

## Targeted Therapy for EBV-Associated B-cell Neoplasms

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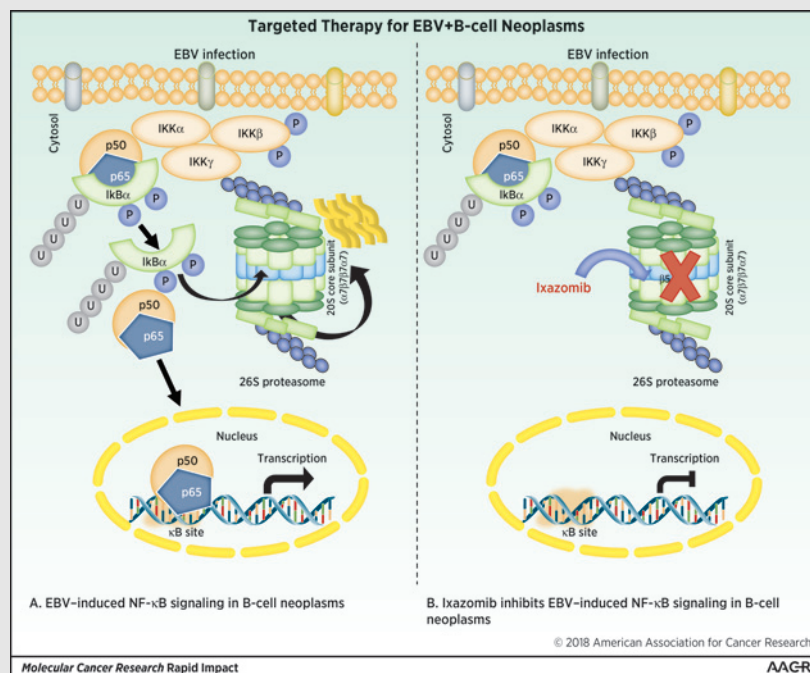


### Abstract

Epstein–Barr virus (EBV) is directly implicated in several B-cell lymphoid malignancies. EBV-associated lymphomas are characterized by prominent activation of the NF- $\kappa$ B pathway and targeting this pathway establishes a rationale for a therapeutic approach. The ubiquitin/proteasome signaling plays an essential role in the regulation of the NF- $\kappa$ B pathway. Ixazomib is an FDA-approved, orally bioavailable proteasome inhibitor. Here we report the first preclinical evaluation of ixazomib-mediated growth-inhibitory effects on EBV-infected B-lymphoblastoid cell lines Raji and Daudi. Ixazomib induced apoptosis in these cell lines in a dose-dependent manner. Cell-cycle analysis demonstrated ixazomib treatment induced cell-cycle arrest at the G<sub>2</sub>–M phase with a concomitant decrease in G<sub>0</sub>–G<sub>1</sub> and S phases. The results further revealed an increase in p53, p21, and p27 levels and a decrease in survivin and c-Myc protein levels. Mechanistically, ixazomib treatment resulted in the accumulation of polyubiquitinated proteins, including phosphorylated I $\kappa$ B $\alpha$  with a significant reduction of p65 subunit nuclear translocation. Altogether, our preclinical data support the rationale for *in vivo* testing of ixazomib in EBV-associated B-cell neoplasms.

**Implications:** This preclinical study supports the use of oral proteasome inhibitor ixazomib for targeting NF- $\kappa$ B signaling in the treatment of EBV-associated B-cell neoplasms.

**Visual Overview:** <http://mcr.aacrjournals.org/content/molcanres/17/4/839/F1.large.jpg>.



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### Introduction

Epstein–Barr virus (EBV) is directly implicated in several B-cell lymphoid malignancies (1). EBV-associated B-cell neoplasms occur in both solid organ and allogeneic hematopoietic stem cell transplantations due to the necessity of immunosuppression in these patients to modulate organ rejection and graft versus host disease (GVHD), respectively (2). Treatment of these B-cell neoplasms is limited to a reduction in immunosuppression, administration of immunotherapeutics, and antineoplastic chemotherapy (3). EBV-associated lymphomas are characterized by prominent activation of NF- $\kappa$ B pathway and targeting this pathway establishes a rationale for an attractive therapeutic

