Vertical Targeting of the Phosphatidylinositol-3 Kinase Pathway as a Strategy for Treating Melanoma

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Abstract

Purpose: Melanoma is relatively resistant to chemotherapy; improved targeting of molecules critical for cell proliferation and survival are needed. Phosphatidylinositol-3 kinase (PI3K) is an important target in melanoma; however, activity of PI3K inhibitors (PI3KI) is limited. Our purpose was to assess mTOR as a cotarget for PI3K.

Methods: Using a method of quantitative immunofluorescence to measure mTOR expression in a large melanoma cohort, we studied associations with PI3K subunits, p85 and p110α. We assessed addition of the mTOR inhibitor rapamycin to 2 PI3KIs, NVP-BKM120 and LY294002. We studied in vitro activity of a novel dual PI3K/mTOR inhibitor NVP-BEZ235 and activity of the combination of NVP-BEZ235 and the MAPK/ERK kinase (MEK) inhibitor AZD6244.

Results: Strong coexpression of mTOR and p110α was observed (r = 0.658; P < 0.0001). Less coexpression was seen with p85 (r = 0.239; P < 0.0001). Strong synergism was shown between rapamycin and both PI3KIs. Activity of both PI3KIs was similarly enhanced with all rapamycin concentrations used. The dual PI3K/mTOR inhibitor effectively inhibited viability in 23 melanoma cell lines (IC50 values in the nanomolar range), regardless of B-Raf mutation status, with resultant reduction in clonogenicity and downregulation of pAkt and pP70S6K. Synergism was seen when combining NVP-BEZ235 and AZD6244, with resultant increases in poly(ADP-ribose) polymerase and caspase-2 cleavage.

Conclusions: mTOR and p110α are coexpressed in melanoma. Rapamycin concentrations as low as 1 nmol/L enhance activity of PI3KIs. The dual PI3K/mTOR inhibitor NVP-BEZ235 is highly active in melanoma cells in vitro, suggesting that concurrent PI3K and mTOR targeting in melanoma warrants further investigation, both alone and in combination with MEK inhibitors. Clin Cancer Res; 16(24); 6029–39. ©2010 AACR.

Introduction

Melanoma is the malignancy with the highest rise in incidence in the past decades (1). More than 68,700 new diagnoses were estimated in 2009 in the United States, and 8,650 patients were estimated to have died (2). Once melanoma has metastasized, systemic therapeutic options are limited (3). A small subset (<10%) of patients has durable responses to immune/cytokine-based therapies, such as high-dose interleukin-2 and ipilimumab (4, 5). Response rates to chemotherapy are somewhat higher, but responses are typically more transient (3). In the past years, improved understanding of cellular processes essential for cell growth, metastases, proliferation, and survival have led to the development of targeted therapies for this disease (6, 7), and additional studies are needed to expand these approaches.

Phosphatidylinositol-3 kinases (PI3K) are a family of intracellular signaling intermediary proteins that are essential for the inhibition of apoptosis. These kinases are active in human cancers and are critical for malignant progression (8–10). Class IA PI3Ks, which consist of a p85 regulatory subunit and a p110 catalytic subunit, are the most widely implicated in cancer and are primarily activated by receptor tyrosine kinases (11). PI3K activity is inhibited by a number of molecules including PTEN, which can be mutated and inactivated in malignant cells (12). PI3K activation results in phosphorylation of Akt and subsequent activation of a number of proteins including GSK3, GSK3β, FOXO transcription factors, MDM2, and BAD, which, in turn, results in cell survival and promote cell-cycle entry (9). In addition, Akt phosphorylation results in the activation of the mTOR (mammalian target of rapamycin)/raptor

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Translational Relevance

We previously showed that phosphatidylinositol-3 kinase (PI3K) is a valuable therapeutic target in melanoma; however, mechanisms of resistance to PI3K inhibitors (PI3Ki) exist. Given the availability of drugs that target both PI3K and mTOR, we showed strong coexpression of the p110α PI3K subunit and mTOR in a large cohort of melanomas. In vitro, addition of rapamycin at concentrations as low as 1 nmol/L sensitized melanoma cells to 2 PI3Kis, LY294002, and NVP-BKM120, a novel clinical grade PI3Ki. The dual PI3K/mTOR inhibitor NVP-BEZ235 currently in clinical trials was active in vitro in a panel of 23 B-Raf mutants and wild-type melanoma cell lines. Synergism was seen between NVP-BEZ235 and the MAP/ERK kinase inhibitor AZD6244, with resultant decrease in clonogenicity and increase in the induction of apoptosis. Taken together, these studies strongly support further in vivo assessment of NVP-BEZ235 alone and in combination with mitogen-activated protein kinase pathway inhibitors in melanoma. Low, intermittent dosing might be better tolerated while preserving activity.

