Dual inhibition of PI3K and mTORC1/2 signalling by NVP-BEZ235 as a new therapeutic strategy for acute myeloid leukemia.

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SM. Maira: Novartis pharma employee and shareholder. The other authors disclosed no potential conflicts of interest.
TRANSLATIONAL RELEVANCE

The mammalian target of rapamycin (mTOR) kinase is implicated in the regulation of initiation of mRNA translation, cell cycle progression, and cellular proliferation. The mTORC1 pathway is commonly activated in acute myeloid leukemia (AML) and represents a major target for AML therapy. However, the efficacy of allosteric mTORC1 inhibition with rapamycin and its analogs is limited in AML. The understanding of the mechanisms leading to rapamycin-resistance led to the development of second generation of mTOR inhibitors which directly suppress the mTOR catalytic activity in both mTORC1 and mTORC2 complexes. We therefore tested the dual PI3K/mTORC1/2 ATP-competitive inhibitor NVP-BEZ235 in AML. Interestingly, this compound has a strong anti-leukemic activity by overcoming mechanisms leading to rapamycin-resistance. Furthermore, NVP-BEZ235 only barely affects the survival of normal CD34+ hematopoietic progenitors, suggesting a favorable therapeutic index in vivo. NVP-BEZ235 represents therefore a promising perspective for AML therapy and should have clinical development in the future.
ABSTRACT

Purpose
The growth and survival of AML cells are enhanced by the deregulation of signalling pathways such as PI3K/Akt, and mTOR. Major efforts have thus been made to develop molecules targeting these activated pathways. The mTOR serine/threonine kinase belongs to two separate complexes, mTORC1 and mTORC2. The mTORC1 pathway is rapamycin-sensitive and controls protein translation through the phosphorylation of 4E-BP1 in most models. In AML however, the translation process is deregulated and rapamycin-resistant. Furthermore, the activity of PI3K/Akt and mTOR are closely related as mTORC2 activates the oncogenic kinase Akt. We therefore tested in this study, the anti-leukemic activity of the dual PI3K/mTOR ATP-competitive inhibitor NVP-BEZ235 compound (Novartis®).

Experimental Design
Activity of NVP-BEZ235 was tested in primary AML samples (n=21) and human leukemic cell lines. The different signalling pathways were analysed by western blotting. The cap-dependent mRNA translation was studied by 7mGTP pulldown experiments, polysomal analysis and [3H]-leucine incorporation assays. The anti-leukemic activity of NVP-BEZ235 was tested by analyzing its effects upon leukemic progenitor clonogenicity, blast cell proliferation and survival.

Results
The NVP-BEZ235 compound was found to inhibit PI3K and mTORC1 signalling and also mTORC2 activity. Furthermore, NVP-BEZ235 fully inhibits the rapamycin-resistant phosphorylation of 4E-BP1, resulting in a marked inhibition of protein translation in AML cells. Hence, NVP-BEZ235 reduces the proliferation rate and induces an important apoptotic response in AML cells without affecting normal CD34+ survival.
Conclusions

Our results clearly demonstrate the anti-leukemic efficiency of the NVP-BEZ235 compound which therefore represents a promising option for future AML therapies.
INTRODUCTION

In acute myeloid leukemia (AML), the constitutive activation of signal transduction pathways enhances the survival and proliferation of the leukemic cells (1). The increased understanding of AML biology in recent years however contrasts with the remaining poor prognosis for patients with this disease (2). The development of new therapies is thus highly desirable and the targeting of signalling pathways that are preferentially activated in transformed AML cells represents a rapidly expanding research field in this regard (1).

The class IA Phosphoinositide 3-Kinase (PI3K) is a lipid kinase that is mainly induced following growth factor receptor activation. Upon PI3K stimulation, the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) induces the activating phosphorylation by PDK1 of the oncogenic kinase Akt on T308. The PI3K signalling pathway is frequently deregulated in cancer cells and its constitutive activity strongly contributes to the oncogenic process (3). In AML, constitutive PI3K/Akt activation, mainly due to the activity of the PI3K p110δ isoform (4, 5), is detected in 50% to 80% of samples at diagnosis (6-8) and has recently been linked to an IGF-1/IGF-1R autocrine loop (9). Although specific inhibition of PI3K p110δ activity barely induces apoptosis in AML (9, 10), the PI3K/Akt pathway markedly contributes to AML cell proliferation (4, 5, 9), therefore representing a potential therapeutic target for this disease.

The serine/threonine kinase mTOR (mammalian target of rapamycin) is a component of two exclusive complexes, mTORC1 and mTORC2, which are defined by both their molecular composition and substrate specificity. The interaction of mTOR with raptor (regulatory associated protein of mTOR) defines mTORC1, whose substrates include the ribosomal protein S6 Kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs) (11, 12). The mTORC1 pathway generally controls the cap-dependent translation of mRNAs through the phosphorylation of the key translation regulator 4E-BP1.
Briefly, hypophosphorylated 4E-BP1 molecules block the formation of the translation initiating complexes (eIF4F) through their interaction with the eIF4E proteins which bind the 7-methyl-GTP (7mGTP) cap structure located at the 5’end of most mRNA molecules (14). Following a complex sequence of phosphorylation events, the release of 4E-BP1 from eIF4E enables the interaction between the eIF4E and eIF4G proteins that in turn initiates eIF4F assembly (15, 16). In contrast, the role of the mTORC2 complex which is based on the interaction between rictor (rapamycin insensitive companion of mTOR) and mTOR (17, 18) has only recently emerged in cancer cell biology and is mainly related to the control of Akt S473 phosphorylation (19).

