Targeting PI3K/mTOR Signaling Displays Potent Antitumor Efficacy against Nonfunctioning Pituitary Adenomas

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Abstract

Purpose: Novel therapeutic approaches are needed to improve the postoperative management of residual nonfunctioning pituitary adenomas (NFPA), given their high relapse rate. Here, we evaluated the antitumor efficacy of the dual PI3K/mTOR inhibitor NVP-BEZ235 in the only available model of spontaneous NFPA (MENX rats).

Experimental Design: Organotypic cultures of rat primary NFPA were incubated with NVP-BEZ235 and assessed for cell viability, proliferation, apoptosis, and PI3K/mTOR inhibition. NVP-BEZ235, or placebo, was administered to MENX rats and tumor response was monitored noninvasively by diffusion weighted-magnetic resonance imaging (DW-MRI).

Following treatment, tumor tissues were investigated for cell proliferation, apoptosis, and PI3K/mTOR inhibition. Genes mediating the cytotoxic activity of NVP-BEZ235 were identified by gene-expression profiling. Among them, Defb1, encoding beta-defensin 1, was further studied for its role in pituitary cells and in human pancreatic neuroendocrine tumor (NET) cells.

Results: NVP-BEZ235 showed antiproliferative and pro-cell death activities against NFPA both in vitro and in vivo, and the response to the drug correlated with inhibition of the PI3K pathway. DW-MRI identified early functional changes (decreased cellularity) in the adenomas before their size was affected and emerged as a useful modality to assess therapy response. The cytotoxic effect of PI3K/mTOR blockade in NFPA was mediated by several genes, including Defb1. NVP-BEZ235 treatment induced Defb1 expression in NFPA in vitro and in vivo, and in pancreatic NET cells. High Defb1 levels sensitized NET cells to PI3K/mTOR inhibition.

Conclusions: Our findings provide rationale for clinical investigation of PI3K/mTOR inhibition in NFPA and identify novel effectors of PI3K-mediated neuroendocrine cell survival. Clin Cancer Res; 1–12. ©2015 AACR.

Introduction

Pituitary adenomas account for 20% of all primary intracranial tumors (1). They can be hormonally active or clinically nonfunctioning. Nonfunctioning pituitary adenomas (NFPA) represent 30% to 35% of all pituitary adenomas and about 80% of them are gonadotropinomas (2).

Transsphenoidal surgery is the mainstay treatment of patients with pituitary adenomas. Because of the lack of signs and symptoms secondary to hormone hypersecretion, NFPA are often diagnosed late when they cause mass effects. At this stage, more than 40% of NFPA are invasive (3), so that complete surgical removal cannot be achieved. Reported recurrence rate varies between series, with up to 50% of patients experiencing long-term relapse (4, 5). Management of patients with symptomatic residual and recurrent disease is challenging because no medical therapies are currently available. Radiotherapy remains the only post-operative option, but it is not curative. Chemotherapy is only used as salvage treatment, but usually with disappointing results (6, 7). Therefore, novel therapeutic approaches are required.

Activation of the PI3K–AKT–mTOR signaling cascade plays a pivotal role in the initiation and progression of many human malignancies by enhancing cell survival, stimulating cell proliferation, and inhibiting apoptosis (8, 9). Constitutive activation of the PI3K–AKT–mTOR signaling cascade is also a feature of pituitary adenomas, and is secondary to mutations or amplification of PI3K (10, 11) or to overexpression of PI3K (12). NFPA also display activation of the PI3K–AKT–mTOR pathway (13). Thus, agents inhibiting PI3K signaling might represent an effective therapeutic option for these tumors. In support of this hypothesis, the mTOR inhibitor everolimus was reported to inhibit the viability of primary cultures of human NFPA in vitro (14, 15). Several compounds have been generated to inhibit the PI3K–AKT–mTOR signaling cascade. The mTOR inhibitor rapamycin and its analogs (rapalogs) are currently used to treat several solid tumors (16, 17). However, the development of drug resistance may limit their efficacy (18). A well-documented mechanism of resistance to rapamycin and rapalogs is the activation of feedback loops on AKT, which can re-activate the PI3K pathway (19). To escape the feedback resistance, compounds able to inhibit both mTOR and the upstream PI3K kinase were generated, including...
Translational Relevance

