

Levels of p27 Sensitize to Dual PI3K/mTOR Inhibition

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Abstract

Constitutive activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling cascade occurs in a variety of human malignancies, where it sustains tumor cell proliferation and survival. Pharmacologic blockade of this pathway exerts antineoplastic activity by triggering apoptosis and/or cell-cycle arrest. Pituitary adenomas show activation of the PI3K/AKT/mTOR pathway, but only a fraction of them respond *in vitro* to the antiproliferative action of rapamycin and RAD001 (mTOR inhibitors), possibly because of the described negative feedback loop on AKT which reactivates the signaling cascade. Rats affected by the multiple endocrine neoplasia-like syndrome (MENX) develop pituitary adenomas showing increased activated AKT. In this study, we comparatively investigated the antitumor potential of the novel dual PI3K/mTOR inhibitor NVP-BEZ235 and the single mTOR inhibitor RAD001 on rat pituitary adenoma cells in primary culture. NVP-BEZ235 inhibits the PI3K pathway both upstream and downstream of AKT, thereby preventing the negative feedback loop. NVP-BEZ235 was more effective than RAD001 in reducing cell viability of pituitary adenomas. Consistently, NVP-BEZ235 treatment decreased Akt and S6 phosphorylation and triggered apoptosis. Because MENX is caused by a germline loss-of-function mutation in the cell-cycle inhibitor p27Kip1, we investigated the relationship between this defect and response to NVP-BEZ235 treatment. The levels of p27Kip1 positively correlate with the response to NVP-BEZ235 treatment. Combined treatment with NVP-BEZ235 and the proteasome inhibitor bortezomib, which increases p27Kip1 amount, shows synergistic antiproliferative effects on pituitary adenoma cells. Our data suggest that NVP-BEZ235 may represent an effective therapeutic modality for pituitary adenomas and that p27Kip1 levels represent a potential predictor of response to dual PI3K/mTOR inhibition. *Mol Cancer Ther*; 10(8); 1450–9. ©2011 AACR.

Introduction

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway plays a pivotal role in the initiation and progression of many human malignancies by enhancing cell survival, stimulating cell proliferation, and inhibiting apoptosis (1, 2). AKT is the primary mediator of PI3K-initiated signaling by phosphorylation of several downstream effectors such as mTOR, ribosomal protein S6 kinase (S6K1), the forkhead family of transcription factors (FKHR/FOX), glycogen synthase kinase, and the proapoptotic Bcl2-associated death promoter protein (3).

As a consequence of its importance in key cellular processes, the PI3K/AKT/mTOR signaling cascade has become an important therapeutic target in cancer, and compounds have been generated that inhibit this pathway at different levels. The mTOR inhibitors rapamycin and its analogs (also called rapalogs) are currently used with success for treating several solid tumors (4, 5). However, resistance to the treatment with rapamycin/rapalogs has been reported (6). This phenomenon has been in part ascribed to a feedback loop triggered by rapamycin, which leads to the activation of the AKT kinase through S6K1, thereby, counteracting the antitumor potential of mTOR inhibition (7, 8). To bypass this problem, pharmaceutical research has recently developed a new class of compounds able to inhibit both mTOR and the upstream PI3K kinase and prevent the negative feedback loop and AKT activation. Among these agents is NVP-BEZ235, a synthetic small molecule that inhibits both PI3K and mTOR kinase activity by binding to the ATP-binding cleft of these enzymes (9). NVP-BEZ235 has shown potent antiproliferative activity in several preclinical models of various tumor types (9–13) and is currently evaluated in phase I clinical trials in solid tumor patients.

Constitutive activation of the PI3K pathway associates with increased levels of phosphorylated (activated) AKT.

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One important target of AKT is the cyclin–cyclin dependent kinase (CDK) inhibitor p27Kip1 (hereafter p27; ref. 14). p27 is a key regulator of the progression from G₁ to S phase of the cell cycle and acts by binding and inhibiting the CyclinE-CDK2 complex in the G₁ phase (15). AKT-mediated phosphorylation of p27 causes cytoplasmic relocalization and degradation of the protein, thereby, ultimately abrogating the cell-cycle inhibitory functions of p27 (16). Reversal of this effect by pharmacologic blockade of the PI3K signaling cascade is predicted to restore cell-cycle regulation and consequently diminish tumor cell growth and improve the clinical outcome of the patient (17). Indeed, an increase in nuclear p27 upon drug administration has been associated with induction of G₀–G₁ arrest following treatment of tumor cells with several chemotherapeutic compounds, including the tyrosine kinase enzymes inhibitors imatinib mesylate (Gleevec), lapatinib (Tyverb), and mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 (18–20).

p27 is rarely deleted or mutated in human cancers but its level of expression is reduced in the vast majority of human cancers, phenomenon that associates with poor clinical outcome in several malignancies, including cancers of the breast, colon, prostate, and ovary, among others (21). Because tumor samples with high or low p27 protein levels show similar amounts of the *CDKN1B* transcript (22, 23), it is believed that decrease of p27 expression in human tumors is mainly because of enhanced proteasome-mediated degradation of the protein, the mechanism shown to be responsible for p27 downregulation in colon cancer (24).

Although the use of p27 as a prognostic biomarker has long been established, its importance in the response to therapy in human cancers has started only recently to be investigated. Moneo and colleagues reported that the levels of p27 sensitize sarcoma cell lines to Aplidin, a macrocyclic depsipeptide showing antitumor activity in various human tumors (25). Higher p27 levels were recently found to be associated with improved response of breast cancer cell lines to 2 rapalogs, temsirolimus and everolimus (RAD001; ref. 26).