Rapamycin and its derivates (RAD001, CCI-779), referred to as rapalogs, are highly specific mTORC1 inhibitors that have been developed as anti-cancer drugs, due to the frequent activation of the mTORC1 pathway in different cancers including AML (20). Rapamycin, through its association with the FK506 binding protein 12 (FKBP-12) (21), induces the disassembly of the mTORC1 complex (22, 23) resulting in the repression of mTORC1 activity. However, although an interesting anti-tumor activity of rapamycin as a monotherapy has been reported in some AML patients (24), mechanisms underlying resistance to rapamycin have now been described. First, the rapamycin-induced inhibition of mTORC1 activity in primary AML cells increases PI3K activity via the stimulation of an IGF-1/IGF-1R autocrine loop which enhances AML cell proliferation (10). Furthermore, allosteric inhibition of mTORC1 does not block protein translation, mainly due to the sustained high level of 4E-BP1 phosphorylation in AML cells treated with rapamycin (25). Moreover, rapamycin generally fails to inhibit mTORC2 activity (17, 18), although evidence has suggested that prolonged treatment with rapamycin may inhibit mTORC2 activity against its substrate Akt S473, depending on the cell type (26, 27). Taken together, these data led to the development of new strategies to efficiently target mTOR signaling such as direct ATP
competitive ‘active-site’ mTOR inhibitors. Recently, different groups have reported the identification and characterization of those molecules (28-31) and observed promising anti-cancer activities both in vitro and in vivo (29, 32). However in AML, the implication of the PI3K/Akt signalling pathway in blast cell proliferation (4, 5, 9) and the oncogenic crosstalk between mTOR and PI3K (10) clearly suggest an additional benefit resulting from the simultaneous blockade of PI3K and mTOR catalytic activities.

In our current study, we report for the first time the effects of the dual PI3K and mTOR inhibitor NVP-BEZ235 (33) (Novartis, Basel, Switzerland) in AML. Our data demonstrate that this molecule inhibits PI3K, mTORC1 and mTORC2 activities in both human AML cell lines and in primary AML samples. Moreover, in contrast to rapamycin, NVP-BEZ235 causes a complete blockade of the phosphorylation of the translation repressor 4E-BP1. This results in a marked inhibition of protein translation in AML cells. NVP-BEZ235 also demonstrates potent anti-leukemic activities in AML without affecting normal hematopoiesis ex vivo. Given these results, NVP-BEZ235 shows great promise for testing in clinical trials as a novel AML therapeutic agent.
MATERIALS AND METHODS

PATIENTS
Bone marrow (BM) samples were obtained from 21 patients with newly diagnosed AML (at the exclusion of AML3, AML6 and AML7 phenotypes); all included in various chemotherapy trials initiated by the French Multicenter Group, Groupe Ouest Est des Leucémies et Autres Maladies du Sang (GOELAMS). Normal peripheral CD34+ cells were purified from healthy allogenic donors after informed consent. All biological studies were approved by the GOELAMS Institutional Review Board, and signed informed consent was obtained in accordance with the Declaration of Helsinki.

CELLS CULTURES AND REAGENTS
Blast cells were isolated from bone marrow aspirates from AML patients at diagnosis by a Ficoll-Hypaque density gradient separation. Only bone marrow samples with more than 80% of blast cells were used. Cell viability was assessed by trypan blue assay, and only samples with less than 5% trypan blue-positive cells were further processed. The human leukemic cell lines were purchased from DSMZ (Braunschweig, Germany) for MV4-11 and from ATCC for MOLM-14 and OCI-AML3. We used from 1 to 1000nM NVP-BEZ235 (Novartis, Basel, SW), 10nM rapamycin (Sigma, Saint Louis, MO), 10µM UO126 (Cell Signalling technology, Danvers, MA), 25µM LY294002 (Sigma).

[3H] LEUCINE AND [3H] THYMIDINE INCORPORATION ASSAYS
The global protein synthesis and cell proliferation were assessed by respectively [3H] leucine and [3H] thymidine pulse assays, as reported (25).

7METHYL GUANOSINE CAP AFFINITY ASSAY
7mGTP pulldown experiments were performed as reported (25). Briefly, cell lysates were incubated at 4°C for 2h with 7mGTP-Sepharose beads (Amersham, Piscataway, NJ), then washed and resuspended in boiling Laemmli sample buffer.

POLYSOME ANALYSIS
Polysome analyses were done as reported (25), in the MV4-11 leukemic cell line. After separation through 10%-50% sucrose density gradients, the polysome profile was generated by the continuous measure of the optic density at 254nm.