approach (4). The ubiquitin/proteasome signaling pathway plays an important role in the regulation of NF- $\kappa$ B pathway (5). Bortezomib is the first FDA-approved proteasome inhibitor for treating both newly diagnosed and relapsed/refractory multiple myelomas, and mantle cell lymphomas. Bortezomib acts through inhibition of the 26S proteasome, a large protease complex that degrades ubiquitinated proteins. Bortezomib stabilizes various cellular proteins involved in cell-cycle arrest and apoptosis including p21, p27, p53, and I $\kappa$ B $\alpha$  by inhibiting proteasome function (6). Stabilization of I $\kappa$ B $\alpha$  results in inhibition of the NF- $\kappa$ B signaling pathway, which promotes tumor cell survival, growth, and angiogenesis (7). The major limiting factor for long-term administration of bortezomib through intravenous or subcutaneous routes is a risk of peripheral neuropathy (8). There was a need to develop an orally bioavailable proteasome inhibitor with a low toxicity profile to overcome this conundrum. Ixazomib is structurally a dipeptidyl leucine boronic acid and the only approved orally bioavailable proteasome inhibitor. Ixazomib inhibits proteasome activity at low concentrations by binding to the  $\beta_5$  subunit of the 20S catalytic core subunit of the proteasome and is currently in clinical trials for patients with relapsed/refractory multiple myeloma and solid tumors (9–11). Compared with bortezomib, ixazomib has demonstrated similar efficacy with better pharmacokinetic/pharmacodynamic parameters in multiple myeloma. Ixazomib in combination with lenalidomide and dexamethasone sensitize bortezomib-resistant myeloma phenotype. From the existing literature review, there are no reports on the preclinical or clinical use of ixazomib in EBV-associated B-cell neoplasms. Therefore, using ixazomib for targeting NF- $\kappa$ B activation is a novel therapeutic approach and our preclinical data suggest the potential of ixazomib use as monotherapy or in combination for the treatment of EBV-associated B-cell neoplasms.

## Materials and Methods

### Cell lines and culture conditions

Human EBV-positive Burkitt lymphoma–derived cell lines Raji and Daudi were purchased from ATCC. Cells were grown in Hyclone RPMI1640 medium supplemented with 10% FBS (Corning Life Sciences) and 1% penicillin/streptomycin (Corning Life Sciences) at 37°C with 5% carbon dioxide.

### Isolation of normal B cells

Healthy individual blood samples were procured from Bio-repository Core Facility at the University of Kansas Medical Center (Kansas City, KS). Normal B cells were isolated from the blood samples using CD19 Positive Selection Kit from Stemcell Technologies.

### Reagents and antibodies

The following antibodies were used:  $\beta$ -actin-612656, p65-610868, p21-556431, PARP-556494, and p53-554293; (BD Biosciences); phospho-I $\kappa$ B $\alpha$ -2859, I $\kappa$ B $\alpha$ -9247, GAPDH-5174, Survivin-28023, and p27-3688 (Cell Signaling Technology); Lamin B-6216 and c-Myc-40 (Santa Cruz Biotechnology); and polyubiquitin-SIG-39500 (Covance). Propidium iodide (PI) and RNase were purchased from Sigma-Aldrich and ixazomib from Selleck Chemicals.

### Flow cytometry

FACS analysis was carried out as described previously (12). Briefly, normal B cells, Raji, and Daudi cells were treated with

various concentrations of ixazomib for 48 hours, and the cells were harvested, washed with PBS, and incubated with the FITC-Annexin V antibody (556419 BD Biosciences) and TO-PRO-3 stain (Molecular Probes). Samples were analyzed using BD Accuri C6 Plus flow cytometer (BD Biosciences).

### Cell-cycle analyses

After indicated treatments, cells were harvested, washed with PBS, and fixed in ethanol overnight at  $-20^{\circ}\text{C}$ . The cell-cycle analysis was performed as described previously (12).

