Modulation of Glucocorticoid Resistance in Pediatric T-cell Acute Lymphoblastic Leukemia by Increasing BIM Expression with the PI3K/mTOR Inhibitor BEZ235

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Abstract

Purpose: The aim of our study is to evaluate the preclinical therapeutic activity and mechanism of action of BEZ235, a dual PI3K/mTOR inhibitor, in combination with dexamethasone in acute lymphoblastic leukemia (ALL).

Experimental Design: The cytotoxic effects of BEZ235 and dexamethasone as single agents and in combination were assessed in a panel of ALL cell lines and xenograft models. The underlying mechanism of BEZ235 and dexamethasone was evaluated using immunoblotting, TaqMan RT-PCR, siRNA, immunohistochemistry, and immunoprecipitation.

Results: Inhibition of the PI3K/AKT/mTOR pathway with the dual PI3K/mTOR inhibitor BEZ235 enhanced dexamethasone-induced anti-leukemic activity in vitro (continuous cell lines and primary ALL cultures) and systemic in vivo models of T-ALL (including a patient-derived xenograft). Through inhibition of AKT, BEZ235 was able to alleviate AKT-mediated suppression of dexamethasone-induced apoptotic pathways leading to increased expression of the proapoptotic BCL-2 protein BIM. Downregulation of MCL-1 by BEZ235 further contributed to the modulation of dexamethasone resistance by increasing the amount of BIM available to induce apoptosis, especially in PTEN-null T-ALL where inhibition of AKT only partially overcame AKT-induced BIM suppression.

Conclusions: Our data support the further investigation of agents targeting the PI3K/mTOR pathway to modulate glucocorticoid resistance in T-ALL. Clin Cancer Res. 22(3); 621–32. ©2015 AACR.

Introduction

Glucocorticoids represent an important component of pediatric acute lymphoblastic leukemia/lymphoma (ALL) treatment. By themselves, glucocorticoids can achieve clinical remission in ALL, and with the addition of other chemotherapeutic agents, contribute to more than 80% of patients achieving long-term event-free and overall survival (1, 2). In addition, early response to glucocorticoids is a positive prognostic indicator and those patients who lack a robust response more frequently experience negative outcomes (3–5). Therefore, investigating the mechanisms leading to glucocorticoid resistance may identify biomarkers and targets that potentially improve overall outcomes.

Inappropriate activation of the oncogenic PI3K/AKT/mTOR signaling pathway (PI3K/AKT/mTOR pathway) contributes to the pathogenesis and chemotherapy resistance observed in many cancers, including hematologic malignancies (6–8). Activation of the PI3K/AKT/mTOR pathway in ALL depends on a large number of mechanisms, including deletion/inactivation of the phosphatase and tensin homologue (PTEN), SH2 domain–containing inositol phosphatase 1 (SHIP1) inactivation, and mutations in PI3K, AKT, RAS, NOTCH1, or receptor tyrosine kinases (RTK; refs. 9–12). In both higher risk B-cell (B-ALL) and more notably T-cell ALL (T-ALL) primary bone marrow samples, AKT hyperactivation has been observed (12, 13). In one study of T-ALL, 85% of primary patient samples were characterized by increased AKT phosphorylation when compared with normal thymocyte controls (12). Consequently, this AKT hyperactivation has been correlated with reduced responses to induction therapy and poor overall and relapse-free survival (13). Two frequent mutations associated with increased AKT activity in T-ALL occur in NOTCH1, which is represented in greater than 50% of all T-ALL (14), and PTEN, which is estimated to be present in 8% to 30% of all T-ALL (9, 10, 15, 16). Interestingly, NOTCH1 activating mutations can downregulate PTEN via HES1 (15), suggesting that PTEN dysfunction is an important biologic characteristic of T-ALL. Reduced PTEN activity leads to increased PI3K/AKT pathway activity and has also been associated with poor outcomes (9, 10, 12, 16). PTEN is a negative regulator of the PI3K/AKT/mTOR pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2) counteracting the action of PI3K and eliminating AKT recruitment to the cell membrane where it is phosphorylated by phosphoinositide-dependent
protein kinase (PDK1) at Thr308 and by mTORC2 at Ser473 to be activated (17). Activated AKT phosphorylates more than 100 different substrates ultimately promoting increased cell survival, proliferation, growth, protein synthesis, and metabolism (8, 17, 18).