Nonfunctioning pituitary adenomas (NFPA) are the second most common type of pituitary adenomas. They are often invasive and not amenable to complete surgical resection. When incompletely removed, their long-term relapse rate is up to 50%. Currently, no effective medical therapy for NFPAs exists. As these tumors show hyperactivation of the PI3K–AKT–mTOR pathway, we tested the efficacy of a dual PI3K/mTOR inhibitor, NVP-BEZ235, in the only spontaneous model of NFPA (MENX rats), which closely recapitulate human adenomas. Our preclinical in vitro and in vivo experiments demonstrate cytostatic and cytotoxic action of NVP-BEZ235 in NFPA, and prove the efficacy of targeting PI3K/mTOR in these tumors. Our results may have translational relevance for the medical treatment of patients with residual and recurrent disease. Furthermore, we unveil the molecular readouts of NVP-BEZ235–dependent cell death and identify novel putative therapeutic targets and/or predictors of treatment response in NFPAs and other neuroendocrine tumors.

NVP-BEZ235, a synthetic small molecule that inhibits both PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes (20). NVP-BEZ235 has shown potent antiproliferative activity in preclinical models of several tumor types (20–24), and is currently evaluated in phase I/II clinical trials in patients with advanced solid tumors.

MENX is a multitumor syndrome in the rat caused by a biallelic loss-of-function mutation in Cdkn1b (25). MENX–affected rats (mutant) develop gonadotroph pituitary adenomas with complete penetrance and represent the only spontaneous model of such tumors. Rat adenomas closely resemble human NFPA based on histologic, immunohistochemical, molecular and ultrastructural data, and are a suitable model for pharmacologic studies of pituitary adenomas (26–28).

Purpose of our study was to define the efficacy of PI3K/mTOR inhibition in NFPA using the MENX rat model of spontaneous gonadotroph pituitary adenomas. We first assessed the effects of NVP-BEZ235 on 3D organotypic cultures of rat primary pituitary adenoma cells in vitro. Functional assays suggested that PI3K/mTOR inhibition promotes cytostatic, but also cytotoxic effects on pituitary adenoma cells. NVP-BEZ235 was then tested in vivo in affected rats. Molecular analyses in combination with functional imaging modalities (diffusion weighted-magnetic resonance imaging; DW-MRI) further pointed to a cytotoxic effect of NVP-BEZ235. DW-MRI emerged as a useful approach for early therapy monitoring of NFPA following PI3K/mTOR inhibition. Expression array analyses identified the DEFB1 gene (encoding beta-defensin 1) as a novel mediator of PI3K blockade-dependent cell death and as a potential predictor of therapy response to PI3K inhibitors.

Materials and Methods

Compound preparation

NVP-BEZ235 was kindly supplied by Novartis Pharma. For in vitro studies, stock solutions of NVP-BEZ235 were prepared in 100% DMSO and stored at −20 °C. Dilutions to the final concentration of 1 μmol/L were made in the culture medium immediately before use. For in vivo experiments, NVP-BEZ235 (45, 30, and 20 mg/kg) was suspended in 1 volume of 1-methyl-2-pyrrolidone (Sigma-Aldrich) and 9 volumes of PEG300 (Sigma Aldrich).

Organotypic culture

Primary pituitary adenoma cells from mutant rats were isolated as previously reported (26), and organotypic cultures were established using the 3D GravityPLUS (InSphero) system. Cells were seeded in a 96-well hanging drop culture platform (GravityPLUS™; InSphero) in 3D InSight Cell Line Maintenance Medium (InSphero) to form spheroids. The spheroids were then transferred to a spheroid-specific 96-well microtissue receiver plate (GravityTRAP™, InSphero) and further cultivated in the GravityTRAP™ plates. The spheroids were analyzed in an inverted microscope and their size was estimated following treatment using a Hitachi camera HW/C20 installed in a Zeiss Axioplan microscope with Intellicam software (Carl Zeiss MicroImaging GmbH).

Immunostaining

Spheroids and pituitary tumor tissue from MENX rats were collected after 2 weeks of treatment with NVP-BEZ235 or placebo (PEG). They were fixed with 4% paraformaldehyde and embedded in paraffin. IHC was performed on an automated immunostainer (Ventana Medical Systems) as previously described (27). Primary antibodies were directed against monoclonal phospho (p)-S6 (S6-S240/244; 1:500; Cell Signaling Technology), monoclonal p-AKT (Ser473; 1:75; Cell Signaling Technology), monoclonal Ki67 (clone B56, 1:100; Dako), monoclonal p27 (1:100; BD Biosciences), polyclonal activated caspase-3 (1:100; Cell Signaling Technology), monoclonal α-SU (clone 1:1,000; supplied by Dr. Parlow, NHPP, UCLA). Antibodies were diluted in Dako REAL™ antibody diluent (Dako). The SuperSensitive IHC detection system from BioGenex was used to visualize the antibody binding following the manufacturer’s instructions. Images were recorded using a Hitachi camera HW/C20 (Hitachi) installed in a Zeiss Axioplan microscope with Intellicam software (Carl Zeiss MicroImaging).

