Activity of the Novel Dual Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Inhibitor NVP-BEZ235 against T-Cell Acute Lymphoblastic Leukemia

Francesca Chiarini1, Cecilia Grimaldi1, Francesca Ricci3, Pier Luigi Tazzari3, Camilla Evangelisti1, Andrea Ognibene3, Michela Battistelli6, Elisabetta Falcieri1,6, Fraia Melchionda2, Andrea Pession2, Pasqualepaolo Pagliaro3, James A. McCubrey7, and Alberto M. Martelli1,5

Abstract

Recent findings have highlighted that constitutively active phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling is a common feature of T-cell acute lymphoblastic leukemia (T-ALL), where it upregulates cell proliferation, survival, and drug resistance. These observations lend compelling weight to the application of PI3K/Akt/mTOR inhibitors in the therapy of T-ALL. Here, we have analyzed the therapeutic potential of the novel dual PI3K/mTOR inhibitor NVP-BEZ235, an orally bioavailable imidazoquinoline derivative, which has entered clinical trials for solid tumors, on both T-ALL cell lines and patient samples. NVP-BEZ235 was cytotoxic to a panel of T-ALL cell lines as determined by MTT assays. NVP-BEZ235 treatment resulted in cell cycle arrest and apoptosis. Western blots showed a dose- and time-dependent dephosphorylation of Akt and mTORC1 downstream targets in response to NVP-BEZ235. Remarkably, NVP-BEZ235 targeted the side population of both T-ALL cell lines and patient lymphoblasts, which might correspond to leukemia-initiating cells, and synergized with chemotherapeutic agents (cyclophosphamide, cytarabine, dexamethasone) currently used for treating T-ALL patients. NVP-BEZ235 reduced chemoresistance to vincristine induced in Jurkat cells by coculturing with MS-5 stromal cells, which mimic the bone marrow microenvironment. NVP-BEZ235 was cytotoxic to T-ALL patient lymphoblasts displaying pathway activation, where the drug dephosphorylated eukaryotic initiation factor 4E-binding protein 1, at variance with rapamycin. Taken together, our findings indicate that longitudinal inhibition at two nodes of the PI3K/Akt/mTOR network with NVP-BEZ235, either alone or in combination with chemotherapeutic drugs, may be an efficient treatment of those T-ALLs that have aberrant upregulation of this signaling pathway for their proliferation and survival. Cancer Res; 70(20); 8097–107. ©2010 AACR.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive disorder of precursor cells committed to the T-cell lineage (1). Over the past 20 years, survival rates of T-ALL patients have improved, mainly because of advances in chemotherapy protocols. Survival rates at 5 years for children and adolescents with T-ALL are 70% to 75%, whereas for adults, the rates are 35% to 40% (2). In spite of these improvements, novel and less toxic treatment strategies for T-ALL are needed (3). Novel therapies may target aberrantly activated signaling pathways influencing the proliferation, survival, and drug resistance of these T-ALLs. One such pathway is represented by the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling network (4, 5). Pathway upregulation in T-ALL is due to several reasons, which include Notch1 activation leading to HES1 (hairy and enhancer of split 1)-mediated transcriptional suppression of the PTEN (phosphatase and tensin deleted on chromosome 10) gene (6, 7), PTEN phosphorylation or oxidation (8), interleukin (IL)-4 secreted by bone marrow stromal cells (9), or mutations affecting PI3K, PTEN, or Akt (10). About 85% of T-ALL patients display increased PI3K/Akt/mTOR activation at diagnosis (8, 11). mTOR exists as two complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2; ref. 12). Allosteric mTOR inhibitors, which include rapamycin and its analogues (rapalogues), mainly target mTORC1. These inhibitors display promising effects in preclinical models of T-ALL (13, 14).
However, rapamycin/rapalogues are mainly cytostatic (15) and could hyperactivate Akt due to the existence of feedback loops between mTORC1, PI3K, and Akt (16). Moreover, it is beginning to emerge that there are mTORC1 functions, including phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; which controls translation), which are insensitive to rapamycin/rapalogues (17, 18). Recently, dual PI3K/mTOR inhibitors have been synthesized, which, unlike rapamycin/rapalogues, target the catalytic site of both kinases (19). As such, these compounds are pro-apoptotic and dephosphorylate 4E-BP1. We have recently shown the cytotoxic effect of the dual PI3K/mTOR inhibitor PI-103 in preclinical settings of T-ALL (20). PI-103 has proven efficacy also in vivo against models of human tumors xenografted in mice, where it displayed low toxicity and was well tolerated (19). However, PI-103 did not enter clinical trials, mainly because of its rapid in vivo metabolism (21). Here, we have analyzed the therapeutic potential of the novel dual PI3K/mTOR inhibitor NVP-BEZ235, an orally bioavailable imidazoquinoline derivative (22), which has entered clinical trials for solid tumors, in both T-ALL cell lines and patient samples. We have shown that this drug displayed strong cytotoxic activity against T-ALL cells. Rapid commitment of T-ALL cells to death was triggered by NVP-BEZ235. Importantly, healthy donor peripheral blood CD4+ T cells were much less sensitive than the T-ALL cell lines tested, suggesting a favorable therapeutic index. Combinations of NVP-BEZ235 with conventional anti-T-ALL chemotherapeutic agents showed a strong synergistic activity, implying that these NVP-BEZ235–based combinations could be feasible in the clinic. Thus, our results provide the framework for clinical trials of the dual PI3K/mTOR inhibitor NVP-BEZ235 to improve patient outcome in T-ALL.