### Cell fractionation and immunoblot analyses

After indicated treatments, the cells were harvested, and nuclear and cytosolic fractionations were made using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology). For immunoblot analyses, total cell lysates were made with a non-denaturing lysis buffer with protease inhibitors (cOmplete Mini, Roche Diagnostics), protein concentrations were determined using BCA Protein Assay Kit (Pierce Biotechnology), and the samples were separated by SDS-PAGE as described previously (12). The blots were scanned using the Odyssey IR scanner (LI-COR Biosciences).

### Immunofluorescence

After designated treatments, cells were cytospun onto glass slides (Wescor Cytopro Cyto centrifuge), fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X-100 in PBS, and blocked with 5% BSA/PBS. The slides were then incubated with p65 antibody for 2 hours, washed with PBS and incubated with secondary antibody conjugated with Dylight-594 for an hour (D1-2594, Vector Laboratories), and finally mounted with Vectashield antifade mounting medium with DAPI (H-1200, Vector Laboratories). Images were acquired using with an Eclipse E1000 microscope (Nikon).

### Statistical analyses

Significant differences in values between treated and untreated lymphoma cells in different experimental conditions were determined by one-way ANOVA with Dunnett test method (SigmaPlot, version 13.0; Systat Software). Significance was defined as  $P < 0.05$ .