For immunofluorescence, we used the primary antibodies used for IHC and the antibody ab115813 against BD-1 (AbCam), and secondary anti-mouse Alexa Fluor 555 Conjugate (Cell Signaling Technology) or anti-rabbit FITC-conjugated (Invitrogen) antibodies (27). Sections were then analyzed with a Zeiss Axiobrevo 200 epifluorescence microscope, including Apotome unit (Carl Zeiss MicroImaging).

Quantification of P-S6 and cleaved caspase-3 (cc3) staining intensity was performed using ImageJ (NIH). Images were subjected to the threshold function, and we used the same threshold for all images obtained with the same antibody. Then, the percentage of positive area (P-S6) or the intensity of the staining (cc3) was determined. The Ki67 labeling index (LI = percentage of positive nuclei) was estimated as previously reported (28).

Animals and in vivo treatment

This study was approved by the ethics committee on animal research of the government of Upper Bavaria, Germany. MENX–affected rats were maintained as previously reported (29) in agreement with the procedures approved by the Helmholtz Zentrum München, by the Technische Universität München, and by the local government authorities.
Three doses of NVP-BEZ235 were tested in MENX rats: 20, 30, and 45 mg/kg. As the two higher doses caused a weight loss >10% after 10 days of treatment, the dose of 20 mg/kg was used for further studies. For MRE studies, MENX-affected rats at 7 to 8 months of age (with sizeable adenomas but still in good general health) were treated for 14 days with NVP-BEZ235 (20 mg/kg) or placebo (PEG) administered daily per oral gavage. The side effects of the drug were observed was mild diarrhea in the last days of the treatment (4/8 rats). Being this our first in vivo study of spontaneous rat pituitary adenomas, functional/molecular changes in the tumors were considered more objective and measurable end points (primary end points) compared to size and/or survival (secondary end points).

Pathologic analysis
Pituitary tumor tissues of PEG- or NVP-BEZ235-treated rats were fixed in 4% buffered formalin and paraffin-embedded. Three μm sections were cut and stained with hematoxylin and eosin (H&E), and were evaluated by an experienced neuropathologist (F. Roncaroli).

Magnetic resonance imaging
MRI was performed using a 3.0 Tesla clinical MRI system (Ingenia 3.0T; Philips Healthcare) prior and 2 weeks after treatment with NVP-BEZ235. Anesthetized animals (2.5% isoflurane, administered in pure oxygen) were placed in a standard human wrist coil (SENSE Wrist coil 8 elements; Philips Healthcare) in a prone position. T2-weighted (T2w) turbo spin echo sequence (slice thickness = 0.7 mm, in plane resolution 0.3 × 0.3 mm², TR/TE = 3399/106 ms, averages = 12) was performed to assess the tumor volume before and after treatment. Tumor volume was manually segmented and calculated by Osirix (http://www.osirix-viewer.com). Statistical analysis (paired t test) was performed using Prism GraphPad 4 (GraphPad Software, Inc.).

Following morphologic T2w imaging, diffusion weighted-MRI (DW-MRI) was performed using a multishot spin echo EPI sequence with a total of 6 diffusion weightings: $b_{0.5}$ values = 0, 50, 100, 200, 400, and 600 s/mm², slice thickness 1.4 mm, in plane resolution 0.62 × 0.78 mm², EPI factor = 7, TR/TE = 4907/62 ms, averages = 2. Three center slices in sagittal orientation, covering the pituitary gland were selected to assess the median apparent diffusion coefficient (ADC) value before and after treatment. Segmented tumors were analyzed by in-house software written in IDL (ITI VIS).

RNA extraction
For RNA extraction from organotypic cultures, spheroids ($n = 30$) were pooled and total RNA was extracted using the automated Maxwell 16 Cell Total RNA Purification Kit with the Maxwell 16 Instrument (Promega). From rat tissues, 8-μm sections of freshly fixed pituitary adenomas were stained with toluidine blue using standard manufacturer's protocols with minor modifications. The sections were air-dried and microdissected with a Leica AS LMD Laser Capture Microdissection System using laser pulses of 7.5-μm diameter, 20 to 40 mW, and with 2 to 3 ms duration (Leica). Laser-captured tumor tissues were dissolved in 1-Thioglycerol/Homogenization Solution (Promega) and stored at −80°C. RNA extraction was performed as described above.