Materials and Methods

Materials

NVP-BEZ235 was kindly provided by Novartis. For Western blotting, primary antibodies were purchased from Cell Signaling Technology. Rapamycin, vincristine, dexamethasone, cyclophosphamide, cytarabine (Ara-C), Hoechst 33342, and fumitremorgin C were purchased from Sigma-Aldrich. PI-103 was purchased from Alexis Biochemicals. KO143 was purchased from Axon Medchem BV. Antibody to ABCG2 was from Millipore/Upstate, antibody to 170-kDa P-glycoprotein (P-gp) was from Kamiya, and antibody to MRPI was from BD Biosciences Pharmingen. AlexaFluor-conjugated antibodies to PTEN, Ser473 p-Akt, Thr37/46 p-4E-BP1, and Ser235/236 p-S6 ribosomal protein (p-S6RP) were from Cell Signaling Technology.

Cell culture and primary samples

The T-ALL cell lines Jurkat, MOLT-4, CEM-S, CEM-R (CEM VBL100, drug-resistant cells overexpressing P-gp; ref. 23), RPMI-8402, and BE-13 were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). All cell lines were from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and were characterized as specified (http://www.dsmz.de/human_and_animal_cell_lines/main.php?contentleft_id=21). Patient samples or peripheral blood CD4+ T lymphocytes from healthy donors were obtained with informed consent according to institutional guidelines and isolated using Ficoll-Paque (Amersham Biosciences) for patient lymphoblasts or by magnetic labeling (Miltenyi Biotech) for CD4+ T lymphocytes. CD4+ T lymphocytes (105 per well) were cultured in RPMI 1640 containing 10% FBS and stimulated for 24 hours with a mixture of 10 μg/mL phytohemagglutinin-M (PHA-M) and 50 ng/mL human recombinant IL-2 to induce proliferation.

Cell viability analysis

MTT assays were performed to assess the sensitivity of cells to drugs, as previously described (24).

Annexin V-FITC/propidium iodide staining

To determine the extent of apoptosis induction, flow cytometric analysis of Annexin V-FITC/propidium iodide (PI)–stained samples was performed (24). Samples were analyzed by an EPICS XL flow cytometer (Beckman Coulter) with the appropriate software (System II, Beckman Coulter). At least 15,000 events per sample were acquired.

Cell cycle analysis

Flow cytometric analysis was performed using PI/RNase A staining according to standard procedures, as described previously (24).

Transmission electron microscopy

This was performed as described previously (25).