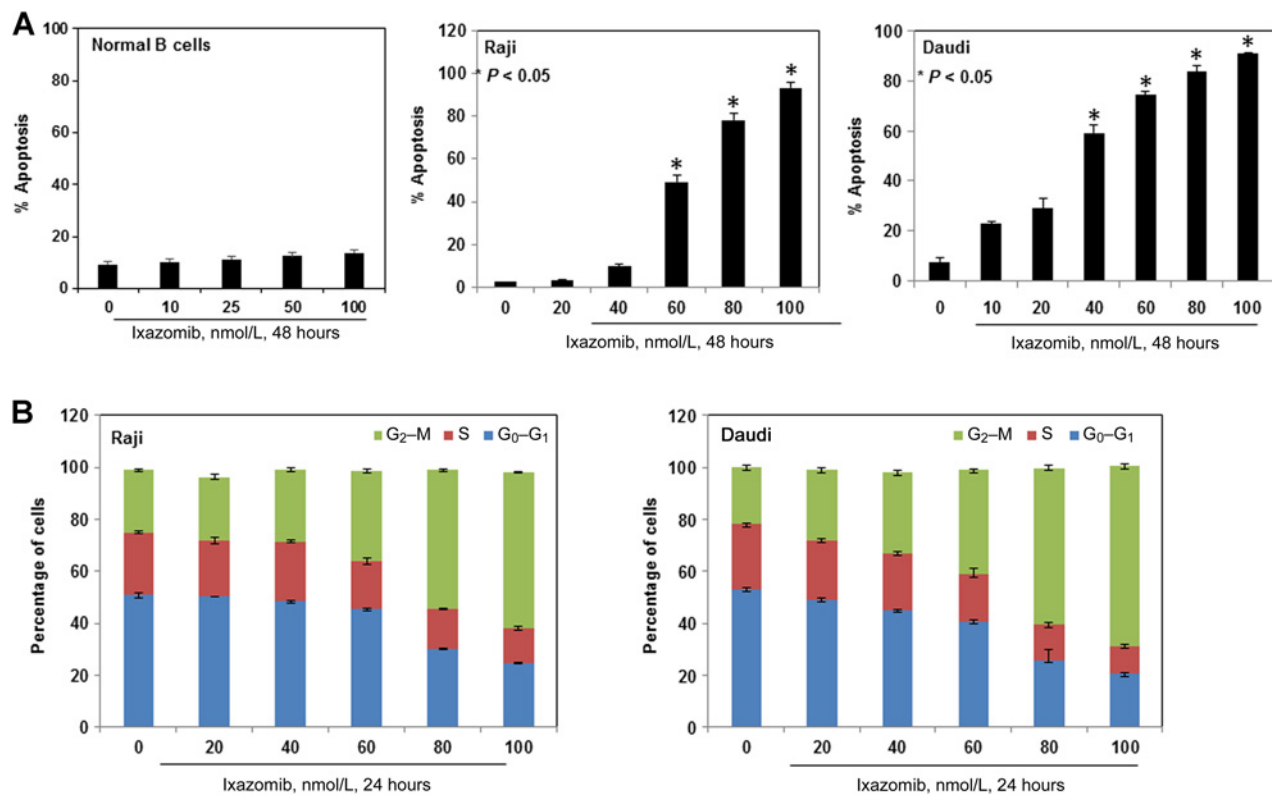
## Results

### Ixazomib induces apoptosis in EBV-associated lymphoma cells

We determined the apoptotic effect of ixazomib in EBV-associated lymphoma cell lines Raji and Daudi. The normal B cells and the lymphoma cell lines were treated with increasing concentrations (0 to 100 nmol/L) of ixazomib for 48 hours. After treatment, cells were washed with PBS and incubated with FITC-Annexin V and To-Pro-3 stain. The percentage of apoptotic cells was then determined by flow cytometry. As demonstrated in Fig. 1A, Raji and Daudi cell lines showed induced apoptosis in a dose-dependent manner ( $P < 0.005$ ; Fig. 1A), but no significant effect was seen on normal B cells. The two tested cell lines are sensitive to ixazomib. Altogether, these results suggest that ixazomib induces apoptosis in EBV-associated lymphoma cell lines.

### Ixazomib induces G<sub>2</sub>-M cell-cycle arrest in EBV-associated lymphoma cells

We also determined the effect of ixazomib on the cell-cycle status in Raji and Daudi cells. Because of the high percentage of



**Figure 1.**

Proteasome inhibitor ixazomib induces apoptosis and cell-cycle arrest in EBV-positive lymphoma cells. **A**, Ixazomib induces apoptosis in EBV-associated lymphoma cells. Normal B cells ( $n = 3$ ), Raji, and Daudi cells were treated with the indicated concentrations of ixazomib for 48 hours. Cells were harvested, washed, and stained with FITC-Annexin V and TOPRO-3 and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three independent experiments; bars represent the SEM. **B**, Ixazomib induces G<sub>2</sub>-M cell-cycle arrest in EBV-associated lymphoma cells. Raji and Daudi cells were treated with indicated concentration of ixazomib for 24 hours. Cells were fixed and stained with propidium iodide and cell-cycle status was determined by flow cytometry. Values represent the mean of three independent experiments.

apoptotic cells at higher concentrations and extended treatment periods, we restricted the treatment duration to 24 hours. After 24 hours of treatment, cells were washed, fixed, and then cell-cycle analysis was performed using flow cytometry. The addition of ixazomib inhibited the growth of cells in a dose-dependent manner in both cell lines (Fig. 1B). Indeed at 100 nmol/L of ixazomib concentration, the proportion of cells actively replicating DNA (S phase) decreased by 10% and the fraction of 2N DNA (G<sub>0</sub>-G<sub>1</sub>) reduced significantly by 25% compared with the control. Then, we had cells at 4N DNA (G<sub>2</sub>-M), which increased markedly by more than 30% (Fig. 2). These results suggest that ixazomib treatment inhibits the cellular DNA synthesis leading to cell-cycle arrest at the G<sub>2</sub>-M phase of the EBV-associated lymphoma cell lines.

