean and SEM. Equal protein loading was examined by detection of β-actin, and data are expressed as a percentage of control. Data were analyzed statistically by Mann–Whitney test, and values shown are the mean ± SEM: *, $P < 0.05$; ***, $P < 0.001$ compared with corresponding control.

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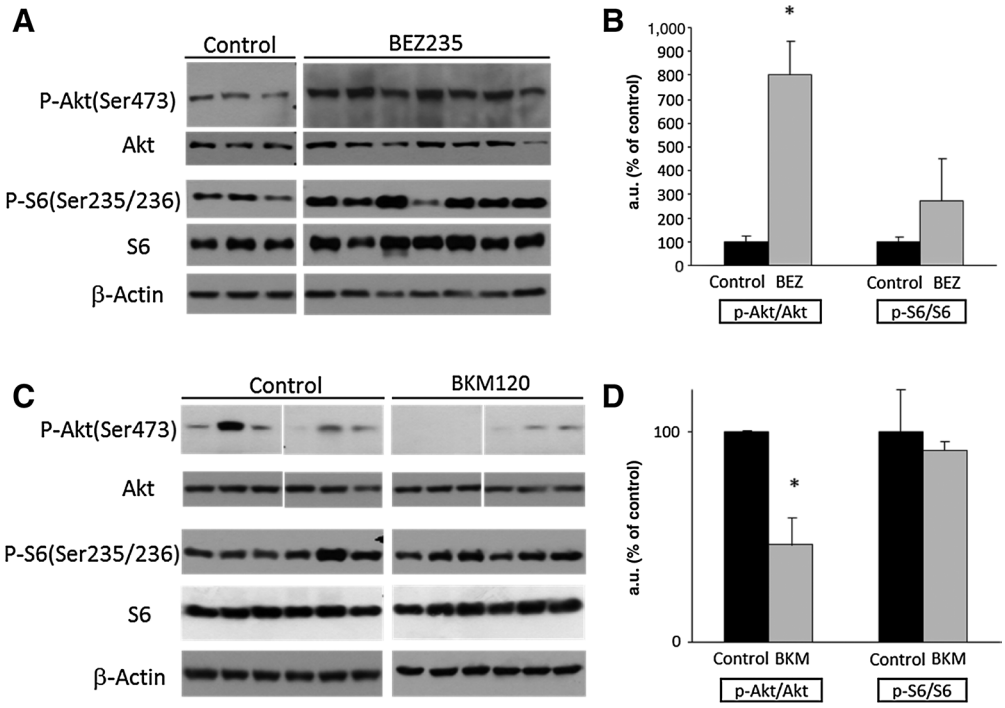

Figure 5.

In vivo effects of NVP-BEZ235 and NVP-BKM120 treatments on Akt and S6 ribosomal protein phosphorylation in the SMtTW3 tumor model. Rats were treated 5 weeks after graft placement (A and B) with NVP-BEZ235 20 mg/kg/d, 5 days a week, for 6 weeks (control-6, $n = 7$ and BEZ-6, $n = 10$) and (C and D) with NVP-BKM120 5 mg/kg/d, 5 days a week, for 4 weeks (control-4, $n = 8$ and BKM-4, $n = 9$). Each Western blot (A–C) for the phosphorylated protein was followed by blotting for the total protein after stripping in Tris buffer, pH 2.0. Signal represents mean ± SEM, calculated as phosphorylated-to-total protein ratio (B–D) and presented as a percentage of control. a.u., arbitrary units. *, $P < 0.05$.

having been reached (Fig. 3B). This reduction was accompanied by significantly reduced PRL levels in the NVP-BKM120–treated group compared with the control group (2,007 ± 231 μg/L vs. 5,666 ± 1,141 μg/L, $P < 0.001$) at 4 weeks (Fig. 3D). In contrast, PRL concentrations remained similar between the NVP-BEZ235 group and control group after 3 and 6 weeks of treatment (17,530 ± 3,600 μg/L vs. 23,850 ± 6,438 μg/L at 3 weeks and 33,900 ± 12,780 μg/L vs. 31,760 ± 14,090 μg/L at 6 weeks; Fig. 3C).

