Cancer Therapy: Preclinical

The Efficacy of the Novel Dual PI3-Kinase/mTOR Inhibitor NVP-BEZ235 Compared with Rapamycin in Renal Cell Carcinoma

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Abstract

Purpose: Inhibitors of TORC1 have been shown to be active in patients with metastatic renal cell carcinoma (RCC). As the phosphatidylinositol 3-kinase (PI3K) pathway activates numerous other kinases, transcription factors, and proteins associated with cell growth and survival besides mammalian target of rapamycin (mTOR), disruption of this pathway upstream of mTOR may be more effective than inhibition of TORC1 alone.

Experimental Design: To investigate this possibility, the dual PI3K/mTOR inhibitor NVP-BEZ235 was compared with rapamycin in RCC cell lines and xenografts generated from 786-O and A498 cells.

Results: Treatment of RCC cell lines with NVP-BEZ235 in vitro resulted in the nuclear translocation of p27, greater reduction in tumor cell proliferation, and more complete suppression of Akt, Mnk-1, eIF4E, and 4EBP-1 phosphorylation and cyclin D1 and hypoxia-inducible factor 2α (HIF2α) expression than that achieved with rapamycin. The reduction of HIF2α levels correlated with reduced HIF activity as determined by luciferase assay. NVP-BEZ235 induced growth arrest in both the 786-O and A498 xenografts that was associated with inhibition of Akt and S6 phosphorylation as well as the induction of apoptosis and reduction in markers of tumor cell proliferation. In contrast, rapamycin induced only minimal growth retardation.

Conclusion: Dual inhibition of PI3K/mTOR with NVP-BEZ235 induced growth arrest in RCC cell lines both in vitro and in vivo more effectively than inhibition of TORC1 alone. These results provide the rationale for the clinical assessment of agents such as NVP-BEZ235 in patients with advanced RCC.

The kinase mammalian target of rapamycin (mTOR) exists in two functionally distinct multiprotein complexes, TORC1 and TORC2. TORC1, a complex including mTOR and raptor (regulatory-associated protein of mTOR), regulates protein translation and many aspects of metabolism (1). TORC2, a complex including mTOR and rictor (rapamycin-insensitive companion of TOR), regulates the activity of the kinase Akt (1). Inhibitors of TORC1 have significant activity in patients with renal cell carcinoma (RCC), and two such agents, temsirolimus and everolimus, are now approved by the Food and Drug Administration (FDA) for the therapy of advanced RCC (2, 3). However, responses to TORC1 inhibitors are infrequent and typically short lived, and all patients treated with these drugs eventually develop progressive disease.

One of the proposed shortcomings of TORC1 inhibitors is the feedback activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Treatment with TORC1 inhibitors has been shown in some cases to result in the activation of PI3K through a feedback loop involving the insulin-like growth factor I (IGF-I) receptor (4). TORC1 inhibitors also can activate Akt directly through the derepression of TORC2, which results in TORC2-mediated phosphorylation of Akt on Ser473 (5). The primary mechanism of action of TORC1 inhibitors is thought to be the dephosphorylation and activation of eukaryotic translation initiation factor (eIF4E) binding proteins (4EBP), which function to sequester and block eIF4E from carrying out cap-dependent translation of certain "difficult to translate" mRNAs such as those of VEGF, cyclin D, c-Myc, and survivin (6, 7). The feedback activation of PI3K may directly undermine the efficacy of TORC1 inhibitors by promoting the phosphorylation of eIF4E by Mnk1, thereby enhancing its affinity for the mRNA cap structure and activating cap-dependent translation (8).
Translational Relevance

Although two inhibitors of TORC1, temsirolimus and everolimus, are approved by the Food and Drug Administration for the treatment of renal cell carcinoma (RCC), these agents induce only modest tumor regression and extend progression-free survival only a few months in most patients. Our data suggest that agents that inhibit either phosphatidylinositol 3-kinase (PI3K)/Akt and/or TORC2 simultaneously with TORC1 may have advantages over selective TORC1 inhibitors in the treatment of RCC. The results of our studies with NVP-BEZ235, a dual inhibitor of PI3K and mammalian target of rapamycin, in RCC cell lines support this hypothesis. As several such agents are now in early-stage clinical testing, our results provide the rationale to assess these agents in patients with RCC.