We recently identified a strain of rats that consistently and with high penetrance develops multiple endocrine tumors. We named this disease multiple endocrine neoplasia-like syndrome (MENX). Among the neoplasms associated with MENX are pituitary adenomas that, in affected rats, occur with complete penetrance by the age of 6–8 months (27). The genetic mutation causing MENX is a germline biallelic frameshift mutation in the *Cdkn1b* gene, encoding p27 (27). This mutated allele encodes a very unstable mutant p27 protein that is rapidly degraded, at least in part, through the proteasome (28). As a consequence, the tissues/cells of affected rats have a very low amount of protein, although p27fs177 maintains several characteristics of the wild-type (wt) protein such as intracellular localization, protein binding (27).

Pituitary adenomas account for about 10% of intracranial tumors. Although they are usually benign, they can cause severe morbidity secondary to the hyperproduction of a pituitary hormone or in clinically nonfunctioning adenomas due to local invasion. Surgery is currently the treatment of choice in the majority of cases, apart from prolactinomas and to a lesser extent, somatotroph adenomas (29). Adenomas of the pituitary are among the tumors showing hyperactivation of the PI3K/AKT/mTOR pathway (30). Because of the activation of this signaling cascade, pituitary adenomas should be sensitive to treatment with mTOR inhibitors. Rapamycin and its analog RAD001 (everolimus) have shown antiproliferative activity against pituitary adenomas grown as dispersed primary cultures *in vitro* (31, 32), but a proportion of these tumors ranging from 30% to 70% were resistant to these compounds (33). Resistance to rapalogs treatment, as indicated above, should be bypassed by an upstream blockade of the PI3K pathway, such as that seen following treatment with the dual PI3K/mTOR inhibitor NVP-BEZ235. Although this agent has shown antitumor effects in neuroendocrine tumor cell lines of various origins, where it induces apoptosis and G₀–G₁ arrest (34), it has not been tested so far in pituitary adenomas.

In this study, we tested the dual PI3K/mTOR inhibitor NVP-BEZ235 for its efficacy against MENX-associated pituitary adenomas and then compared the results with the antitumor potential of the single mTOR inhibitor, RAD001. We, here, report that NVP-BEZ235 is a highly effective antineoplastic agent in our experimental system, as it is able to inhibit the viability of 100% of rat pituitary adenomas grown as primary cultures. In contrast, RAD001 elicits only partial response from the rat pituitary adenomas. Because of the genetics of this animal model, we also assessed the role of p27 in the sensitivity to NVP-BEZ235. Interestingly, we observed that the amount of p27 positively correlates with the efficacy of NVP-BEZ235 as an antitumor agent, suggesting that the expression of p27 in tumor cells may be a predictor of response to dual PI3K/mTOR inhibition.

Materials and Methods

Drugs and chemicals

RAD001, NVP-BKM120, and NVP-BEZ235 were kindly provided from Novartis Pharma. Bortezomib was purchased from LC Laboratories. All compounds were dissolved in dimethyl sulfoxide and stored at –20°C. Dilutions to the final concentration were made in the culture medium immediately before use. The chemical structure of the compounds we used is shown in Supplementary Fig. S1.

Cell culture

Fresh pituitaries were isolated from 7 to 8 months old MENX-affected and wt control rats at autopsy. Affected rats had histologically confirmed adenomas, expressing the common alpha subunit and to a lesser extent the

follicle stimulating hormone and luteinizing hormone specific beta subunits of the gonadotroph hormones (unpublished results). The tissues were washed with Hank's Balanced Salt Solution (Invitrogen), minced, and digested with collagenase V (Worthington) for 90 minutes at 37°C, and then mechanically dispersed. Dispersed cells were centrifuged and resuspended in RPMI1640 (Invitrogen) supplemented with 10% FBS, 100 units/mL of penicillin G sodium, 100 µg/mL streptomycin, Fungizone antimycotic (Invitrogen), and D-valine for removal fibroblast contamination (35). Cell viability was over 80% as assessed by trypan blue staining. The cells were seeded in 96-well plates (25,000 cells per well) and left for 36 hours at 37°C in a humidified incubator with 5% CO₂ in air before beginning the treatments.

GH3 cells were purchased from the American Type Culture Collection (Nr. CCL-82.1), which uses short tandem repeat analysis (DNA profiling) for intraspecies identification. GH3 cells were grown in F12 medium supplemented with 15% horse serum, 2.5% FCS, 20 mmol/L L-glutamine, 100 units/mL of penicillin G sodium, and 100 µg/mL streptomycin. Primary rat fibroblast cells (REF) were established in our laboratory as previously reported (28). Primary mouse embryonic fibroblasts (MEF) were obtained from p27^{-/-}, p27^{+/-}, and p27^{+/+} mouse embryos as described (28). These primary cells were used at early passages and only tested for the expression level of p27. REFs and MEFs were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 20 mmol/L L-glutamine, 100 units/mL of penicillin G sodium, 100 µg/mL streptomycin, and Fungizone antimycotic. During the experiments, cells were grown in the absence of antibiotics. The primary cells were not specifically tested. Primary cells and GH3 cells were tested every month for mycoplasma contamination and found to be mycoplasma-free cultures.

Cell viability

The effect of RAD001 and NVP-BEZ235 was tested on pituitary adenoma cells, embryonic primary fibroblast, and GH3 cells, plated in 96-well plates and 36 hours later treated with test substances or vehicle. Cell viability was assessed 24 to 48 hours after incubation with the test substances by using the Vialight Plus Kit (Lonza), which is based on the bioluminescent measurement of ATP present in metabolically active, proliferating cells. ATP was measured by using the a luminometer (Berthold) and a second integrated reading for each well. The reported results represent the mean of 6 replicates.

We measured cell viability and not replication of dispersed rat pituitary adenoma cells, because in our experimental conditions these cells do not replicate, as determined by counting seeded cells every 24 hours over a period of 72 hours postseeding (data not shown).

