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Original research

Oncogene-independent resistance in Philadelphia chromosome - positive (Ph^+) acute lymphoblastic leukemia (ALL) is mediated by activation of AKT/mTOR pathway

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Abstract

Tyrosine kinase inhibitors (TKIs) such as imatinib, nilotinib, dasatinib, and ponatinib have significantly improved the life expectancy of Philadelphia chromosome-positive (Ph^+) acute lymphocytic leukemia (ALL) patients; however, resistance to TKIs remains a major clinical challenge. Point mutations in the tyrosine kinase domain (TKD) of BCR-ABL1 have emerged as the predominant cause of acquired resistance. In approximately 30% of patients, the mechanism of resistance to TKIs remains elusive. This study aimed to investigate mechanisms of nonmutational resistance in Ph^+ ALL. Here we report the development of a nonmutational resistance cell line SupB15-RT; conferring resistance to approved ABL kinase inhibitors (AKIs) and allosteric inhibitors GNF-2, ABL001, and crizotinib, except for dasatinib (IC90 50nM), a multitarget kinase inhibitor. We found that the AKT/mTOR pathway is activated in these cells and their proliferation inhibited by Torin-1 with an IC50 of 24.7 nM. These observations were confirmed using 3 different ALL patient-derived long term cultures (PDLTCs): (1) HP (BCR-ABL1 negative), (2) PH (BCR-ABL1 positive and responsive to TKIs) and (3) BV (BCR-ABL1 positive and nonmutational resistant to TKIs). Furthermore, Torin-1 and NVP-BEZ235 induced apoptosis in PH and BV cells but not in HP cells.
Our experiments provide evidence of the involvement of AKT/mTOR pathway in the evolution of nonmutational resistance in Ph^+ ALL which will assist in developing novel targeted therapy for Ph^+ ALL patients with BCR-ABL1 independent nonmutational resistance.

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Introduction

The Philadelphia chromosome (Ph) is the der(22) of the reciprocal translocation t(9;22)(q34;q11), which leads to the formation of the chimeric BCR-ABL1 fusion gene, and encodes the breakpoint cluster region andabelson tyrosine kinase (BCR-ABL1) fusion protein (1,2). It is the oncogenic driver in 95% of chronic myeloid leukemia (CML) and approximately 30% of adult acute lymphoblastic leukemia (ALL) patients (3). Ph^+ ALL is an acute leukemia from the onset and is characterized by leukemic blasts with a differentiation block at the prelymphatic stage. Patients suffering from acute disease constitute a high-risk group with a poor prognosis and general resistance to therapy.

Tyrosine kinase inhibitors (TKI) such as imatinib, designed to target BCR-ABL1, have resulted in durable cytogenetic and molecular remissions in most early CML patients and increased remission rates and survival in Ph^+ ALL patients (4). Unfortunately, resistance to imatinib occurs frequently in Ph^+ ALL, and the outcome remains dismal in these patients (5,6). Several next-generation TKIs, like nilotinib, dasatinib, and ponatinib were developed

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to address TKI resistance and intolerance (7). The outcome of Ph+ ALL patients has improved significantly with the incorporation of TKIs into chemotherapy and improved survival in young adults (8–10). This combined approach has resulted in the achievement of a complete response (CR) in >90% of patients with newly diagnosed Ph+ ALL. However, survival is impaired by the relapse after initial response (11). The 5-year disease-free survival and overall survival (OS) rates were 43% when imatinib was combined with intensive therapy (12). When dasatinib was combined with intensive chemotherapy, the 3-year event-free survival and OS rates were 55% and 69%, respectively. In case of ponatinib a complete molecular response was increased to 74% in Ph+ ALL patients (13).

Despite the development of the advanced TKIs several TKI resistance mechanisms, including BCR-ABL1 mutations, have been identified. However, in many patients who relapse, the mode of TKI resistance remains elusive (14). In most cases, continuous exposure to TKIs selects for resistant clones, particularly in advanced disease, that is, CML-BC and Ph^+ ALL. This resistance is attributable to either point mutations in the BCR-ABL1 kinase domain or a nonmutational mechanism which continues to pose a clinical challenge (15).

Allosteric inhibition of BCR-ABL1 is another approach of targeting BCR-ABL1 in CML and Ph^+ ALL. Different BCR-ABL1 allosteric inhibitors for example, GNF-2, crizotinb and ABL001 have been identified or developed (16,17) which binds to/target myristoyl-binding pocket and functions as nonadenosine-5'-triphosphate-competitive inhibitor of BCR-ABL.

Ph^+ ALL patients with no known BCR-ABL1 kinase domain mutations unresponsive to multiple TKI treatments represent a population of ALL patients with BCR-ABL1-independent resistance. The treatment options for such patients are limited. In the PACE trial, only 27% of "resistant/intolerant" patients treated with ponatinib, a third-generation TKI, achieved major molecular response (MMR) (18). A genetic study has shown that this type of resistance can vary between patients, often proposing re-activation of signaling pathways involved in the pathogenesis of Ph^+ leukemia (19).

BCR-ABL1 fusion kinase constitutively activates multiple downstream signaling pathways resulting in aberrant proliferation and inhibition of apoptosis. These pathways include RAS/MAPK, JAK/STAT, PI3K/AKT, and MYC (1,2,20).

The PI3K/AKT is a major survival and growth pathway commonly implicated in the pathogenesis of various malignancies (21). It is a downstream signaling network which in normal physiological conditions relays signals from receptor tyrosine kinases, cytokine receptors, integrins, and G protein-coupled receptors on the binding of ligands such as hormones, cytokines and growth factors (22).

PI3K/AKT/mTOR is downstream of BCR-ABL1 and has a crucial role in BCR-ABL1 mediated leukemogenesis (23). Constitutive activation of this pathway has been shown to be involved in the development of resistance of BCR-ABL1 expressing cells to TKIs (24). Activation of PI3K results in the phosphorylation of AKT, which in turn activates mTOR, a distal element of the PI3K/AKT/mTOR pathway (25). mTOR is a serine/threonine kinase that comprises of 2 different complexes, mTORC1 and mTORC2 that vary in their substrate specificity and functionality (25). mTORC1 induces cell growth in response to nutrients and growth factors by regulating the translational regulators S6K1 and 4E-BP1, whereas mTORC2 mediates cell proliferation and survival by phosphorylating AKT (25,26).

The mechanism of TKIs resistance in CML has been intensively investigated while the mechanism of treatment-resistance in Ph^+ ALL has been for the most part ignored. The aim of this study was to determine the mechanism of BCR-ABL1-independent andnonmutational resistance in Ph^+ ALL and identify potential molecular pathways that can be targeted pharmacologically. In this paper, we demonstrate the role of AKT/mTOR pathway in the evolution of nonmutational resistance in Ph^+ ALL.

Material and methods

Cell lines and cell culture

All cell lines were obtained from our lab stocks generated from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) vials. K562, Nalm-6 and Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Gibco/Invitrogen, Karlsruhe, Germany) while SupB15 cells were grown in RPMI 1640 containing 20% FCS. To select for the imatinib-resistant phenotype, SupB15 cells were cultured with gradually increasing concentrations of imatinib for 6 mo as described previously by our group (27).

Ph^+ and Ph^- ALL patient-derived long-term cultures (PDLTCs) (HP, PH, and BV) were maintained in a serum-free medium as described previously (28).