Not surprisingly, inhibition of PI3K and/or TORC2 simultaneously with TORC1 has emerged as a therapeutic strategy that may negate activation of this feedback loop and more effectively suppress the translation of these critical mRNAs.

The ability to avoid the feedback activation of PI3K is not the only theoretical advantage of PI3K inhibitors over TORC1 inhibitors. PI3K/Akt signaling activates an array of kinases, transcription factors, and other proteins besides mTOR that promote cell growth and survival (9, 10). These prosurvival effects include the phosphorylation of fas ligand, Bim, and other proapoptotic proteins. PI3K activation also results in the downstream activation of NF-κB and the inactivation of prosurvival proteins such as BAD and procaspase 9. Disruption of any of these prosurvival signals may have therapeutic benefits that complement the effects of TORC1 inhibition and enhance antitumor activity.

In addition to PI3K inhibition, attenuation of TORC2 is yet another potential strategy in the treatment of RCC, particularly the clear cell variant. The majority of these tumors possess biallelic alterations in the von Hippel Lindau (VHL) gene (11, 12), resulting in the accumulation of hypoxia-inducible factors (HIF) 1 and 2, and the subsequent activation of their target genes, including VEGF, PDGF, TGF-α, and CXCR4 (13–15). Although the two HIFs have overlapping effects on gene expression, HIF-2α is thought to be the more relevant HIF with respect to the development and progression of RCC (16–19). Recent studies have shown that the translation of HIF-2α is completely dependent upon the activity of TORC2 (19). These data suggest the potential value of TORC2 inhibition in the treatment of RCC.

A new generation of agents with activity against PI3K and mTOR is currently in clinical development. NVP-BEZ235 is a novel, orally bioavailable imidazoquinoline that potently and reversibly inhibits class I PI3K activity by binding to its ATP-binding domain (20). NVP-BEZ235 also binds directly to the mTOR ATP-binding domain and directly inhibits its catalytic activity, thereby inhibiting both TORC1 and TORC2. NVP-BEZ235 has been shown to inhibit PI3K and mTOR activity and tumor growth in numerous preclinical models, including prostate, breast, and pancreatic carcinoma, glioblastoma, and multiple myeloma, and is currently in phase I testing in patients with solid tumors (21–23).

Although RCC is the only malignancy for which TORC1 inhibitors have gained FDA approval, the benefit derived from the use of these drugs in RCC patients is modest. Agents that block PI3K or TORC2 in addition to TORC1 have numerous theoretical advantages over TORC1 inhibitors that may translate into superior antitumor activity. In this report, we compared the effects of the novel dual PI3K/mTOR inhibitor NVP-BEZ235 with those of the selective TORC1 inhibitor rapamycin on intracellular signaling in vitro and in vivo and on tumor growth in RCC xenografts. We show that NVP-BEZ235 downmodulates cyclin D, survivin, and HIF-2α to a far greater extent than does rapamycin. It induces the nuclear translocation of p27, blocks the phosphorylation of eIF4E and Mnk1, and inhibits tumor cell proliferation both in vitro and in vivo better than rapamycin does. Our data reinforce the view that the concurrent suppression of PI3K and/or TORC2 in addition to TORC1 is likely to be a more effective strategy in the treatment of RCC than the inhibition of TORC1 alone.

Materials and Methods

Cell lines and reagents

The human RCC cell lines (786-O, 769-P, A498, Caki-1, and Caki-2) were obtained from the American Type Culture Collection and were passaged for <6 months after they were received. The 786-O and 769-P lines were maintained in RPMI 1640, A498 in MEM, and Caki-1 and Caki-2 in McCoy’s 5A. All media contained 10% fetal bovine serum (FBS), 4 mmol/L glucose, and 50 μmol/L gentamicin. Cells were incubated at 37°C at 5% CO₂. NVP-BEZ235 (Novartis Pharmaceuticals) and rapamycin (Santa Cruz Biotechnology) were solubilized for in vitro assays in DMSO.