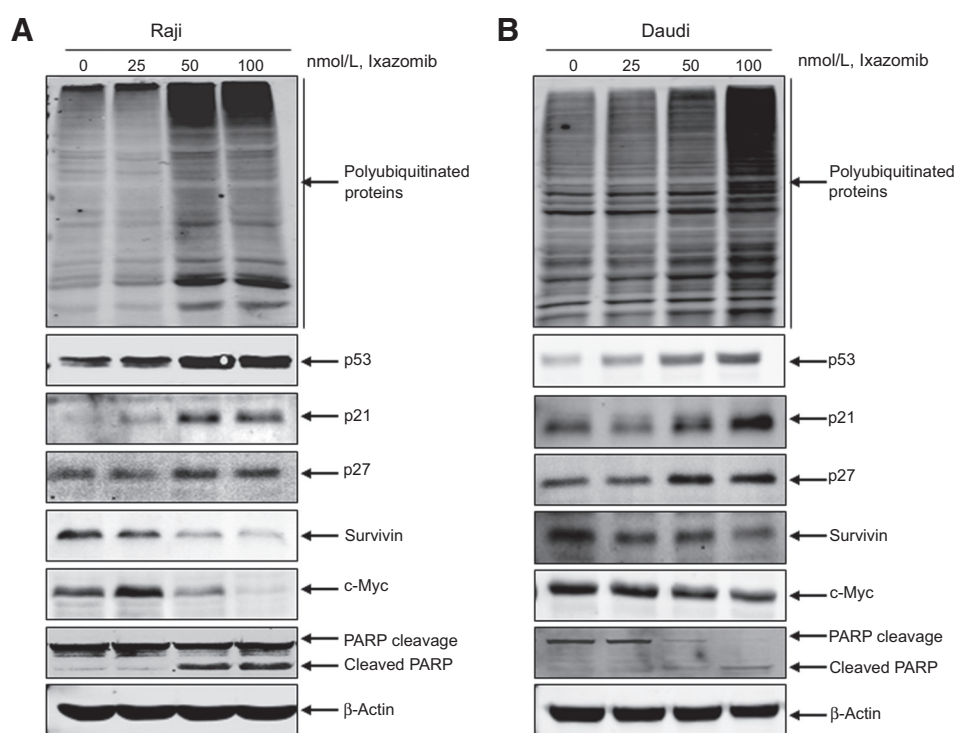
#### Ixazomib upregulates cell-cycle regulators and inhibits antiapoptotic proteins

After observing induced apoptosis and cell-cycle arrest with ixazomib treatment, we explored the changes in the key regulatory molecules involved in the process of cell cycle and apoptosis. Both cell lines, Raji and Daudi, were treated with ixazomib (0 to 100 nmol/L) and incubated for 24 hours. As demonstrated in Fig. 2A and B, both cell lines showed increased polyubiquitinated proteins with induction of key cell-cycle regulators p53, p21, and p27 in a dose-dependent manner (Fig. 2A and B). Compared with

control, the antiapoptotic protein amounts of survivin and c-Myc were decreased in ixazomib-treated lymphoma cells. Also, we observed cleaved PARP in a dose-dependent manner in both cell lines (Fig. 2A and B). These observations clearly confirm the effect of ixazomib in cell-cycle regulation and apoptosis by differentially regulating p53, p21, p27, survivin, c-Myc, and PARP activity.

#### Ixazomib inhibits proteasomal degradation of phosphorylated I $\kappa$ B- $\alpha$

From the previous results, it is clearly evident that ixazomib treatment leads to accumulation of polyubiquitinated proteins. On the basis of previous literature, it is clear that EBV-induced NF- $\kappa$ B activation plays a crucial role in the transformation of lymphoma cells (1). To confirm, we have examined the expression status of pI $\kappa$ B $\alpha$  (S32/36) after 100 nmol/L of ixazomib treatment in a time-dependent manner for up to 8 hours. We observed the inhibition of pI $\kappa$ B $\alpha$  proteasomal degradation, which is a key regulatory subunit of NF- $\kappa$ B (Fig. 3A). However, no changes were detected in protein amounts of I $\kappa$ B $\alpha$  and p65 in a time-dependent manner in both tested cell lines (Fig. 3A). These results suggest that ixazomib blocks proteasomal degradation of pI $\kappa$ B $\alpha$  and inhibits the NF- $\kappa$ B pathway in EBV-associated lymphoma cell line.



**Figure 2.**

**A and B,** Ixazomib upregulates cell-cycle regulators and inhibits antiapoptotic proteins: Raji and Daudi cells were treated with the indicated concentrations of ixazomib for 24 hours. At the end of treatment, total cell lysates were prepared and immunoblot analyses were performed for cell-cycle-regulatory and antiapoptotic proteins. The expression levels of  $\beta$ -actin in the lysates served as the loading control.

