Targeted Therapy for EBV-Associated B-cell Neoplasms

Siddhartha Ganguly1,2, Sudhakiranmayi Kuravi1,2, Satyanarayana Allebona1,2, Giridhar Mudduluru1,2, Roy A. Jensen2,3,4, Joseph P. McGuirk1,2, and Ramesh Balusu1,2,4

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-kB 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-kB 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 G2–M phase with a concomitant decrease in G0–G1 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 IκBα 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-kB signaling in the treatment of EBV-associated B-cell neoplasms.

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

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 immunotheartapeutics, and antineoplastic chemotherapy (3). EBV-associated lymphomas are characterized by prominent activation of NF-kB pathway and targeting this pathway establishes a rationale for an attractive therapeutic approach.
approach (4). The ubiquitin/proteasome signaling pathway plays an important role in the regulation of NF-κB signaling 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 p65. Bortezomib is the first FDA-approved proteasome inhibitor for treating both newly diagnosed and relapsed/refractory multiple myelomas, and mantle cell lymphomas.

Ixazomib induces apoptosis in EBV-associated lymphoma cells

We determined the apoptotic effect of ixazomib in EBV-associated lymphoma cells. 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 cells.

Ixazomib induces G2/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

Growing evidence suggests the potential of ixazomib use as monotherapy or in combination for the treatment of EBV-associated B-cell neoplasms. Therefore, using ixazomib for targeting myeloma phenotype. From the existing literature review, there are limited data suggesting the potential of ixazomib use as monotherapy or in combination for the treatment of EBV-associated B-cell neoplasms.

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).
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 (G0–G1) reduced significantly by 25% compared with the control. Then, we had cells at 4N DNA (G2–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 G2–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 IkB-α

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-κB activation plays a crucial role in the transformation of lymphoma cells (1). To confirm, we have examined the expression status of pIkBz (S32/36) after 100 nmol/L of ixazomib treatment in a time-dependent manner for up to 8 hours. We observed the inhibition of pIkBz proteasomal degradation, which is a key regulatory subunit of NF-κB (Fig. 3A). However, no changes were detected in protein amounts of IkBz and p65 in a time-dependent manner in both tested cell lines (Fig. 3A). These results suggest that ixazomib blocks proteasomal degradation of pIkBz and inhibits the NF-κB pathway in EBV-associated lymphoma cell line.
Ixazomib reduces nuclear translocation of NF-κB subunit

Removal of pIkBβ subunit from the p65 complex leads to the activation of NF-κB signaling. Inhibition of pIkBβ degradation by the proteasome inhibitor ixazomib decreases p65 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 p65 (red color). In both cell lines, immunofluorescence showed an increased cytosolic accumulation of p65 after 6 hours (Fig. 3B). Western blot analysis from nuclear and cytosolic fractionations showed that ixazomib inhibited the p65 translocation from cytoplasm to the nucleus in a time-dependent manner (Fig. 3C), which is concomitant with increased pIkBβ 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-κB pathway by actively reducing p65 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 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 administrated 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-κB pathway by interfering p65 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 are significant in cancer (15). Ixazomib signaling plays a critical role in NF-κB activation and regulation. Furthermore, B-cell lymphoid malignancies are directly influenced by EBV, which are known to activate the NF-κB pathway (17). In this study, we examined the effects of...
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). G1–S and G2–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-κB signaling axis, which is also a transcriptionally repressed antiapoptotic gene by p53 (23).

As discussed, NF-κB plays a critical role in proteasome-mediated cellular functions and consists of NF-κB1/p50, NF-κB2/p52, RelA/p65, RelB, and c-Rel as family members (Fig. 3, visual overview). NF-κB forms various homo- and heterodimeric complexes. Activation of NF-κB signaling mediates through translocation of the p65 complex into the nucleus. NF-κB activation is regulated by IκBα, which masks the nuclear localization signal

Figure 3.

Ixazomib reduces nuclear translocation of NF-κB subunit. A, Ixazomib inhibits proteasomal degradation of phosphorylated IκB-α. 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 pIκBα, t-IκBα, and p65 proteins. The expression levels of β-actin in the lysates served as the loading control. B, Ixazomib reduces nuclear translocation of NF-κB subunit demonstrated by immunostaining. Raji and Daudi cells were treated with 100 nmol/L ixazomib for indicated time points and cyto spun onto glass slides. The cells were fixed, permeabilized, and stained for p65 and DAPI. Images were acquired with a fluorescent microscope using a 60× oil immersion lens. C, Ixazomib reduces nuclear translocation of NF-κB 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-κB signaling inhibition by ixazomib.
motif of p65. When IkB kinases (IKKα, IKKβ, and IKKγ) phosphorylate IkBα, they were also simultaneously ubiquitinated and degraded by proteasome machinery (Fig. 3, visual overview). Thus, the free NF-κB dimers can translocate to the nucleus, where they dysregulate different tumorigenic genes (25). Ixazomib treatment stabilized the phosphorylated IkBα protein amounts without altering the total IkBα 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-κB–regulated genes c-Myc and survivin were also downregulated (22–23). Collectively, these preclinical findings suggest that inhibition of NF-κB 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, Bellumc 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.

References

Authors’ Contributions
Conception and design: S. Ganguly, S. Alleboina, R.A. Jensen, R. Balusu
Development of methodology: S. Ganguly, S. Kuravi, S. Alleboina, R. Balusu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ganguly, S. Alleboina, R.A. Jensen, R. Balusu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Ganguly, S. Kuravi, S. Alleboina, G. Mudduluru, J.P. McGuirk, R. Balusu
Writing, review, and/or revision of the manuscript: S. Ganguly, S. Kuravi, S. Alleboina, G. Mudduluru, R.A. Jensen, J.P. McGuirk, R. Balusu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ganguly, S. Alleboina, R.A. Jensen, R. Balusu
Study supervision: S. Ganguly, R. Balusu

Acknowledgments
The authors would like to thank Ms. Sophia McCormick and Mrs. Cassaudra Shipman from the Biospecimen Repository Core Facility (BBRC), the University of Kansas Medical Center (Kansas City, KS) for providing healthy donor blood to isolate normal B cells. R. Balusu acknowledges the pilot award from American Cancer Society (ACS-IRG-16-194-07), Soslod Family Foundation Research Award, Hale Family Foundation, and Frontiers Clinical and Translational Pilot Award U19TR000001. We are very grateful to Dr. Janice Cheng [lab member] for thorough proofreading of the manuscript.

Received August 29, 2018; revised October 16, 2018; accepted November 14, 2018; published first November 28, 2018.
Targeted Therapy for EBV-Associated B-cell Neoplasms

Siddhartha Ganguly, Sudhakiranmayi Kuravi, Satyanarayana Alleboina, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-18-0924

Visual Overview
A diagrammatic summary of the major findings and biological implications:
http://mcr.aacrjournals.org/content/17/4/839/F1.large.jpg

Cited articles
This article cites 25 articles, 11 of which you can access for free at:
http://mcr.aacrjournals.org/content/17/4/839.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/17/4/839.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.