RNAi

SiRNA targeting human rictor (L-016984-00, Dhharmacon, Chicago, IL) and control non-targeted siRNAs (Dharmacon, Inc., Chicago, IL) were transfected using the AMAXA nucleofector (AMAXA Biosystems, Cologne, Germany) as previously described (25).

WESTERN BLOT

Whole cell extracts and western blots were performed as previously described (25) using antibodies listed in Supplemental Table 1. The images were captured using a CCD camera (LAS3000 from FujiFilm). The signal intensity was quantified using Multigauge software from FujiFilm®

FLOW CYTOMETRY

Apoptosis was quantified by annexinV-PE staining (Becton Dickinson, Le Pont-De-Claix, France) as previously described (34) and cell cycle was assessed using DNA staining with Draq5 (Alexis Biochemicals, Lausen, Switzerland). Detailed cycle analysis of MV4-11 cells was performed by quantifying GO/G1, S and G2/M phases by propidium iodide (PI) staining using CycleTEST™ PLUS kit (Becton-Dickinson) according to the manufacturer recommendations. For the quantification of G0 and M phases, 10⁶ cells were permeabilized with 1mL of ice cold ethanol (1 h, 4°C). Following two washes with PBS, 1% FBS, 0.25% Triton X-100 (PFT), the cells were stained in 200 µL of PFT for 30 min at room temperature in the dark, either with 1 µg of 7-aminoactinomycin D (7-AAD, Sigma) and 5 µL of Alexa Fluor®488-conjugated anti-human Ki67 mAb (B56, Becton-Dickinson), or with 2 µL of Alexa Fluor®488-conjugated anti-phospho(ser10)-histone H3 polyclonal antibody (Cell Signaling Technology, Danvers, MA), respectively.

COLONY ASSAYS

Clonogenic assays of normal CD34+ hematopoietic progenitors

The formation erythroid (BFU-E), granulo-macrophagic (CFU-GM) and granulo-erythroid–megakaryocytic-monocytic (CFU-GEMM) colony forming units from normal CD34+
hematopoietic cells, exposed or not to NVP-BEZ235 during 12h, was assessed as reported (34).

**Colony Forming Unit-Leukemia (CFU-L) assays**

CFU-L assays from primary AML cells exposed or not to NVP-BEZ235 during 12h were performed, as reported (24, 34).

**STATISTICAL ANALYSIS**

Data are expressed as mean values and s.d. Statistical significance of differences observed between experimental groups were determined using Student’s t-test. *, ** and *** mean P<0.05, P<0.01 and P<0.001 respectively. The experiments performed in AML cell lines were done in triplicate. In each case using primary AML cells, independent experiments means i) that the experiments were performed in different days and ii) that cells from different patients were used.
RESULTS

NVP-BEZ235 inhibits PI3K and mTORC1 signalling in AML cells.

The dual PI3K/mTOR inhibitory activity of NVP-BEZ235 was assessed both in human AML cell lines (MV4-11, MOLM-14 and OCI-AML3) and in primary leukemic cells sampled from AML patients at diagnosis. Dose-dependent experiments (from 1 to 1000nM) revealed that PI3K activity is strongly inhibited by NVP-BEZ235 treatment in all three AML cell lines tested. Indeed, Akt phosphorylation on T308, which directly reflects the inhibition of PI3K activity, was suppressed by a 10nM dose of NVP-BEZ235 (Fig. 1A). Moreover, NVP-BEZ235 was found to inhibit P70S6K T389 phosphorylation from 25nM in a similar manner to 10nM rapamycin, reflecting an inhibition of mTORC1 activity (Fig. 1A). In these experiments, the broad-spectrum kinase inhibitor LY294002 was used as a control for the inhibition of Akt T308 and P70S6K T389 phosphorylation (Fig. 1A). Finally, NVP-BEZ235 did not suppress ERK T202/Y204 phosphorylation, in contrast to the specific MEK1/2 inhibitor UO126, and did not decrease Stat5 Y694 phosphorylation in the MV4-11 and MOLM-14 cell lines (the OCI-AML3 cell line is deficient in STAT5 Y694 phosphorylation and was used as a negative control), even at the maximal concentration of 1000nM (Fig. 1A). These results emphasize the specificity of NVP-BEZ235 against class IA PI3K and mTOR activities in AML cells. Based on these results, all subsequent experiments were performed using 10, 100 and 1000nM concentrations of NVP-BEZ235.

In a cohort of 10 primary AML samples, treatment with NVP-BEZ235 was also found to induce a dose-dependent decrease in Akt T308 and P70S6K T389 phosphorylation without affecting the ERK/MAPK pathway (Fig. 1B, left panel and Supplementary Fig. S1). Quantification by western blotting of these 10 AML samples (Fig. 1B right panel) indicated a significantly decreased phosphorylation of P70S6K T389 at all concentrations of NVP-
BEZ235 (decreases from 54% to 90% using 10 to 1000nM NVP-BEZ235. We also determined that NVP-BEZ235 still markedly inhibited P70S6K T\(^{389}\) phosphorylation after a 24 hour incubation in both primary AML cells and the MV4-11 cell line (Supplementary Fig. S2). NVP-BEZ235 thus behaves as a potent cell-permeable PI3K and mTORC1 inhibitor in leukemic cells.