As constitutive activation of the PI3K/AKT/mTOR pathway occurs so frequently in T-ALL and is associated with treatment resistance, this makes it an attractive target to improve treatment responses in this disease. We and others previously demonstrated that rapamycin enhanced dexamethasone activity in in vitro and in vivo models of T-ALL (19, 20). Despite the promising activity seen with the latter combination, the immunosuppressive effects of rapamycin in combination with dexamethasone were concerning and therefore not pursued further (20). Over the last decade, various targeted inhibitors of the PI3K/AKT/mTOR pathway have been developed. The first to enter clinical trials was BEZ235, a dual PI3K/mTOR inhibitor that is currently on hold in development due to low oral bioavailability after phase I/II clinical trials in adults with solid and hematologic malignancies as a single agent and in combination (21–25). As a single agent, BEZ235 was well tolerated in adult leukemia patients (21), providing a possible alternative to rapamycin. In this study, we show that BEZ235 increased dexamethasone-induced apoptosis through increasing BIM in T-ALL models. This activity is due to BEZ235 inhibition of AKT1, which is a crucial mediator suppressing BIM expression in response to dexamethasone. We also show that BEZ decreases MCL-1 through a mechanism distinct from AKT inactivation. The decrease in MCL-1 by BEZ235 may represent importance in T-ALL, which is a crucial mediator suppressing BIM expression in T-ALL models. This activity is due to BEZ235 inhibition of increased dexamethasone-induced apoptosis through increasing alternative to rapamycin. In this study, we show that BEZ235 is tolerated in adult leukemia patients (21), providing a possible corticoid resistance in pediatric patients with T-ALL.

Translational Relevance

Glucocorticoids represent a critical component of pediatric acute lymphoblastic leukemia (ALL) treatment, as glucocorticoid resistance has been associated with poor outcomes. T-ALL (T-ALL) frequently manifests constitutive activation of the PI3K/AKT/mTOR pathway which may contribute to the glucocorticoid resistance observed in these patients. We demonstrated that the PI3K/mTOR inhibitor BEZ235 potentiates dexamethasone cytotoxic activity in a panel of T-ALL cell line and xenograft models. BEZ235, through inhibition of AKT1, increased expression of proapoptotic protein BIM. BEZ235, independently of AKT, also decreased expression of antiapoptotic protein MCL-1. In addition to demonstrating BEZ235 to be a possible adjunct to glucocorticoid treatment in T-ALL, identification of BIM and MCL-1 as important molecular mediators of drug sensitivity informs future studies seeking to improve glucocorticoid cytotoxic activity in T-cell ALL.

Materials and Methods

In vitro cell lines and culture

Human pediatric T-ALL cell lines established from children at diagnosis (COG-LL-329h) and relapse (COG-LL-317h, COG-LL-332h) and B-ALL cell lines established at diagnosis (COG-LL-319h, COG-LL-402h) and relapse (COG-LL-355h) were provided by the Children’s Oncology Group (COG) Cell Line and Xenograft Repository (www.cogcell.org). Human T-ALL cell line TX-LY-172h (established from a child at relapse) was obtained from the Texas Cancer Cell Repository (TXCCR, www.txccr.org). COG and TXCCR cell lines were cultured in Iscove’s Modified Dulbecco’s medium (IMDM; Thermo-Scientific) supplemented with 3 mmol/L L-glutamine, 5 μg/mL insulin, and 20% heat-inactivated FBS. Nalm-6 [pre-B ALL, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany] and RS4;11 (pre-B ALL), T-ALL cell lines (CCRF-CEM, MOLT-3, and MOLT-4) from ATCC were cultured in RPMI-1640 medium (Thermo-Scientific) supplemented with 10% heat-inactivated FBS. All cell lines were maintained and treated with drugs in a 37°C incubator with 5% O2 (bone marrow–level hypoxia), 5% CO2, and 90% N2. Karyotype information of the cell lines is included in Supplementary Table S1. Cell line identities were confirmed after each expansion and before freezing by short tandem repeat (STR) profiling. Profiles for each cell line were unique except for pairs of cell lines established from the same patient at different stages of the disease (COG-LL-329h and COG-LL-332h, MOLT-3 and MOLT-4) and were verified in the COG cell line + Xenograft STR database (www.COGCell.org). All cultures were confirmed to be free of mycoplasma and Epstein-Barr virus (EBV) contamination. Studies using tissues from human subjects were approved by the Institutional Review Board (IRB) of Texas Tech University Health Sciences Center.

Primary cells

Clinical specimens were obtained with consent via a biobanking protocol approved by the TTUHSC committee for protection of human subjects. Ficoll-separated mononuclear cells from peripheral blood of pediatric patients diagnosed with ALL (obtained with informed consent under COG biobanking protocol ABTR04B1) were obtained from COG or the TXCCR. All patients gave informed consent; the study using the primary samples was approved by the TTUHSC Institutional Review Board. Mononuclear cells were verified to contain more than 80% leukemic blasts by flow cytometry using CD45 and CD7 for T-ALL samples or CD45 and CD19 for B-ALL samples. Isolated cells were cultured over a layer of immortalized HS-5 human bone marrow stromal cells (ATCC) in IMDM (Thermo-Scientific) supplemented with 3 mmol/L L-glutamine, 5 μg/mL insulin, 20% heat-inactivated FBS, recombinant IL7 (10 ng/mL; R&D Systems), recombinant stem cell factor (50 ng/mL; R&D Systems), and recombinant Flt-3 ligand (20 ng/mL; R&D Systems). All primary cells were maintained and treated with drugs in a 37°C incubator with 5% O2 (bone marrow–level hypoxia; refs. 26, 27), 5% CO2, and 90% N2. Cytotoxicity experiments were performed in 24-well plates. Viability of the cells was assessed by flow cytometry after staining with the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) following manufacturer's protocol. Viability was calculated as the percentage of cells not staining for Annexin V or propidium iodide.