Protein extraction and Western blotting

For protein extraction, cells were collected after treatments, washed twice in PBS, and lysed in lysis buffer

essentially as previously reported (27). Protein concentration was assessed by the bicinchoninic acid assay (Pierce). Total extracts were subjected to polyacrylamide gel electrophoresis by using Bis-Tris 4% to 12% NuPAGE gels, blotted, and probed with the following antibodies: monoclonal, against phosphorylated (p)-Akt (S473), polyclonal against p-Akt (T308), polyclonal against total Akt, polyclonal against phospho-S6 (S240/244), polyclonal against total S6 (all from Cell Signaling); monoclonal anti-p27 (BD Bioscience); monoclonal anti- α -tubulin (Sigma). Immunoreactive proteins were visualized by using West Pico chemiluminescent substrates (Pierce).

Immunohistochemistry

Rat pituitary tissues were fixed in 10% buffered formalin and embedded in paraffin. After antigen retrieval, immunohistochemistry was carried out by using the following antibodies: monoclonal anti-total AKT and anti-phospho-AKT (S453; 1:30; Cell Signaling). Tissues were counterstained with hematoxylin. Images were recorded by using a Hitachi camera HW/C20 installed in a Zeiss Axioplan microscope with Intellicam software (Zeiss MicroImaging).

Plasmid constructs and transfection

The constructs expressing wt p27 or the mutant p27fs177 as green fluorescent protein (GFP) fusion proteins have been previously described (28). For transient transfections, GH3 cells were grown in 48- or 6-well plates and transfected when they were 70% to 80% confluent by using the FuGeneHD reagent (Roche) in Opti-MEM I (Invitrogen), following the manufacturer's instructions.

Apoptosis assay

Apoptosis was measured by assessing the activity of caspase-3/7 by using Caspase-Glo 3/7 Assay Kit (Promega). Primary cells or transfected GH3 cells were plated in 96-well plates and 36 hours later were treated with NVP-BEZ235 (10 and 100 nmol/L) or vehicle. Twenty-four to 48 hours after treatment, caspase-3/7 enzymatic activity was assessed with a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. Luminescence was measured by using a luminometer (Berthold).

Statistical analysis

Results of the cell viability assays are shown as the mean of values obtained in independent experiments $\pm$ SEM. A paired 2-tailed Student's *t*-test was used to detect significance between 2 series of data and *P* < 0.05 was considered statistically significant.

Results

The PI3K/AKT/mTOR pathway is hyperactivated in pituitary tumor cells of MENX rats

To determine whether the PI3K/AKT/mTOR pathway is activated in rat pituitary adenomas as it occurs

in human pituitary adenomas (30), we assessed the Akt phosphorylation levels in adenomas of adult mutant rats and age-matched wt controls by using immunohistochemistry with antibodies against phosphorylated (p)-Akt-Ser(S)473 protein. Pituitary tissues from adult mutant rats (bearing tumors) and age-matched wt controls were analyzed. Adenoma cells showed cytoplasmic immunoreaction for p-Akt-S473, whereas the adjacent normal tissues and pituitary glands from unaffected rats were virtually negative (Fig. 1A). In contrast, no difference in the expression of total Akt between pituitary adenomas and normal pituitary glands was detected (Fig. 1A). We also analyzed protein extracts from primary pituitary cell cultures established from mutant and wt rats for Akt, p-Akt-S473, and p-Akt-Thr(T)308 expression. We also assessed the phosphorylation of the ribosomal protein S6 (S6-S240/244), an effector of S6K1 (thus of mTOR) signaling. The protein extracts showed variable but consistently appreciable increase of p-Akt-S473 as well as p-Akt-T308 and p-S6-S240/244 in tumors compared with wt pituitary cells (Fig. 1B and C), thereby, confirming and extending the immunohistochemistry data. The presence of normal cells within the primary tumor cultures likely causes underestimation of the amount

of p-Akt-S473 in the protein extracts when compared with immunostaining.

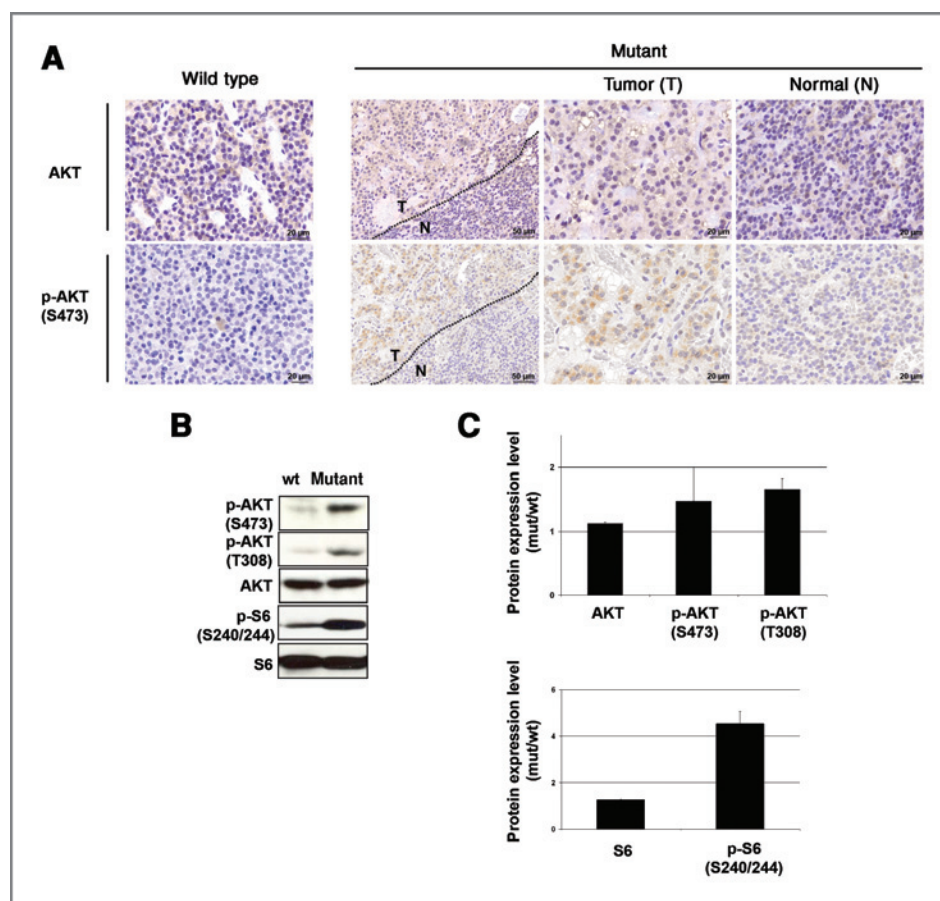
Together, these results indicate the activation of Akt signaling in MENX-associated pituitary adenomas.