**NVP-BEZ235 inhibits mTORC2 activity in AML cells.**

We further wished to examine mTORC2 activity in AML cells. We first assessed the mTOR autophosphorylation site on S\(^{2481}\) (35, 36), as this closely reflects its catalytic activity in both mTORC1 and mTORC2 complexes (29). Interestingly, we found that in the AML cell lines, NVP-BEZ235 induced a dose-dependent inhibition of mTOR S\(^{2481}\) phosphorylation, in contrast to rapamycin, (Fig. 2A) which suggested that not only mTORC1 but also mTORC2 could be targeted by this compound. The Akt and SGK serine/threonine kinases are the best characterized mTORC2 substrates (27, 29, 37). As expected, NVP-BEZ235 suppressed Akt S\(^{473}\) phosphorylation in a dose-dependent manner (Fig. 2A). In addition, the phosphorylation of FOXO3a at S\(^{253}\), which directly reflects the activation state of Akt on S\(^{473}\), was also inhibited to a similar extent by NVP-BEZ235 (Fig. 2A). The activity of SGK was then assessed by testing the phosphorylation of its substrate NDRG1 on T\(^{346}\) (38). Accordingly, NVP-BEZ235 strongly inhibited NDRG1 T\(^{346}\) phosphorylation (Fig. 2A). Not surprisingly, rapamycin did not inhibit Akt (S\(^{473}\)), FOXO3a (S\(^{253}\)) or NDRG1 (T\(^{346}\)) phosphorylation under the same conditions, which confirms that the allosteric inhibition of mTORC1 does not repress mTORC2 activity in short-term experiments (Fig. 2A).

NVP-BEZ235 also suppressed Akt S\(^{473}\), FOXO3a S\(^{253}\) and NDRG1 T\(^{346}\) phosphorylation in primary AML samples at a dose of 10nM (Fig. 2B, left panel and Supplementary Fig. S3A). Western blotting quantification of the signals from the AML
samples tested indicated a mean decrease from 56% to 83% and from 71% to 89% for the bands detected with the anti-Akt S\textsuperscript{473} (n=8) and anti-NDRG1 T\textsuperscript{346} antibodies (n=5), using 10 to 1000nM NVP-BEZ235, respectively (Fig. 2B; right panel).

It has been established that full Akt and SGK activation also requires PI3K activity (39-41) and to therefore more precisely assess the biochemical activity of the mTORC2 complex in AML cells, we tested the phosphorylation status of paxillin on Y\textsuperscript{118}, reported as an indirect substrate for mTORC2 activity in non-transformed cells (17). We verified that this phosphorylation was indeed dependent on mTORC2 activity in primary AML cells by disrupting the mTORC2 complex using rictor siRNA. As shown in Figure 2C, paxillin phosphorylation on Y\textsuperscript{118} decreased when rictor expression was knocked down by siRNA, thus indicating that paxillin Y\textsuperscript{118} phosphorylation reflects mTORC2 activity in primary AML cells. Interestingly, NVP-BEZ235 induced a dose-dependent decrease in paxillin Y\textsuperscript{118} phosphorylation in primary AML cells (Fig. 2D; left panel and Supplementary Fig. S3B) and the quantified immunoblotting signals from four AML samples indicated a mean decrease of paxillin Y\textsuperscript{118} phosphorylation of 43% and 73% using 100 and 1000nM NVP-BEZ235, respectively (Fig. 2D; right panel). Taken together, these data clearly show that NVP-BEZ235 represses mTORC2 activity in AML cells.

**NVP-BEZ235 induces the complete dephosphorylation of the translation regulator 4E-BP1 in AML cells.**

We have recently shown that the limitations in the anti-leukemic activity of the rapalogs are mainly due to the rapamycin-resistant phosphorylation of the translation regulator 4E-BP1, leading to a deregulation of cap-dependent mRNA translation (25). We thus hypothesized that a direct targeting of mTOR catalytic activity in an FKBP-12-independent manner would induce the inhibition of the oncogenic translation process,
resulting in a more potent anti-leukemic effect. We therefore compared the effects of rapamycin and NVP-BEZ235 upon 4E-BP1 phosphorylation in AML cells. Interestingly, and in contrast to rapamycin, NVP-BEZ235 induced a strong dephosphorylation of 4E-BP1 on the T\(^{37/46}\), T\(^{70}\) and S\(^{65}\) residues in the AML cell lines (Fig. 3A). Similar results were obtained in primary AML samples (Fig. 3B; left panel and Supplementary Fig. S4). Quantification of the western blotting signals obtained from 14 AML samples indicated a mean decrease from 46% to 97% using 10 to 1000nM NVP-BEZ235, respectively (Fig. 3B; right panel). Furthermore, this blockade of 4E-BP1 phosphorylation was sustained after 24 hours of exposure of NVP-BEZ235 (Supplementary Fig. S1). NVP-BEZ235 is thus a potential inhibitor of protein translation in AML.

NVP-BEZ235 inhibits cap-dependent mRNA translation and oncogenic protein synthesis in AML cells.