Cytotoxicity, apoptosis, and cell-cycle assays

The in vitro cytotoxic activities of dexamethasone, BEZ235, and the BEZ235 plus dexamethasone combination were determined using the DIMSCAN digital imaging microscopy cytotoxicity system as described previously (28). One day prior to drug addition, cells were seeded in 96-well plates at a density previously determined to result in confluence in nontreated wells. Cells...
were exposed to DMSO, BEZ235, dexamethasone, or BEZ235 plus dexamethasone for a total of 48 to 96 hours before addition of fluorescein diacetate (FDA) and eosin Y. Fluorescence was measured in each well and quantified by DIMSCAN software. To detect apoptosis, treated cells were harvested and stained using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) following manufacturer’s protocol. Cell-cycle analysis was performed using a propidium iodide staining protocol as described previously (29).

Immunohistochemistry
Sections (4 μm) from formalin-fixed, paraffin-embedded spleens were processed for immunohistochemistry and stained with hematoxylin and eosin. BIM was detected using BIM antibody (Cell Signaling) and the VECTASTAIN ABC Kit for Rabbit IgG (Vector Laboratories) according to manufacturer’s instructions. The sections were examined using a light microscope (BX51; Olympus). Images were captured using CellSens Standard 1.6 (Olympus).

RNA interference
ON-TARGETplus AKTI SmartPool siRNA and ON-TARGETplus Non-Targeting Control Pool were purchased from Thermo Scientific. BCL2L11 Silencer Select siRNA and MCL1 Silencer Select siRNA were purchased from Life Technologies. Transfections were performed using the Nucleofector 4D System (Lonza Biologics) with conditions optimized for the respective cell lines (Solution SF; Program CM-137: 1 μm/L siRNA). Three million COG-LL-317h or RS4;11 cells were used per transfection. Following transfection, cells were plated and incubated for 16 hours before experiments.

In vivo experiments
Mice were housed in specific pathogen-free animal facilities located at Texas Tech University Health Sciences Center, and all mouse procedures were in accordance with the guidelines of Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee. For bioluminescent models, 2 × 10⁶ COG-LL-317h expressing the luciferase gene [pCCL-cMNDU3c-LUC-PGK-bH7 vector using human B7.1 (CD80) as a selection marker] and sorted to greater than 90% CD80 positivity were injected intravenously via tail vein into female nonobese diabetic (NOD) severe-combined immunodeficient (SCID)/IL2Rγ knockout (NSG) mice aged 6 to 8 weeks (purchased from Charles River Laboratories). Mice were initially evaluated after 14 days for engraftment by in vivo bioluminescent imaging after the intraperitoneal injection of n-luciferin–K⁺ salt bioluminescent substrate (Perkin-Elmer; 150 mg/kg) dissolved in 1× PBS with the IVIS Lumina XR (Perkin-Elmer). Following initial imaging, mice were randomized into groups of 5 mice injected intraperitoneally with vehicle (5% glucose) or dexamethasone (7.5 mg/kg/d) and/or orally gavaged with vehicle [NMP:PEG300 (1:9, v/v)] or BEZ235 (10 mg/kg/d) daily for 7 days. One day after last treatment, mice were reimaged and posttreatment luminescence was compared with initial imaging to determine leukemia progression for each mouse in each treatment group. Radiance was quantified using Living Image Software version 4.3.1 (Perkin-Elmer).

Event-free survival (EFS) in response to treatment was determined in 2 ALL xenograft models, COG-LL-317x (T-ALL patient–direct xenograft) and RS4;11 (Pre B-ALL). Female NOD/SCID mice aged 6–8 weeks were purchased from Charles River Laboratory.
Bar graphs represent mean ± SD.