Western blot analysis

This was performed by standard methods, as previously reported (24). Analysis with an antibody to β-actin showed equal protein loading.

Coculture with mouse MS-5 stromal cells

Jurkat cells (1 × 10⁶/mL) were seeded on top of MS-5 mouse stromal cells (at ∼70% confluence) for 3 hours before the addition of NVP-BEZ235 alone, vincristine alone, or a combination of NVP-BEZ235 and vincristine. After 24 hours of incubation at 37°C, cells were harvested with trypsin/EDTA, washed, and resuspended in binding buffer containing Annexin V-FITC. Cells were counterstained with a phycocerythrin (PE)–conjugated anti-CD45 antibody or with an irrelevant isotypic control antibody and analyzed by flow cytometry after electronic gating on CD45+ leukemia cells (26).

Combined drug effect analysis

The combination effect and a potential synergy were evaluated from quantitative analysis of dose-effect relationships as described previously (24). For each combination experiment, a combination index (CI) number was calculated using the Biosoft CalcuSyn software. This method of analysis generally defines CI values of 0.9 to 1.1 as additive, 0.3 to 0.9 as synergistic, and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.
Flow cytometric analysis of PTEN, p-Akt (Ser473), p-4E-BP1 (Thr37/46), and p-S6RP (Ser235/236) levels in MS-5 cells or T-ALL patient samples

MS-5 cells or lymphoblasts from pediatric patients with T-ALL were fixed in reagent 1 of the Intraprep Kit (Beckman Coulter) and permeabilized with saponin-based reagent 2, as reported elsewhere (20). Cells were incubated with primary antibodies conjugated to AlexaFluor 488 or AlexaFluor 647. A rabbit IgG conjugated to AlexaFluor 488 or AlexaFluor 647 was used as an irrelevant antibody. Cells were analyzed on a FC500 flow cytometer (Beckman Coulter). At least 5,000 events per sample were acquired.

Flow cytometric detection of T-ALL side population cells

Cells were resuspended at $1 \times 10^6$/mL in prewarmed RPMI 1640 with 2% FBS. Hoechst 33342 dye was added to a final concentration of 5 μg/mL in the presence or the absence of KO143 (0.1 μmol/L) or fumitremorgin C (10 μmol/L) and the cells were incubated at 37°C for 90 minutes with intermittent shaking. In some experiments, cells were stained with monoclonal antibodies to ABC membrane transporters (P-gp, MRP1, and ABCG2), followed by a PE-conjugated secondary antibody. Control samples were analyzed with an irrelevant isotypic PE-conjugated antibody. Cells were then washed with PBS containing 2% bovine serum albumin and processed for Hoechst 33342 staining. Samples were analyzed with a Cell Lab Quanta SC (Beckman Coulter) flow cytometer equipped with UV lamp and 488 solid state laser. The Hoechst 33342 dye was excited at 366 nm. Side population (SP) cells were gated by the FL1/FL3 histogram, whereas ABC transporter staining was evaluated on the FL2 channel (27, 28).

Figure 1. NVP-BEZ235 induces cytotoxicity and apoptosis in T-ALL cell lines. A, MTT assays of T-ALL cell lines treated with NVP-BEZ235 for 24 h. B, comparison between NVP-BEZ235 and PI-103. The results of MTT assays performed at 24 h are shown. Points and columns, mean of at least three different experiments; bars, SD. *, $P < 0.01$. C, flow cytometric analysis of Annexin V-FITC/PI–stained T-ALL cells treated with increasing NVP-BEZ235 concentrations. The percentages of early apoptotic cells (Annexin V–FITC+/PI−; bottom right quadrant) and late apoptotic/necrotic cells (Annexin V–FITC+/PI+; top right quadrant) are indicated. The histograms are representative of three separate experiments performed in duplicate. D, Western blot analysis documenting activation of caspase-8, caspase-9, and caspase-3 by NVP-BEZ235 (200 nmol/L). Cells were treated with NVP-BEZ235 for the indicated times, collected, and then lysed. Fifty micrograms of each lysate were electrophoresed on SDS-PAGE gels followed by transfer onto a nitrocellulose membrane. CTRL, untreated sample.
Results