Western blots

Cells were treated as described in Results and then lysed in lysis solution (Cell Signaling) supplemented with sodium fluoride (10 μmol/L) and phenylmethylsulfonyl fluoride (100 μg/mL). Lysates were fractionated on polyacrylamide gels and transferred to nitrocellulose. The blots were probed with specific antibodies followed by a second antibody-horseradish peroxidase conjugate and then incubated with SuperSignal substrate (Pierce). Phospho- and total Akt, S6, eIF4E, Mnk1, 4EBP, GSK3β, and phospho-extracellular signal-regulated kinase (ERK), and cyclin D1 antibodies were purchased from...
Cell Signaling. The total ERK and c-Myc antibody was purchased from Santa Cruz. The survivin, vinculin, and HIF-2α antibodies were purchased from R&D Systems, Sigma, and Novus Biologicals, respectively.

Cell proliferation assays
MTS proliferation studies were carried out using the Promega CellTiter 96 AQueous Cell Proliferation Assay (Promega). RCC cell lines were grown in tissue culture–coated 96-well plates and treated as described in Results. Cells were then treated with the MTS/phenazine methosulfate solution for 1 hour at 37°C. Absorbance at 490 nmol/L for each condition was determined using an enzyme-linked immunosorbent assay plate reader. Data are reported as percent viable tumor cells in each condition as compared with cells treated with DMSO alone.

Cell death assay
Cells were treated as described in Results. In each assay, the adherent cells were detached by treatment with trypsin and combined with the nonadherent cells. Propidium iodide (5 ng/mL) was added to the cells, and after 20 minutes at room temperature the cells were analyzed by flow cytometry with a Coulter FC 500 cytometer. The percentage of cells staining positive was recorded.

HIF reporter assay
The HIF luciferase reporter construct, encoding firefly luciferase under control of a cytomegalovirus promoter and three tandem copies of the vascular endothelial growth factor (VEGF) hypoxia response element, was provided by Andrew Kung (Children's Hospital, Boston, MA). RCC cell lines were transiently cotransfected with the reporter and a construct encoding renilla luciferase using the TransIT-TKO system. After 24 hours, the transfected cells were treated with DMSO, rapamycin (100 nmol/L), or NVP-BEZ235 (250 nmol/L) for 24 hours. Renilla and firefly activities were then determined by luminometry using the Dual-Luciferase Reporter Assay System (Promega) and the ratio calculated. Results were expressed as the ratio of firefly to renilla luciferase activity.

Localization of p27
Nuclei were isolated from the cytosolic fraction in 786-O and A498 cells treated with DMSO, rapamycin (100 nmol/L), or NVP-BEZ235 (250 nmol/L) for 6 hours using the Nuclei EZ Prep Nuclei Isolation Kit (Sigma). Lysates from nuclear and cytosolic fractions were analyzed for p27 by Western blot. To determine localization of p27 by immunofluorescence, 786-O and A498 cells were grown on tissue culture–treated slides, treated as above, and fixed with 0.5% zinc chloride and 0.5% zinc acetate for 15 minutes. Cells were then treated with rabbit anti-p27 (1:100; Cell Signaling), followed by goat anti-rabbit coupled to Alexa Fluor 488 (1:100; Cell Signaling). Nuclei were detected with Hoechst 33342 (Sigma). Slides were then mounted and analyzed by fluorescence microscopy.

Xenograft model
Eight-week-old female, nude/beige mice were purchased from Charles River Laboratories. Approximately 5 × 106 786-O or A498 cells were injected into the flanks, and tumors were allowed to reach 10 mm in maximal diameter. Mice were then treated once daily by gavage with either vehicle, rapamycin (3.5 mg/kg), or NVP-BEZ235 (40 mg/kg). Bidimensional tumor measurements were taken every other day and mice were weighed once weekly. Tumor volume was estimated using the standard formula: (length × width^2)/2. Mice were sacrificed after 3 or 21 days of treatment and the tumors were excised. Tumors were divided and either flash frozen in liquid nitrogen or placed in 10% buffered formalin. Rapamycin was initially solubilized as a stock solution of 20 mg/mL in ethanol. Prior to gavage, rapamycin was brought up to volume (0.2 mL) in PBS with 0.5% Tween 80 and 2.5% N, N-dimethyacetamide. Prior to gavage, NVP-BEZ235 was initially solubilized in one part N-methylpyrrolidone and brought up to volume (0.2 mL) in nine parts PEG 300.

