Selective Toxicity of Investigational Ixazomib for Human Leukemia Cells Expressing Mutant Cytoplasmic NPM1: Role of Reactive Oxygen Species

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Abstract

Purpose: This study was performed to determine whether the investigational proteasome inhibitor ixazomib demonstrated selective antineoplastic activity against acute myelogenous leukemia cells expressing a mutated nucleophosmin-1 gene and to gain a better understanding of its mechanisms of action.

Experimental Design: The cytotoxic effects of ixazomib treatment were analyzed in human acute myelogenous leukemia (AML) cell lines and primary AML samples expressing wild-type or mutated NPM1 (NPMc+). The potential roles of oxidative stress in mediating cytotoxic activity were determined using flow cytometry, enzyme-based assays, and Western blots.

Results: Apoptosis induced by ixazomib was abrogated by knockdown of NPM1/NPMc+ expression using an inducible shRNA construct and enhanced by NPMc− overexpression. Cytotoxicity was associated with superoxide generation and was reduced by the addition of the antioxidant N-acetylcysteine. AML cells expressing NPMc+ had significantly reduced levels of intracellular glutathione and NADPH associated with reduced antioxidant responses to drug treatment. Treatment of patients with relapsed NPMc+ AML resulted in an antileukemic effect in 1 patient as demonstrated by a marked reduction of leukemic blasts in the peripheral blood. Efficacy was associated with superoxide generation, reduced glutathione levels, and reduced mRNA and protein expression of antioxidant effectors in responding cells.

Conclusions: In this study, a direct association was observed between NPMc+ expression in AML, reduced antioxidant responses, and enhanced sensitivity to an oral proteasome inhibitor that induces oxidative stress. These data suggest that intracellular determinants of antioxidant responses may be good predictors of therapeutic response to ixazomib.

Introduction

Molecular characterization of underlying genetic defects is of both prognostic and therapeutic importance in the management of acute myelogenous leukemia (AML). Heterozygous mutation of nucleophosmin-1 (NPM1) is one of the most frequent genetic lesions in adult AML and is detected in over 60% of patients with a normal karyotype (1, 2). NPM1 is a multifunctional nucleolar phosphoprotein that shuttles between the nucleus and cytoplasm. Although wild-type (WT) NPM1 is primarily localized in the nucleolus, a mutation that results most commonly from a four base pair (TCTG) insertion at the C-terminus of the protein leads to its aberrant cytoplasmic localization. This dyslocalization is attributable to the addition of a nuclear export signal, the loss of two tryptophan residues (288 and 290), and to the acquisition of a cysteine residue at position 288 (3, 4).

With the exception of all-trans-retinoic acid (ATRA) that is used to treat acute promyelocytic leukemia, there are no currently approved therapies that selectively target specific genetic subtypes of AML. Although conventional cytotoxic chemotherapy benefits many patients with de novo AML expressing NPMc+ in the absence of a FLT3-ITD mutation (5), NPM1 mutations represent an attractive target for therapeutic intervention. We have recently demonstrated that NPMc+ expression sensitizes AML cells to apoptosis induced by reactive oxygen species (ROS)-generating drugs that include the proteasome inhibitor bortezomib (Velcade) and arsenic trioxide (ATO; ref. 4). Data from Falini and colleagues (6) further supported this finding by demonstrating growth inhibition and apoptosis in AML cells expressing mutated NPM1 after treatment with ATO alone and in combination with ATRA. This result is also supported by the recent observation that CD34+ AML cells, an immunophenotypic feature associated with AML expressing NPMc+ (2), are more sensitive to bortezomib treatment than are CD34− AML cells (7). However, the clinical use of bortezomib in a heavily pretreated population of AML patients has been limited by adverse events, including peripheral neuropathy and orthostatic hypotension, and durations of therapy have been short (8, 9).

Ixazomib, a small-molecule boronate proteasome inhibitor, is the first oral proteasome inhibitor under clinical investigation in multiple myeloma (10). Ixazomib (MLN2238), the biologically
Translational Relevance

Acute myelogenous leukemias (AML) remain a group of diseases in dire need of additional therapies. The treatment of these disorders is increasingly guided by the underlying genetic aberrations. Mutations in NPM1 are the most frequent molecular aberration in AML. The discovery that the expression of mutated NPM1 (NPMc+) results both in reduced antioxidant responses and in enhanced sensitivity to a proteasome inhibitor that induces superoxide provides a new insight into the mechanism of action of ixazomib and of other drugs in this class such as bortezomib. Furthermore, it suggests that monitoring oxidative stress responses in leukemic cells before and during treatment with proteasome inhibitors may provide prognostic information on the potential for a therapeutic response.

Cytotoxicity of Ixazomib for NPMc+ AML

Primary AML samples

Ficoll-purified, viably frozen mononuclear cells from the bone marrow or peripheral blood of AML patients were obtained after informed consent according to the institutional guidelines [Stanford University Institutional Review Board (IRB) No. 6453 and 27687]. Patients treated with single-agent ixazomib (including patients AML1, AML2, and AML3) were enrolled on an active clinical protocol at the Stanford University Medical Center for relapsed and refractory AML expressing the mutated NPM1 gene (NCT02030405, Stanford IRB Protocol ID 28771). Over a 14-month period, 3 of 4 patients accrued had samples available for study. Patients received ixazomib at a flat dose of 4 mg or at a dose of 2 mg/m² if body surface area was < 2 m² on days 1, 4, 8, and 11 of a 21-day cycle. This clinical trial is ongoing, and complete efficacy and safety results are not yet available. Peripheral blood and bone marrow samples were collected from these patients prior to ixazomib therapy, 22 hours after first dose of ixazomib in cycle 1, and at indicated time points during cycles 1 and 2. Hydroxyurea was only permitted for symptomatic patients with peripheral leukocyte count > 25,000 at the discretion of the investigators during cycle 1.