Targeting the PI3K/AKT/mTOR pathway in pituitary tumor cells of MENX rats

Because rat pituitary adenomas show activation of AKT signaling, we evaluated the effects of the mTOR inhibitor RAD001 and the dual PI3K/mTOR inhibitor NVP-BEZ235 on the survival of adenoma cells. Primary cultures were treated with RAD001 (0.1–100 nmol/L) or NVP-BEZ235 (0.1–100 nmol/L). We considered as responders those cultures displaying a 20% or more cell viability reduction versus control and nonresponders the remaining ones, using a widely used response cutoff (31, 33).

As shown in Fig. 2A, the rapalog RAD001 caused a dose-dependent reduction in cell viability that reached statistical significance at the 100 nmol/L concentration (-36% ; $P < 0.05$ vs. control) in 5 out of 11 primary cultures. In contrast, incubation with the dual PI3K/mTOR inhibitor NVP-BEZ235 reduced cell viability up to 25% at the 10 nmol/L concentration ($P < 0.001$ vs. control) and up to 37% at 100 nmol/L concentration

Figure 1. Activation of the PI3K/AKT/mTOR pathway in rat pituitary adenomas. **A**, immunohistochemistry was carried out on paraffin-embedded pituitary tissues from wt (control) and mutant rats by using antibodies against total Akt and p-Akt (Ser473). Original magnifications: $\times 20$ (scale bar, 50 $\mu\text{mol/L}$) and $\times 40$ (scale bar, 20 $\mu\text{mol/L}$). **B**, immunoblotting of protein extracts from primary pituitary cells from wt and mutant rats. Membrane was probed with anti-p-Akt (S473), anti-p-Akt (T308), anti-total Akt, anti-p-S6 (S240/244), and anti-total S6 antibodies. **C**, graphs show the average band intensities, as determined by band densitometry, obtained following Western blotting of protein extracts from 2 wt and 4 mutant (= tumors) primary pituitary cultures analyzed as in B.



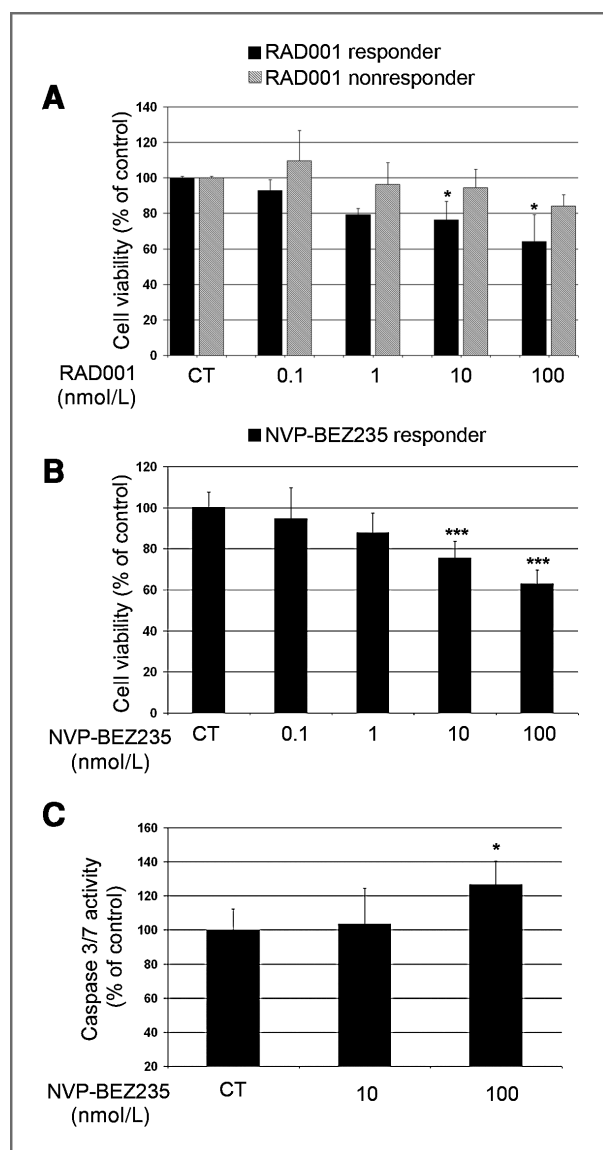


Figure 2. Effect of the single mTOR inhibitor (RAD001) and the dual PI3K/mTOR inhibitor (NVP-BEZ235) on primary cultures from rat pituitary adenomas. Primary rat pituitary cells were incubated in 96-well plates for 48 hours in culture medium supplemented with increasing concentrations (0.1–100 nmol/L) of RAD001 (A) or NVP-BEZ235 (B). Untreated control (CT) cells were incubated with vehicle solution. Cell viability was assayed by measuring ATP levels. Data are shown as percentage of control. Primary cultures were divided into responder and nonresponder according to the reduction of cell viability (at least 20%). Data from primary cultures were analyzed independently with 6 replicates each and were expressed as the mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.001$ versus CT. C, caspase 3/7 activity was measured to monitor apoptosis in 3 rat primary pituitary adenoma cultures treated with NVP-BEZ235 (10 or 100 nmol/L) for 48 hours or left untreated (CT). *, $P < 0.05$ versus CT.