RT² profiler PCR array and quantitative TaqMan RT-PCR
For expression analysis, the first-strand cDNA synthesis was performed with the pooled mRNA from organotypic cultures using the RT² First Strand Kit (Qiagen). This cDNA was then added to the RT² SYBR Green qPCR Master Mix (Qiagen), and then each sample was onto the RT² Profiler PCR Array Rat Cell Death PathwayFinder (Qiagen). Real-time PCR detection was performed by heating the plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes. Data analysis was performed using the manufacturer's software (http://www.sabiosciences.com/prcarraydataanalysis.php).

Quantitative RT-PCR was performed using TaqMan inventoried primers and probes for the genes indicated in the article and for the rat beta 2-microglobulin gene, mouse beta 2-microglobulin gene or human $TBP$ gene as internal controls (Applied Biosystem). The data were analyzed with $2^{-\Delta\Delta C_T}$ method as previously reported (28).

Cell culture and transfection
The BON1 and QGP1 cell lines were bought from LGC Standards in 2014 and grown as recommended by the manufacturer. The gonadotroph cell line αT3 was a gift of P. Mellon (University of California, San Diego). Since receipt, the cells have not been subsequently authenticated. Cells were grown in DMEM + GlutaMAX-I (Invitrogen) with 10% (v/v) FBS (Invitrogen) and 1% (v/v) penicillin-streptomycin (Invitrogen).

BON1 cells and QGP1 cells were transfected with 20 pmol of scrambled (ONTARGETplus Non-targeting siRNA, Dharmacon) or pooled siRNA against human $DEFB1$ gene (ONTARGET plus SMART pool siRNA, Dharmacon) by Amaxa 4D-Nucleofector (Lonza) following the manufacturer's instructions. The target sequences of pooled siRNA against human $DEFB1$ gene are: CUGAACGGGAAAGCACA, CGLIGIJUILLACICLICGJUUA, CGCCUGACGUGUACUJUU and CUGCCCCAGUICLIUJUACAAA.

Cell proliferation and apoptosis assays
Proliferation of BON1, QGP1, and αT3 cells after treatment was measured with the WST-1 colorimetric assay (Roche) as previously described (30). Cell proliferation in 3D spheroids was measured with the CellTititer Glo Cell Viability Assay (Promega) according to the manufacturer's recommendations. Apoptosis was measured by assessing the activity of caspase-3/7 using the Caspase-Glo 3/7 Assay Kit (Promega). BON1 cells and QGP1 cells were transfected with scrambled or anti-$DEFB1$ siRNA as above, and 24 hours later treated with NVP-BEZ235 or DMSO for additional 24 hours. Caspase-3/7 activity was then assessed with a luminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. Luminescence was measured with a luminometer (TECAN).

Statistical analysis
Results of the cell assays and TaqMan analysis are shown as the mean of values obtained in independent experiments ± SEM. A paired two-tailed Student $t$ test was used to detect significance between two series of data, and $P < 0.05$ was considered statistically significant.

Results
PI3K/mTOR inhibition decreases cell proliferation and promotes apoptosis of organotypic 3D cultures of rat primary NFPA cells
Similar to human NFPA (13), MENX-associated pituitary adenomas show activation of the PI3K–AKT–mTOR pathway.

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DMSO control; Fig. 1A) and a decrease in cell viability (−43%; P < 0.01 vs. DMSO control; Fig. 1B) were observed in the spheroids treated with NVP-BEZ235, but not in those incubated with DMSO. Drug-treated 3D cultures showed both a dramatic decrease in P-S6 and reduced cell proliferation, as assessed by Ki67 staining (Fig. 1C). Treatment of organotypic cultures with NVP-BEZ235 also induced the activation of caspase-3 (cleaved caspase-3, cc3) whereas no increase was seen in spheres treated with DMSO (Fig. 1C). These experiments confirmed the inhibition of the PI3K–AKT–mTOR pathway by NVP-BEZ235 and its antiproliferative and pro-cell death effects in vitro on NFPAs cells.