Although both treatments significantly reduced the tumor mitotic index compared with controls (NVP-BEZ235: 37 ± 3 vs. 106 ± 13, $P < 0.001$; Fig. 4A and for NVP-BKM120: 29 ± 6 vs. 58 ± 7, $P < 0.05$; Fig. 4B), this effect was only transient with NVP-BEZ235. Indeed, the significant effect of NVP-BEZ235 was lost after 6 weeks (43 ± 7 vs. 58 ± 12). Moreover, although both treatments significantly increased cleaved PARP levels, an indicator of apoptosis, NVP-BEZ235 had only a transient significant effect observed only after 3 weeks of treatment ($P < 0.05$). This NVP-BEZ235—induced effect had lost its significance after 6 weeks, whereas NVP-BKM120 remained potent throughout the treatment period ($P < 0.05$; Fig. 4C and D).

Western blot analysis revealed increased phosphorylated Akt-Ser473 levels in the tumors derived from the NVP-BEZ235–treated rats (6 weeks, $P < 0.01$), whereas phosphorylated S6 remained unchanged or was slightly increased in some cases (Fig. 5A and B). In contrast, phosphorylated Akt-Ser473 levels were reduced in most NVP-BKM120–treated tumors ($P < 0.01$; Fig. 5C and D).

These data show that the initial efficacy of the dual PI3K/mTOR inhibitor NVP-BEZ235 was lost during treatment, an effect that was accompanied by an inability to decrease Akt and S6 phosphorylation. In contrast, the single PI3K inhibitor suppressed Akt phosphorylation and displayed rapid and effective antitumor efficacy.

Effects of NVP-BEZ235 and NVP-BKM120 on primary cell cultures of human PRL tumors

To test the efficacy of the two inhibitors in human pituitary tumors, we used primary cell cultures of PRL-secreting pituitary tumors. NVP-BEZ235 treatment at both 10 and 100 nmol/L significantly decreased PRL secretion (% suppression 37 ± 6 and 59 ± 8.7, respectively, $P < 0.001$; Fig. 6A) in seven human prolactinomas. In contrast, NVP-BKM120 was effective at the 100 nmol/L concentration only (33 ± 23, $P < 0.05$). Regarding cell viability, only 100 nmol/L NVP-BEZ235 led to a reduction (%suppression 38 ± 12, $P < 0.05$) with the lower doses having no significant effect and NVP-BKM120 remaining ineffective within this nanomolar range (Fig. 6B).

Discussion

Since the recent reclassification of endocrine pituitary tumors (28, 29), not all of these tumors are considered as benign. Indeed, around 10% of them are aggressive and suspected of malignancy, and some progress to carcinomas with metastases.

Aggressive pituitary tumors that are resistant to conventional treatments have a poor prognosis. Their management requires

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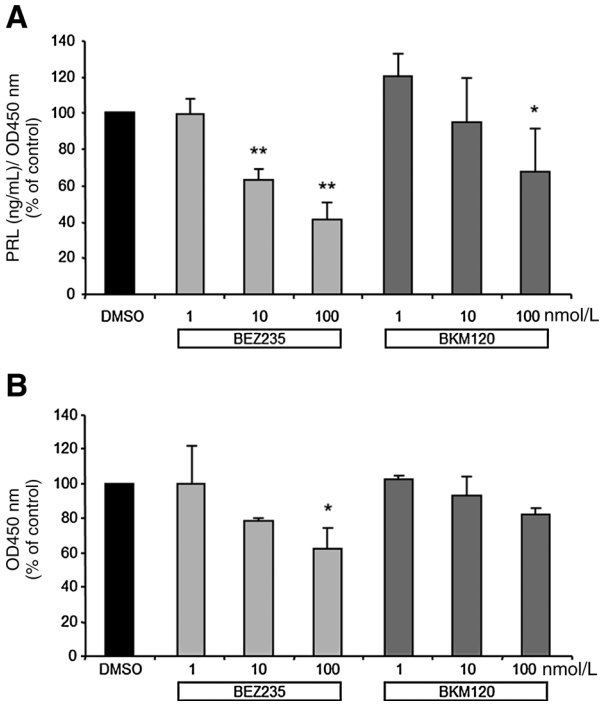