Immunohistochemistry
All specimens were kept in 10% buffered formalin for 48 hours, after which they were embedded in paraffin and 4-μm-thick slides were prepared and stained with H&E. Sections were dewaxed, soaked in alcohol, and after pressure cooker treatment in antigen unmasking solution at 125°C for 30 seconds, incubated in 3% hydrogen peroxide for 5 minutes. Sections were incubated with the appropriate antibody and epitopes detected using the DAKO EnVision+ horseradish peroxidase detection kit (Dako). Slides immunostained for CD34, Ki67, and caspase 3 were scanned using the ScanScope slide scanning system (Aperio Technologies) and analyzed using the Microvessel Analysis, Nuclear Image Analysis, and Pixel Analysis Algorithms (Aperio), respectively. The caspase 3, Ki67, and CD34 antibodies were purchased from Dako, Cell Signaling, and Abcam, respectively. Human tonsilar tissue was used as a positive control for caspase 3 staining.

Statistical analysis
Results for proliferation and reporter assays were reported as mean ± SD. Statistical comparisons for mean final tumor volumes in the xenograft studies were made using a one-way ANOVA. P values of pairwise comparisons were adjusted using Tukey's method. Comparison of luciferase activity between treatment conditions and immunohistochemical staining quantification between tumors from various treatment groups were made using two-sample t-tests. P < 0.05 was considered significant.

Results
Concentration-dependent effects of NVP-BEZ235 on intracellular signaling and proliferation in RCC cell lines
To assess the effects of NVP-BEZ235 on intracellular signaling in RCC, two human RCC cell lines, namely, 786-O
(VHL\(^{1-/-}\), PTEN-null) and 769-P (VHL\(^{1-/-}\), PTEN-WT), were exposed to increasing concentrations of NVP-BEZ235 \textit{in vitro} for 6 hours, lysed, and analyzed by Western blot. As shown in Fig. 1A, NVP-BEZ235 inhibited TORC1/2 activity at low nanomolar (<25 nmol/L) concentrations as evidenced by the dephosphorylation of S6, 4E-BP, and Akt (Ser\(^{473}\)). A higher concentration of NVP-BEZ235 was required to suppress the phosphorylation of Akt (Thr\(^{308}\)) and GSK3β (Ser\(^{9}\)), suggesting that the IC\(_{50}\) for NVP-BEZ235 for PI3K may be higher than that for either TORC1 or TORC2 in RCC cell lines. NVP-BEZ235 also induced the activation of the mitogen-activated protein (MAP) kinase as indicated by enhanced ERK phosphorylation. Both the hierarchical suppression of PI3K and TORC1/2 and the activation of MAP kinase by NVP-BEZ235 have been previously reported in other tumor types (21, 24).

To assess the activity of NVP-BEZ235 on tumor cell proliferation, the RCC cell lines were exposed to increasing concentrations of the drug \textit{in vitro} for 48 hours and then analyzed by MTS assay. As shown in Fig. 1B, treatment with NVP-BEZ235 resulted in a concentration-dependent reduction in viable tumor cells. To determine if this effect might be due to the induction of apoptosis, the treated cells were stained with propidium iodide and analyzed by flow cytometry. Drug exposure failed to increase the percentage of propidium iodide-staining (apoptotic) cells (data not shown), suggesting the reduction in viable tumor cells \textit{in vitro} was due to suppression of proliferation rather than the induction of apoptosis.

**Comparison of the effects of NVP-BEZ235 versus rapamycin in RCC cell lines**

To determine if NVP-BEZ235 might downmodulate the cap-dependent translation of difficult-to-translate mRNAs such as those for cyclin D1, c-Myc, and survivin more effectively than a TORC1 inhibitor, five different RCC cell...
lines, namely, 786-0, 769-P, A498 (VHL−/−, PTEN-WT), Caki-1 (VHL+/−, PTEN-WT), and Caki-2 (VHL+/−, PTEN-WT), were treated with either DMSO, rapamycin (100 nmol/L), or NVP-BEZ235 (250 nmol/L) for 24 hours and subsequently analyzed by Western blot. The concentration of rapamycin was chosen based on similar studies in the literature and, as shown in Supplementary Fig. S1, was at least 10× greater than that required to suppress the phosphorylation of S6 in 786-O cells (25). The NVP-BEZ235 concentration was chosen based on the complete inhibition of Akt (Thr308) phosphorylation as well as maximal suppression of tumor cell proliferation achieved at this dose. As shown in Fig. 2A, both drugs completely suppressed S6 phosphorylation, but only NVP-BEZ235 consistently blocked both Akt and 4E-BP1 phosphorylation and reduced cyclin D1 and survivin expression. The expression of c-Myc was unaffected by either drug. NVP-BEZ235 also reduced the phosphorylation of eIF4E (Ser209) and Mnk1 (Thr197/204), neither of which was appreciably affected by rapamycin.