Inhibitors/compounds

The details of the compounds andinhibitors used are listed in Table S1a. All these compounds were dissolved in dimethyl sulfoxide (Sigma, Steinheim, Germany) to a 1000X stock solution, which were further diluted to 1X working concentrations for the experiments.

Proliferation/cytotoxicity assay and apoptosis assay

The effect of different inhibitors on cellular proliferation of SupB15 WT/RT and PDLTCs was measured using XTT cell proliferation assay kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. Briefly, 20,000 and 50,000 cells were plated in 96-well plates in the presenceor absence of the inhibitors, either alone or in combinations, at various concentrations as listed in Table S1a for 72 and 96 h (SupB15 WT/RT and PDLTCs respectively) in their specified medium. For control, medium was added to the cells containing only vehicle.

The cell growth was assessed by counting cells using hemocytometer with dye exclusion (dead cells) as described previously (29). The apoptosis was evaluated by the 7-amino-actinomycin D (7-AAD) (30,31) and Annexin-V staining methods as previously described (31). The IC50 was calculated using CalcuSyn software (Biosoft, Cambridge, United Kingdom).

Western blotting/immunoblotting

Western Blotting was performed as described in Sambrook's protocols (32) using Bio-Rad WB system; the details of antibodies used is detailed in Table S1b. The lysates were prepared using phospho-lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA pH 8.0, 100mM Sodium Fluoride, 10mM Sodium Pyrophosphate, 200 μM Sodium Ortho Vanadate, 10% Glycerol, 1% Triton X and 1x Protease inhibitor (Roche, Basel, Switzerland) after overnight incubation of the test cell lines with the inhibitors and/or drugs. Blocking was performed in 5% low-fat dry milk and antibody incubation in 0.5% low-fat dry milk. Washing was carried out in Tris-buffered saline (TBS) (10mM Tris-HCl pH 8.0, 150 mM NaCl) containing 0.1% Tween-20 (TBS-T). The blots were stained with ECL (Amersham Biosciences, Bucks, United Kingdom) and visualized using ChemiDoc imaging system (Bio-Rad Laboratories Inc., Hercules, USA).

Karyotyping/M-FISH

Multicolor FISH was performed using the 24XCyte MetaSystems color kit (MetaSystems, Altlussheim, Germany) to identify precisely complex chromosomal rearrangements in SupB15-WT and SupB15-RT cells according to the manufacturer's recommendations (33).

Immunophenotyping

Expression of surface markers was analyzed using phycoerythrin (PE)–conjugated antihuman CD19 and CD33 (Becton Dickinson [BD], Heidelberg, Germany), and fluorescein isothiocyanate (FITC)–conjugated anti-human CD34 and CD38 (BD) specific monoclonal antibodies. Control staining was performed using corresponding isotype control antibodies (BD).

Detection and analysis of the fluorescent-labeled cells was performed using the FACSCanto II system and FACS Diva software (BD).

Real-time PCR

Total RNA was isolated by TRI Reagent method according to manufacturer's instructions (34). After RNA isolation, all samples were treated with rDNase I (Life Technologies, South San Francisco, CA) for 30 min. Later, extracted RNA was reverse transcribed to cDNA using SuperScript IV VILO (Invitrogen, Karlsruhe, Germany) as per manufacturer's recommendations. The TaqMan real-time PCR was conducted in triplicates following standard protocols using the Quant Studio V (Applied Biosystems, Darmstadt, Germany). The master mix for qPCR used was Takyon Low Rox Probe 5X MasterMix dTTP (Eurogentics, Belgium). The RT-PCR program consisted of an initial cycle of 50°C for 02 min and 95°C for 03 min followed by 40 cycles at 95°C for 10 sec and at 60°C for 1 min. The details of the primers and probes for the expression of BCR-ABL1 gene in SupB15-WT, SupB15-RT, PH, and BV cells are described in Table S1c (35). The Ct values were exported into a Microsoft Excel worksheet for calculation of fold changes according to the comparative Ct method. The expression of the target normalized to endogenous ABL1 gene is given by $2^{-\Delta\Delta Ct}$ (36).

Sequencing

For the detection of mutations in the ABL1 kinase domain, RNA was extracted from SupB15-WT, and SupB15-RT cells using QIAamp RNA Kit (Qiagen, Düsseldorf, Germany) and cDNA was synthesized using SuperScript IV VILO (Invitrogen, Karlsruhe, Germany) kit as mentioned earlier. For exclusive BCR-ABL1 kinase domain amplification the primers listed in Table S1c were used (37,38).

PCR products were sequenced by Seqlab (Göttingen, Germany) using the AN4 (Table S1c) (38,39). The sequence data were analyzed for mutations with Clone Manager Professional (Sci ED Software, Morrison, NC, USA).

Statistical analysis

Experiments were performed 3 times, and the means ± SD of triplicates from one representative experiment out of 3 performed are shown. Statistical analyses were carried out using GraphPad Prism5 (GraphPad Software Inc. San Diego, CA).

Results

Nonmutational resistance model

To investigate the mechanisms of oncogene independent resistance, we used a previously developed model for nonmutational resistance with acquired BCR-ABL1-independent resistance and aimed to characterize it further (27).

As shown in Figure 1A and Table S2, the SupB15-WT cells were inhibited by imatinib with an IC50 of 180 nM, whereas the resistant cell line SupB15-RT cells were able to grow in up to 10 μM imatinib (Fig. 1B). SupB15-RT cells showed cross-resistance against all approved TKIs (imatinib, nilotinib, ponatinib) except dasatinib (IC50 90 nM), (Fig. 1B). These inhibitors efficiently inhibited the proliferation of SupB15-WT cells in a dose-dependent manner (IC50 7.1-180 nM) (Fig. 1A and Table S2).

We further investigated the growth inhibitory effect of 3 allosteric inhibitors (GNF-2, ABL001 and crizotinib) () on SupB15-WT and SupB15-RT. Both the cell lines were grown in increasing concentrations of the 3 inhibitors. SupB15-RT cell line was resistance to all these inhibitors, whereas the SupB15-WT cell line growth was inhibited (IC50 12-405 nM) (Fig. S1A-B and Table S2).

We then went on to determine if the resistance of SupB15-RT cells is oncogene (BCR-ABL1) independent. Western blots were performed to evaluate the kinase activity of BCR-ABL1 using ABL1 and phospho-ABL1. Effect on cellular proliferation was undertaken by XTT assay.

We found that imatinib abrogated the kinase activity of BCR-ABL1 in SupB15-WT and SupB15-RT cells but had no effect on the proliferation of the SupB15-RT cells. In contrast, the growth of SupB15-WT cells was completely inhibited at this concentration (Fig. 1C and D). This strongly suggested that the mechanism of resistance observed in the SupB15-RT was oncogene independent.

SupB15-RT cells have a novel mechanism of resistance

The mechanisms of drug resistance mechanisms include aberrant karyotypes or additional translocation, deletion or insertion, ABL kinase domain mutation, or oncogene amplification (29,40-43).

We set out to determine if the resistance observed in the SupB15-RT cells was conferred by one of the known mechanisms or a novel mechanism.

We undertook chromosomal karyotyping on single-cell genomes using multicolor FISH (M-FISH) technology and found that SupB15-RT cells were identical to parental SupB15-WT cells when karyotyped (Fig. 2A).