($P < 0.001$ vs. control) in 10 out of 10 cultures tested (Fig. 2B), making this compound more effective in our experimental model.

Incubation with NVP-BEZ235 at concentrations that suppress cell viability also decreased the phosphoryla-

tion of both Akt (Akt-Ser473) and S6 (S6-S240/244) in agreement with the role of this drug as a dual PI3K/mTOR inhibitor (Fig. 3A).

To investigate the extent and specificity of suppression of downstream signaling, we compared the effect of the single PI3K inhibitor NVP-BKM120, of the single mTOR inhibitor RAD001, and of NVP-BEZ235 on rat primary pituitary adenoma cultures. As expected, NVP-BKM120 downregulated Akt-S473 phosphorylation but did not affect p-S6-S240/244. The mTOR inhibitor RAD001 reduced S6-S240/244 phosphorylation but increased Akt-S473 phosphorylation, because of the mentioned negative feedback loop on Akt (Fig. 3B). In contrast, NVP-BEZ235 reduced the phosphorylation of both Akt and S6, as expected. These data illustrate that the cells from MENX-affected rats respond to PI3K and/or mTOR inhibition similarly to *in vitro* and *in vivo* human cancer models (11, 36, 37).

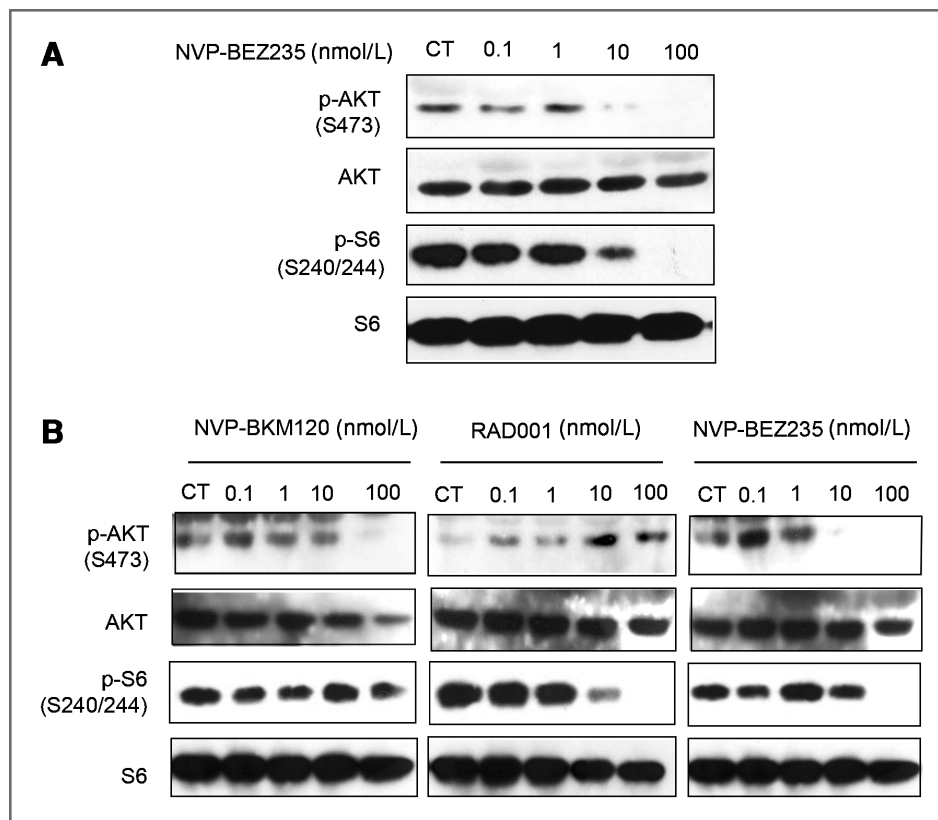
The limited amount of cells obtained from adenomas precluded the evaluation of the cell-cycle distribution of the treated primary cultures by fluorescence-activated cell sorting. However, following incubation with NVP-BEZ235 (+24 hours, 100 nmol/L), we observed an increase in caspase-3/7 activity (+24% vs. control, $P < 0.05$) in 3 rat primary cultures, which suggests that this drug reduced the cell viability of adenoma cells at least in part by promoting apoptosis (Fig. 2C).

p27 expression sensitizes to NVP-BEZ235 treatment

The germline *Cdkn1b* mutation in MENX-mutant rats translates into a p27 mutant protein (named p27fs177) that maintains several characteristics of the wt p27 protein (intracellular localization and protein binding), but it is highly unstable and consequently expressed at low level in cells/tissues of MENX-mutant rats (27). This is why we consider it a loss-of-function mutation. Because p27 is a target of AKT, we investigated next the relationship between the amount of p27 and the response to a PI3K/mTOR blockade. To this aim, we used several genetically defined cell lines characterized by different amounts of endogenous p27. We used primary embryonic fibroblasts established from MENX-affected rats (REF10 cells) or from wt rats (REF7 cells) and MEFs from wt (p27^{+/+}, MEF12 cells), homozygous (p27^{-/-}, MEF19 cells), or heterozygous (p27^{+/-}, MEF 21 cells) knockout mice. These cell lines have the different expression levels of p27 (Supplementary Fig. S2A). As previously reported for other MEF cells (38), these primary cultures show a low basal level of Akt phosphorylation (Supplementary Fig. S2B; data not shown).