**NVP-BEZ235 has cytotoxic proapoptotic effects on T-ALL cell lines**

The effects of NVP-BEZ235 on T-ALL cells were analyzed by first treating the cells with increasing concentrations of the drug and analyzing the rates of survival by MTT assays. Most cell lines displayed an IC$_{50}$ for NVP-BEZ235 ranging from 80 to 400 nmol/L, whereas the IC$_{50}$ for BE-13 cells (which do not display p-Akt; see following discussion) was 1 μmol/L. The drug-resistant CEM-R cell line had the highest sensitivity to NVP-BEZ235 (IC$_{50}$, 80 nmol/L; Fig. 1A). Next, a comparison was made between NVP-BEZ235 and PI-103, another dual PI3K/mTOR inhibitor. When used at equimolar concentrations, PI-103 was less effective than NVP-BEZ235 in affecting T-ALL cell line growth (Fig. 1B) at all the concentrations tested.

The effects of NVP-BEZ235 on the induction of apoptosis were determined with Annexin V-FITC/PI staining in MOLT-4, Jurkat, and CEM-R cells. After 6 hours of NVP-BEZ235 treatment, flow cytometry analysis documented an increase in apoptosis in all the cell lines analyzed (Fig. 1C). Western blotting analysis of extracts from MOLT-4, Jurkat, and CEM-R cells treated with NVP-BEZ235 for 1, 3, and 6 hours showed cleavage of procaspase-8, procaspase-9, and procaspase-3 (Fig. 1D).

**NVP-BEZ235 blocks cells in the G$_0$/G$_1$ phase of the cell cycle**

Given the fundamental role played by PI3K/Akt/mTOR signaling in cell proliferation (29), the effects of NVP-BEZ235 on cell cycle progression were also investigated. Flow cytometric analysis of PI-stained T-ALL cells treated with NVP-BEZ235 for 16 hours documented an increase in G$_0$/G$_1$ phase cells and a concomitant decrease in S and G$_2$-M phases in both MOLT-4 and CEM-R cell lines (Supplementary Fig. S1A). As pRb is a critical regulator of the cell cycle transition from G$_1$ to S phase (30), its phosphorylation state was analyzed by Western blotting. We detected a decrease in the amount of Ser807/811 pRb in MOLT-4 and CEM-R cells treated with 200 nmol/L NVP-BEZ235 for 16 hours, whereas total pRb levels remained unchanged (Supplementary Fig. S1A).

In contrast, CD4$^+$ T lymphocytes isolated from the peripheral blood of healthy donors and stimulated with PHA-M plus IL-2 were much less sensitive to NVP-BEZ235 concentrations up to 500 nmol/L. The analysis, carried out by means...
of flow cytometry of PI-stained samples, documented only a slight increase in G0/G1 cells (Supplementary Fig. S1B). No apoptosis was detected in these cells (data not shown). Overall, these findings showed that NVP-BEZ235 potently reduced the growth of T-ALL cell lines and that this effect was due to both apoptosis and cell cycle arrest. Moreover, these results also suggested that the drug could have a favorable therapeutic index, as it did not significantly affect the proliferation of normal CD4+ T lymphocytes.

NVP-BEZ235 induces autophagy

As mTORC1 is an inhibitor of autophagy (31), it was investigated whether NVP-BEZ235 could indeed induce autophagy in T-ALL cells. Induction of autophagy was confirmed by transmission electron microscopy analysis, the golden standard for autophagy studies, which documented the presence of autophagic vacuoles in the cytoplasm of NVP-BEZ235–treated CEM-S cells. Similar results were detected in MOLT-4 cells (Supplementary Fig. S2A). In CEM-S cells treated with NVP-BEZ235 for 16 hours, we observed an increase in Beclin-1, a well-established autophagy marker (ref. 32; Supplementary Fig. S2B). In addition, we studied the cleavage of LC3, which is the only known mammalian protein that stably associates with the autophagosome membranes (33). Indeed, LC3 can be detected as LC3-A (cytosolic) and LC3-B (membrane bound and enriched in the autophagic vacuole fraction) forms. NVP-BEZ235 treatment caused an increase in LC3-B (cleaved fragment; Supplementary Fig. S2B). Because it is now emerging that autophagy could represent a mechanism of protection implemented by cells treated with mTORC1 inhibitors (34), it was investigated if an autophagy inhibitor, chloroquine, could potentiate the proapoptotic effects of NVP-BEZ235. However, chloroquine actually slightly decreased the number of apoptotic CEM-S cells when used in combination with NVP-BEZ235, even if this decrease was not statistically significant (Supplementary Fig. S2C). These results implied that autophagy induced by NVP-BEZ235 has no protective effect against apoptosis.