#### Ixazomib reduces nuclear translocation of NF- $\kappa$ B subunit

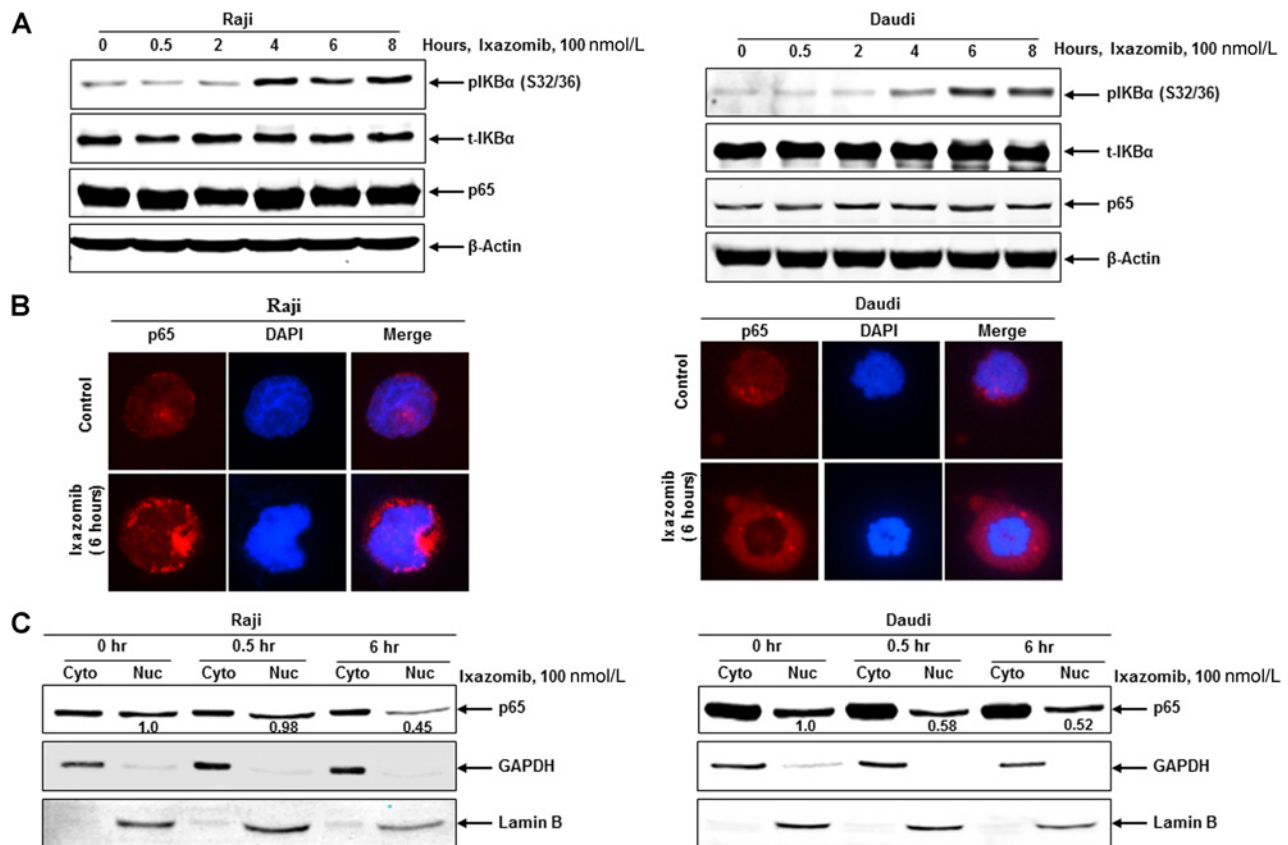
Removal of p18 $\beta$  subunit from the p53 complex leads to the activation of NF- $\kappa$ B signaling. Inhibition of p18 $\beta$  degradation by the proteasome inhibitor ixazomib decreases p53 translocation from the cytosol into the nucleus. On the basis of the time course results, we chose to use the 6th hour as the time point and 100 nmol/L as the treatment concentration for immunofluorescence and Western blot analysis to show the cellular localization of p53 (red color). In both cell lines, immunofluorescence showed an increased cytosolic accumulation of p53 after 6 hours (Fig. 3B). Western blot analysis from nuclear and cytosolic fractionations showed that ixazomib inhibited the p53 translocation from cytoplasm to the nucleus in a time-dependent manner (Fig. 3C), which is concomitant with increased p18 $\beta$  in both tested cell lines (Fig. 3A). The purity of fractions was assessed using GAPDH as cytosolic and lamin B as the nuclear fraction controls. In conclusion, these results reveal that ixazomib treatment inhibits the NF- $\kappa$ B pathway by actively reducing p53 translocation to the nucleus in EBV-associated lymphoma cell lines.