To compare the effects of rapamycin and NVP-BEZ235 on proliferation, the same five RCC cell lines were treated with either DMSO, rapamycin (100 nmol/L), or NVP-BEZ235 (250 nmol/L) for 48 hours and analyzed for viability by MTS assay. As shown in Fig. 2B, treatment with NVP-BEZ235 resulted in significantly greater reduction in proliferation than rapamycin in all RCC cell lines.

### Effects of NVP-BEZ235 and rapamycin on HIF-2α expression and HIF transcriptional activity

To investigate the hypothesis that an agent with activity against both TORC1 and TORC2 would be more effective than a TORC1 inhibitor at suppressing HIF-2α expression, lysates from RCC cell lines treated with DMSO, rapamycin (100 nmol/L), or NVP-BEZ235 (250 nmol/L) for 24 hours were analyzed by Western blot for HIF-2α expression. As
shown in Fig. 3A, NVP-BEZ235 was significantly more effective than rapamycin in reducing HIF-2α expression in all five RCC cell lines. To assess the effect of NVP-BEZ235 on overall HIF activity in RCC cell lines (many of which express only HIF-2α) the five cell lines were transiently transfected with a HIF-luciferase reporter construct, treated with drug, and analyzed by luminometry for luciferase activity. As shown in Fig. 3B, NVP-BEZ235 was significantly more effective than rapamycin (P < 0.05) in inhibiting overall HIF activity in all of the RCC cell lines except Caki-2, in which the two drugs were equally effective.

**Effect of NVP-BEZ235 and rapamycin on p27 localization**

One mechanism by which PI3K blockade results in cell cycle arrest is induction of p27 nuclear translocation (26). To investigate whether NVP-BEZ235 and rapamycin may have differential effects on p27 localization, the intracellular distribution of p27 in RCC cells treated with DMSO, rapamycin, or NVP-BEZ235 was assayed by both Western blot and immunofluorescence. As shown in Fig. 4A, treatment of both 786-O and A498 cells with NVP-BEZ235 resulted in the translocation of p27 to the nucleus. This finding was corroborated in intact cells by immunofluorescence (Fig. 4B).

**Antitumor activity of NVP-BEZ235 compared with that of rapamycin in 786-O and A498 RCC xenografts**

To assess the in vivo efficacy of NVP-BEZ235, nude/beige mice bearing either 786-O or A498 xenografts were treated daily with vehicle, rapamycin, or NVP-BEZ235 as described in Materials and Methods for 21 consecutive days. As shown in Fig. 5A, in both xenograft models, NVP-BEZ235 treatment resulted in growth arrest whereas rapamycin had only a modest retarding effect on tumor growth. In both xenograft models, there was a significant difference in the average tumor volume among the three groups on day 21 (P < 0.01 for each pairwise comparison). The NVP-BEZ235–treated group had the lowest tumor volume in both 786-O and A498 xenograft models (418.8 ± 108.1 mm³ and 550.5 ± 96.3 mm³, respectively), which was significantly different from both the rapamycin-(1,394.9 ± 192.5 mm³; 1,263.9 ± 141.2 mm³) and vehicle-treated (1,858.2 ± 239.4 mm³; 1,840.8 ± 292.0 mm³) groups.