**PI3K/mTOR inhibition reduces cell proliferation and promotes cell death in a model of endogenous NFPAs in vivo**

To understand the effect of NVP-BEZ235 in spontaneous pituitary adenomas, we treated MENX rats with the drug. We first verified whether the drug reached the pituitary and repressed the PI3K pathway, as shown in vitro. NVP-BEZ235 (45 mg/kg) or PEG (placebo) was administered to MENX rats (n = 2 each group) by oral gavage and animals were sacrificed 1 or 6 hours after treatment. Immunostains for P-AKT and P-S6 showed considerable reduction of the two proteins, particularly of P-S6, 6 hours after administration of NVP-BEZ235 when compared with PEG-treated rats (Supplementary Fig. S2A). In contrast, decrease in P-AKT and P-S6 was not significant at 1 hour after treatment (Supplementary Fig. S2A). The same tissues were also analyzed by Imaging Mass Spectrometry (MALDI Imaging). At 6 hours after treatment, but not at 1 hour after treatment, the pituitary adenomas of NVP-BEZ235-treated rats had a proteomic profile significantly different from the tumors of placebo-treated rats (Supplementary Fig. S2B), thereby providing indirect evidence that the drug had reached the pituitary.

These results led us to treat mutant rats for 2 weeks with 20 mg/kg NVP-BEZ235 or PEG (placebo) both administered daily per oral gavage (Fig. 2A). At the end of the treatment, ex vivo tissue analysis showed that P-S6 expression and Ki67 LI were similar to the tumors of placebo-treated rats (Supplementary Fig. S2B), there-
treated tumors contained macrophages in variable number but none of them showed overt necrosis.

Diffusion weighted MRI shows a reduction in cellularity in NVP-BEZ235–treated rats

We then asked the question of whether we can monitor the response to NVP-BEZ235 noninvasively in living MENX rats. DW-MRI with calculated mean ADC values were used to assess changes in tumor cellularity (31) as indication of response to therapy. ADC values were obtained for all mutant rats \( n = 12 \) at day 0 (pretherapy scans), and then 14 days after daily administration of 20 mg/kg NVP-BEZ235 to a subset of animals \( n = 8 \), or of placebo (PEG) to the remaining ones \( n = 4 \) (post-therapy scans). Three sagittal center slices were used to calculate a mean ADC for each pituitary gland. As shown in Fig. 3, we observed significantly increased ADC values after NVP-BEZ235 treatment (mean ADC pre = 0.6325 vs. mean ADC post = 0.8146; \( P = 0.0013 \)) whereas no significant changes were observed in placebo-treated rats (mean ADC pre = 0.7059 vs. mean ADC post = 0.7058; \( P = 0.999 \)).

In parallel to the DW-MRI, we measured volumetric changes of the pituitary tumors before and after treatment with NVP-BEZ235 or with PEG by conventional T2w MRI. A slight decrease in tumor size was observed in NVP-BEZ235–treated animals (mean tumor volume pre = 0.0359 cm\(^3\) vs. mean tumor volume post = 0.0348 cm\(^3\)), albeit it did not reach the statistical significance by the paired t-test (Fig. 3B). Conversely, a slight increase in tumor size was observed in placebo-treated rats (mean tumor volume pre = 0.0355 cm\(^3\) vs. mean tumor volume post = 0.0399 cm\(^3\)). Probably, a 14-day course of treatment is not long enough to elicit tumor shrinkage to an extent detectable by neuroimaging. Thus, DW-MRI, assessing a functional parameter such as the induction of cell death, might be useful for early response tumor monitoring following PI3K/mTOR inhibition in pituitary adenomas before changes in tumor volume take place.

Gene-expression array analysis of NVP-BEZ235–treated 3D pituitary adenoma cultures identifies novel targets of the PI3K pathway

We then explored the molecular mechanisms mediating NVP-BEZ235–induced cell death in NFPAs. We performed gene-expression profiling of organotypic cultures derived from five independent rat pituitary adenomas following 2 weeks of treatment with NVP-BEZ235 or with DMSO. Genetic signatures were obtained using PCR-based arrays containing 84 cell death–related genes. By using a >1.5 fold-change cutoff for gene-expression changes, a total of 26 genes were found to be differentially expressed between the two groups. Twelve genes were upregulated in drug-treated versus placebo-treated cells, and 14 genes were downregulated (Supplementary Fig. S4). On the basis of their functional
annotation 11, including Foxi1, Defb1, and Tnfrsf8, were involved in necrosis. Seven genes, including Tnfrsf10b, Cd40lg, and Bcl2a1, were related to apoptosis and the remaining ones play a role in autophagy.