To investigate whether any BCR-ABL1 kinase domain mutation is present in SupB15-RT cells that could confer resistance to these cells, the BCR-ABL1 kinase domain was sequenced using Sanger sequencing method and it was found that SupB15-RT cells do not carry any BCR-ABL1 mutations (Fig. 2B).

To determine if the BCR-ABL1 gene was amplified in SupB15-RT, TaqMan Real-Time PCR and western blot analysis using BCR-ABL1 specific antibody were performed. As shown in Figure 2C and D, no difference was found in oncogene expression between SupB15-WT and SupB15-RT cells (Fig. 2C and D).

Flow cytometric, comparative immunophenotypic analysis of SupB15-RT and SupB15-WT cells, were performed using CD19, CD34, CD38, and CD33 antibodies. As shown in Figure 2E, SupB15-RT cell line displayed a leukemic immune phenotype that was similar to SupB15-WT cells in terms of B cell marker, for example, CD19 (>99%) and mature lymphocytic marker for example, CD38 (~0%). Expression of CD34 was, however, reduced to one third in SupB15-RT cells. The expression of myeloid marker CD33 was increased in SupB15-RT cells (27.02%) when compared to SupB15-WT cells (1.19%) (Fig. 2E).

Taken together, these data show that both SupB15-WT and SupB15-RT cell lines have identical karyotypes, BCR-ABL1 kinase domain sequence and oncogene expression suggesting that the SupB15-RT cells might have a novel mechanism of acquired resistance. The only difference we observed was in the expression of hematopoietic stem cell marker (CD34) and myeloid differentiation marker (CD33).

SupB15-RT cell line showed constitutive activation of AKT/mTOR pathway

To investigate the potential mechanisms of resistance in SupB15-RT cells, a targeted screening was carried out by western blot analysis using anti-

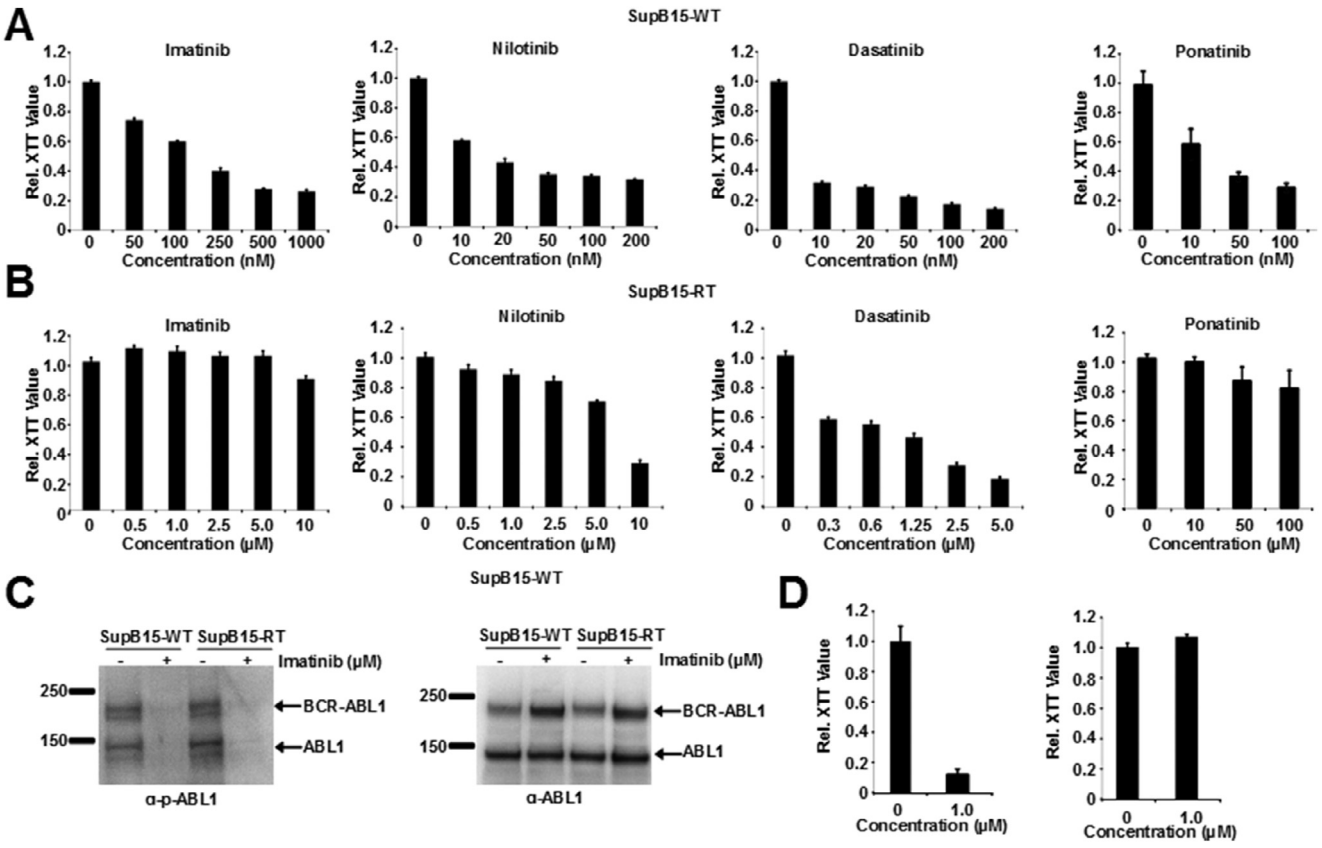

Fig. 1. Development of nonmutational resistance model. (A) SupB15-WT cells were exposed to TKIs, 0, 50, 100, 250, 500 and 1000 nM imatinib, 0, 10, 20, 50,100 and 200 nM niloatinib, 0, 10, 20, 50,100 and 200 nM dasatinib, 0, 10, 50,100, 200 nM ponatinib and XTT assay was performed. (B) XTT assay for SupB15-RT cells upon exposure to TKIs, 0, 0.5, 1, 2.5, 5 and 10, μM imatinib, 0, 0.5, 1, 2.5, 5 and 10 μM niloatinib, 0, 0.3, 0.6, 1.25, 2.5 and 5 μM dasatinib, 0, 10, 50 and100 nM ponatinib (C) Western blot analysis of lysates of SupB15-WT and SupB15-RT cells treated with 1μM imatinib using antibodies directed against: Y245-ABL and ABL1. Molecular mass references (KDa) are presented. (D) XTT assay for SupB15-WT and SupB15-RT cells upon exposure to 1 μM.

CASPASE-9, CASPASE-3, BCL-xL, PARP, phospho-4E-BP1, phospho-AKT, phospho-PI3K, phospho-LYN, phospho-STAT5, phospho-ERK1/2, α-TUBULIN, and β-ACTIN (Fig. 3A-C, S2A and data not shown). As shown in Figure 3A and B, 4E-BP1 and AKT were activated in SupB15-RT cells revealing the constitutive activation of the AKT/mTOR pathway. To validate whether AKT/mTOR pathway was central in developing acquired nonmutational resistance, SupB15-WT and SupB15-RT cells were treated with increasing concentration of imatinib and western blot was performed using phospho-AKT, AKT, phospho-STAT5, STAT5, phospho-PI3K, PI3K, phospho-LYN, LYN, phospho-ERK1/2, ERK1/2. We found that inhibiting BCR-ABL1 with imatinib led to the abrogation of AKT in SupB15-WT cells but not in SupB15-RT cells. Constitutive activation of SRC kinases like LYN, JAK/STAT and RAS/MAPK pathways were not observed in either SupB15-RT or -WT cells. Although PI3K is an upstream component of the AKT/mTOR pathway, surprisingly its activation was not observed in the resistant cell line model, suggesting a feedback mechanism of AKT activation (Fig. 3C and S2A)

Collectively, these data show that the AKT/mTOR pathway is constitutively activated in the SupB15-RT cells suggesting its involvement in acquired nonmutational resistance.