REF7 and REF10 primary rat fibroblasts were incubated with NVP-BEZ235 (10 nmol/L) and then their viability was assessed. NVP-BEZ235 significantly reduced cell viability of REF7 (wt p27) cells by $\sim 37\%$ ($P < 0.05$ vs. control). In contrast, no significant inhibition of survival was observed for REF10 cells that express low levels of p27fs177 (Fig. 4A). This result suggested that normal p27

Figure 3. Downregulation of downstream target proteins after NVP-BEZ235 treatment in rat primary adenomas. **A**, protein extracts from rat primary pituitary cells incubated with increasing concentrations (0.1–100 nmol/L) of NVP-BEZ235 for 24 hours or left untreated (CT) were used for Western blotting with anti-p-Akt (S473), anti-Akt, anti-p-S6 (S240/244), and anti-S6. **B**, rat primary pituitary adenoma cells from 1 affected rat were incubated with different concentrations (0.1–100 nmol/L) of NVP-BKM120, RAD001, or NVP-BEZ235. After 24 hours, Western blotting was carried out to monitor the expression level of the indicated proteins as in **A**.



expression enhances the response of primary rat fibroblasts to NVP-BEZ235. Indeed, following incubation with NVP-BEZ235, p27 expression increased slightly in REF7 cells whereas no change in the amount of mutant p27fs177 was observed in REF10 cells (Supplementary Fig. S2B). Levels of p-S6-S240/244 decreased in both REF7 (wt p27) and REF10 (mutant p27) cells, confirming that both cell types are sensitive to NVP-BEZ235 administration at the molecular level. Surprisingly, for both REF7 and REF10 cells, incubation with NVP-BEZ235 increased phosphorylated Akt levels similar to what we observed in cultures of adenoma cells after treatment with RAD001.

To confirm the role of p27 in sensitizing cells to NVP-BEZ235, MEFs from p27^{+/+}, p27^{+/-}, and p27^{-/-} mice were incubated with 10 nmol/L NVP-BEZ235. p27^{+/+} MEFs showed a more pronounced reduction in cell viability than cells from p27^{-/-} mice, whereas heterozygous p27^{+/-} MEFs showed an intermediate survival inhibition (Fig. 4B).

GH3 rat pituitary adenoma cells do not express endogenous p27 because of hypermethylation of the *Cdkn1b* promoter (39). We, therefore, transfected these cells with vectors expressing wt p27 or p27fs177 (Fig. 4C) and observed that GH3 expressing normal levels of p27 responded better to NVP-BEZ235 treatment compared with those expressing the mutant protein. The result of this experiment further supports our hypothesis that normal p27 expression sensitizes to NVP-BEZ235

(Fig. 4C). Incubation with NVP-BEZ235 reduced phosphorylation of both Akt-S473 and S6-S240/244 in both types of GH3-transfected cells, excluding unspecific disabling action of the mutant p27 on the upstream signaling pathways mediating the response to the drug (Supplementary Fig. S2C). Earlier in the article, we showed that NVP-BEZ235 induced apoptosis in primary pituitary adenoma cells (Fig. 2C). To determine whether the amount of p27 has an effect on the induction of apoptosis following NVP-BEZ235 treatment, we measured the amount of caspase-3/7 (a marker of apoptosis) in treated GH3 cells having different p27 expression levels. As shown in Fig. 4D, NVP-BEZ235 induced apoptosis in GH3 cells transfected with wt p27, but much less so in cells expressing the mutant p27fs177. Taken together, these results suggest that higher levels of functional p27 enhance the antiproliferative effect of NVP-BEZ235 in susceptible rodent cells (fibroblasts, pituitary adenoma).

Combined treatment with the proteasome inhibitor bortezomib enhances the antiproliferative effect of NVP-BEZ235

On the basis of what we illustrated above about the association between levels of p27 and sensitivity to NVP-BEZ235, we predicted that the increase in p27 amount would further sensitize rat primary pituitary tumor cells to NVP-BEZ235. In a previous study, we showed that