Figure 1.
BEZ235 enhances dexamethasone cytotoxicity in several T-ALL models. A and C, dose–response curves for 7 T-ALL cell lines (A) and 5 B-ALL cell lines (C) exposed to BEZ235 (BEZ; dose range, 100–1,000 nmol/L), dexamethasone (DEX; dose range, 20–200 nmol/L), or BEZ235 plus dexamethasone (BEZ+DEX; dose range, 100–1,000 and 20–200 nmol/L, respectively) at a fixed ratio. Cytotoxicity was evaluated after 72 hours of exposure by DIMSCAN, and relative survival (%) was determined by mean fluorescence of the treated cells/mean fluorescence of control cells × 100. CI values are displayed for cell lines displaying strong synergy (CI < 0.1). Each point represents mean ± SD. B and D, primary T- (B) and B-ALL cells (D) were treated with BEZ235 (1 μmol/L), dexamethasone (200 nmol/L), or BEZ235 plus dexamethasone (1 μmol/L and 200 nmol/L, respectively) for 36 hours. Cells were stained for anti-Annexin V–FITC-conjugated antibody and propidium iodide before analysis by flow cytometry. Viability (%) represents percentage of cells not staining for Annexin V or propidium iodide. Bar graphs represent mean ± SD.

BEZ235 enhances in vitro cytotoxic activity of dexamethasone preferentially in T-ALL.

The antileukemic activity of BEZ235, dexamethasone, and BEZ235 plus dexamethasone was further assessed in systemic in vivo models of T- and B-ALL. In vivo activity was first assessed in a systemic bioluminescent model of T-ALL (COG-LL-317x expressing luciferase reporter) where mice were imaged 14 days after inoculation and again following 7 days of treatment. Initial bioluminescence demonstrated no significant difference in day 0 leukemia burden observed in mice from different treatment groups (Fig. 2A and B). Compared with vehicle-treated mice, mice with BEZ235 only treatment did not demonstrate significant changes in final leukemia burden. Although mice treated with dexamethasone demonstrated a significant reduction in final leukemia burden compared with control, mice treated with BEZ235 plus dexamethasone showed significantly greater antileukemic activity, indicating that BEZ235 enhances dexamethasone antileukemic activity in vivo as well. Confirming the in vivo bioluminescence results, spleens isolated from mice treated with BEZ235 plus dexamethasone showed significant reduction in weight compared with mice treated with vehicle, BEZ235, or dexamethasone alone (Fig. 2C).

In a separate study, EFS in response to these agents was assessed in systemic xenograft models of T- and B-ALL. In the systemic T-ALL patient–derived xenograft COG-LL-317x, BEZ235 plus dexamethasone also increased EFS compared with dexamethasone alone (Fig. 2D). While treatment with dexamethasone alone significantly prolonged EFS in the systemic B-ALL model RS4;11, there was no significant increase in survival in mice treated with BEZ235 plus dexamethasone (Fig. 2E).

BEZ235 enhances dexamethasone-induced apoptosis

Glucocorticoids by themselves exert their antileukemic activity through inducing both apoptosis and cell-cycle arrest (32). A cell-cycle analysis in the COG-LL-317h T-ALL cell line revealed an increase in G0–G1 and a decrease in S-phase cell populations following treatment with BEZ235, dexamethasone, or the BEZ235 plus dexamethasone combination (Supplementary Fig. S2).
However, G0–G1 arrest was not significantly greater after treatment with BEZ235 plus dexamethasone compared with either single agent alone, indicating that the increased cytotoxic activity of BEZ235 plus dexamethasone is not due to cell-cycle arrest. Therefore, apoptosis was assessed by measuring Annexin V positivity following treatment with BEZ235, dexamethasone, or BEZ235 plus dexamethasone in T-ALL model COG-LL-317h. As single agents compared with vehicle, only dexamethasone induced a small increase in percentage of apoptotic lymphoblasts (Fig. 3A and B). BEZ235 as a single agent had no significant effect on induction of apoptosis compared with vehicle. Treatment with BEZ235 plus dexamethasone, however, significantly increased the percentage of apoptotic lymphoblasts when compared with vehicle, dexamethasone, or BEZ235 alone. In contrast, RS4;11, a dexamethasone-sensitive B-ALL model, demonstrated a robust apoptotic response to dexamethasone alone, but did not further increase with the addition of BEZ235 (Fig. 3A and B). Surprisingly, the addition of BEZ235 significantly decreased the percentage of apoptotic lymphoblasts compared with dexamethasone-only treated lymphoblasts. In COG-LL-317h, activation of caspase-9 and -3 was assessed following treatment by Western blotting for their respective cleavage products. Treatment with BEZ235 plus dexamethasone led to increased cleavage of both caspase-9 and -3, whereas no cleavage of caspases was detected in BEZ235, dexamethasone, or vehicle-treated lymphoblasts (Fig. 3C). These data show that BEZ235 plus dexamethasone antileukemic activity is primarily working through increasing apoptosis.
BEZ235 enhances dexamethasone-induced apoptosis through increased expression of the proapoptotic BCL-2 protein BIM