Dual mTORC1 & C2 inhibitor (Torin-1) can overcome nonmutational resistance in SupB15-RT cell line

Constitutive activation of the AKT/mTOR cascade in SupB15-RT cells prompted us to investigate the mechanism of this pathway activation. Constitutive activation of AKT has been attributed to either PI3K signaling or autonomously by mTOR complex 2 (mTORC2) (44). Furthermore, increased phosphorylation of 4E-BP1, a downstream target of mTORC1, was also observed in SupB15-RT cells. To dissect the level at which the AKT/mTOR signaling is activated and to determine the mechanism of AKT activation, we used 2 pharmacological inhibitors with distinct inhibitory properties: Torin-1, a combined mTORC1/C2 inhibitors and NVP-BKM120, a selective PI3K inhibitor (31).

We found that Torin-1 inhibited cellular proliferation in a dose-dependent manner with an IC-50 of 21nM in SupB15-WT and 24.7nM in SupB15-RT cells with a maximum inhibition of cell growth at 100nM in both the lines (Fig. 4A). In contrast, no effect on proliferation was observed in SupB15-RT cell line while SupB15-WT cells showed minor response when treated with NVP-BKM120 (Fig. 4B).

To investigate whether the antiproliferative effect of mTOR inhibition occurs in conjunction with dephosphorylation of the 4E-BP1, SupB15-WT and SupB15-RT cells were exposed to increasing concentrations of Torin-1 and western blot analysis was performed using anti-ABL1, anti-phospho-4E-BP1 and α-TUBULIN antibodies. As depicted in Figure 4C, Torin-1 inhibited phosphorylation of 4E-BP1 in a dose-dependent manner in both the cell lines.

As dasatinib and Torin-1 independently inhibited the growth of SupB15-RT cells, we examined the effect of dasatinib and Torin-1, alone and in combination on SupB15-WT and SupB15-RT. As shown in Figure 4D, dasatinib and Torin-1 inhibited the growth of SupB15-WT and RT cells in an additive manner.

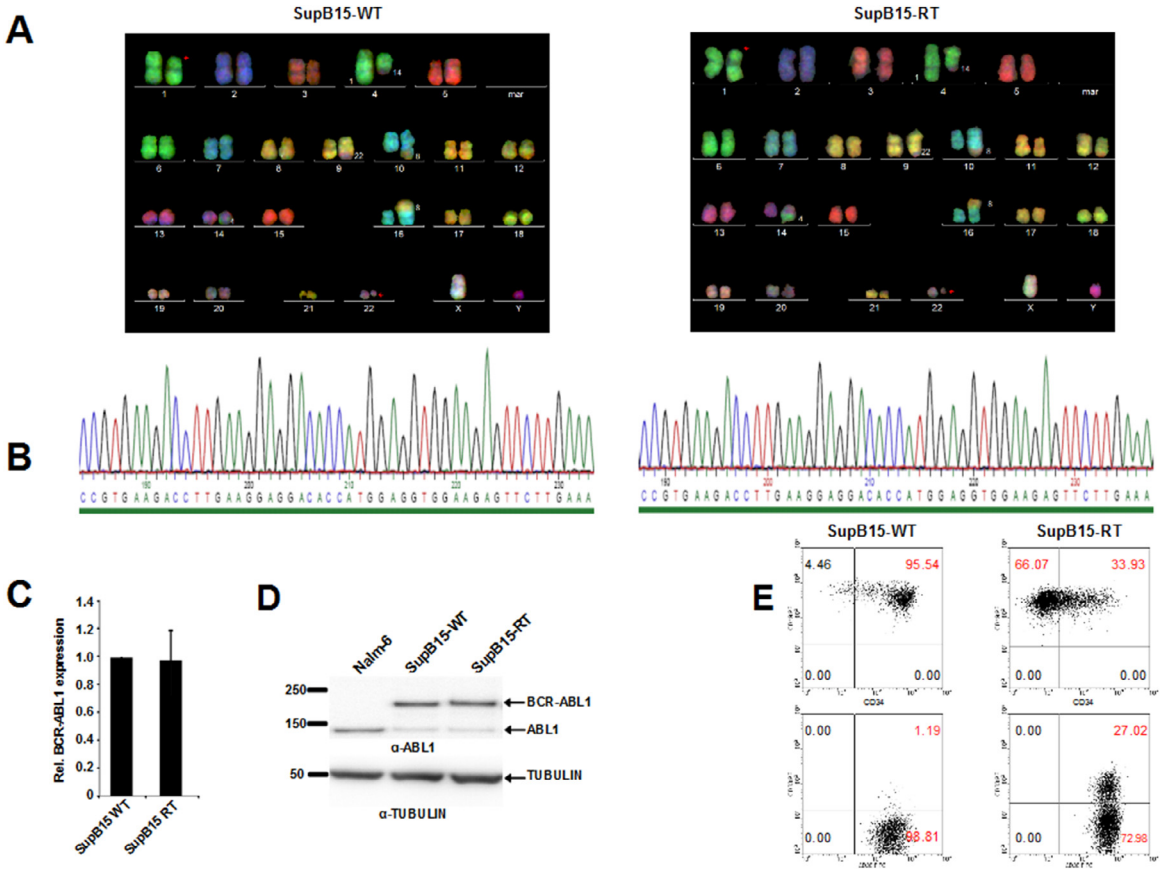

Fig. 2. Karyotyping, ABL kinase domain mutational profile, BCR-ABL1 expression and immunophenotypes of SupB15-WT and SupB15-RT (A) Multicolor FISH was performed on SupB15-WT and SupB15-RT cells (B) Sanger sequence was performed for the detection of primary mutations, as well as of additional mutations using cDNA from SupB15-WT cells and SupB15-RT cells. (C) Total RNA was isolated from SupB15-WT and SupB15-RT cells. The expression levels of BCR-ABL1 was analyzed using q-RT-PCR. The Ct values were normalized to that of ABL gene and results are represented as $2^{-\Delta\Delta Ct}$. (D) Western blot analysis of lysates of SupB15-WT and SupB15-RT using antibodies directed against: ABL1. Molecular mass references (KDa) are presented, and α-tubulin was used as a loading control. (E) Immunophenotypes of SupB15-WT and SupB15-RT were determined by FACS using CD34, CD19, CD38 and CD33 antibodies.