complex, which, in turn, activates downstream mediators including p70S6K, resulting in the regulation of protein synthesis and cell growth (12). Activation of this pathway in malignant cells can occur due to multiple mechanisms, including activating mutations, decreased expression of pathway suppressors such as PTEN, amplification of PI3K, amplification of Akt, and activation of receptors or oncogenes upstream of PI3K. Given the critical role of the PI3K/Akt/mTOR pathway in human cancer, targeting this pathway is the focus of intense research, and drugs that target members of this pathway are in preclinical and clinical development.

There are a number of lines of evidence that support the importance of the PI3K pathway in melanoma in clinical and preclinical models, as detailed elsewhere (11, 13). Overexpression of Akt can convert radial growth melanoma to vertical growth melanoma (14). Drugs that target PI3K have shown activity in melanoma cells in preclinical models. For example, inhibitors of the p110α PI3K subunit result in growth inhibition in melanoma cells (15). A highly specific PI3K inhibitor (PI3KI), ZSTK474, was shown to be very active in a B16 melanoma mouse model as a single agent, with minimal associated toxicity (16). In our previous work, we showed that PI3K expression was upregulated in melanomas compared with nevi and that expression was significantly higher in metastatic than in primary specimens (11). Taken together, these findings strongly support further clinical development of PI3Kis for melanoma.

One of the possible limitations of specific PI3K inhibition as a single modality for the treatment of melanoma cells is development of escape mechanisms via activation of parallel pathways, particularly the Ras–Raf–MAPK (mitogen-activated protein kinase) pathway. This pathway is constitutively active in approximately 70% of melanomas due to activating mutations in B-Raf or N-Ras (17, 18). In our prior work, we showed that the degree of sensitivity to the PI3KI LY294002 is unrelated to B-Raf mutational status (11); however, the MAPK pathway can also be activated by other mechanisms in melanoma, and members of this pathway such as extracellular signal-regulated kinase (ERK) and ribosomal s6 kinase (RSK) inhibit TSC2, resulting in mTOR activation and PI3K pathway activation that bypasses PI3K and Akt (19). Another potential mechanism of resistance to specific PI3K inhibition is downregulation of S6, a negative regulator of PI3K, through inhibition of insulin receptor substrate 1, causing a negative feedback loop (20). Downstream mediators, including mTOR, can activate Akt via PDK2 (21).

mTOR kinases are key downstream components of the PI3K pathway and include mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2). mTORC1 activates PI3K/Akt/mTOR pathway in human cancer, targeting this pathway is the focus of intense research, and drugs that target members of this pathway are in preclinical and clinical development.

A relatively small study of 107 melanomas reported S6 phosphorylation as a surrogate for mTOR activity and showed that S6 was phosphorylated in 73% of melanomas (23), but no published studies assess expression of mTOR on large cohorts of melanomas and nevi.

A number of inhibitors of mTOR are in clinical development, some of which have been approved by the Food and Drug Administration for other malignancies (24). We therefore hypothesized that although single-agent mTOR inhibitors have limited efficacy in melanoma, cotargeting mTOR might be a valuable strategy for overcoming the escape mechanisms that can limit the activity of PI3Kis in melanoma. This approach has been explored by Marone et al. (25). Our purpose was to expand the studies of Marone by utilizing early-passage, patient-derived melanoma cell strains, to study clinical quality PI3Kis in addition to LY294002 and assess coexpression of PI3K subunits and mTOR in melanoma tumors. To obtain objective, quantitative measures of mTOR expression, we employed a new method of automated, quantitative analysis (AQUA) of in situ protein expression, which has been validated and used in a number of previous melanoma studies (11, 26). We found that mTOR and the p110α subunit of PI3K were strongly coexpressed in human melanoma specimens and...
that cotargeting mTOR and p110α was highly synergistic. A novel dual PI3K/mTOR inhibitor was also studied alone and in combination with a MAP/ERK kinase (MEK) inhibitor.

**Materials and Methods**

**Tissue microarray construction**

Tissue microarrays (TMA) were constructed as previously described (11). Cohorts of 230 primary melanomas, each measuring 0.6 mm in diameter, were spaced 0.8 mm apart on glass slides. For comparison of expression, specimens from a series of 293 metastatic patients were included in the array. Specimens and clinical information were collected with approval of the Yale Institutional Review Board. Specimens were resected from 1959 to 2000. The cohort has been described and validated in numerous publications (11). Pellets of 15 melanoma cell lines were embedded as described (27) for normalization across slides. The benign nevus array contained 540 nevi as well as 40 melanomas and cell lines that were also present on the tumor array, used both for controls and for normalization.