To evaluate the in vivo effects of NVP-BEZ235 and rapamycin on intracellular signaling, three mice per treatment group were sacrificed after three days of treatment and tumors were excised for analysis by Western blot and immunohistochemistry. S6 phosphorylation was suppressed in tumors from mice treated with either drug (Figs. 5B and 6). Akt phosphorylation was inhibited only in the tumors from mice treated with NVP-BEZ235. In both xenograft models, tumors from mice treated with NVP-BEZ235 also showed significantly lower expression of phospho-eIF4E, phospho-4E-BP1, cyclin D1, and HIF-2α than tumors from saline- or rapamycin-treated mice. These in vivo data recapitulate the in vitro results obtained with drug-treated RCC cell lines (Figs. 2 and 3). In both xenograft models, immunohistochemical analyses revealed reduced Ki67 staining and increased cleaved caspase 3 staining in the tumors from NVP-BEZ235–treated mice relative to the saline controls. Quantification of Ki67 staining showed a significant decrease in mean percent cells positive for nuclear staining in tumors from NVP-BEZ235–treated mice compared with saline controls in the A498 model (17.1 versus 26.3; n = 3 per group; P = 0.026) and a nearly significant decrease in the 786-O model (31.3 versus 44.7; n = 3 per group; P = 0.059). Quantification of cleaved caspase 3 staining showed modest but significantly higher staining in tumors from NVP-BEZ235–treated mice compared with...
saline controls in both xenograft models (29.0 versus 15.3 pixels/μmol/L²; \( P = 0.017 \) in 786-O, and 9.75 versus 3.57 pixels/μmol/L²; \( P = 0.024 \) in A498). Microvessel density determined by quantification of CD34 staining revealed no significant difference in NVP-BEZ235–treated mice compared with saline controls (2.41 \( \times 10^{-5} \) versus 2.53 \( \times 10^{-4} \) vessels/μmol/L²; \( P = 0.85 \)) in the 786-O xenografts and a slight decrease in A498 xenografts that did not meet criteria for significance (4.5 \( \times 10^{-4} \) versus 6.0 \( \times 10^{-4} \) vessels/μmol/L²; \( P = 0.17 \)). These results suggest that the failure of the tumors in the NVP-BEZ235–treated mouse to grow was due to a combination of antiproliferative and proapoptotic effects of the drug on tumor cells rather than through inhibition of angiogenesis.

**Discussion**

Although both temsirolimus and everolimus have been approved by the FDA for the treatment of RCC, neither agent induces substantial tumor regression except in a minority of patients. There are numerous factors that could account for the limited efficacy of these selective TORC1 inhibitors, among which is the feedback activation of the PI3K pathway. Our data, however, do not support the hypothesis that this is a frequent limiting factor in RCC because rapamycin treatment resulted in an unequivocal increase in Akt phosphorylation (Ser473) in only one of the five RCC cell lines examined (Fig. 2A) and the drug had no effect on Akt phosphorylation in 786-0 or A498 xenografts (Fig. 5B). These data suggest that rapalogue-induced PI3K or Akt activation may be uncommon in RCC and therefore not a dominant mechanism underlying treatment failure.

One clear distinction between rapamycin and NVP-BEZ235 is the differential effects of the two drugs on 4E-BP1 phosphorylation. As shown in Fig. 2A, rapamycin had virtually no effect on this parameter in any of the RCC cell lines tested. The basis for the failure of rapamycin to suppress 4E-BP1 phosphorylation in the RCC cell lines is unclear but cannot be attributed to an inadequate drug concentration used in the study as the concentration tested (100 nmol/L) was ≥10-fold higher than that required to inhibit S6 phosphorylation (Supplementary Fig. S1). Although 4E-BP1 is a canonical TORC1 substrate, its phosphorylation has long been recognized to be less responsive to rapalogues than that of S6 (27). In fact, Choo et al. have recently shown that the suppression of 4E-BP1 phosphorylation by rapamycin is reversed within a few hours of drug exposure (28). Regardless of the mechanism, the failure of rapamycin to block 4E-BP1 phosphorylation is likely to limit the extent to which the drug suppresses elf4E function and cap-dependent translation and to contribute to the apparent inferiority of rapalogues to agents such as NVP-BEZ235.