The phosphorylation of 4E-BP1 is the limiting step in the assembly of the translation initiating complex eIF4F, and is initiated by the interaction between the eIF4E and eIF4G proteins (25). We thus performed \(^7\)mGTP pulldown assays using the MV4-11 cell line to examine whether a 24 hour exposure to NVP-BEZ235 modulates the interaction between eIF4E and eIF4G (active translation) or 4E-BP1 (inactive translation). As shown in Figure 4A, NVP-BEZ235 decreased the levels of eIF4G but increased the amounts of 4E-BP1 associated with eIF4E, indicating an inhibition of eIF4F assembly.

We additionally performed polysome analysis in MV4-11 cells from which we extracted a non-polysomal fraction corresponding to ribosomes not engaged in translation and a polysomal fraction containing the ribosomes undergoing active translation (25). After centrifugation through a sucrose gradient, continuous measurements at a 254 nm optical density revealed three peaks corresponding to 40S and 60S ribosome subunits and 80S
ribosomes, respectively (arrows, Fig. 4B). The polysomes had sedimented below (horizontal two-point arrow, Fig. 4B). Treatment of the MV4-11 cell line with NVP-BEZ235 clearly blocked translation initiation, as indicated by the large to small polysome shift and a concomitant increase in the free ribosome concentration (Fig. 4B). We further performed [3H]-leucine assays, in which the radioactivity levels are proportional to the neo-synthesized protein concentrations. NVP-BEZ235 (from 100nM) markedly decreased the global protein synthesis levels in the MV4-11 cell line and this closely correlated with the suppression of phosphorylation of the translation regulator 4E-BP1 (Fig. 4C). Moreover, the expression of three highly oncogenic proteins known to be regulated at the translation initiation level, c-Myc, Cyclin D1 and Bcl-xL (42), was markedly reduced in these cells after 24 hours of exposure to NVP-BEZ235. These data further demonstrate that NVP-BEZ235 is a potent inhibitor of the translation process in AML cells via the inhibition of the rapamycin-resistant phosphorylation of 4E-BP1.

**NVP-BEZ235 inhibits the proliferation of AML cells**

We investigated the anti-leukemic activity of the NVP-BEZ235 compound and assayed the cell proliferation rates using [3H]-thymidine pulse assays. We consistently observed a dose-dependent decrease in cell proliferation, with an IC50 of 59nM in primary AML cells (n=4), and of 25nM in MV4-11, 45nM in MOLM-14 and 60nM in OCI-AML3 cells (Fig. 5A).

Consistent with its anti-proliferative effects, the cell cycle analysis using PI staining showed a significant increase of the proportion of MV4-11 cells in the G0/G1 phase of the cell cycle and a decrease of the proportion of cells in the S and G2/M phases of the cell cycle upon NVP-BEZ235 treatment (Fig. 5B 1st column). Moreover, using a more detailed cell cycle analysis by 7AAD/Ki67 and phospho(ser10)-histone H3 staining, we observed that
NVP-BEZ235 led to the accumulation of cells in the G0 phase (16-fold increase; Fig. 5B 2nd column) and to a strong reduction of cells in the M phase (7-fold decrease, Fig. 5B 3rd column). Similar results were obtained from the same experiments in MOLM-14 cells (data not shown). We further found by western blotting that NVP-BEZ235 also down-regulates the expression of the cell cycle activators CDK2 and SCF^K2p^ and increases the expression of the cell cycle inhibitor p27kip1 (Fig. 5C).

**NVP-BEZ235 inhibits the clonogenic growth of AML progenitors without affecting normal hematopoiesis.**

We generated clonogenic cultures of primary AML cells and incubated these with 10 to 1000nM NVP-BEZ235. The subsequent levels of clonogenic leukemic colony formation (CFU-L) from three different AML samples were dramatically reduced with a mean decrease from 69% to 96% upon treatment with 10 to 1000nM NVP-BEZ235, respectively (Fig. 6A). In contrast, NVP-BEZ235 barely affected the clonogenic growth and differentiation of normal CD34+ hematopoietic progenitors, even at the maximal concentration of 1000nM. The number of mixed (CFU-GEMM), erythroid (BFU-E) or granulo-monocytic (CFU-GM) colonies was not significantly decreased by NVP-BEZ235 (Fig. 6A).

**NVP-BEZ235 induces apoptosis in AML cells but not in normal CD34+ cells.**

We next determined the impact of NVP-BEZ235 upon AML cell survival. The NVP-BEZ235 was found to induce from a 100nM dose a significant apoptosis response in primary AML samples and in MV4-11 cells, as evidenced by an increase in annexin V staining (Fig. 6B). Conversely, NVP-BEZ235 barely affected the survival of normal immature CD34+ cells, even at the higher concentration of 1000nM (Fig. 6B). The pro-apoptotic effects of NVP-BEZ235 in AML cells correlated with a decreased expression of the anti-apoptotic protein...
MCL-1, suggesting an induction of the mitochondrial apoptotic pathway. We also detected a cleavage of caspase-3 and of its substrate PARP by western blotting in primary AML samples and in MV4-11 leukemic cells (Fig. 6C). Taken together, these results indicate that NVP-BEZ235 preferentially impairs the survival of leukemic cells when compared with normal hematopoietic cells, suggesting a favorable therapeutic index within the hematopoietic system in vivo for this compound.
DISCUSSION