NVP-BEZ235 affects PI3K/Akt/mTOR signaling in T-ALL cell lines

Western blot analysis showed a dose-dependent decrease in Ser473 p-Akt after 24 hours of treatment with the drug in all the cell lines (Fig. 2A). BE-13 cells did not display Akt phosphorylated on Ser473. Total Akt levels were unaffected by NVP-BEZ235. mTORC1 downstream substrates (p70S6K, 4E-BP1, and S6RP) were also efficiently dephosphorylated by NVP-BEZ235, whereas their total levels did not change (Fig. 2A). A time-dependent study was also performed, which documented that NVP-BEZ235 (200 nmol/L) dephosphorylated Akt and S6RP already after 1 hour of treatment, whereas p70S6K and 4E-BP1 dephosphorylation was detected after 6 hours of treatment (Fig. 2B).

NVP-BEZ235 synergizes with chemotherapeutic drugs

It was investigated whether NVP-BEZ235 could synergize with drugs commonly used for treating T-ALL patients.
T-ALL cell lines were incubated for 24 hours with the drugs alone, NVP-BEZ235 alone, or the drugs plus NVP-BEZ235 at a fixed ratio (NVP-BEZ235/cyclophosphamide, 1:10; NVP-BEZ235/Ara-C, 1:1). MTT assays were then performed. Analysis of the results proved that NVP-BEZ235 synergized with all the tested drugs; however, the synergism was particularly strong with Ara-C. CI analysis documented the existence of a strong synergism (CI < 0.3) at NVP-BEZ235 concentrations that were well below its IC_{50} (Fig. 3). Moreover, in RPMI-8402 cells, NVP-BEZ235 synergized with dexamethasone (data not shown).

NVP-BEZ235 reduces chemoresistance to vincristine induced in Jurkat cells by coculturing with MS-5 stromal cells

Because interactions between bone marrow stromal cells and leukemic cells are thought to be responsible for decreased chemotherapeutic drug sensitivity (35), we investigated whether NVP-BEZ235 could increase the cytotoxicity of vincristine in a coculture system. We used the murine stromal cell line MS-5, which is known to provide long-term support for primitive hematopoietic progenitors and to mimic the bone marrow microenvironment (36). Coculture with stromal cells only slightly decreased the sensitivity of Jurkat cells to NVP-BEZ235, with the percentage of apoptotic cells decreasing from 15% to 11% (Fig. 4A). Flow cytometric analysis of CD45+ cells showed that coculture with stromal cells significantly (P < 0.01) decreased the sensitivity to vincristine of Jurkat cells when compared with cells growing as suspension cultures, as documented by the percentage of Annexin V–FITC–positive cells. However, NVP-BEZ235 restored vincristine sensitivity to the levels measured in suspension cultures treated with vincristine alone. Nevertheless, the efficacy of the combined treatment in the presence of stromal cells was inferior to that detected in suspension cultures (Fig. 4A). This probably reflects activation of additional survival pathways by stromal cells. To determine whether the effect of NVP-BEZ235 on Jurkat cells was direct or indirect via an effect on MS-5 signaling or viability, we performed the following experiments. When PI3K/Akt/mTORC1 signaling was studied by flow cytometry in MS-5 stromal cells cocultured with Jurkat cells, it was possible to document that NVP-BEZ235 did not affect the phosphorylation levels of both Ser473 p-Akt and Thr37/46 p-4E-BP1 (Fig. 4B). Moreover, either vincristine or NVP-BEZ235 alone did not affect in a statistically significant manner MS-5 cell viability, whereas with the combined treatment, 85% of stromal cells were still viable (Fig. 4C).