Next, we verified whether three of the genes found to be downstream of the PI3K–AKT–mTOR pathway in pituitary adenomas cells in vitro (i.e., Defb1 and Tnfrsf10b upregulated, Bcl2a1 downregulated) are targets of this signaling cascade also in vivo.

MENX mutant rats were treated with NVP-BEZ235 (n = 4) or placebo (PEG; n = 4) for 2 weeks, and then the expression of these three genes was assessed in their pituitary glands by quantitative (q)RT-PCR analyses. As shown in Fig. 4, Defb1 and Tnfrsf10b were upregulated by a +7.68 and +2.62 fold change (P < 0.05), respectively, in the tumors of NVP-BEZ235–treated rats when compared with tumors of placebo-treated animals. The antiapoptotic gene Bcl2a1 was downregulated in pituitary tumors after
NVP-BEZ235 treatment (Δ−0.5 fold change vs. placebo; P < 0.05; Fig. 4). These in vivo data are consistent with those obtained analyzing pituitary adenoma organotypic cultures in vitro.

In conclusion, gene-expression array analysis identified novel genes mediating the cytotoxic effect of PI3K/mTOR inhibition in NFPAs.

**Defb1** is a target of PI3K/mTOR inhibition in pituitary adenomas and in other neuroendocrine tumor cells

Among the cell death–related genes induced by PI3K/mTOR inhibition in rat NFPAs, **Defb1** is particularly interesting. This gene encodes beta-defensin 1 (BD-1), a member of a highly conserved group of host defense peptide (32). Defensins play an important role in processes other than innate immunity (33). In human cancers, BD-1 has been proposed to act as a tumor suppressor, as it inhibits cell growth and promotes apoptosis (34–37). Currently, the extent of expression and role of **Defb1** in neuroendocrine tumors (NET), and in pituitary adenomas in particular, is unknown.

For this reason, we determined the level of expression of **Defb1** in human NFPAs (n = 6) by qRT-PCR and compared it with normal human pituitary tissues (n = 3). The results showed that **Defb1** is extremely downregulated in human NFPAs when compared with normal pituitary (Fig. 5A). We also checked the expression of the BD-1 protein in human clinically nonfunctioning gonadotroph adenomas (n = 10) by immunofluorescence. Only one out of 10 tumors showed weak expression whereas the others were negative (Fig. 5B). In the normal human anterior pituitary, BD-1 was expressed and was found to colocalize with luteinizing hormone (LH; Supplementary Fig. S5), indicating that BD-1 is expressed in normal gonadotroph cells and suggesting that BD-1 is lost in gonadotroph adenomas.

Next, we investigated the role of **Defb1** in NFPAs by performing functional *in vitro* studies. Currently there are no established human/rodent NFPAs/gonadotroph adenoma cell lines, so we tested αT3, a mouse immortalized gonadotroph cell line (38). Similar to the rat primary adenoma cells, also αT3 cells showed an increase in beta-defensin 1 expression at both the mRNA and protein level (by immunofluorescence) upon NVP-BEZ235 treatment (Fig. 5C and D). Concomitantly, a reduction in the levels of both P-AKT and P-S6 was observed in response to drug treatment (Fig. 5E), confirming the inhibition of PI3K–mTOR signaling in αT3 cells.

To verify whether BD-1 plays a more general role in NETs, we also analyzed two well-characterized cell lines, BON1 and QGP1, both derived from human pancreatic endocrine tumors. Both cell lines express **Defb1** (Fig. 6C). Similar to rat primary pituitary adenoma cells and mouse gonadotroph cells, also treatment of BON1 and QGP1 with NVP-BEZ235 induced the levels of both **Defb1** mRNA and BD-1 protein (Fig. 6A–C). In agreement with previously published data (39), we observed that incubation with NVP-BEZ235 decreased the proliferation and increased the apoptosis of both cell lines, this latter assessed by measuring caspase-3/7 activity (Fig. 6D–G).

We then wondered whether the induction of **Defb1** sensitizes NET cells to PI3K/mTOR inhibition. Thus, we silenced **Defb1** expression by siRNA-mediated gene knockdown in BON1 and QGP1 cells, and we then treated them with NVP-BEZ235. We could demonstrate that the knockdown of **Defb1** by specific si-**Defb1** molecules reduces the antitumor effect of the drug. Indeed, there was a more prominent decrease in cell proliferation (Fig. 6D and F) and increase in apoptosis (caspase-3/7 activity) in cells transfected with unspecific scrambled siRNA than in cells transfected with si-**Defb1** (Fig. 6E and G). Efficient **Defb1** gene silencing was confirmed by qRT-PCR (Supplementary Fig. S6).