Despite the considerable improvements in our knowledge of AML biology in recent years, the treatment of this disease remains a challenge for clinicians. Major efforts have been made to develop new compounds that target the preferentially activated signalling pathways implicated in AML cell proliferation and survival. The PI3K/Akt/mTOR axis represents a promising therapeutic target in a number of cancers as many components of these signalling pathways are frequently deregulated in tumor cells. We recently showed that a constitutive activation of PI3K is detected in about 50% of primary AML samples at diagnosis (8), mostly due to an autocrine stimulation of the IGF-1 receptor (9). Accordingly, either the blockade of this IGF-1 autocrine production or the specific inhibition of PI3K p110δ activity leads to a strong decrease of AML cell proliferation (4, 5, 9). The constitutive activation of the mTORC1 pathway is detectable in virtually almost all AML patient samples, which emphasized the potential of using mTOR inhibitors as therapeutic agents in these tumors. However, first generation mTOR inhibitors of the rapalog family, which are highly specific mTORC1 allosteric inhibitors, produced heterogeneous results in clinical trials (20). In AML, the therapeutic value of rapalogs appears weak, at least in monotherapy settings (24). One possible strategy to enhance their effectiveness consists on dual inhibition of both the PI3K and mTOR signalling pathways (10, 34). Indeed, although the constitutive activation events for PI3K and mTORC1 are independent (25), the PI3K and mTOR signalling networks are closely related and are subject to complex cross-talk and feedback interactions. In this regard, mTORC1, through the phosphorylation of P70S6K on T389, decreases PI3K activity by inducing the degradation of molecular adaptors of the IRS family (43). Accordingly, the RAD001-mediated inhibition of mTORC1 increases PI3K activity, which contributes to a reduction in the anti-leukemic activity of RAD001 (10). Moreover, both PI3K and mTORC2 regulate the full activity of the oncogenic kinase Akt via the phosphorylation of T308 and S473,
respectively (29, 37). These observations underscore the rationale for a dual PI3K and mTORC1 inhibition strategy to treat AML.

Accordingly, in our current study we tested the anti-leukemic activity of the NVP-BEZ235 compound, an ATP-competitive inhibitor that directly targets the PI3K and mTOR catalytic domains. This compound has already been tested in other cancer models (44, 45) or other hematological malignancies (46-48) and promising preclinical data were reported. Interestingly in AML, NVP-BEZ235 demonstrated marked anti-leukemic effects in vitro as it induced a dose-dependent decrease in AML cell proliferation, inhibited the clonogenic growth of AML progenitors, and significantly induced apoptosis in AML cells. Furthermore, these effects were highly specific to primary AML cells as NVP-BEZ235 did not affect normal hematopoiesis.

As expected, NVP-BEZ235 strongly inhibited the PI3K and mTORC1 signalling pathways which was evidenced by the decrease of Akt T³⁰⁸ and P70S6K T³⁸⁹ phosphorylation, respectively, in AML cells. Moreover, we did not observed an S6K-dependent increase in Akt T³⁰⁸ phosphorylation in NVP-BEZ235-treated AML cells due to the concomitant inhibition of PI3K and mTOR kinases. Furthermore, NVP-BEZ235 did not induce off-target effects against other signalling pathways that are frequently deregulated in primary AML cells, such as ERK/MAPK and Stat5, emphasizing the specificity of this compound against class IA PI3K and mTOR activities. We also demonstrate from our present data that NVP-BEZ235 inhibits mTORC2 activity in AML. This was first suggested by the fact that NVP-BEZ235 fully inhibits mTOR S²⁴⁸¹ phosphorylation, which reflects the mTOR catalytic activity regardless of its association to mTORC1 or mTORC2 complexes. Secondly, NVP-BEZ235 strongly suppresses the phosphorylation of the well characterized mTORC2 substrates Akt S⁴⁷³ and NDRG1 T³⁴⁶ in AML cells. However, Akt and NDRG1 phosphorylation are also dependent on PI3K activity (39-41) and accordingly, the blockade of PI3K by the specific p110δ
inhibitor IC87114 in primary AML cells also suppressed Akt S\(^{473}\) phosphorylation (4, 5). To more precisely elucidate the activity of NVP-BEZ235 against mTORC2, we employed paxillin Y\(^{118}\) phosphorylation as a marker of mTORC2 activity as the siRNA-mediated decrease of rictor expression markedly inhibits this phosphorylation event in primary AML samples. Interestingly, a parallel was observed between decreased Akt S\(^{473}\) and paxillin Y\(^{118}\) phosphorylation in AML cells treated with NVP-BEZ235, which confirmed the inhibition of the mTORC2 activity. Although only recently suggested, the role of mTORC2 in oncogenesis is now established (19). In AML, Zeng and colleagues have shown that mTORC2 controls Akt activation by demonstrating that the disruption of mTORC2 following prolonged exposure to rapalogs inhibits Akt S\(^{473}\) phosphorylation (27). This suggests that mTORC2 inhibition plays a role in the anti-leukemic activity of these molecules (27). We thus speculate that the targeting of mTORC2 may contribute to the anti-leukemic activity of NVP-BEZ235.