AKT/mTOR pathway is activated in ALL patient-derived long term cultures (PDLTCs) with nonmutational resistance

The biology of Ph+ ALL is not fully represented by cell lines. To further validate the findings in more clinically relevant models, we utilized a unique culture system for primary cells from Ph− and Ph+ ALL patients (28,31,45,46). Two PDLTCs were used (i.e., PH and BV) that express p185 $^{BCR\text{-}ABL1}$ and present a different response to TKIs without any known BCR-ABL1 kinase domain mutations. To avoid the bias of nonspecific toxicity of TKIs, the HP, a Ph− PDLTCs, was used as a negative control. These cells were treated with different concentrations of imatinib, GNF-2 and ponatinib and cytotoxicity or proliferation was assessed by XTT assay. We found that BV cells were resistant to all 3 inhibitors while PH cells responded well (Fig. 5A, B and C and Table S2).

To address the question, of whether BV cells display the same AKT/mTOR activation pattern observed in SupB15-RT cells we examined the AKT/mTOR pathway in PH (TKIs sensitive) and BV (TKIs resistant) cells by western blot analysis using specific phospho-AKT-Ser473 (which was used in SupB15-RT cells), a master kinase in AKT/mTOR pathway, and total AKT antibodies. As depicted in Figure 5D, AKT activation was significantly more pronounced in BV cells compared to PH and HP cells.

These data suggest that BV has nonmutational resistance similar to our *in vitro* generated SupB15-RT cell line.

Inhibition of mTOR can overcome nonmutational resistance in PDLTCs

To investigate whether inhibition of the AKT/mTOR pathway can arrest the growth of TKI responsive and resistant PDLTCs. We exposed HP, PH, and BV cells to NVP-BKM120, NVP-BEZ235 (dual mTOR and PI3K inhibitor) and Torin-1 and performed XTT assay to dissect the role of AKT/mTOR pathway activation in the proliferation of the BV cells. Inhibition of mTOR by Torin-1 and NVP-BEZ235 efficiently arrested the growth of all PDLTCs including BV cells at low concentrations (IC50 < 100 nM). At the same time, the selective PI3K inhibitor NVP-BKM120 did not affect the growth of these cells (Fig. 6A-C and Table S2).

These data show that the activation of the AKT/mTOR pathway is indispensable for the growth of resistant BV cells confirming its exclusive role in resistance independent of PI3K.

Further, the effect of Torin-1 was investigated in combination with dasatinib (second-generation TKI) on PDLTCs. These cells were treated with different concentration of Torin-1 and dasatinib (i.e., 0, 10, 50, and 100 nM). Interestingly, dasatinib alone was enough to completely abrogate the growth of PH and BV cells, while HP cells did not show significant response

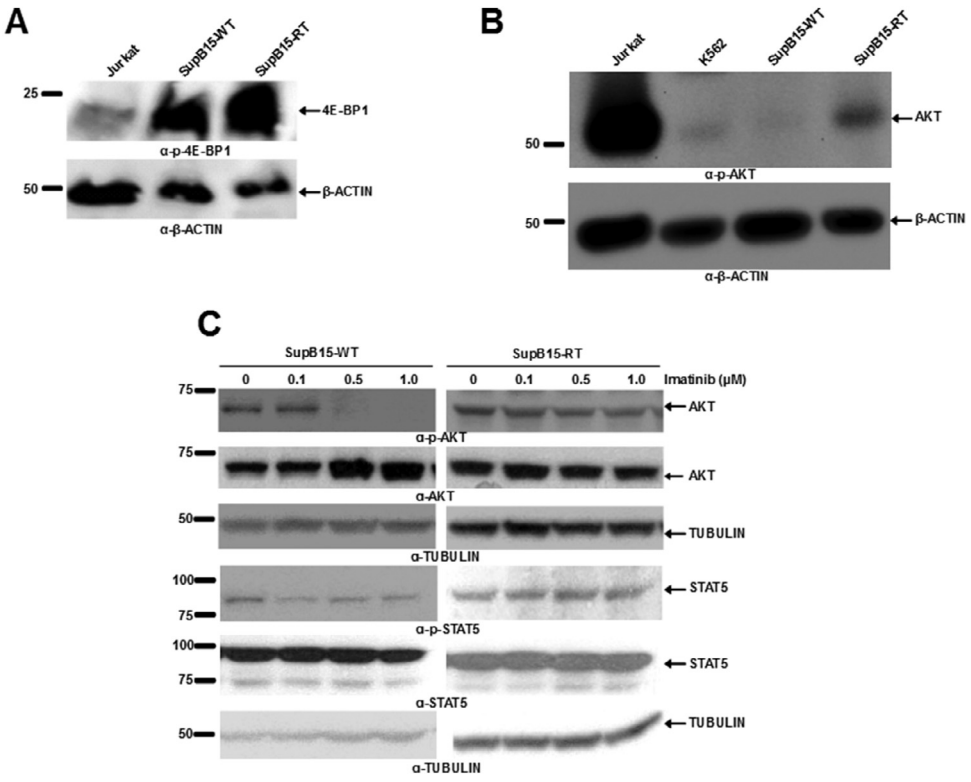

Fig. 3. Role of mTOR/AKT pathway in nonmutational resistance acquired by SupB15 cells (A) Western blot analysis of lysates of SupB15-WT and SupB15-RT using antibodies directed against: phospho-4E-BP1. Molecular mass references (KDa) are presented, and β-actin was used as a loading control. (B) Western blot analysis of lysates of SupB15-WT and SupB15-RT treated with imatinib (0, 0.1, 0.5, and 1μM) using antibodies directed against: phospho-AKT serine 473, AKT, phospho-STAT5 and STAT5. Molecular mass references (KDa) are presented, and β-actin was used as a loading control. (C) Western blot analysis of lysates of SupB15-WT and SupB15-RT using antibodies directed against: phospho-AKT serine 473 and AKT. Molecular mass references (KDa) are presented, and β-ACTIN was used as a loading control. Western blot analysis of lysates of SupB15-WT and SupB15-RT either untreated or treated with imatinib (0.1, 0.5, and 1μM) using antibodies directed against: phospho-AKT serine 473, AKT, phospho-STAT5 and STAT5. Molecular mass references (KDa) are presented, and β-ACTIN was used as a loading control.

to dasatinib. The combination of dasatinib and Torin-1 inhibited the growth of BV cells in an additive manner. Torin-1 was able to inhibit the proliferation in HP cells but in combination with dasatinib no added effect was observed (Fig. 6D-F).

We went on to determine if Torin-1 and NVP-BEZ235 induced apoptosis in the PDLTCs (HP, PH and BV lines). PDLTCs were treated with increasing concentrations of Torin-1 and NVP-BEZ235. Apoptosis was assessed by 7-AAD and Annexin-V after 96 h. We observed that increasing concentrations of Torin-1 and NVP-BEZ235 induced apoptosis in PH and BV cells but were not able to induce apoptosis in either HP cells (Fig. S2B).

Discussion

In the current study we hypothesized that the Ph+ ALL patients who experience poor response to TKI treatment, have short survival and present no known mechanisms of resistance might share an alternative drug target or pathway that can be inhibited with a novel compound.

We therefore used a previously developed method to generate imatinib resistant cell line SupB15-RT (27,47). We demonstrated that SupB15-RT conferred BCR-ABL1-independent resistance against TKIs and allosteric inhibitors except for dasatinib, a multitarget ABL/SRC kinase inhibitor. The effect of dasatinib on the proliferation of SupB15-RT cells may be attributed to the fact that dasatinib, originally developed as an SRC-kinase inhibitor, not only inhibits the BCR-ABL1 kinase but also targets a broader range of kinases compared to imatinib and nilotinib, the inhibition spectrum of which is mainly limited to ABL, c-KIT and PDGFR (7,45). Additionally, dasatinib exclusively targeted ACK1, ALK1/ACVRL1, BRK, BTK, KHS/MAP4K5, SIK1, SIK2, and TEC kinases in a cell-free kinase assay while ponatinib, a potent and multitarget kinase inhibitor is unable to target them (39). These kinases may have a role in the resistance developed by SupB15-RT cells. The role of these kinases in BCR-ABL1 independent resistance needs further investigation.