Altogether, these data suggest that **Defb1** is a downstream target of the PI3K/mTOR pathway in human NETs, where it mediates proapoptotic signals, and is a putative predictor of therapy response.

**Discussion**

We have previously shown that NVP-BEZ235 can potently inhibit cell proliferation of both dispersed rat primary pituitary adenoma cells and established adenoma cell lines *in vitro* (26). Here, we expanded these studies to include organotypic cultures of rat primary pituitary tumors, better models of the situation in tissues. We demonstrated that drug treatment induces potent antiproliferative and proapoptotic effects in NFPAs. Moreover, we have established the utility of NVP-BEZ235 as a cytotoxic agent in a preclinical and *in vivo* model of spontaneous NFPAs. In our tumor model, the *in vitro* sensitivity of NFPAs to NVP-BEZ235 corresponds to the *in vivo* sensitivity and both correlate with inhibition of downstream effectors of the PI3K pathway. To date, NVP-BEZ235 has been shown to behave as a cytostatic or cytotoxic antitumor agent, depending on the tumor type. In NFPAs, this compound displays both activities. Similarly, Dai and colleagues...
reported that the PI3K/mTOR inhibitor XL765, alone or in combination with temozolomide, inhibits proliferation and induces caspase-3/7 activity in a xenograft model of pituitary somatotroph adenoma cells (GH3 cells). In our animal model, NVP-BEZ235 can reduce the proliferation and induce apoptosis of the pituitary adenomas in their natural anatomical location.

For many solid tumors, it typically takes several months to evaluate therapy response based on RECIST criteria (41) when using solely morphologic imaging methods with soft tissue contrast (e.g., CT or MRI). Thus, the identification of parameters that could be used as surrogate markers of response to therapy is of pivotal importance. Their assessment by functional imaging modalities could help to quickly identify nonresponders, thus minimizing potential side effects (and costs) by early discontinuation of an ineffective therapy. In our study, we showed that noninvasive DW-MRI is a useful imaging modality for the early therapy response monitoring of NFPA treated with a PI3K/mTOR inhibitor. DW imaging can characterize tumor physiology and morphology and provide information about cellular consistency, which reflects in lower or higher ADC values. Indeed, NVP-BEZ235 administration in vivo resulted in a significant increase in ADC values, mirroring reduced cellular density and enhanced cell death, after only 2 weeks of treatment, whereas no changes in ADC values were detected in rats treated with placebo only. Importantly, changes in ADC values following drug treatment preceded significant changes in pituitary tumor volume, as measured by anatomical MRI. Recent studies performed in other tumor entities, including gastrointestinal stromal tumors, hepatocellular carcinomas, and breast cancer, propose DW-MRI as a surrogate marker of response to chemotherapy (42–44). As shown in a xenograft model of prostate cancer, changes in water molecule diffusion and ADC values can be detected already 24 hours after photodynamic therapy (45). On the basis of our data, DW-MRI has great potential as an imaging biomarker for early prediction of the response of NFPA to PI3K/mTOR inhibition.

In an attempt to identify the genes mediating the cytotoxic role of PI3K inhibition in our model, we performed gene-expression analyses of organotypic cultures treated with NVP-BEZ235, or
placebo-treated, focusing on cell death-related genes. Several genes were found differentially expressed between the two sample groups, supporting the hypothesis of an important role of active PI3K signaling in pituitary adenoma cell survival. Defb1, encoding BD-1, was among the genes significantly upregulated following NVP-BEZ235 treatment (but not placebo treatment), and was never so far studied in the context of pituitary adenomas. Besides its role as the most important antimicrobial peptide in epithelial tissues, BD-1 was recently found to be involved in a variety of processes other than innate immunity, including immunomodulation, development, wound healing, and cancer (33). In human cancers, BD-1 has been proposed to act as a tumor suppressor in renal clear cell carcinoma and prostate cancer because its expression is lower in the tumors compared with preneoplastic or normal tissues, and its ectopic overexpression induces caspase-3-mediated apoptosis (35, 37). The mechanism of downregulation of Defb1 in the above tumors has not been fully elucidated, but methylation does not seem to play an important role (37). Consistent with these studies, we found that both Defb1 and BD-1 are virtually not expressed in human NFPAs, suggesting that Defb1 might represent a novel tumor-suppressor gene in NFPAs.