The proapoptotic BCL-2 family protein BIM (encoded by BCL2L11) is an indirect glucocorticoid target and major mediator of glucocorticoid-induced apoptosis (33–37). Consequently, in both preclinical models and primary lymphoblasts, there are inferior responses to glucocorticoids and poorer prognosis associated with reduced increases in BIM expression following glucocorticoid exposure (38, 39). We therefore examined the effect of BEZ235 on dexamethasone-induced BIM expression in the BEZ235 plus dexamethasone-responsive T-ALL cell line COG-LL-317h following treatment and compared with the dexamethasone-sensitive B-ALL cell line RS4;11. After treating COG-LL-317h lymphoblasts with BEZ235, dexamethasone, or BEZ235 plus dexamethasone, there was an observed increase in expression of the 3 dominant isoforms of BIM with BEZ235 plus dexamethasone relative to dexamethasone alone is due to the changes in stability of the protein (Fig. 4D). A similar trend was observed in another B-ALL cell line COG-LL-319h, although dexamethasone had minimal impact on BIM expression as a single agent (Fig. 4C).

Splenic tissue isolated from mice engrafted with COG-LL-317h and treated with agents for 7 days were fixed and stained with antibody against BIM. Mice treated with BEZ235 plus dexamethasone demonstrated increased BIM staining compared with mice treated with vehicle or either single agent alone, consistent with data obtained from in vitro studies (Fig. 4E).

As BIM (encoded by BCL2L11) is necessary for dexamethasone-induced apoptosis, we investigated whether it contributes to BEZ235 plus dexamethasone antileukemic activity. Using an siRNA against BCL2L11, BCL2L11 was knocked down in COG-LL-317h (Fig. 5A), and the changes in apoptosis and cytotoxicity in response to BEZ235, dexamethasone, or BEZ235 plus dexamethasone were observed. Following treatment with BEZ235 plus dexamethasone, BCL2L11-knockdown lymphoblasts showed a significant reduction in caspase-3 cleavage compared with non-targeting siRNA control (Fig. 5A). Correspondingly, both apoptosis (Fig. 5B) and cytotoxicity (Fig. 5C) were significantly

![Graph](image-url)
reduced following treatment with BEZ235 plus dexamethasone in BCL2L11-knockdown lymphoblasts when compared with non-targeting siRNA control.

**AKT1 inhibition alleviates suppression of basal and dexamethasone-induced BIM expression**

PTEN inactivation/deletion and concomitant PI3K/AKT/mTOR pathway activation are common abnormalities in primary T-ALL (9). Therefore, we investigated the role of PTEN status and PI3K/AKT pathway activity on glucocorticoid responses in T- and B-ALL cell lines. We examined PTEN protein expression in 5 patient-derived T-ALL cell lines (COG-LL-317h and TX-LY-172h) as well as MOLT3 and MOLT-4 were derived from the same patients) and compared with 5 patient-derived B-ALL cell lines (Supplementary Fig. S3A). Analysis of PTEN protein expression in ALL cell lines revealed undetectable protein in 3 of 5 T-ALL (COG-LL-317h, CCRF-CEM, MOLT-4) models examined. Sequence analysis of exons 5 and 7 revealed truncating mutations in each of those 3 cell lines (Supplementary Table S1). COG-LL-332h had a substantial reduction in PTEN expression and TX-LY-172h demonstrated comparable levels in expression when compared with B-ALL cell lines. The T-ALL models with undetectable levels of PTEN by Western blotting demonstrated substantially increased phosphorylation of AKT at both Thr308 and Ser473. COG-LL-332h, which shows reduced PTEN expression, has a comparable level of AKT phosphorylation compared with B-ALL.
Fig. 5.
AKT1 contributes to dexamethasone-resistance by suppressing BIM. A, Western blot analysis examining BIM expression and caspase cleavage in COG-LL-317h cells following transfection with nontargeting siRNA control or siRNA against BCL2L11 and treatment for 36 hours with vehicle (0.01% DMSO) or BEZ235 plus dexamethasone (1 μmol/L and 200 nmol/L, respectively). B, flow cytometric analysis examining apoptosis as measured by Annexin V positivity in COG-LL-317h cells following transfection with non-targeting siRNA control or siRNA against BCL2L11 and treatment for 36 hours with vehicle (0.01% DMSO), BEZ235 (BEZ; 1 μmol/L), dexamethasone (DEX; 200 nmol/L), or the BEZ235 plus dexamethasone (BEZ + DEX; 1 μmol/L and 200 nmol/L, respectively). The mean values and SDs (error bars) of Annexin V-FITC-positive cells are shown for triplicate samples assayed over 2 separate experiments. C, cytotoxicity as determined by DIMSCAN in COG-LL-317h cells following transfection with nontargeting siRNA control or siRNA against BCL2L11 and treatment with vehicle (0.01% DMSO), BEZ235 (BEZ; 1 μmol/L), dexamethasone (DEX; 200 nmol/L), or the BEZ235 plus dexamethasone (BEZ + DEX; 1 μmol/L and 200 nmol/L, respectively). The mean values and SDs (error bars) relative to vehicle only treated cells are shown for 12 replicates. D, Western blot analysis of COG-LL-317h cells transfected with nontargeting siRNA or siRNA against AKT1 after incubation for 48 hours demonstrating sustained knockdown of AKT activity. E, Western blot analysis examining BIM induction in COG-LL-317h cells following transfection with nontargeting siRNA control or siRNA against AKT1 and treatment for 24 hours with vehicle (0.01% DMSO), BEZ235 (BEZ; 1 μmol/L), dexamethasone (DEX; 200 nmol/L), or BEZ235 plus dexamethasone (BEZ + DEX).