**Immunohistochemistry**

Staining was carried out for automated analysis of melanoma specimens as previously described (11). Slides were incubated at 4°C overnight in a humidity tray with a primary antibody cocktail containing rabbit anti-human mTOR (Cell Signaling Technology), at a dilution of 1:100 with goat anti-mouse IgG conjugated to Alexa 546 (Molecular Probes) to identify the S100 mask. Goat anti-rabbit horseradish peroxidase (HRP)-decorated polymer (EnVision; Dako Corporation) was used as a secondary reagent. The target was visualized with Cy5-tyramide (Perkin Elmer). Coverslips were mounted using ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen).

**Automated image acquisition and analysis**

Images were acquired using our previously described automated method (11). S100-conjugated Alexa 546 defines the tumor compartment from stroma. Coalescence of S100 at the cell surface was used to localize cell membranes, and DAPI was used to identify nuclei. mTOR was visualized with Cy5. The mTOR signal pixels within the cytoplasm was normalized to the area of tumor mask and scored on a scale of 0–255 (the AQUA score).

**Statistical analysis**

JMP Version 5 and Statview (SAS Institute Inc.) were used to do data analysis. Associations with clinical and pathologic parameters were assessed by ANOVA. Associations between mTOR and PI3K subunits were assessed using Spearman’s rank correlation.

**Human cell lines**

Nineteen low-passage, patient-derived melanoma cell lines were obtained from the Cell Culture Facility of the Yale Skin Disease Research Core Center. Metastatic cell lines: YUMAC (locally recurrent metastasis), YUSAC2, YULAC, YUROB, YUJKSl, YUVON, YUFIR, YUSIV, YUSTE, YUCAS, YUROL (distant soft-tissue metastases), YUFIC, YUKIM, YUHOIN, YUSIK (lymph node metastases), YUGEN8 (brain metastasis), YUSOC (in-transit cutaneous metastasis), YUHEF (lung metastasis), and YUPLA (in-transit cutaneous metastasis) were maintained in 15-cm dishes and OptiMEM media (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 1% antibiotic-antimycotic (penicillin, streptomycin, amphotericin B; Invitrogen). The human primary melanoma cells WW165 were maintained in OptiMEM supplemented with 10% heat-inactivated FBS, 0.1 mmol/L 3-isobutyl-1-methyl-xanthine (Sigma Aldrich), and 1% antibiotic-antimycotic. Established cell lines mel 501, mel 928, and mel 624 were obtained from Dr. Steven Rosenberg, Surgery Branch, National Cancer Institute, and were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2. V600K or V600E mutations in B-Raf were found in YUMAC, YUSAC, YULAC, YUJKSl, YUSIK, YUFIR, YUSTE, WW165, mel 501, mel 928, and mel 624. All remaining cell lines were wild type for B-Raf. One (YUJIFC) was found to harbor an N-Ras mutation.

**Synergism studies**

At a density of 104, cells were plated in triplicate in 96-well plates with growth medium and allowed to adhere overnight. Two PI3Ks, LY294002 (LC Laboratories) and NVP-BKM120 (Novartis Pharmaceuticals), were used alone and in combination with the mTORC1 inhibitor, rapamycin (LC Laboratories) at concentrations of 3–50, 0.313–2.5, and 0.001–1 μmol/L, respectively, for 48 hours. Combinations of NVP-BEZ235 and the dual MEK inhibitor AZD6244 (Selleck Chemicals), were studied at concentrations of 1–50 nmol/L and 0.05–5 μmol/L, respectively. The relative number of viable cells was assessed by the luminometric Cell-Titer Glo assay (Promega), and luminescent quantification was measured using a Viktor plate reader (Perkin Elmer). Using CalcuSyn software (Biosoft), results were analyzed for synergistic, additive, or antagonistic effects. Synergism is indicated by combination index (CI) of <0.9, additive by CI values between 0.9 and 1.1, and antagonism by CI of >1.1 (28).