The critical mechanism underlying NVP-BEZ235 action, however, may also result from the inhibition of protein translation. We recently emphasized that the weak anti-leukemic activity of the rapalogs was mainly due to the sustained high level of 4E-BP1 phosphorylation in AML cells treated with these compounds (25). Conversely, we show herein that in contrast to rapamycin, NVP-BEZ235 strongly blocks mTOR catalytic activity. This results in the inhibition of 4E-BP1 T\(^{37/46}\) phosphorylation, residues which have been reported to be direct substrates for mTOR catalytic activity (49). As 4E-BP1 phosphorylation follows a hierarchical process in which the priming phosphorylation on T\(^{37/46}\) residues is rate limiting (16, 50), the inhibition of mTOR catalytic activity results in a complete inhibition of 4E-BP1 phosphorylation on S\(^{65}\) and T\(^{70}\). These results thus provide a basis for understanding the differential effects between rapalogs and mTOR catalytic inhibitors toward 4E-BP1 phosphorylation and translation inhibition in AML. Indeed, phosphorylation of the translation regulator 4E-BP1 is the limiting step for the assembly of the translation initiating complexes.
As a consequence of 4E-BP1 dephosphorylation, NVP-BEZ235 markedly inhibits the assembly of eIF4F-initiating complexes and reduces the recruitment of mRNA molecules to polysomes. This results in a global inhibition of protein synthesis and thus a decreased expression of oncogenic proteins regulated at the translation initiation level, e.g. c-Myc, Cyclin D1 and Bcl-xL. As we have recently shown that the deregulation of oncogenic mRNA translation is of major importance in the oncogenic process in AML (25), we now speculate that an important component of the anti-leukemic activity of NVP-BEZ235 in AML is the inhibition of oncogenic mRNA translation.

Overall, our present findings firmly establish that NVP-BEZ235 fulfills all of the biological criteria for rapid clinical testing. Indeed, NVP-BEZ235 inhibits the PI3K, mTORC1 and mTORC2 signalling pathways, leading to the death of AML cells. Moreover, from the perspective of clinical development, NVP-BEZ235 barely impacts upon the differentiation or survival of normal CD34+ hematopoietic progenitors, strongly indicative of a favorable therapeutic index for this molecule in vivo. A phase I/II clinical trial with NVP-BEZ235 is now in progress for advanced solid tumors (ClinicalTrials.gov NCT00620594) and the preliminary data from these trials suggest that the general tolerance of this orally bioavailable molecule is good. We provide here a strong rationale for undertaking clinical trials of NVP-BEZ235 in AML patients also in the very near future.
REFERENCES

FIGURES LEGENDS

Figure 1. NVP-BEZ235 inhibits PI3K and mTORC1 signalling in AML. A. The human leukemic cell lines were cultured during 1h without or with UO126, rapamycin, LY294002 and NVP-BEZ235. Signalling pathways were tested by western blot using specific antibodies. B. Left panel: Primary blast cells from 10 different AML patients were cultured during 1h without or with NVP-BEZ235. Signalling pathways were tested by WB. Right panel: Phospho-P70S6K T389 was quantified and normalized to P70S6K signal intensity in 10 AML samples. Results are expressed as a ratio to the control incubation without NVP-BEZ235.

Figure 2. NVP-BEZ235 is a potent inhibitor of mTORC2 activity in AML cells. A. The human leukemic cell lines were cultured during 1h without or with rapamycin, LY294002 and NVP-BEZ235. Signalling pathways were tested by WB. B. Left panel: Primary blast cells from different AML patients were cultured during 1h without or with NVP-BEZ235. Signalling pathways were tested by WB. Right panel: Phospho-Akt S473 and phospho-NDRG1 T346 were quantified and normalized to Akt and actin signal intensity in respectively 8 and 5 AML samples. Results are expressed as a ratio to the control incubation without NVP-BEZ235. C. AML blast cells from patients #5 and #7 were transfected with Rictor siRNA or control siRNA and then analyzed by WB. D. Left panel: Primary blast cells from 4 different AML patients were cultured during 1h without or with NVP-BEZ235. Signalling pathways were tested by WB. Right panel: Phospho-Paxillin Y118 was quantified and normalized to actin signal intensity in 4 AML samples. Results are expressed as a ratio to the control incubation without NVP-BEZ235.