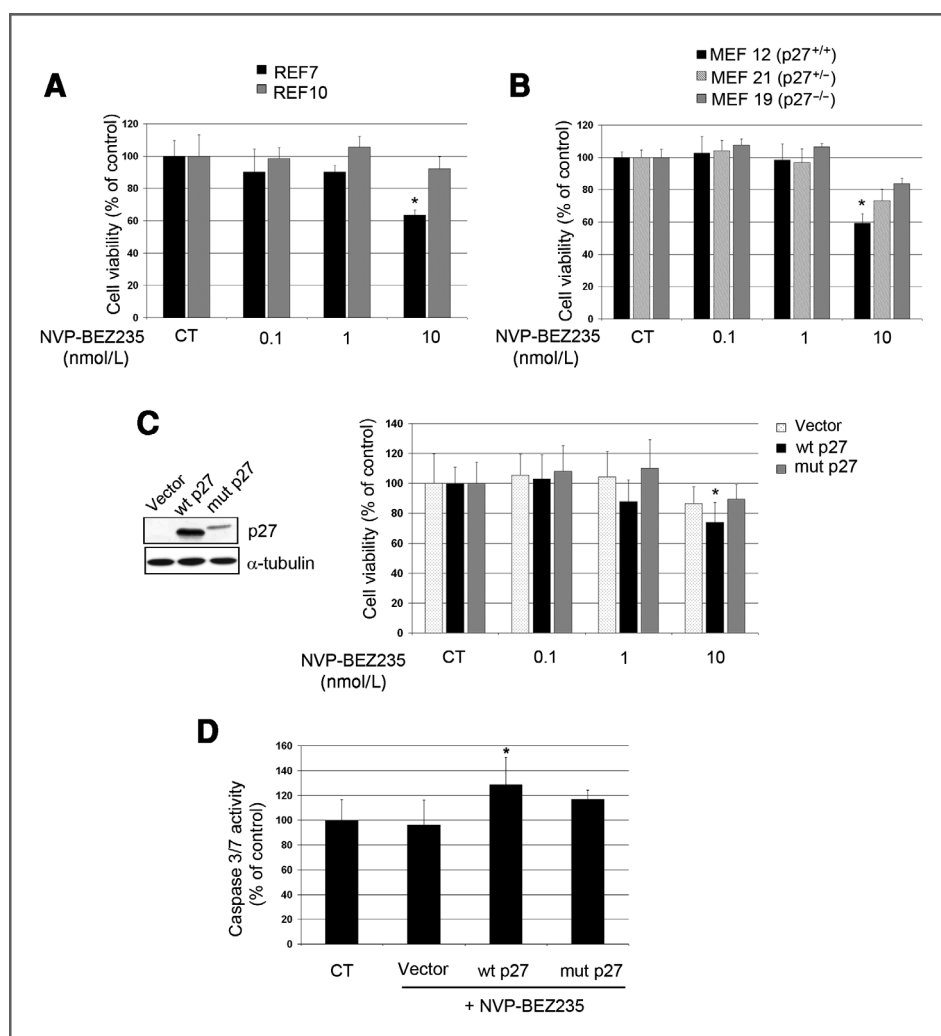


Figure 4. Higher p27 expression levels enhance sensitivity to NVP-BEZ235. **A**, embryonic fibroblasts from wt (REF7) or mutant (REF10) rats were incubated with different concentrations (0.1–10 nmol/L) of NVP-BEZ235 for 24 hours. Cell viability was assayed by measuring ATP levels. Data from primary cultures were analyzed independently with 6 replicates each and were expressed as the mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.001$ versus CT. **B**, MEFs from p27^{+/+}, p27^{+/-}, and p27^{-/-} mice were treated with different concentrations (0.1–10 nmol/L) of NVP-BEZ235 for 24 hours, or left untreated (CT). Cell viability was assessed as in **A**. **C**, p27-negative GH3 cells were transfected with GFP-vector, GFP-wt p27, and GFP-mut p27 (p27fs177) constructs. Twenty-four hours after transfection, cells were harvested, proteins extracted, and immunoblotting was carried out probing for p27 and α -tubulin as a loading control. Then, cells were incubated with different concentrations (0.1–10 nmol/L) of NVP-BEZ235 for additional 24 hours or left untreated (CT). Cell viability was assessed as in **A**. **D**, caspase 3/7 activity was measured to monitor apoptosis in GH3 cells transfected as in **C** and 24 hours later incubated with NVP-BEZ235 (10 nmol/L) for additional 24 hours or left untreated (CT).

proteasome inhibitor drugs, such as MG132, epoxomicin, and bortezomib, are able to recover the expression of the unstable mutant p27fs177 protein in REF10 primary fibroblasts (28). We then tested whether bortezomib would rescue p27fs177 expression also in primary adenoma cells from MENX rats; incubation with 10 nmol/L bortezomib increases the amount of the mutant protein (Supplementary Fig. S3).

To further clarify whether increased p27 levels might enhance the sensitivity of rat pituitary adenoma cultures to NVP-BEZ235, tumor cells from 3 mutant rats were incubated with this drug in the presence/absence of bortezomib. The combined treatment determined a more potent inhibition of pituitary adenoma cell viability as compared with the incubation with each individual drug (Fig. 5A), and this synergistic action was not dependent on the mode of bortezomib administration (given alongside NVP-BEZ235 or 8 hours earlier; data not shown). Levels of phosphorylated Akt-S473 and S6-S240/244 were reduced more after the combined treatment with 10 nmol/L NVP-BEZ235 and 10

nmol/L bortezomib than after the individual treatments (Fig. 5B). Concomitantly, we observed an increase in p27 levels upon incubation with bortezomib (Fig. 5B). Similar results were also obtained following cotreatment of REF10 fibroblasts (mutant p27) with bortezomib and NVP-BEZ235 (Fig. 5C). Taken together, these data further point to a role of the amount of p27 in mediating the antiproliferative action of NVP-BEZ235.

Discussion

This study shows that dual PI3K/mTOR inhibition leads to a more pronounced reduction in viable rat pituitary adenoma cells than single mTOR inhibition (RAD001). Consistently, at the molecular level, NVP-BEZ235 prevented the negative feedback activation of Akt that was instead observed after treatment with RAD001. Exposure of rat pituitary adenoma cells to NVP-BEZ235 promotes the same molecular events already observed in many human cancer cell lines of