#### Discussion

Inhibition of proteasome activity disrupts the regulation and stability of intracellular proteins like cell-cycle regulators, proapoptotic proteins, and many others (5). Bortezomib is a known and well-characterized proteasome inhibitor, which is clinically used to treat several hematologic malignancies. The major limiting factor of bortezomib is long-term administration through intravenous or subcutaneous routes with the associated risk of peripheral neuropathy (8). Carfilzomib is another FDA-approved proteasome inhibitor but administered intravenously similar to bortezomib (11). Ixazomib is the only alternative proteasome inhibitor to overcome this bortezomib-mediated clinical toxicity

(11). Ixazomib is the first clinically available oral proteasome inhibitor and has been approved for use as a single agent or in combination with other drugs in myeloma therapy (13). A phase I study was conducted to determine the pharmacokinetic parameters in patients with multiple myeloma (13). On the basis of these results, several phase II and III clinical trials have been conducted in patients with relapsed/refractory multiple myeloma. Results indicate progression-free survival is longer in ixazomib-treated patients compared with a placebo group. A recent double-blind, phase III clinical trial demonstrated ixazomib plus lenalidomide and dexamethasone resulted in a significantly longer progression-free survival with limited toxic effects in patients with multiple myeloma compared with the control arm (13). In this study, we tested the preclinical efficacy of the next-generation orally administered proteasome inhibitor ixazomib in EBV-associated lymphoma cell lines. We found that ixazomib significantly enhances apoptosis and cell-cycle arrest by stabilizing p53 and inhibiting the NF- $\kappa$ B pathway by interfering p53 translocation to the nucleus.

The cell transforms to a cancerous cell by gaining the sustainable control of different cellular functions such as apoptosis, differentiation, growth, replication, angiogenesis, invasion, and metastasis, which are mediated by different molecular network mechanisms (14). The ubiquitin-proteasome degradation pathway (UPP) is one such cellular function. UPP has been demonstrated to be involved in cell-cycle progression, apoptosis, transcription, inflammation, as well as immune surveillance (15). These essential functional roles of UPP have made it an important druggable pathway to treat different types of cancer (16). Proteasome signaling plays a critical role in NF- $\kappa$ B activation and regulation. Furthermore, B-cell lymphoid malignancies are directly influenced by EBV, which are known to activate the NF- $\kappa$ B pathway (17). In this study, we examined the effects of



**Figure 3.**

Ixazomib reduces nuclear translocation of NF- $\kappa$ B subunit. **A**, Ixazomib inhibits proteasomal degradation of phosphorylated I $\kappa$ B- $\alpha$ . Raji and Daudi cells were treated with 100 nmol/L concentration of ixazomib for indicated time points. At the end of treatment, total cell lysates were prepared, and immunoblot analyses were performed for pI $\kappa$ B $\alpha$ , t-I $\kappa$ B $\alpha$ , and p65 proteins. The expression levels of  $\beta$ -actin in the lysates served as the loading control. **B**, Ixazomib reduces nuclear translocation of NF- $\kappa$ B subunit demonstrated by immunostaining. Raji and Daudi cells were treated with 100 nmol/L ixazomib for indicated time points and cytospun onto glass slides. The cells were fixed, permeabilized, and stained for p65 and DAPI. Images were acquired with a fluorescent microscope using a 60 $\times$  oil immersion lens. **C**, Ixazomib reduces nuclear translocation of NF- $\kappa$ B subunit demonstrated by cellular fractionation. Raji and Daudi Cells were treated with 100 nmol/L ixazomib for indicated time points, and immunoblot analysis of p65 was performed on subcellular fractions (nuclear and cytosolic). The expression of GAPDH and Lamin B served as the cytosolic and nuclear fraction controls, respectively. Visual overview, Schematic diagram of NF- $\kappa$ B signaling inhibition by ixazomib.

proteasome inhibitor ixazomib on apoptosis and cell-cycle checkpoints in EBV-associated lymphoma cell lines Raji and Daudi. Ixazomib treatment significantly induced apoptosis in these two cell lines in a dose-dependent manner. Our results are in line with the data of Chauhan and colleagues, where they demonstrated that ixazomib treatment induced apoptosis in human multiple myeloma cell lines (18). Furthermore, ixazomib-induced apoptosis results are comparable with bortezomib in EBV-associated B-cell lymphoma cells (1).