In contrast with the adenomas, BD-1 is expressed in gonadotroph cells of the normal pituitary gland. BD-1 is generally expressed in epithelial cells of the gastrointestinal and broncho-respiratory tract, tissues involved in host defense, and only one study so far has reported BD-1 expression in the posterior lobe of the pituitary (neurohypophysis) of the fish orange-spotted grouper, where it plays a still unidentified role (46).

We here show, for the first time, that BD-1 expression modulates the response to antitumor drugs. Indeed, we demonstrate that, concomitantly with promoting antitumor activities, PI3K/mTOR blockade upregulates Defb1/DEFB1 in various neuroendocrine cell
models (pituitary and pancreatic) in vitro, as well as in MENX-associated pituitary adenomas in vivo. Silencing of the DEFBl gene in human BON1 and QGP1 cells abolished in part the antiproliferative and proapoptotic effect of NVP-BEZ235, suggesting that DEFBl is among the factors mediating the cytostatic and cytotoxic effect of the drug. On the basis of these findings, we propose that the increase in DEFBl expression upon treatment with PI3K/mTOR inhibitors sensitizes human NET cells, and possibly NFPAs, to these compounds.

Renal clear cell carcinoma, prostate cancer, as well as NFPAs, are characterized by hyperactivation of the PI3K pathway (12, 47, 48), and concomitantly were found not to express DEFBl (35, 37 and this study). It is tempting to speculate that the downregulation of DEFBl in these tumors might be mediated by activation of the PI3K–AKT–mTOR pathway, possibly through modulation of transcription factors activity. Consistent with this hypothesis, we observed that pharmacologic blockade of PI3K signaling rescues DEFBl expression in NFPAs and NET cells. Currently, there are no data directly connecting the PI3K pathway and the transcriptional regulation of the DEFBl gene. It has been reported that DEFBl expression is repressed by the transcription factor PAX2 in prostate cancer cells (49). Inhibition of PI3K activity leads to a down-regulation of PAX2 expression in renal tubular cells (50). PAX2 is expressed in the endocrine pancreas (from which BON1 and QGP1 cells derive; ref. 51), but no information on the pituitary gland is available. Thus, we could envision a mechanism whereby inhibition of PI3K signaling in NET cells decreases PAX2 expression, which, in turn upregulates DEFBl. Further studies are required to verify this hypothesis.

Bcl2a1/BF1-1 was also among the genes differentially expressed in NVP-BEZ235-treated versus placebo-treated primary pituitary adenoma cells. In contrast with DEFBl, its expression was reduced by the drug treatment in vitro and in vivo. BF1-1 is an antiapoptotic member of the Bcl-2 family of cell death regulators. In a physiologic context, BF1-1 is mainly expressed in the hematopoietic system, where it facilitates the survival of selected leukocytes subsets. BF1-1 has been found overexpressed in a subset of chemoresistant tumors, where it protects tumor cells from chemotherapy-induced apoptosis (52). Recently, peptide aptamers specifically targeting BF1-1 have been generated and shown to sensitize B-cell lymphoma cell lines to chemotherapeutic drugs through induction of apoptosis (53, 54). Given that NFPAs are usually resistant to traditional cytotoxic chemotherapeutic agents (2, 6), downregulation of BF1 by NVP-BEZ235 might represent a useful strategy to sensitize these tumors to apoptosis induced by conventional chemotherapy.

Altogether, our gene-expression studies unveiled novel putative targets in NFPAs that deserve to be further evaluated for their therapeutic potential.

Of the several inhibitors of the PI3K signaling cascade, only everolimus (a mTOR inhibitor) has been so far evaluated in one patient with an aggressive pituitary tumor, specifically, with an ACTH-secreting pituitary carcinoma (55). Everolimus was ineffective at normalizing hormone secretion and at controlling tumor growth. The lack of efficacy of everolimus could be ascribed to the well-documented feedback loop of rapalogs on AKT phosphorylation, already extensively associated with drug resistance in a variety of human cancers (18). To overcome tumor cell resistance to these drugs, dual inhibitors have been developed that block mTOR, but also the upstream PI3K kinase, such as NVP-BEZ235. Here, we demonstrate that dual PI3K/mTOR inhibition is highly effective against NFPAs in vitro and in vivo. These preclinical trials performed on an endogenous model of NFPAs provide the rationale for targeting PI3K–mTOR signaling in patients with pituitary adenomas, especially those with large and invasive NFPAs at high risk for tumor relapse.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

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