**Cell viability assays**

Cells were exposed to NVP-BEZ235 (Novartis Pharmaceuticals) for 48 hours at concentrations ranging from 1 to 500 nmol/L. Dimethyl sulfoxide (DMSO) was used in the control wells. Relative number of viable cells was assessed by the luminometric Cell-Titer Glo assay (Promega). Luminescent quantification of present ATP was determined using a Viktor plate reader (Perkin Elmer). To determine the IC50, we used XLfit statistical software (IDBS).
**Immunoblot assays**

After treatment with LY294002, rapamycin, NVP-BEZ235, and AZD6244, cells were lysed using standard methods (11). DMSO was used alone for the control cells. The following primary antibody anti-human antibodies were used: poly(ADP-ribose) polymerase (PARP), phosphorylated AKT Ser473, and phosphorylated P70S6K Thr389 at 1:1,000 (Cell Signaling Technologies). Mouse anti-human anti-caspase-2 (BD Pharmigen) was used at a concentration of 1:1,000. A monoclonal mouse anti–β-actin antibody (Sigma Aldrich) was used at 1:10,000 for normalization of protein gel loading.

**Clonogenic assays**

Cells were seeded at a density of 500 cells per well in 6-well plates and allowed to adhere overnight. Cells were incubated with NVP-BEZ235 at concentrations of 1, 10, and 40 nmol/L for 7 days. Combination studies were done using NVP-BEZ235 at 5, 20, and 50 nmol/L and AZD6244 at 50, 500, and 5,000 nmol/L. Medium and drugs were then replaced on the eighth day. On day 11, cell colonies were fixed with 25% glutaraldehyde (Sigma Aldrich) and 1% methanol, stained with 0.5% crystal violet (Sigma Aldrich), and colonies composed of more than 50 cells were counted. Experiments were done in triplicate and results were averaged. Cytotoxicity was determined relative to the control (untreated) colonies.

**Results**

**mTOR expression in human melanoma tumors and coexpression with PI3K**

To assess patterns of mTOR expression in melanoma specimens, we stained 230 primary and 293 metastatic melanomas with an anti-mTOR antibody. To account for intratumor heterogeneity in mTOR expression, 2 separate melanoma TMA's, each containing a core from a different area of tumor for each patient, were stained. Figure 1A and B shows examples of strong and weak immunoreactivity of mTOR, respectively, which was expressed only in the cytoplasmic region and not in the nuclear compartment. Expression levels in the 2 arrays were tightly correlated ($r = 0.658, P < 0.0001$), and scores were averaged for each case to generate a composite score used for the analyses. AQUA scores ranged between 11.12 and 69.36. mTOR expression was not associated with survival among either the primary or metastatic patients (data not shown). We assessed the association between mTOR expression and previously published expression of the p85 and p110α subunits of PI3K (11). Using Spearman’s nonparametric rank correlation coefficient, a strong association was found between mTOR expression and p110α expression ($r = 0.658, P < 0.0001$) whereas a weaker association was found between levels of mTOR and p85 ($r = 0.239, P < 0.0001$).

**Synergism between PI3K and mTOR inhibition**

Using concentrations of 5, 25, and 50 μmol/L of the PI3K LY294002, we studied synergism with a range of concentrations of rapamycin (1, 100, and 1,000 nmol/L) in 5 melanoma cell lines (3 B-Raf mutants and 2 wild types). Synergism was seen in all 5 cell lines at a concentration of 5 μmol/L LY294002 with all 3 concentrations of rapamycin (Table 1). No synergism was seen at the higher concentrations of LY294002. Interestingly, the decrease in viability seen with the addition of all concentrations of rapamycin was similar; viability seen with adding 1 nmol/L rapamycin to LY294002 was similar to that of 1 μmol/L. In 1 B-Raf mutant and 1 B-Raf wild-type cell line, we assessed the effects of LY294002 and rapamycin, alone and in combination, on the downstream PI3K targets, pAkt, and pP70S6K. Although treatment with rapamycin resulted in an increase in pAkt compared with untreated cells, treatment with LY294002 both alone and in combination with rapamycin resulted in downregulation of pAkt and pP70S6K, as shown in Figure 2A. We note that the degree of decrease in pAkt and pP70S6K was the same or better with the lower concentration of rapamycin compared with the higher concentrations (100 and 1,000 nmol/L).

Given the poor pharmacologic qualities of LY294002, we then studied synergism between rapamycin and a clinical quality PI3KI, NVP-BKM120, being developed by Novartis. The IC_{50} values for the panel of 5 cell lines for NVP-BKM120 alone ranged from 1.06 to 2.28 μmol/L. In all 5 cell lines, synergism was seen at lower concentrations of NVP-BKM120 (313 and 625 nmol/L), as shown in Table 1, and at the highest concentration (1,250 nmol/L) YULAC and YUSIK remained synergistic. YUKSI and YUVON were additive with 1 mol/L of rapamycin but synergistic with higher concentrations (100 and 1,000 nmol/L). YUCAS remained synergistic with NVP-BKM120 at 1,000 nmol/L but antagonistic with rapamycin at 1 and 100 nmol/L. As shown in Figure 2B, the combination of LY294002 and rapamycin (1, 100, and 1,000 nmol/L) and that of NVP-BKM120 and rapamycin (at all concentrations) resulted in comparable decreases in cell viability, using YULAC as an example.