T-ALL lymphoblasts are sensitive to NVP-BEZ235

To better assess the effectiveness of NVP-BEZ235 as a potential therapeutic agent in T-ALL, we examined seven pediatric T-ALL patient samples isolated from the bone marrow or peripheral blood for the levels of PTEN, Ser473 p-Akt, Thr37/46 p-4E-BP1, and Ser235/236 p-S6RP, as well as for their sensitivity to NVP-BEZ235, using flow cytometric and MTT assays. All of the patients displayed enhanced phosphorylation of Ser473 p-Akt, Thr37/46 p-4E-BP1, and Ser235/236 p-S6RP (not shown). As to PTEN, one patient had very low levels whereas the six others had higher levels (Fig. 5A). These results were in agreement with the gene expression profiles (not shown). T-ALL lymphoblast samples were treated with increasing concentrations of NVP-BEZ235, and cell survival was analyzed by MTT assays. A marked reduction of cell viability at 96 hours was detected. The IC_{50} for patient samples ranged between 10 and 50 nmol/L NVP-BEZ235 (Fig. 5B). Flow cytometric analysis documented a decrease in the levels of p-4E-BP1 and p-S6RP in NVP-BEZ235–treated samples (Fig. 5C). Interestingly, rapamycin treatment did not result in dephosphorylation of 4E-BP1, although it efficiently dephosphorylated S6RP. These results were confirmed by Western blot analysis (Fig. 5C and D). Overall, these findings show that NVP-BEZ235 has a potent cytotoxic activity also against primary cells from T-ALL patients with upregulated PI3K/Akt/mTORC1 signaling.

NVP-BEZ235 targets the SP of T-ALL cell lines and patient lymphoblasts

Finally, we sought to determine whether NVP-BEZ235 could target the T-ALL SP. This subpopulation, which overexpresses ABCG2 (also referred to as breast cancer resistance protein or BCRP) and other ABC plasma membrane transporters, is thought to share some properties of cancer stem cells (CSC; ref. 37). This has been shown in solid tumors (38, 39) and acute myelogenous leukemia (40). However, candidate CSCs have been successfully identified in the SP of a murine model of adult T-cell leukemia/lymphoma (41). This suggests that the SP of T-ALL shares some properties with CSCs. The SP of T-ALL cells was identified by the live-cell DNA-binding dye Hoechst 33342. As a control, Hoechst 33342 staining could be inhibited after incubation with either of the high-specificity ABCG2 transporter inhibitors fumitremorgin C or KO143. A decrease in the amount of SP cells was evident in samples treated with either NVP-BEZ235 or rapamycin for 24 hours (Fig. 6A) in MOLT-4, CEM-S, and BE-13 cells. Next, we analyzed by flow cytometry the plasma membrane levels of the most important ABC transporters in the SP of CEM-S cells, which displayed no expression of both P-gp and MRP1 but high levels of ABCG2 (Fig. 6B).

Both NVP-BEZ235 and rapamycin targeted the SP of lymphoblasts from T-ALL patients (two representative patients are shown). Expression of ABCG2 on SP cells was documented by double staining with Hoechst 33342 and an antibody that recognizes an extracellular epitope of the transporter (Fig. 6C and D).