In addition to 4E-BP1, the phosphorylation of Mnk1 and its substrate elf4E is differentially affected by NVP-BEZ235 and rapamycin (Fig. 2A). The combined effect of reduced phosphorylation of both elf4E and its binding partner (4E-BP) resulting from exposure to NVP-BEZ235 would be predicted to have major consequences for the
translation of certain difficult-to-translate mRNAs that by virtue of their extended, complex 5’ untranslated region, are ignored by the ribosome except when eIF4E is available and functional. The translation of the survivin and cyclin D transcripts are cap dependent and as shown in Fig. 2A, the levels of these proteins are profoundly downmodulated in NVP-BEZ235–treated but not rapamycin–treated RCC cells. In addition to its role in cap-dependent translation, eIF4E regulates the nuclear export of certain mRNAs based on a sequence present in the transcript 3’ untranslated region. The cyclin D mRNA has such a 4E-sensitivity element (4ESE), and it is possible that the nuclear retention of the transcript in response to eIF4E dephosphorylation contributes to the cyclin D downmodulation achieved with NVP-BEZ235 (29). Although there are numerous possible mechanisms for the reduction of tumor cell proliferation induced by NVP-BEZ235, reduction of cyclin D levels and the induced nuclear translocation of p27 may be the dominant mechanisms for the antiproliferative effects of this drug. Our data suggest that the failure of rapamycin to suppress both 4E-BP and eIF4E phosphorylation or to translocate p27 to the nucleus may be responsible for its limited effect on tumor cell proliferation and tumor growth.

Despite the inhibition of 4EBP and eIF4E phosphorylation and suppression of survivin and cyclin D1 levels, NVP-BEZ235 had no effect on c-Myc levels. The persistence of this gene product in circumstances in which cap-dependent translation should be blocked raises the possibility that the c-Myc mRNA may be translated through a cap-independent mechanism involving an internal ribosome entry-site (IRES). Indeed, the utilization of IRES to translate this mRNA as well as that of cyclin D1 has been described in the setting of TORC1 inhibition (30). Furthermore, utilization of IRES was shown to be promoted by low basal Akt activity, a condition induced pharmacologically by NVP-BEZ235. Whether this mechanism can account for the failure of NVP-BEZ235 to...
suppress c-Myc levels as well as the apparent enhancement of HIF activity induced by rapamycin in some cell lines requires further study.

Our data suggest that the predominant antitumor effect of NVP-BEZ235 is a combination of slowed tumor proliferation and the induction of apoptosis. Akt inhibition would be expected to induce the expression of fas ligand and numerous proapoptotic BH3-only Bcl-2 family members (e.g., Bim, BNIP) as well as suppress the translation of inhibitors of apoptosis (IAP) family members such as survivin, all of which should predispose tumor cells to undergo apoptosis. Although we did not observe significant induction of cell death in vitro by NVP-BEZ235 as determined by propidium iodide staining, immunohistochemical analyses of the xenografts from NVP-BEZ235–treated mice showed a modest but significantly greater activation of caspase 3 compared with those from saline-treated mice.

The various components of the PI3K signaling pathway can exert either proangiogenic or antiangiogenic effects, depending on the experimental circumstances. As a result, the consequences of inhibiting the pathway on the tumor vasculature are likely to be complex and variable from one model to the next. As an illustration of this point, mice in which the dominant endothelial Akt isoform (i.e., Akt1) has been knocked out are hypersensitive to exogenous VEGF, possibly due to an impaired ability to produce thrombospondin (31). Tumors implanted into these mice grow more rapidly than similar implants in control animals and have an exaggerated vasculature. This suggests that treatment with a PI3K inhibitor, which should block...
endothelial Akt1 activity, might actually result in an increase in tumor angiogenesis, a prediction in fact validated in a 786-0 xenograft study using the PI3K inhibitor LY294002 (32). On the other hand, treatment of mice bearing murine breast carcinomas or rat C6 gliomas with rapamycin at doses sufficient to block Akt in the tumor endothelium resulted in vascular normalization (33, 34). Treatment of rats bearing orthotopic BN472 mammary carcinomas with NVP-BEZ235 resulted in marked reduction in tumor vascular permeability and interstitial pressure, further illustrating the variability in vascular response to PI3K inhibition (35). Although we showed a marked reduction in HIF-2α in RCC cell lines exposed to NVP-BEZ235 and xenografts from mice treated with NVP-BEZ235, we were unable to show any reduction in tumor angiogenesis, suggesting that the proangiogenic effects of Akt inhibition may have been able to compensate for the reduced HIF activity in the tumor cells in this model.

The results of our investigation into the mechanism of action of NVP-BEZ235 suggest that the antitumor activity of the drug is due primarily to suppression of tumor cell proliferation as well as the induction of apoptosis rather than through the disruption of tumor angiogenesis. Our data support the view that agents that block PI3K and/or TORC2 in addition to TORC1 are likely to do better than selective TORC1 inhibitors in the treatment of RCC.

Disclosure of Potential Conflicts of Interest


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