Although BEZ235 inhibits phosphorylation and activation of various components of the PI3K/AKT/mTOR pathway, there are limited data describing the effects glucocorticoids have on this pathway in human ALL. To demonstrate the effect of dexamethasone, BEZ235, and BEZ235 plus dexamethasone on this pathway, COG-LL-317h lymphoblasts were treated for 12, 24, and 36 hours before assessing expression and phosphorylation status of AKT, FOXO, mTOR, S6K1, S6, and 4EBP1 (Supplementary Fig. S3B). Interestingly, with dexamethasone treatment, we found a time-dependent increase in phosphorylation of AKT at both Ser473 and Thr380. BEZ235 as a single agent and in combination with dexamethasone potently reduced phosphorylation of AKT at both sites to undetectable levels as well as one of its targets FOXO4. In contrast, mTORC1 activity as indicated by phosphorylation of its target, S6K1, showed a time-dependent reduction in phosphorylation by dexamethasone alone. The ability of dexamethasone to reduce activation of mTORC1 and its targets further provide evidence that AKT plays a role in glucocorticoid resistance.

Of the 3 AKT isoforms, AKT1 is the most dominant isoform expressed ubiquitously throughout all tissues and thought to be the primary isoform involved in regulating cell survival (40). To investigate the role of AKT1 on dexamethasone activity, COG-LL-317h lymphoblasts were transfected with siRNA specifically against AKT1. AKT1 knockdown decreased AKT activity for at least 48 hours (Fig. SD). Compared with nontargeting control
BEZ235 and dexamethasone in ALL

MCL-1 downregulation by BEZ235 increases dexamethasone response in T-ALL

With only a modest increase in dexamethasone cytotoxicity after AKT1 knockdown, it is likely that another mechanism plays a role in BEZ235 plus dexamethasone cytotoxicity. It has been previously suggested that the antiapoptotic BCL-2 family protein MCL-1 plays a role in glucocorticoid resistance in ALL (41, 42). In our studies, when comparing BIM protein expression between COG-LL-317h and RS4;11 after treatment with BEZ235, dexamethasone, or BEZ235 plus dexamethasone, the increased BIM expression observed after treatment in COG-LL-317h was less than basal expression of BIM in RS4;11, suggesting that increasing BIM, although necessary, is not sufficient to induce apoptosis in models with hyperphosphorylated AKT (Supplementary Fig. S4B). We therefore hypothesized that the increased glucocorticoid-induced BIM expression following AKT1 knockdown is being sequestered by MCL-1, further inhibiting the ability to induce dexamethasone-induced apoptosis. No distinctive differences were seen in constitutive MCL-1 levels between sensitive and insensitive cell lines or T- and B-ALL cell lines (Fig. 6A). However, as observed with various other PI3K/AKT/mTOR pathway inhibitors (43, 44), a strong reduction in MCL-1 expression was observed in COG-LL-317h following treatment with BEZ235, suggesting that MCL-1 plays a role in the BEZ235 plus dexamethasone cytotoxic activity in our models (Fig. 6B). RT-PCR of MCLI demonstrated modest increases in MCLI expression, indicating that BEZ235 regulates MCLI posttranscriptionally (Supplementary Fig. S4C). Other BCL-2 family proteins, such as BCL-2 and BCL-xL, have also been implicated in preventing glucocorticoid-induced apoptosis (42); however, in COG-LL-317h, BEZ235 did not cause any substantial changes in BCL-2 expression, and BCL-xL was under the measurable level by Western blotting, suggesting they do not have a significant role in the combination mechanism in this model (Fig. 6B). Knockdown of MCLI by siRNA increased dexamethasone-induced cytotoxicity (Supplementary Fig. S4D). In addition, MCL1 knockdown was able to induce apoptosis and enhance dexamethasone-induced apoptosis (Fig. 6D). Despite the ability of BEZ235 to decrease MCL1 protein expression, AKT1 knockdown was unable to recapitulate this effect (Supplementary Fig. S4E), suggesting that AKT1 inhibition is not regulating MCL1 and explains the lack of robust cytotoxic response observed previously. As MCL-1 can bind to and sequester BIM, we treated COG-LL-317h with BEZ235, dexamethasone, or BEZ235 plus dexamethasone before immunoprecipitating for MCL-1.