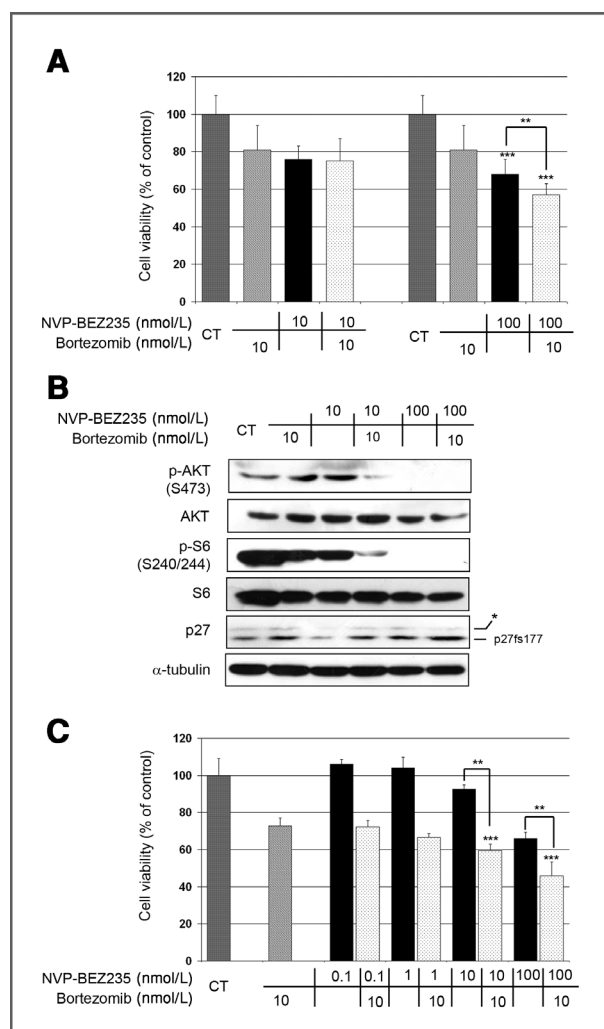


Figure 5. Effect of combined bortezomib and NVP-BEZ235 treatment on rat pituitary adenomas and REF10 cells. **A**, primary pituitary cells were incubated from *MENX*-mutant rats with NVP-BEZ235 (10 or 100 nmol/L) in the presence of bortezomib (10 nmol/L). Untreated control (CT) cells were incubated with DMSO vehicle. Cell viability was measured 48 hours later. Data from primary cultures were analyzed independently with 6 replicates each and were expressed as the mean $\pm$ SEM percent cell viability inhibition versus untreated control (CT) cells. **B**, using samples parallel to **A**, p-Akt (S473), total Akt, p-S6 (S240/244), total S6, and p27 expression levels were monitored by Western blotting. α -Tubulin was used as a loading control. **C**, REF10-mutant rat fibroblasts were incubated with NVP-BEZ235 (0.1–100 nmol/L) in the presence/absence of 10 nmol/L bortezomib as in **A**. Data from primary cultures were analyzed independently with 6 replicates each and were expressed as the mean $\pm$ SEM. **, $P < 0.01$ comparing single versus combined treatment; ***, $P < 0.001$ versus untreated control (CT). *, unspecific band.

various origins (i.e., reduced Akt and S6 phosphorylation), indicating that the germline defect in p27 does not impair upstream events in the signaling cascade.

Previous studies with various neuroendocrine tumor cell lines other than pituitary showed that NVP-BEZ235 triggers G₀–G₁ cell-cycle arrest and apoptosis (34). In the present study, we observed an increase in apoptosis in adenoma cell cultures following incubation with NVP-

BEZ235, but the effect was not as dramatic as observed in GH3 transfected with wt p27 because of the low level of p27fs177 in adenoma cells.

We have observed that the level of p27 positively correlates with the sensitivity to NVP-BEZ235. By using genetically defined MEF and REF isogenic cell lines with different p27 status, and GH3 cells transfected wt p27, p27fs177 or mock vector, we documented a positive correlation between the level of p27 and the response to NVP-BEZ235. The enhanced sensitivity to NVP-BEZ235 associates with a more pronounced inhibition of downstream signaling pathways in cells expressing normal levels of p27 and increased induction of apoptosis, as mentioned above. In the context of our pituitary adenoma model, the use of bortezomib, to stabilize mutant p27fs177, together with NVP-BEZ235 leads to a synergistic antiproliferative effect of the drugs. A similar synergistic effect was also observed by treating REF10 mutant primary rat fibroblasts with both compounds. This drug combination concurrently caused a significant inhibition of Akt-S473 and S6-S240/244 phosphorylation. The synergistic effect of bortezomib and PI3K/mTOR pathway inhibition could be because of events other than the stabilization of p27, because a wide array of molecular events is elicited by this proteasome inhibitor agent. However, in line with our hypothesis that p27 plays a crucial role in mediating the antitumor effect of bortezomib, it was recently shown that this drug increases the efficiency of trastuzumab against Her2-positive breast cancer cells and that the synergistic effect of both agents correlates with the ability of bortezomib to induce nuclear accumulation of p27 (40). Our results suggest that combination treatment with bortezomib may be used to enhance the antiproliferative effect of NVP-BEZ235 in tumor cells having low levels of p27 because of enhanced ubiquitin-mediated degradation, as it occurs in breast and colorectal cancers, among others (21).

MENX-associated pituitary adenomas belong to the group of nonfunctioning adenomas. In humans, nonfunctioning adenomas represent about 30% of pituitary adenomas. Although they are usually benign (29), nonfunctioning adenomas can grow to a considerable size causing signs and symptoms of mass effects and extend or invade the parasellar structures causing severe morbidity to patients (41). Transsphenoidal surgery is the treatment of choice but is rarely curative when tumors are invasive, and if residual tumor is present, they require postoperative radiotherapy (42). Medical therapy of nonfunctioning adenomas is still a matter of great debate, and both somatostatin analogs and dopamine agonists have shown limited effect (43, 44). Partial response has been observed by using the more recently developed multireceptor ligand somatostatin analogue (SOM230) and dopastatin (45, 46), but the use of these drugs is still far from routine clinical practice. The identification of novel therapeutic approaches is, therefore, necessary to treat nonfunctioning adenomas. The results obtained in our model of nonfunctioning adenoma using dual PI3K/mTOR inhibition

confirm and expand the results published by Zatelli and colleagues and Cerovac and colleagues. They showed that dual inhibition is more effective than single mTOR inhibition by using rapamycin and RAD001 (31, 33). Human nonfunctioning adenomas express p27 but at reduced levels compared with normal pituitary cells (47, 48). No mutations have been documented. Low level of p27 depend on posttranslational changes (48), suggesting that nonfunctioning adenomas could successfully respond to combined therapy with NVP-BEZ235 and bortezomib.

In conclusion, we have documented a potent anti-proliferative effect of NVP-BEZ235 against pituitary adenoma cells and showed that the levels of expression of p27 influence the sensitivity of adenoma cells to NVP-BEZ235. This observation provides additional insights into the mechanism of action of this antitumor compound currently in phase I/II clinical trials for solid tumors. The article by Chen and colleagues in which they report that higher p27 levels associate with improved response of breast cancer cell lines to temsirolimus and everolimus (RAD001; ref. 26) is in line with our findings on the role of p27 as a potential therapy, response biomarker.

Compounds that show promising antitumor activity *in vitro* or in animal models often show modest response in clinical practice. The selection of patients that are more likely to respond to treatment is key to improve

the outcome for novel drugs and such selection is based on the identification of biomarkers that allow the stratification of patients and the individualization of treatment. Our studies suggest that the amount of p27 might be a predictive biomarker of the sensitivity of tumor cells to NVP-BEZ235. Clinical validation of p27 as a biomarker of dual PI3K/mTOR inhibition therapeutic efficacy is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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