**Activity of a dual PI3K/mTOR inhibitor in melanoma cell lines**

Given the synergism seen between PI3KIs and rapamycin in melanoma cell lines, we studied the activity of a dual PI3K/mTOR inhibitor, NVP-BEZ235, that has been administered to solid tumor patients in phase I clinical trials. To verify broad activity of the dual PI3K/mTOR inhibitor in melanoma cells, we expanded our panel of cell lines to include a total of 23 cell lines; 1 harboring an N-Ras mutation, 12 harboring B-Raf mutations, and 10 wild type for both. In all 23 melanoma cell lines the IC_{50} values for NVP-BEZ235 were in the nanomolar range, as shown in Table 2.

One of the proposed mechanisms of resistance to PI3KIs is mutation in the Ras–Raf pathway, which are found in more than half of melanomas. By ANOVA, no association was found between the IC_{50} values of NVP-BEZ235 and the presence or absence of B-Raf mutations (Fig. 3A).

The targets of NVP-BEZ235, pAkt and pP70S6K, were both decreased with exposure to the drug in a time- and
dose-dependent fashion, as shown in Figure 3B for YUVON and YUSIK cell lines. Relative to untreated cells, pAkt and, to a greater degree, pP70S6K were downregulated at 1 and 4 hours, and levels of pAkt started to increase after 4 hours with 100 nmol/L of NVP-BEZ235.

Clonogenicity was studied in YUVON and YUSIK cells with exposure to the dual PI3K/mTOR inhibitor. As shown in Figure 3C, NVP-BEZ235 effectively inhibits clonogenicity at low nanomolar concentrations.

Synergism between the dual PI3K/mTOR inhibitor NVP-BEZ235 and the MEK inhibitor AZD6244 in B-Raf mutant and wild-type cell lines

Given that mTOR blockade is necessary to enhance the inhibition of the PI3K pathway, we finally studied the efficacy of dual PI3K/mTOR inhibition and MAPK pathway inhibition in the same subset of 5 melanoma cell lines used for studying synergism between rapamycin and PI3KIs. We added AZD6244 to NVP-BEZ235 and synergism was seen in 4 of the 5 cell lines studied, as shown in Supplementary Table 1. The only cell line in the panel for which we did not see synergism was YULAC, which was highly resistant to AZD6244. An example of cell kill with the combination of these 2 drugs in YUVON and YUSIK cells is shown in Supplementary Figure 1. Clonogenic assays show similar enhanced effects with the addition of AZD6244 to NVP-BEZ235 (Fig. 4A and B).

Induction of apoptosis

Previous studies with NVP-BEZ235 have shown that it causes PARP cleavage and induces apoptosis via activation of caspase-2, with no effect on caspase-3, -7, and -9 (29).

Fig. 1. A, AQUA of in situ protein levels for mTOR. It uses S100 to create a tumor mask (2 top left quadrants at x10). S100 staining was both nuclear and cytoplasmic, and the mask is made by filling in holes (bottom left quadrants on left). DAPI defines the nuclear compartment within the tumor mask, which is then subtracted from the tumor mask to create a cytoplasmic compartment within the tumor mask. mTOR expression is measured within the cytoplasmic compartments, within the tumor mask (bottom right quadrants), and each clinical case is assigned a score on the basis of pixel intensity per unit area within the tumor mask. Examples of a histospot with strong mTOR staining (A) and weak mTOR staining (B).
Table 1. Combination indexes assessing synergism/additivity/antagonism in rapamycin, LY294002, and NVP-BKM120