Figure 3. NVP-BEZ235 induces a complete dephosphorylation of the translation regulator 4E-BP1 in AML cells. A. The human leukemic cell lines were cultured during 1h without or with rapamycin, LY294002 or NVP-BEZ235. Signalling pathways were tested by WB. B. Left panel: Primary blast cells from 14 different AML patients were cultured during 1h without or with NVP-BEZ235. Signalling pathways were tested by WB. Right panel: Phospho-4E-BP1 S65 was quantified and normalized to actin signal intensity in 14 AML samples. Results are expressed as a ratio to the control incubation without NVP-BEZ235.
Figure 4. NVP-BEZ235 inhibits cap-dependent mRNA translation in AML. A. MV4-11 cells were cultured during 24h without or with NVP-BEZ235. 7mGTP affinity assay was then performed and signalling pathways were tested by WB. B. MV4-11 cells were cultured 12h without or with NVP-BEZ235 and were then subjected to polysome analysis. C [3H] leucine pulses were performed to determine global protein synthesis rates in MV4-11 cells cultured without or with NVP-BEZ235. Results are presented as the ratio to the control incubation without inhibitor. D. MV4-11 cells were cultured during 24h without or with NVP-BEZ235. Expression of Cyclin D1, C-myc and Bcl-XL protein was tested by WB.

Figure 5. NVP-BEZ235 inhibits proliferation and cell cycle in AML. A. [3H] Thymidine pulses were performed to determine proliferation rates in primary blast cells from 4 AML samples and human leukemic cell lines cultured without or with NVP-BEZ235. Results are expressed as a ratio between each condition and the control condition. Broken lines indicate NVP-BEZ235 IC50. B. Detailed cell cycle analysis was performed on MV4-11 cells exposed during 24h to NVP-BEZ235. One representative experiment (n=3) is shown. C. Primary AML cells and MV4-11 cells were cultured during 24h without or with NVP-BEZ235. Expression of Skp2, CDK2 and p27 proteins was tested by WB.

Figure 6. NVP-BEZ235 inhibits the clonogenic growth of AML progenitors and induces apoptosis in AML cell without affecting normal hematopoiesis. A. Primary blast cells from 3 AML samples were cultured with or without NVP-BEZ235. After 7 days incubation, CFU-L were scored under inverted microscope. Normal CD34+ cells purified from 3 healthy donors were plated without or with NVP-BEZ235. The BFU-E, CFU-GM, and CFU-GEMM colony forming units were counted under an inverted microscope. Results are expressed as a ratio between each condition and the control condition. B and C. Primary blast cells from 4 AML samples, normal CD34+ cells from 3 healthy donors and MV4-11 cells were cultured 48h without or with NVP-BEZ235. Apoptosis was determined by annexinV binding in flow cytometry. D. Primary blast cells and MV4-11 cells were cultured during 24h without or with NVP-BEZ235. Expression of MCL-1, Caspase 3 and PARP proteins was tested by WB.
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<td></td>
<td>1</td>
<td>10</td>
</tr>
<tr>
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**MV4-11**

**MOLM-14**

**OCI-AML3**
2B

BEZ-235 (nM) [1h]

0 10 100 1000

p-mTOR S2481
mTOR
p-Akt S473
Akt
P-NDRG1 T346
Actin

AML#12

Ratio to Control

n=8

n=5

p-Akt S473
p-NDRG1 T346

Control 10nM 100nM 1000nM

*** *** *** ***
**2C**

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<td>Control siRNA</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Rictor siRNA</td>
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<td>+</td>
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- rictor
- p-Paxillin Y118
- actin

**2D**

**BEZ-235 (nM) [1h]**

- p-Akt S473
- Akt
- p-Paxillin Y118
- Actin

**Ratio to Control**

- n=4
- Control
- 10nM
- 100nM
- 1000nM

- *p < 0.05
- **p < 0.01

AML#9
4C

[Bar chart showing [3H] leucine incorporation (Ratio to Control) for MV4-11 cells treated with BEZ-235 (nM) [2h].

- **BEZ-235 (nM) [2h]**
  - 0
  - 10
  - 100
  - 1000

- **[3H] leucine incorporation**
  - 0
  - 0.2
  - 0.4
  - 0.6
  - 0.8
  - 1
  - 1.2

4D

- **BEZ-235 (nM) [24h]**
  - 0
  - 10
  - 100
  - 1000

- **Protein expression**
  - Cyclin D1
  - c-Myc
  - Bcl-xL
  - actin

MV4-11
**5B**

**Control**
- Ap: 6.5%, G0/G1: 60.4%, S: 17.9%, G2/M: 15.2%

**BEZ235 10nM**
- Ap: 6.9%, G0/G1: 63.6%, S: 17.1%, G2/M: 12.4%

**BEZ235 100nM**
- Ap: 15.3%, G0/G1: 76.5%, S: 5.8%, G2/M: 2.4%

**BEZ235 1000nM**
- Ap: 26.5%, G0/G1: 64.1%, S: 7.0%, G2/M: 2.4%
**BEZ-235 (nM) [24h]**

- **Actin**
- **p27kip1**
- **CDK2**
- **Skp2**

**AML#12**

**MV4-11**

- 0 10 100 1000
- 0 10 100 1000
AML# 325

MV4-11

FS Control

BEZ-235 1000 nM

Annexin V

SS

FS

25%

51%

53%

4%
Dual inhibition of PI3K and mTORC1/2 signalling by NVP-BEZ235 as a new therapeutic strategy for acute myeloid leukemia

Nicolas Chapuis, Jerome Tamburini, Alexa S Green, et al.

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