Several other studies have reported that proteasome inhibitors lead to stabilization of polyubiquitinated proteins. Here in, we observed the same with ixazomib in a dose-dependent manner. p53, a tumor suppressor and a cell-cycle regulator, is one such specific molecule studied for polyubiquitinated protein stabilization (19). Upon ixazomib treatment, both the studied cell lines showed polyubiquitinated proteins and the stabilization of p53 protein, which lead to differential regulation of cell-cycle regulators such as p21, p27, and antiapoptotic molecules like c-Myc and survivin (20). G<sub>1</sub>-S and G<sub>2</sub>-M cell-cycle transitions are controlled by multiple proteins. Transcription of a plethora of cell-cycle-

promoting genes is maintained in an inactive state by interaction or transcription inhibition by p21 and p27 (21). In different cancer entities, it is established that p53 activation transcriptionally represses c-Myc required for cell-cycle regulation and apoptosis (22). Survivin is a downstream regulatory molecule of the NF- $\kappa$ B signaling axis, which is also a transcriptionally repressed antiapoptotic gene by p53. (23). Cleavage of PARP is a known molecular event for activating apoptosis signaling (24). Our data suggest that ixazomib induces expression of cell-cycle regulators p21 and p27, where they inhibit the cell cycle at G<sub>1</sub>-S and G<sub>2</sub>-M. Furthermore, reduced antiapoptotic proteins c-Myc and survivin, with the association of PARP cleavage, is a clear indication of its positive role in apoptosis.

As discussed, NF- $\kappa$ B plays a critical role in proteasome-mediated cellular functions and consists of NF- $\kappa$ B1/p50, NF- $\kappa$ B2/p52, RelA/p65, RelB, and c-Rel as family members (Fig. 3, visual overview). NF- $\kappa$ B forms various homo- and heterodimeric complexes. Activation of NF- $\kappa$ B signaling mediates through translocation of the p65 complex into the nucleus. NF- $\kappa$ B activation is regulated by I $\kappa$ B $\alpha$ , which masks the nuclear localization signal

motif of p65. When I $\kappa$ B kinases (IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ ) phosphorylate I $\kappa$ B $\alpha$ , they were also simultaneously ubiquitinated and degraded by proteasome machinery (Fig. 3, visual overview). Thus, the free NF- $\kappa$ B dimers can translocate to the nucleus, where they dysregulate different tumorigenic genes (25). Ixazomib treatment stabilized the phosphorylated I $\kappa$ B $\alpha$  protein amounts without altering the total I $\kappa$ B $\alpha$  protein amount in both treated cell lines Raji and Daudi. Furthermore, it is clearly evident that in both these cell lines, RelA/p65 accumulated in the cytoplasm rather than in the nucleus. Second, RelA/p65 nuclear presence decreased markedly in the treated cells. As expected, the screened NF- $\kappa$ B-regulated genes *c-Myc* and *survivin* were also downregulated (22–23). Collectively, these preclinical findings suggest that inhibition of NF- $\kappa$ B signaling using ixazomib may be an attractive therapeutic approach in patients with EBV-associated B-cell neoplasms.

### Disclosure of Potential Conflicts of Interest

S. Ganguly has received speakers bureau honoraria from Seattle Genetics and is a consultant/advisory board member for Amgen, Takeda, Janssen, Kite Pharma, and Daiichi Sankyo. J.P. McGuirk reports receiving commercial research grants from Novartis, Fresenius Biotech, Astellas Pharma, Bellicum Pharmaceuticals, Gamida Cell, and Pluristem Ltd, reports receiving other commercial research support from ArticulateScience LLC, and has received speakers bureau honoraria from Kite Pharma. No potential conflicts of interest were disclosed by the other authors.

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