Approximately 80% of patients with Ph+ ALL in relapse conferring resistance to imatinib have BCR-ABL1 mutations, with a predominance of P-loop and T315I mutations (38,48). SupB15-RT exhibited similar properties as SupB15-WT, but the only difference found was that SupB15-RT had lost the expression of CD34 while the expression of CD33 was increased in these cells. These findings are in line with our previous studies where SupB15 cells lost expression of CD133, another stem cell marker, after treatment with imatinib or nilotinib for an extended period (49). CD33 is a marker for myeloid cells. It is also expressed in myeloid leukemia and in > 55% of AML patients. The AKT/mTOR pathway is also found to be constitutively active. Together this suggests a direct correlation of CD33 expression, with AKT/mTOR activation (50,51). BV cells which demonstrated a resistance profile similar to that observed in SupB15-RT cells also have very low expression of CD34 and high expression of CD19 (28). This aligns with the study of a large cohort of 2028 children with ALL, where the expression of CD34+ had more favorable prognosis (52). Moreover, in other studies, increased expression of CD33 is correlated with higher resistance of ALL patients (53).

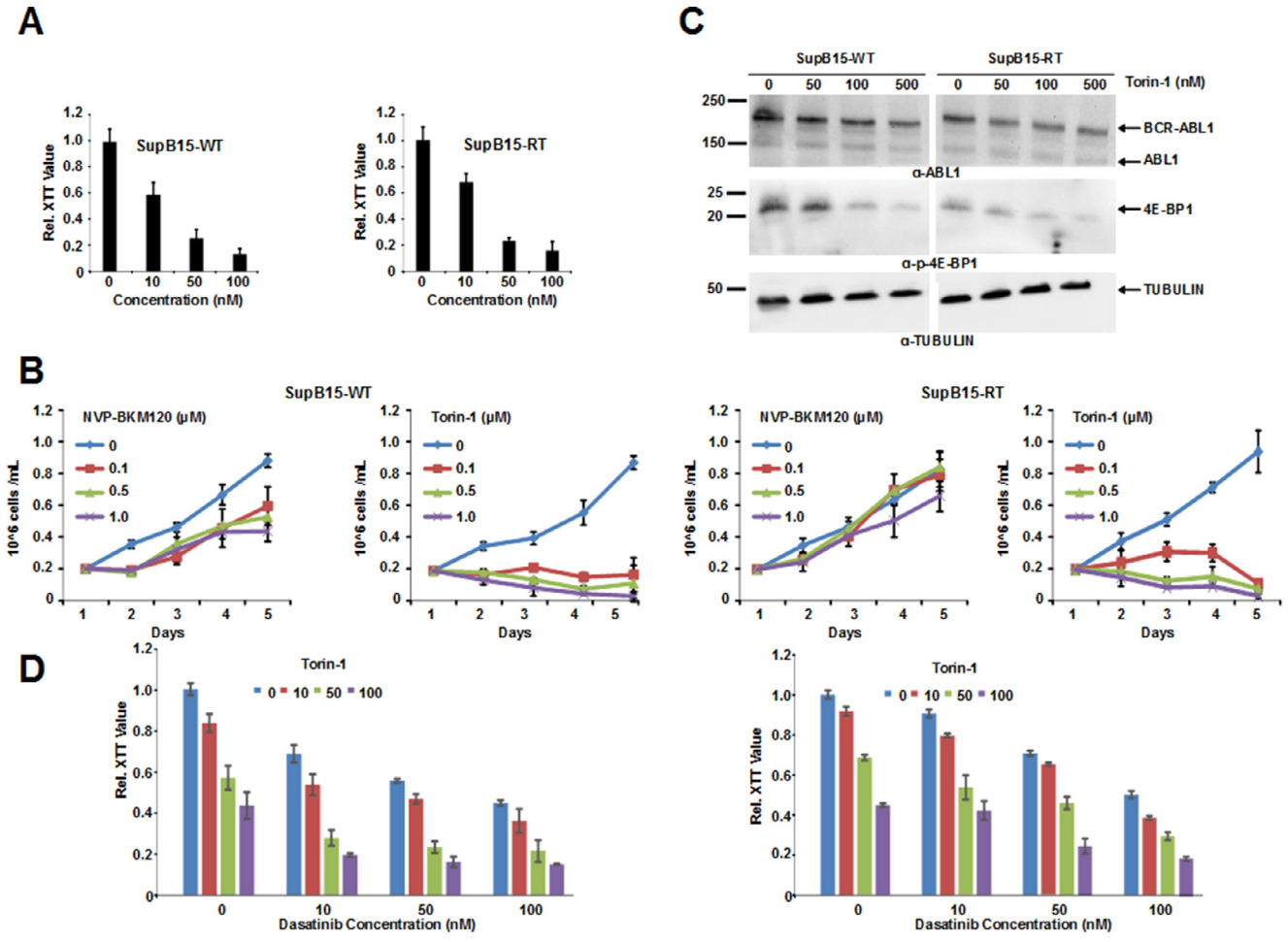

Fig. 4. Effect of Torin-1 on resistant SupB15 cells. (A) XTT assay for SupB15-WT and SupB15-RT cells upon exposure to 0, 10, 50, and100 nM of Torin-1. The mean ± SD of triplicates from one representative experiment out of 3 performed is given. (B) For the viability test, the number of viable cells was determined daily by trypan blue dye exclusion. Data represent the mean and SD of 3 independent experiments. (C) Western blot analysis of lysates of SupB15-WT and SupB15-RT cells either untreated or treated with 10, 50, and 100 nM Torin-1 using antibodies directed against: ABL1 and phospho-4E-BP1. Molecular mass references (KDa) are presented. (D) XTT assay using SupB15-WT and SupB15-RT upon exposure to 0, 10, 50, and 100 nM Torin-1 and 0, 10, 50, and 100 nM dasatinib. Proliferation status was determined by the metabolic activity of cells given by the reduction rate of XTT to formazan. The means ± SD of triplicates from one representative experiment out of 3 performed are given.

The western blot screening revealed that the AKT/mTOR pathway is constitutively activated in SupB15-RT (Fig. 3A and B). It prompted us to ask the question: which of the individual components of the PI3K/AKT/mTOR pathway are most relevant for supporting leukemic cell growth and thus are the most attractive targets for therapeutic intervention in the resistant cells. Our data strongly suggests that mTOR is an essential mediator of 4E-BP1 phosphorylation, despite that, we could not further discriminate between the relative contributions of mTORC1 and mTORC2 because of the lack of selective, ATP-competitive inhibitors of these 2 mTOR components. Thus, both mTORC1 and mTORC2 may phosphorylate 4E-BP1 so that inhibition of either one alone would most likely not dephosphorylate 4E-BP1 because of compensatory phosphorylation by the other. In conjunction with the biochemical data, inhibition of leukemic cell proliferation was most pronounced with the dual inhibitors Torin-1 that resulted in 4E-BP1 dephosphorylation. The inhibition of proliferation in SupB15-RT cells is in agreement with previous studies where mTOR inhibitors, such as OSI-027, PP242, Torin-1 and NVP-BEZ235, have shown an antiproliferative effect in Ph+ acute lymphoblastic leukemia cells (31). The mTOR inhibitors not only targeted the CML cells but also sensitized them to nilotinib (54), and overcame ponatinib-resistance in CML cells (55). In a recent study, Guillen et al (56) have shown that targeting mTORC1 & 2 by either NVP-BEZ235 or Torin-1 could efficiently arrest the growth of leukemic cells in poor prognostic t(6;9) translocation associated Acute Myeloid Leukemia (AML) at low concentrations .