Figure 6. BEZ235 decreases MCL-1 suppression of BIM-induced apoptosis in COG-LL-317h. A, Western blot analysis comparing basal expression of MCL1 in T-ALL (left) and B-ALL (right) cell lines while in their log phase of growth. B, Western blot analysis examining MCL1 and BCL2 expression in T-ALL model COG-LL-317h after treatment with vehicle (0.01% DMSO), BEZ235 (BEZ; 1 μmol/L), dexamethasone (DEX; 200 nmol/L), or the BEZ235 plus dexamethasone (BEZ + DEX; 1 μmol/L and 200 nmol/L, respectively) for 12, 24, or 36 hours. C, Western blot analysis examining MCL1 expression in COG-LL-317h cells following transfection with nontargeting siRNA control or siRNA against MCL1 and incubation for 48 hours. D, flow cytometric analysis examining apoptosis by measuring Annexin V positivity in COG-LL-317h cells following transfection with nontargeting siRNA control or siRNA against MCL1 and treatment with or without dexamethasone (DEX; 200 nmol/L). The mean values and SDs (error bars) of Annexin V+ positive cells are shown for triplicate samples assayed over 2 separate experiments. E, immunoprecipitation of MCL1 with BIM in COG-LL-317h cells treated with vehicle (0.01% DMSO), BEZ235 (BEZ; 1 μmol/L), dexamethasone (DEX; 200 nmol/L), or BEZ235 plus dexamethasone (BEZ + DEX; 1 μmol/L and 200 nmol/L, respectively). Protein lysates from COG-LL-317h cells treated with respective agents were exposed to MCL1 or IgG antibody bound to Protein G. Resulting immunoprecipitate was blotted with antibodies against MCL1 and BIM (right). Input lysate was blotted with antibodies against MCL1 and BIM (left).
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Immunoprecipitation of MCL-1 confirmed that MCL-1 was interacting with BIM in our model, and the increased BIM due to dexamethasone or BEZ235 plus dexamethasone treatment was also being sequestered by MCL-1 (Fig. 4E), indicating that MCL-1 is also playing a role in glucocorticoid resistance. BEZ235 treatment also decreased MCL-1 protein expression in RS4;11 lymphoblasts (Supplementary Fig. S5A), but MCL1 knockdown by siRNA in RS4;11 (Supplementary Fig. SSB) did not significantly increase apoptosis (Supplementary Fig. SSC). Similarly, MCL1 knockdown led to small increases in dexamethasone cytotoxicity but demonstrated only small effects at clinically achievable concentrations of dexamethasone (Supplementary Fig. SSD). These data suggest that decreased MCL-1, although a common effect of BEZ235 across different ALL cell lines, only alters apoptotic response and cytotoxicity in models with low BIM expression and PTEN mutations.

Discussion

Glucocorticoid resistance in the treatment of pediatric ALL remains a major clinical problem and several mechanisms have been suggested (45). Despite the widespread use of glucocorticoids in the treatment of ALL as well as other conditions, our understanding of the molecular basis of their function remains unclear. Our results indicate that AKT1 is partially responsible for suppression of glucocorticoid-induced apoptotic pathways leading to decreases in BIM expression, a known mediator of glucocorticoid-induced apoptosis, which is consistent with previous studies implicating AKT in glucocorticoid resistance and suppression of BIM expression in T-ALL models (46, 47). We also examined the downstream activation of mTORC1 and observed that dexamethasone was able to reduce mTORC1 activation independent of AKT. The observation of increased AKT phosphorylation upon dexamethasone treatment may indicate there is activation of the known negative feedback loop consisting of S6K1 phosphorylation and downregulation of IRS1 (48). This activity could also contribute to glucocorticoid resistance by increasing AKT activation and therefore further inhibiting apoptosis and warrants further investigation. It is also worth noting that inhibition of mTORC1 by dexamethasone treatment over time correlated with progressive G0–G1 arrest, suggesting that mTORC1 inhibition may be involved in the cytostatic effects of glucocorticoids. This would indicate that the cytotoxic and cytostatic mechanisms of glucocorticoids may be independent of each other.