<table>
<thead>
<tr>
<th>PI3KI, (conc., nmol/L)</th>
<th>Rapamycin, mol/L</th>
<th>YUCAS (Cl)</th>
<th>YULAC (Cl)</th>
<th>YUKSI (Cl)</th>
<th>YUSIK (Cl)</th>
<th>YUVON (Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002 (5,000)</td>
<td>1</td>
<td>0.71, syn</td>
<td>0.56, syn</td>
<td>0.25, syn</td>
<td>0.19, syn</td>
<td>0.33, syn</td>
</tr>
<tr>
<td>LY294002 (5,000)</td>
<td>100</td>
<td>0.60, syn</td>
<td>0.39, syn</td>
<td>0.20, syn</td>
<td>0.11, syn</td>
<td>0.27, syn</td>
</tr>
<tr>
<td>LY294002 (5,000)</td>
<td>1,000</td>
<td>0.57, syn</td>
<td>0.28, syn</td>
<td>0.14, syn</td>
<td>0.10, syn</td>
<td>0.22, syn</td>
</tr>
<tr>
<td>BKM120 (313)</td>
<td>1</td>
<td>0.46, syn</td>
<td>0.20, syn</td>
<td>0.27, syn</td>
<td>0.29, syn</td>
<td>0.29, syn</td>
</tr>
<tr>
<td>BKM120 (313)</td>
<td>100</td>
<td>0.47, syn</td>
<td>0.21, syn</td>
<td>0.26, syn</td>
<td>0.28, syn</td>
<td>0.33, syn</td>
</tr>
<tr>
<td>BKM120 (313)</td>
<td>1,000</td>
<td>0.48, syn</td>
<td>0.23, syn</td>
<td>0.38, syn</td>
<td>0.63, syn</td>
<td>0.32, syn</td>
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<tr>
<td>BKM120 (625)</td>
<td>1</td>
<td>0.85, syn</td>
<td>0.40, syn</td>
<td>0.57, syn</td>
<td>0.58, syn</td>
<td>0.59, syn</td>
</tr>
<tr>
<td>BKM120 (625)</td>
<td>100</td>
<td>0.85, syn</td>
<td>0.41, syn</td>
<td>0.53, syn</td>
<td>0.54, syn</td>
<td>0.62, syn</td>
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<tr>
<td>BKM120 (625)</td>
<td>1,000</td>
<td>0.83, syn</td>
<td>0.39, syn</td>
<td>0.59, syn</td>
<td>0.54, syn</td>
<td>0.57, syn</td>
</tr>
<tr>
<td>BKM120 (1,250)</td>
<td>1</td>
<td>1.30, ant</td>
<td>0.35, syn</td>
<td>0.92, add</td>
<td>0.80, syn</td>
<td>0.94, add</td>
</tr>
<tr>
<td>BKM120 (1,250)</td>
<td>100</td>
<td>1.18, ant</td>
<td>0.58, syn</td>
<td>0.83, syn</td>
<td>0.82, syn</td>
<td>0.86, syn</td>
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<tr>
<td>BKM120 (1,250)</td>
<td>1,000</td>
<td>0.80, syn</td>
<td>0.63, syn</td>
<td>0.86, syn</td>
<td>0.68, syn</td>
<td>0.82, syn</td>
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</tbody>
</table>

NOTE: Synergism is indicated by a CI of <0.9, additivity by CI values between 0.9 and 1.1, and antagonism by CI of >1.1.
Abbreviations: CI, combination index; syn, synergism; add, additivity; ant, antagonism.

Fig. 2. A, Western blots showing levels of pAkt and pP70S6K in 2 cell lines with and without exposure to 50 μmol/L LY294002, 0.1 μmol/L rapamycin, and 1 μmol/L rapamycin for 1, 4, and 24 hours, and the combination of LY294002 and the 2 concentrations of rapamycin at 1, 4, and 24 hours. β-Actin is shown as a loading control. Levels of pAkt and pP70S6K decreased in all treatments that contained LY294002, whereas those of pAkt increased with rapamycin alone. B, cell viability assays with rapamycin [Rapa] and LY294002 (LY) or NVP-BKM120 (BKM) alone and in combination in YULAC cells, showing similar enhancement of LY294002 and NVP-BKM120 activity with the addition of 1,100, and 1,000 nmol/L rapamycin.
We therefore studied the effect of NVP-BEZ235 on PARP cleavage and caspase-2 activation alone and in combination with AZD6244 in both a B-Raf wild-type cell line and a B-Raf mutant cell line. As shown in Figure 4C, NVP-BEZ235 alone results in PARP cleavage and caspase-2 activation. AZD6244 alone has a smaller effect on these 2 apoptotic pathway members. The 2 drugs in combination cause PARP and caspase-2 cleavage at lower concentrations than either drug used alone.

**Discussion**

In this work, we studied dual targeting of PI3K and mTOR in melanoma. Expression of mTOR was assessed on a large cohort of melanomas and nevi, and higher levels were found in the malignant melanocytes, which correlated with high PI3K levels, particularly the p110α subunit. We showed that the addition of rapamycin to the PI3K inhibitor NVP-BKM120 resulted in synergism at all concentrations of rapamycin used, which resulted in pAkt and pP70S6K downregulation, with similar levels seen for the different concentrations of rapamycin used.