Figure 6.
Effects of NVP-BEZ235 and NVP-BKM120 on human PRL pituitary tumors in primary cell culture. NVP-BEZ235 and NVP-BKM120 dose-response (1, 10, 100 nmol/L) on basal PRL secretion (A) and cell viability (B) from human PRL pituitary tumors in primary cell culture ($n = 7$). For all cell culture experiments, each PRL radioimmunoassay value was divided by cell viability counts as determined by WST-1 at OD450 nm. Data are the mean ± SEM from seven cultures and are presented as a percentage of control. *, $P < 0.05$; **, $P < 0.001$.

chemotherapeutics such as temozolomide, the success rate of which is lower than initially believed and for which long-term outcomes are questionable (30). The identification of new therapeutic options is therefore necessary (1, 31).

The PI3K/Akt/mTOR pathway is one of the most commonly overactivated pathways in cancer and represents a promising pharmaceutical target (32). Numerous studies have demonstrated links between aberrant PI3K/Akt/mTOR signaling and the pathogenesis of endocrine tumors (11, 12) and in particular pituitary tumors (for review of refs. 32, 33). However, investigations into potential therapeutic options have been mainly based on *in vitro* studies on cell lines or primary cell cultures of human pituitary tumors, whereas *in vivo* data remain scarce. Currently, only two studies have used a xenograft model of GH3 cells implanted into the flanks of nude mice (32): the first examined the effect on tumor growth of a combined treatment consisting of nelfinavir and radiotherapy (34), whereas the second investigated the combination of temozolomide and XL765 (dual PI3K/mTOR inhibitor; ref. 35). The development of robust xenograft models must ideally consider the tissue-specific microenvironment of the tumor entities they intend to emulate. In the case of the pituitary gland, consideration of its dense vascular network is critical, as these vessels can be compressed during the development of a tumor mass, ultimately providing an escape mechanism from the inhibitory control of the hypothalamus (36). In this respect, the kidney microenvironment in which our SMtTW3 tumor model grows allows us to study pituitary tumor growth in a context of rich vascularization. In addition, we have previously demonstrated that the SMtTW3 tumor grafted under the kidney capsule acquires characteristics of human aggressive PRL tumors with activation of common proliferative pathways (37).

Using the SMtTW3 allograft rat model of aggressive PRL pituitary tumors alongside the immortalized lactosomatotroph GH3 cells, we have shown that both the dual PI3K/mTOR inhibitor NVP-BEZ235 and the single PI3K inhibitor NVP-BKM120 can limit pituitary tumor growth *in vitro* as well as *in vivo*, but to different extents. NVP-BEZ-235, but not NVP-BKM-120, displayed potent antiproliferative action in GH3 cells, by accumulation of cells in the G_1 phase. The G_1–S cell-cycle progression is governed by the cyclin-dependent kinases Cdk2, 4, and 6 and their associated cyclins D and E. Cyclin D-Cdk4/6 and cyclin E-Cdk2 phosphorylate Rb, which releases E2F transcription factors to drive the expression of genes pivotal for the transition to the S phase (38). NVP-BEZ235 treatment at concentrations within the high nanomolar range decreased Cdk4 and cyclin D3 in GH3 cells and at the low nanomolar doses also Cdk2 and cyclin E. NVP-BKM120 on the other hand suppressed only cyclin E, reflecting their different antiproliferative efficacy *in vitro*.

Surprisingly, our *in vitro* results did not reflect the *in vivo* situation, as NVP-BKM120 inhibited tumor growth more effectively compared with NVP-BEZ235, which had only a minimal effect after 6 weeks of treatment. This discrepancy could be due to the bioavailability of NVP-BEZ235 *in vivo* and the concentration used to treat our rats. The dose we used (20 mg/kg/day) was lower to that used in mice (40–45 mg/kg/day; refs. 39, 40) yet similar to that previously published in rats (41). Furthermore, NVP-BEZ235 decreased the tumor mitotic index and increased levels of cleaved PARP, indicating a tumor cell response at least at the beginning of the treatment.