Discussion

The PI3K/Akt/mTOR pathway is a recently identified potential target for therapeutic intervention in T-ALL. Because of its complexity and extensive crosstalk with other signaling cascades, therapeutic targeting of the PI3K/Akt/mTOR network at multiple molecular levels may provide better antitumor effects than selective inhibition of only one component of the pathway. In fact, one potential reason for the limited efficacy of single inhibitors in this pathway is the presence of...
Figure 4. NVP-BEZ235 reduces chemoresistance to vincristine induced in Jurkat cells by coculturing with MS-5 stromal cells, which mimic the bone marrow microenvironment. A, Jurkat cells, growing either as suspension cultures or in a coculture system with MS-5 cells, were treated with vincristine (VCR; 10 nmol/L) or NVP-BEZ235 (NVP; 200 nmol/L), alone or in combination, and then analyzed by Annexin V-FITC/CD45-PE staining using flow cytometry. The percentages of healthy and apoptotic cells are indicated in top left and top right quadrants, respectively. B, MS-5 cells cocultured with Jurkat cells were stained with AlexaFluor 488-conjugated anti-p-Akt or anti-p-4E-BP1. Jurkat cells were excluded from the analysis by electronically gating on CD45-PE+ cells. CTRL, untreated cells. NVP, cells treated for 24 h with 200 nmol/L NVP-BEZ235. C, MS-5 cells were incubated with vincristine (10 nmol/L) or NVP-BEZ235 (200 nmol/L), alone or in combination, and then the percentage of Annexin-V+ cells was analyzed by flow cytometry. *, P < 0.05, versus untreated cells (CTRL). Representative of three experiments (A–C).
signaling feedback loops operating through p70S6K and PI3K (12). Exposure to a dual catalytic PI3K/mTOR inhibitor might therefore be sufficient to avoid PI3K/Akt pathway reactivation (22). Moreover, targeting this pathway at multiple levels may prevent the development of drug resistance (42). In our previous work, we have documented the efficacy of dual PI3K/mTOR inhibition in T-ALL cells using PI-103 (20). Here, we showed the efficacy of the novel dual PI3K/mTOR inhibitor NVP-BEZ235 on T-ALL cell lines and lymphoblasts derived from T-ALL patients. NVP-BEZ235 is an ATP competitor that potently

Figure 5. NVP-BEZ235 cytotoxicity toward T-ALL primary cells displaying constitutive activation of the PI3K/Akt/mTOR pathway. A, histograms showing PTEN status in three representative patient (pt) samples. B, MTT assay of T-ALL blasts treated with NVP-BEZ235 for 96 h. Points, mean of at least two different experiments; bars, SD. Four representative patients are shown. C, patient samples were treated with 100 nmol/L rapamycin or 200 nmol/L NVP-BEZ235 for 48 h and then analyzed by flow cytometry for p-S6RP and p-4E-BP1. D, Western blot analysis of two representative patient samples treated with rapamycin (rapa; 100 nmol/L) or NVP-BEZ235 (NVP; 200 nmol/L) for 48 h, then lysed, collected, and probed with antibodies against p-4E-BP1 and β-actin. Representative of two different experiments. CTRL, untreated sample.
reduces the kinase activity of both p110 PI3K and mTORC1/2, whose efficacy in advanced solid tumors is currently being evaluated in phase 1 and 2 clinical trials (22). A comparison between PI-103 and NVP-BEZ235 documented that the latter compound was significantly more powerful toward T-ALL cells when used on an equimolar basis. NVP-BEZ235 induced apoptosis, which led to a cleavage of both procaspase-8 and procaspase-9, suggesting that both the intrinsic and extrinsic pathways of apoptosis are activated on exposure to the drug. Even if downregulation of PI3K/Akt/mTOR signaling is usually associated with activation of the intrinsic pathway (43), caspase-8 activation by the dual PI3K/mTOR inhibitor PI-103 has been reported in leukemic cells (44). In breast cancer cells, NVP-BEZ235 activated caspase-2, which played an important role in the apoptotic process (45).

Moreover, treatment with NVP-BEZ235 resulted in a robust accumulation in the G1 phase of the cell cycle and dephosphorylation of pRb on Ser807/811, which are two of the most critical residues for pRb activity in cell cycle progression (46). Consistent with its inhibitory action on both mTORC1 and mTORC2, NVP-BEZ235 dephosphorylated p70S6K/4E-BP1/S6RP as well as Akt on Ser473 in T-ALL cells. Evidence indicates that the modulation of autophagy is an important component of tumorigenesis, making it a possible therapeutic target. The PI3K/Akt/mTOR pathway is important in inhibiting autophagy (47). We observed that autophagy is activated in response to NVP-BEZ235 treatment in T-ALL cell lines. This observation could be very important, as some recent studies have documented that tumorigenesis stimulated by constitutively activated PI3K/Akt signaling is linked to the ability to inhibit autophagy but not apoptosis, raising the possibility that autophagy may also be an important mechanism underlying the response to therapeutic agents targeting the PI3K/Akt/mTOR pathway (47).