LY294002 is a relatively weak PI3KI, and its poor pharmacologic properties preclude its use in humans. We therefore verified the synergism between mTOR and PI3K inhibition, using, NVP-BEZ235, a clinical quality compound under development by Novartis Pharmaceuticals. Significant synergism was seen with viability curves overlapping for 1 nmol/L, 100 mol/L, and 1 nmol/L of rapamycin. Finally, we studied the *in vitro* activity of a dual PI3K/mTOR inhibitor (NVP-BEZ235) that is in clinical trials, also developed by Novartis Pharmaceuticals, on a panel of 23 human melanoma cell lines. The dual PI3K/mTOR inhibitor was equally effective in *in vitro* in B-Raf mutant and wild-type cell lines and showed induction of caspase-2 and PARP cleavage. Addition of the MEK inhibitor AZD6244 to NVP-BEZ235 resulted in synergism in 4 of 5 cell lines.

PI3K has been shown to be a good therapeutic target in melanoma by our group and others using a variety of PI3KIs (11, 13, 14, 16, 30–32). Although these agents are clearly active in preclinical models, they have yet to be tested in clinical trials specific for melanoma patients. As is the case with many targeted therapies, resistance to highly specific inhibitors can result from escape mechanisms such as downstream target activation, activation of parallel pathways, acquired mutations, etc. For specific PI3KIs, resistance can be due to a number of possible escape mechanisms. There is ample evidence that downstream PI3K pathway activation can occur by mechanisms other than the activation of PI3K itself. For example, Akt can be activated and phosphorylated by mTORC2 (33).

### Table 2. IC<sub>50</sub> values of 23 melanoma cell lines treated with NVP-BEZ235 and characterization of B-Raf and N-Ras mutation status

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; BEZ235, nmol/L</th>
<th>B-Raf</th>
<th>N-Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL501</td>
<td>20.0</td>
<td>V600E/WT (GAG/GTG)</td>
<td>WT</td>
</tr>
<tr>
<td>MEL624</td>
<td>28.7</td>
<td>V600E/WT (GAG/GTG)</td>
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Abbreviation: WT, wild type.
Resistance to PI3K inhibition can also be due to the significant cross talk between the PI3K and MAPK pathways, and MAPK pathway members can activate the PI3K pathway. For example, ERK and RSK inhibit TSC2, resulting in mTOR activation and downstream PI3K pathway activation that is independent of PI3K and Akt (19). Negative-feedback loops such as the activation of insulin receptor substrate 1 results in further PI3K pathway activation (20). Coinhibition of mTOR and PI3K could overcome all of these mechanisms of resistance to PI3KIs and enhance their activity.

Previous studies have reported synergism between PI3K and mTOR inhibitors in melanoma and other cancers (34, 35). In a study by Marone et al., synergism was shown when treating melanoma cells with the combination of 100 nmol/L of rapamycin and 1 μmol/L of the pure PI3KI ZSTK474 (25). Werzowa et al. showed synergism in 2 melanoma cell lines with the combination of 25 nmol/L rapamycin and 10 μmol/L LY294002 (36). Our studies confirm and expand these findings showing synergism between LY294002 (at 5 μmol/L but not at 50 μmol/L) and a range of concentrations of rapamycin that spans 4 logs. We found similar synergism between rapamycin and the novel, clinical quality PI3KI NVP-BKM120. No major differences in viability were seen with the different concentrations of rapamycin, indicating that minimal mTOR

Fig. 3. A, ANOVA of IC50 values of the dual PI3K/mTOR inhibitor NVP-BEZ235 in 23 melanoma cell lines showing no significant difference in sensitivity to this compound in B-Raf mutant and B-Raf wild-type cells. B, decreases in pAkt and pT70S6K in a dose- and time-dependent fashion in 2 melanoma cell lines treated with NVP-BEZ235. pT70S6K levels are undetectable at all concentrations and time points studied, whereas levels of pAkt start rising again after 4 hours of drug exposure in a dose-dependent fashion. C, clonogenic assays in 2 melanoma cell lines (YUVON and YUSIK) treated with NVP-BEZ235 at different concentrations. NVP-BEZ235 was effective in inhibiting colony formation at low nanomolar concentrations.
inhibition is sufficient to potentiate PI3K inhibition. This finding is particularly important when designing inhibitors for clinical use, as mTOR inhibitors, especially when used in combination with other molecular targeted therapies, can be associated with a fair degree of toxicity (37, 38).