Interestingly, neither NVP-BEZ235 nor NVP-BKM120 effectively suppressed SMtTW3 cell proliferation *in vitro* (Supplementary Fig. S3), yet NVP-BKM120 potently inhibited tumor growth *in vivo*. No changes in MAPK phosphorylation or PTEN levels that could explain these findings were found for either treatment in GH3 cells or in tumors (Supplementary Fig. S4). This last point lends support to the antitumoral effect of NVP-BKM120 *in vivo* being mediated, at least in part, through the tumor microenvironment. The PI3K/Akt/mTOR pathway is a major regulator of tumor metabolism, angiogenesis, and adherence (42). Abnormal tumor vascularization has been associated with decreased response to therapy (43), and may have rendered the somatolactotroph tumor cells resistant to NVP-BEZ235 treatment *in vivo*. SMtTW-3 tumors have been described as hemorrhagic, indicative of their extensive vascularization (44). However, similar to observations in a renal cell carcinoma model (45), NVP-BEZ235–treated tumors showed no macroscopic evidence of an altered vascularization. Another explanation could be that *in vivo* the tumor can evolve and acquire resistance to the chemotherapeutic treatment, a process that cannot take place in the short period of time allowed in *in vitro* culture. The finding of decreased tumor mitotic index and increased cleaved PARP levels in tumors at the initial but not at the later stages of treatment with NVP-BEZ235 supports this hypothesis. Despite their different antiproliferative efficacy, both NVP-BEZ235 and NVP-BKM120 inhibited Akt phosphorylation *in vitro*, supporting previous evidence that suppression of

phosphorylated Akt-Ser473 is not always accompanied by a potent antiproliferative response (46). Contrary to that observed *in vitro*, NVP-BEZ235 significantly increased Akt phosphorylation in our *in vivo* model. Increased Akt phosphorylation has been observed in patient biopsies after treatment with allosteric mTOR inhibitors, and has been proposed to lead to or be indicative of treatment resistance (47). NVP-BEZ235 was found to act as an mTOR inhibitor in some tumor types, and may be the principal mechanism behind its efficacy in pituitary tumors of somatolactotroph origin (48), such as those used in our study GH3 cells were previously shown to effectively respond to allosteric mTOR inhibitors (13, 15), rendering plausible the susceptibility of human somatolactotroph tumors to mTOR inhibition. Indeed, NVP-BEZ235 effectively suppressed cell viability and PRL secretion from human PRL pituitary tumors *in vitro*. NVP-BKM120 was also able to suppress PRL synthesis, despite the lack of effect on cell viability, indicating a role for the PI3K cascade in the regulation of PRL synthesis.

The majority of PRL pituitary tumors can be effectively managed with dopamine agonists, and only 5% of patients undergo surgery for tumor resection, mainly due to dopamine agonist resistance. Usually these tumors are macroadenomas (i.e., >10 mm diameter) and are frequently aggressive (49). The estrogen receptor plays a crucial role in lactotroph physiology, and together with factors such as the bone morphogenic factor 4 (BMP4), it contributes toward their tumorigenesis (50). Interestingly, estradiol was found to block the apoptotic action of PI3K inhibitors in breast cancer cells (51). A similar mechanism may take place in human PRL pituitary tumors and compromise their antiproliferative response to NVP-BEZ235 and NVP-BKM120. As our rat tumor model required the use of females, we were not able to address the question of gender specificity in our study.

In conclusion, our data indicate that two inhibitors of the PI3K/Akt/mTOR pathway showed various inhibitory effects on *in vitro* and *in vivo* growth of human and rat pituitary tumors. Therapies targeting this pathway may therefore be of interest at least for treating PRL-secreting aggressive pituitary tumors and carcinomas.

Disclosure of Potential Conflicts of Interest

G. Raverot has received honoraria from the speakers bureau of and is a consultant/advisory board for Novartis. No potential conflicts of interest were disclosed by the other authors.

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Molecular Cancer Therapeutics

Differential Effects of PI3K and Dual PI3K/mTOR Inhibition in Rat Prolactin-Secreting Pituitary Tumors

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