The PI3K/AKT pathway appears to play a critical role in T-ALL, as common mutations in T-ALL (NOTCH1/PTEN) can lead to constitutive activation of this pathway and could account for the high frequency of AKT activation observed in primary T-ALL patient samples (15). This is particularly highlighted by the contrast in cytotoxic activity observed between T-ALL and B-ALL models in response to treatment with BEZ235 plus dexamethasone. In T-ALL models, BEZ plus dexamethasone synergistic activity appeared to be independent of PTEN or AKT status, as evidenced by the increased BIM expression and cytotoxic activity observed in TX-LY-172h, a PTEN wild-type T-ALL model, suggesting BEZ235 plus dexamethasone synergistic activity is not dependent on presence of PI3K/AKT pathway activation. The 2 T-ALL cell lines without synergistic activity, COG-LL-329h and COG-LL-332h, are derived from the same patient at different phases in treatment and have minimal glucocorticoid receptor expression, suggesting that there may be AKT-independent mechanisms of glucocorticoid resistance. In contrast, increased cytotoxic activity with BEZ235 plus dexamethasone was not observed in B-ALL models. An exception NALM-6, a B-ALL model with synergistic activity, did not demonstrate increased BEZ235 plus dexamethasone–induced BIM expression, suggesting that unique molecular characteristics of this model are responsible for the increased cytotoxicity observed with BEZ235 plus dexamethasone. In addition, BEZ235 plus dexamethasone may be antagonistic in some B-ALL models, as demonstrated in RS4;11 and COG-LL-319h, further demonstrating that the applicability of this drug combination is in T-ALL only.

Although BEZ235 increased dexamethasone cytotoxic activity in T-ALL models independent of PTEN function, PTEN-null cell lines had substantial reductions in basal BIM expression (Supplementary Fig. S5). In addition, reductions in BIM expression were associated with considerable AKT phosphorylation. This agrees with a recent study implicating Akt2 overexpression with reduction in BIM expression in a zebrafish T-ALL model (47). Despite inhibition of AKT with BEZ235, in PTEN-null cell lines, we were unable to reconstitute BIM expression to levels similar to wild-type PTEN ALL cells (Supplementary Fig. S5), which may indicate long-term constitutive activation of AKT results in suppression of BIM expression that is not completely reversed by inhibition of AKT. In addition, AKT1 knockdown in PTEN-null T-ALL model COG-LL-317h increased both dexamethasone- and BEZ235 plus dexamethasone–induced expression of BIM but only minimally increased the cytotoxic effects. This may provide a challenge when developing PI3K/AKT pathway inhibitors for the clinical treatment of pediatric ALL, as glucocorticoid resistance cannot be completely reversed by inhibiting AKT. Alternatively, inhibition of the PI3K/AKT/mTOR pathway has also been shown to decrease MCL-1 protein expression in murine-derived B-cell (43), multiple myeloma (44), and ALL cell lines (19). Our data demonstrated a BEZ235 decreased MCL-1 expression in ALL cell line models. This ability of BEZ235 to reduce MCL-1 expression could account for the inability of AKT1 knockdown to substantially increase dexamethasone- and BEZ235 plus dexamethasone–induced cytotoxicity, as AKT1 knockdown alone failed to substantially decrease MCL-1. MCL-1 can bind to and sequester BIM (19, 42), and thus by decreasing MCL-1 by BEZ235 treatment, there is increased availability of free BIM to induce apoptosis as demonstrated with BEZ235 plus dexamethasone treatment. In T-ALL with substantial reductions in BIM, as observed in PTEN-null models, this may be necessary to achieve glucocorticoid-induced apoptosis and explains why AKT1 knockdown was insufficient to induce apoptosis in PTEN-null T-ALL. As suggested by Ploner and colleagues, by regulation of a "BCL-2 theostat," we may be able to enhance glucocorticoid responses in glucocorticoid-resistant ALL (42).

BEZ235 potentiates dexamethasone cytotoxicity in models of T-ALL by increasing BIM and decreasing MCL-1, providing a possible adjunct in current T-ALL treatment protocols (mechanism summarized in Supplementary Fig. S6). Our data are in agreement with a previous cell line study of BEZ235 as a potential therapy modifier in glucocorticoid-resistant T-ALL (49). Mechanistically, AKT1 plays a role in suppressing glucocorticoid-induced apoptotic pathways, yet the precise AKT1 target remains unclear. Piombo and colleagues have implicated direct phosphorylation of the glucocorticoid receptor as a mechanism of PI3K/AKT pathway-mediated glucocorticoid resistance and may be a
plausible mechanism of action for BEZ235 plus dexamethasone (46). Nonetheless, discovery of this mechanism may provide information about possible therapeutic targets to overcome glucocorticoid resistance while minimizing adverse effects. These data in concert with other studies demonstrating increased activity observed with PI3K/Akt pathway inhibitors in combination with glucocorticoids (19, 20, 46) provide a novel approach to overcoming glucocorticoid resistance in T-ALL. If additional studies in T-ALL xenograft of a PI3K/Akt pathway inhibitor + glucocorticoids confirm the data presented here, early-phase clinical trials of such a combination should be undertaken.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: C.P. Hall, C.P. Reynolds, M.H. Kang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.P. Hall, C.P. Reynolds
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.P. Hall, M.H. Kang

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