Clinical outcome of mTOR inhibitors in solid tumor (Phase II clinical trials; NCT01658436) and dasatinib in Ph+ leukemia show that many patients experience toxicities on recommended doses (57). The intensity of intolerability to TKI is mainly due to the high dose of either inhibitor. Hence, it is strongly suggested to reduce the doses of both inhibitors for safety and to maintain potency using a combination therapy approach (58). With this perspective in mind, we opted for the combination approach for targeting the SupB15-RT cells. dasatinib and Torin-1 showed an antiproliferative effect in both SupB15-WT and SupB15-RT as a single agent with IC50 ranging from 7.1 nM to 90 nM. (Fig. 4A). However, the combination of dasatinib and Torin-1 did not lead to a synergistic growth arrest but rather an additive effect in SupB15-WT as well as SupB15-RT cells (Fig. 4D). Our study may contribute towards identifying the most suitable mTOR inhibitor (in terms

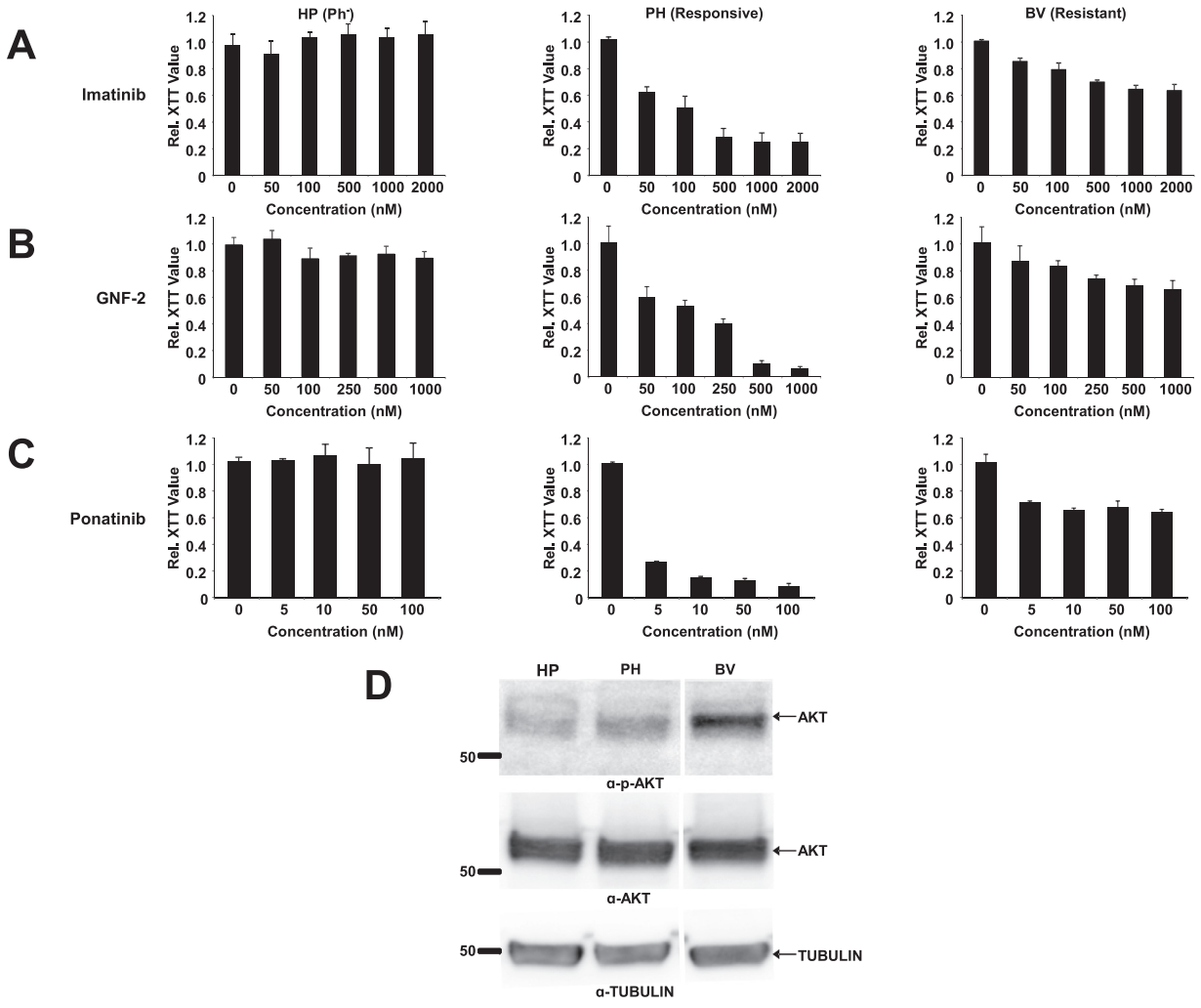

Fig. 5. Role of mTOR/AKT pathway in nonmutational resistance developed by PDLTCs (A) XTT assay for HP, PH, and BV cells upon exposure to 0, 50, 100, 500, 1000, and 2000 nM imatinib. (B) XTT assay for HP, PH and BV cells upon exposure to 0, 50, 100, 250, 500, and 1000 nM GNF-2 (C) XTT assay for HP, PH, and BV cells upon exposure to 0, 5, 10, 50, and 100 nM ponatinib. The mean ± SD of triplicates from one representative experiment out of 3 performed is given. (D) Western blot analysis of lysates of HP, PH, and BV cells using antibodies directed against: phospho-AKT serine 473 and AKT. Molecular mass references (KDa) are presented, and α-TUBULIN was used as a loading control.

of efficacy and tolerability) to be taken forward for combination with potent approved kinase inhibitors such as dasatinib.

A number of ALL cell lines have been established from ALL patients (59). However, these cell lines do not represent the whole spectrum of the disease, and only a few have been fully characterized (60). Furthermore, it is unclear to what extent they still represent the primary material from which they were generated, because some of these cell lines were developed decades ago, and have been cultured for long periods (61). Functional studies involving ALL primary material are hindered by the well-known inability to maintain human ALL blasts in short-term culture and the high basal rate of cell death. Therefore, to further strengthen our findings, we opted to utilize a recently established unique culture system of primary cells from Ph^- and Ph^+ ALL patients for further experiments. This system enables long term cultures of leukemic cells derived directly from patients, which remain genetically as well as immunophenotypically stable for at least 6 mo, without entering senescence or passing the typical crisis of cell lines. To investigate the nonmutational resistance mechanisms and the role of AKT/mTOR pathway in a clinically relevant model, 3 different PDLTCs were selected: HP as a Ph^- ALL patient cell, BV, and PH, both positive for Philadelphia chromosome but differ in response to TKIs (28).