**Figure 6.** NVP-BEZ235 and rapamycin target the SP of T-ALL cells. A, T-ALL cell lines treated with rapamycin (rapa) or NVP-BEZ235 (NVP) for 24 h were stained with Hoechst 33342 dye (5 μg/mL) in the presence or absence of KO143 (0.1 μmol/L) and then analyzed by flow cytometry. The SP, which disappeared in the presence of KO143, was gated and shown as a percentage of the whole viable cell population. B, flow cytometric analysis of CEM-S SP cells stained with PE-conjugated antibodies to ABCG2, P-gp, and MRP1. Black histograms, negative control (irrelevant antibody); white histograms, anti-ABC transporter antibody. C and D, flow cytometric analysis of the SP and ABCG2 in two representative patient samples. T-ALL primary cells were incubated for 24 h with rapamycin (100 nmol/L) or NVP-BEZ235 (200 nmol/L). Fumitremorgin C was used at 10 μmol/L. Representative of two separate experiments. CTRL, cells stained with Hoechst 33342 only. Representative of two separate experiments. D, black histograms, negative control (irrelevant antibody); white histograms, anti-ABCG2 antibody.
Because survival signals generated by cytokines secreted by cells of the bone marrow microenvironment play an important role in the emergence of drug resistance, we asked whether NVP-BEZ235 could diminish these survival signals. Our results suggest a potential clinical activity of a combination therapy (NVP-BEZ235 plus vincristine) even in the presence of bone marrow stromal cells. Remarkably, NVP-BEZ235 displayed a strong (in the low nanomolar range) cytotoxic activity against lymphoblasts from patients with pediatric T-ALL characterized by enhanced levels of p-Akt, p-4E-BP1, and p-S6RP. Moreover, it caused the dephosphorylation of both 4E-BP1 and S6RP in these samples, whereas treatment with rapamycin did not induce 4E-BP1 dephosphorylation. Therefore, as reported for acute myelogenous leukemia cells (16), agents that target mTORC1 activities globally could be more beneficial than rapamycin/rapalogs for T-ALL patients (16).

The difficulty in eradicating tumors might result from the conventional treatments targeting the bulk of the tumor cells, but not the CSCs. Therefore, strategies that eliminate these cells could have significant clinical implications. We have shown that both NVP-BEZ235 and rapamycin markedly reduced the T-ALL SP, which might correspond, at least in part, to CSCs. It is still unclear whether NVP-BEZ235 was directly cytotoxic to the SP cells or it simply blocked ABCG2 activity (and thus Hoechst dye extrusion), as it has been previously documented that membrane localization of ABCG2 in mouse bone marrow cells was dependent on Akt activity (48). However, NVP-BEZ235 targeted the SP of BE-13 cells that do not display Akt activation. Moreover, rapamycin also strongly reduced the SP of T-ALL, and our previous results indicated that rapamycin was not as effective as PI-103 in reducing p-Akt levels in T-ALL cells (20).

These observations suggest that NVP-BEZ235 could be directly cytotoxic to the SP cells due to inhibition of mTORC1 activity. Indeed, the key role played by mTORC1 signaling in the survival of leukemic stem cells in mouse models of acute lymphoblastic leukemia is now beginning to emerge (49, 50). However, additional experiments are required to further address this issue.

Taken together, our preclinical findings strongly suggest that NVP-BEZ235, either alone or in combination with traditional chemotherapeutic drugs, could be a valuable compound in the treatment of those T-ALL patients displaying activation of PI3K/Akt/mTOR signaling and who still face a poor prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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