Dual PI3K/mTOR inhibitors have been shown to be active in a number of malignancies (39–45). Marone et al. showed that NVP-BEZ235 is active in melanoma cell lines and mouse models, as were 2 other dual PI3K/mTOR inhibitors, NVP-BAG956 and NVP-BBD130 (25). In addition, they showed that NVP-BEZ235 was active in a B16 mouse model, causing a decline in tumor burden, increased tumoral necrosis, and decreased tumor vasculature. These findings are fully consistent with ours, in which we showed IC_{50} values in the low nanomolar range in 23 melanoma cell lines, which included 19 early-passage, patient-derived cell lines. Similar to the studies by Marone et al., we found that the NVP-BEZ235 decreases viability and clonogenicity. As shown by Brachmann et al. in breast cancer, we found PARP cleavage and caspase-2 induction but to a lesser degree. Treatment with a combination of these compounds has a greater effect on PARP and caspase-2 cleavage than either drug alone.

**Fig. 4.** A and B, clonogenic assays in 2 melanoma cell lines (YUVON and YUSIK) treated with NVP-BEZ235 (BEZ) and AZD6244 (AZD) alone and in combination. Combinations were more effective in inhibiting colony formation at lower concentrations than either drug alone. C, treatment of YUVON and YUSIK cells with ascending concentrations of NVP-BEZ235 resulted in dose-dependent PARP cleavage and caspase-2 induction. Treatment with AZD6244 also resulted in PARP cleavage and caspase-2 induction but to a lesser degree. Treatment with a combination of these compounds has a greater effect on PARP and caspase-2 cleavage than either drug alone.
mTOR inhibitors have been widely used in patients, and are available for clinical testing in combination with PI3K and Akt inhibitors. Our data suggest that small doses of mTOR inhibitors might be adequate to effectively downregulate pAkt when given in combination with PI3KIs; smaller doses are likely to be associated with less toxicity.

Mutations in c-Kit are relatively rare, and c-Kit-targeting therapies have resulted in durable responses (6, 46). B-Raf mutations are much more common (approximately 60%), and PLX-4032 (Plexxikon), a specific inhibitor of mutated B-Raf, has shown dramatic activity in metastatic melanomas that harbor B-Raf mutations (47). Responses to PLX-4032 are transient, however, and 1 potential mechanism of acquired resistance is the activation of the PI3K pathway. A number of studies have shown that coactivation of the PI3K pathway is necessary for malignant transformation and tumor growth in cells harboring mutations of the Ras–Raf pathway (48, 49). Our findings also suggest that concurrent targeting of the Ras–Raf and the PI3K pathways with drugs such as AZD6244 and NVP-BEZ235 might be more effective in some (but not all) patients than targeting either one of the pathways alone.

In this work, we used 2 novel PI3K pathway inhibitors, the dual PI3K/mTOR inhibitor NVP-BEZ235 and the PI3KI NVP-BKM120. Both are currently being tested in clinical trials for patients with solid tumors (CBEZ235A101, CBKM120A101). The maximum tolerated dose of both compounds has been established and publication of the toxicity data is pending. Our results suggest that further assessment of these drugs alone and in combination with MAPK pathway inhibitors in melanoma patients is warranted. We note that no association was found in our studies between sensitivity to NVP-BEZ235 and B-Raf mutation status, further supporting the critical role of the PI3K inhibition in this disease and suggesting that NVP-BEZ235 is effective in both B-Raf wild-type and mutant phenotypes.

In summary, we previously showed that PI3K is upregulated in melanoma. Here we showed strong coexpression of the p110α catalytic subunit and mTOR, suggesting that cotargeting these 2 molecules might be an effective approach for treating this disease. We further showed strong synergism between 2 PI3KIs and rapamycin, with no major differences seen between different rapamycin concentrations, indicating that minimal mTOR inhibition might be sufficient for potentiating the effects of PI3KIs and might result in less toxicity than larger doses of mTOR inhibitors. A dual PI3K/mTOR inhibitor, NVP-BEZ235, was highly active in vitro in a large panel of B-Raf mutant and wild-type melanoma cell lines, and synergism was seen when adding AZD6244 in most, but not all, cell lines. Further evaluation of NVP-BEZ235 in melanoma is warranted. Studies to identify biomarkers predictive of sensitivity to PI3K/mTOR inhibition are ongoing in our laboratory.

Disclosure of Potential Conflicts of Interest

Drs. D.L. Rimm and R.L. Camp are scientific co-founders, consultants, and stock option owners of HistoRx that has licensed the AQUA technology from Yale University.

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Saadia A. Aziz, Lucia B. Jilaveanu, Christopher Zito, et al.


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