The effective suppression of AKT/mTOR signaling was achieved by utilizing 2 potent, ATP-competitive compounds, Torin-1 and NVP-BEZ235, both of which block the mTOR complexes C1 and C2 downstream of AKT. These compounds inhibited proliferation and induced cell death in PH and BV cells. The magnitude of the anti-leukemic effects of AKT/mTOR inhibitors did not differ in all PDLTCs that were either sensitive or resistant, due to nonmutational resistance (as in BV cells), towards the ABL-directed inhibitors. In conjunction with published data showing that blockage of the PI3K/AKT/mTOR pathway with the dual inhibitor NVP-BEZ235 does not appreciably affect survival, clonogenic growth and differentiation of normal CD34 positive cells (62). The profound antileukemic activity of the dual mTOR inhibitors against Ph^+ ALL indicates a pivotal role of the PI3K/AKT/mTOR pathway in ALL cells. This is consistent with the generally accepted activation of AKT by the BCR-ABL1 oncogene but does not exclude its involvement in BCR-ABL1 negative ALL as well. Aberrant activation of the AKT pathway is one of the most frequent perturbations of signaling pathways in malignancies, including leukemias (63).

In this study, we found that the unresponsiveness of the resistant cells to various inhibitors was not attributable to known causes as BCR-ABL1 mutations or activation of SRC kinases. Furthermore, the BCR-ABL1-

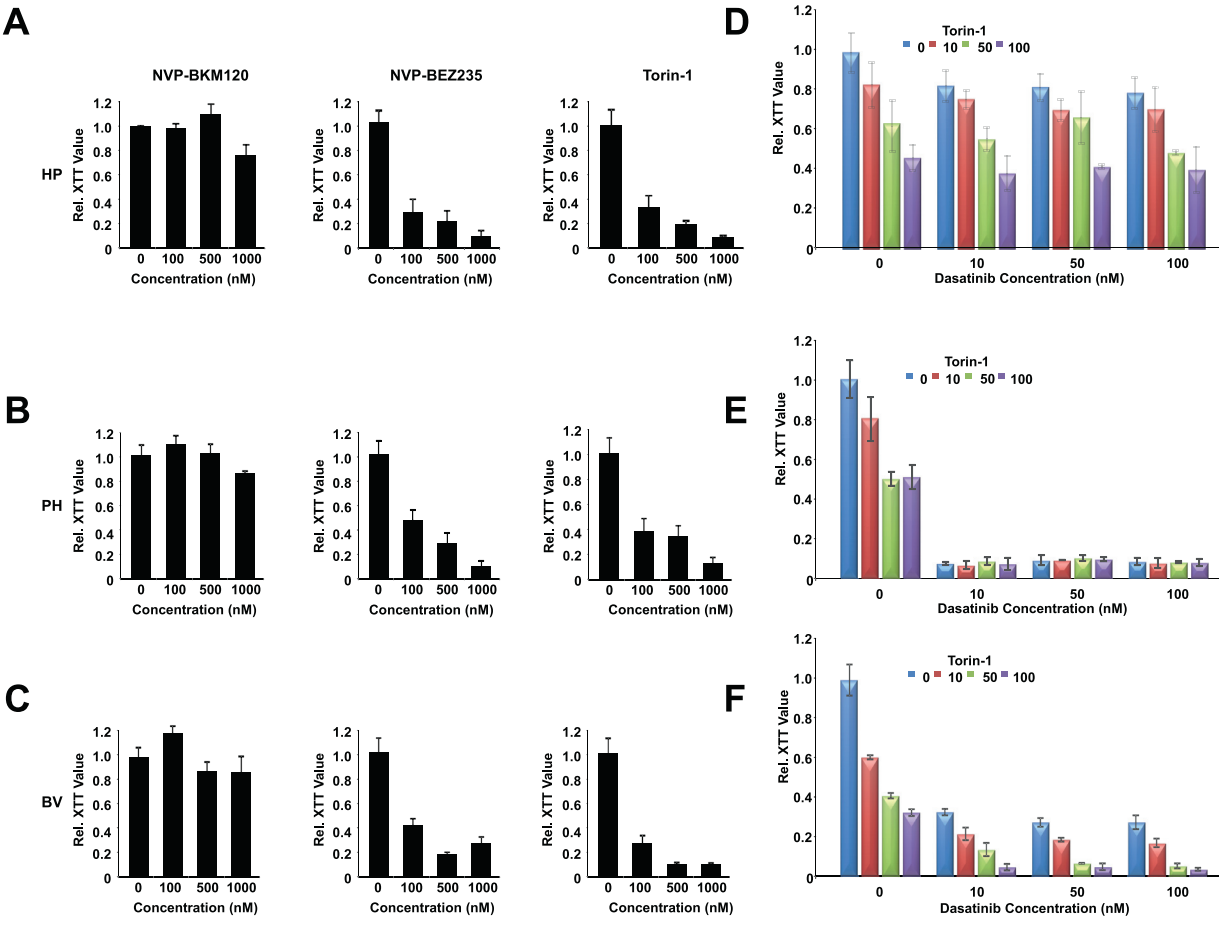

Fig. 6. Effect of dual mTOR inhibitors on the growth resistant PDLTCs (A) XTT assay for HP cells upon exposure to 0, 100, 500, and1000 nM of NVP-BKM120, NVP-BEZ235, and Torin-1. (B) XTT assay for PH cells upon exposure to 0, 100, 500 and1000 nM of NVP-BKM120, NVP-BEZ235, and Torin-1. (C) XTT assay for BV cells upon exposure to 0, 100, 500, and1000 nM of NVP-BKM120, NVP-BEZ235, and Torin-1. The mean ± SD of triplicates from one representative experiment out of 3 performed is given. (D) XTT assay for HP cells upon exposure to 0, 10, 50, and 100 nM Torin-1 and 0, 10, 50, and 100 nM dasatinib. (E) XTT assay for PH cells upon exposure to 0, 10, 50, and 100 nM Torin-1 and 0, 10, 50, and 100 nM dasatinib. (F) XTT assay for BV upon exposure to 0, 10, 50, and 100 nM Torin-1 and 0, 10, 50, and 100 nM dasatinib. The means ± SD of triplicates from one representative experiment out of 3 performed are given.

triggered JAK2/STAT5 and ERK1/2 pathways were also not involved in the resistance conferred by these cells. The only mechanism of resistance unraveled in this study was the constitutive activation of the AKT/mTOR pathway. The mTOR inhibitors Torin-1 and NVP-BEZ235 inhibited cell growth in these models and further sensitized resistant cells to lower doses of dasatinib. Therefore, we can suggest mTOR inhibitors as a therapeutic option for patients with BCR-ABL1-independent resistance and that dasatinib can further enhance their efficacy. This recommendation is important consideration for heavily pretreated patients not responding to currently available TKIs as treatment options for such patients are limited or unavailable.

Competing interests

The authors declare no competing financial interests.

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Author contributions

AAM: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript;

UZ: Performed experiments, collection and/or assembly of data, data analysis and interpretation

SMAA: Performed experiments, collection and/or assembly of data, data analysis and interpretation, manuscript writing

OGO: Data analysis and interpretation, manuscript writing

ENMAL: Data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

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Supplementary materials

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