Reagents

Ixazomib citrate (MLN9708) was kindly provided by Millennium Pharmaceuticals, Inc., and was the reagent used in the preclinical experiments where ixazomib is referenced in the text. Other compounds used included suberoylanilide hydroxamic acid (SAHA; Selleck-Chem), Trichostatin A (TSA; Selleck-Chem), MLN2238 (Selleck-Chem), doxycycline (Calbiochem), and bortezomib (Sigma).

Retro-or lentiviral vector constructs and establishment of stable cell lines

Generation of the lentivirus-infected OCI-AML3 cell line containing a doxycycline-inducible construct to inducibly express NPM1/NPMc+ shRNA was performed as previously described (4). Retroviral infection of K562 cells with GFP, Step/Flag (SF)-tagged wt-NPM1, or SF-tagged NPMc+ was also performed as previously described (4). After 1 week, the 10% of GFP-positive cells with the highest GFP expression were sorted and used within 3 weeks for experiments, as NPMc+ expression declined thereafter.

Cell viability

AML cell lines were seeded in triplicate at 2 x 10⁴ cells per well in 96-well plates and incubated for 24 or 48 hours at 37°C with 5% CO₂. Cells were treated with serial dilutions of ixazomib (4.7 nmol/L to 1,200 nmol/L) and/or SAHA (125 nmol/L to 5,000 nmol/L) and incubated at 37°C with 5% CO₂. Cell viability was assessed at either 24 or 48 hours after the initial drug treatment using CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega) and measured by microplate reader with a 96-well format. IC₅₀ values were calculated using data from at least three independent experiments. For evaluation of N-acetylcysteine (NAC) effects, cells were preincubated with NAC for 4 hours prior to treatment with ixazomib to allow time for GSH biosynthesis (16).

Materials and Methods

Cell lines

The OCI-AML3 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Authentication of the OCI-AML3 cell line included testing for mycoplasma contamination, and the presence of the NPMc+ mutation was confirmed by sequencing using RT-PCR and by Western blot analysis using an NPMc+ specific antibody. The human acute myeloid leukemia cell line K562 was authenticated by and purchased from the ATCC and cultured for fewer than 6 months. Growth curves and morphology were evaluated on both cell lines. All cell lines were maintained in the recommended culture media, and all drug treatment experiments were initiated at a cell density of 1 x 10⁶ to 4 x 10⁵ cells/mL.

Active boronic acid form of ixazomib citrate (MLN9708) that forms immediately upon exposure to aqueous solution or plasma, has a proteasome dissociation half-life that is 6-fold faster than that of bortezomib (11). This rapid-on and rapid-off pharmacology makes this drug potentially more specific for tumor cells and may lead to an improved therapeutic index (12). Its substantial oral bioavailability and weekly or twice-weekly scheduling (13) are additional attractive properties for a disease, such as AML, which primarily affects an elderly population that may not tolerate intensive treatment regimens. In addition, ixazomib has significantly less neurotoxicity than bortezomib and has displayed a manageable safety profile in early-phase clinical trials in refractory and heavily pretreated myeloma populations (14, 15). This improved tolerability profile positions ixazomib as a promising antileukemic agent for maintenance therapy or for combination therapy with other antileukemic drugs in the treatment of AML.

We found that ixazomib has selective toxicity for NPMc+ AML cells and has elucidated novel mechanism underlying the cytotoxicity. Ixazomib is effective in inducing apoptosis in NPMc+ expressing AML that is associated with ROS induction and is attenuated by antioxidant treatment. Indeed, the expression of mutated NPM1 results in a reduction in the Nrf2-mediated antioxidant response leading to reduced intracellular glutathione (GSH) levels. These findings provide new insights into the mechanism of action of proteasome inhibitors as well as of other inducers of ROS in relation to NPMc+ expression.
**In vitro culture of primary AML cells**

Ficoll-purified mononuclear cells were thawed and washed in Iscove’s modified Dulbecco’s medium containing 20% FBS + DNAse (Stem Cell Technology) and cultured for 4 to 6 hours before sorting by FACs. Cells were stained with human CD45 (eBioscience, Inc.) and CD33 (Miltenyi Biotec) in FACS buffer (PBS containing 0.5% FBS and 2 mmol/L EDTA) for 15 minutes in the dark. Cells were washed with Annexin V 10× binding buffer and double stained for Annexin V for 15 minutes in the dark, followed by propidium iodide (PI) according to the manufacturer’s recommendations (eBioscience, Inc.). Cells were resuspended in equal parts of EGM-2 complete medium (Lonza) and SFEM complete medium (Stem Cell Technology) and sorted by FACs. Annexin V–negative and PI-negative viable AML blasts gated by CD45+ and medium side scatter (SSC) profiling were separated on a BD Aria II flow cytometer (BD Biosciences). Primary AML-sorted blasts were plated in duplicate when possible and cultured in modified culture medium consisting equal parts of EGM-2 complete medium and SFEM complete medium at a 1 to 1.5 × 10^5 cells per well in 96-well microplates at 37°C. The components of the culture media are as follows: SFEM complete medium: StemSpan SFEM (Stem Cell Technology) supplemented with 20% FBS and StemSpan CC100 cytokine cocktail (Stem Cell Technology). EGM-2 complete medium: EGM2 medium supplemented with SingleQuote kit Suppl. and growth factors (Lonza).

**Flow cytometric analysis of apoptosis and ROS**

Apoptosis was determined using Annexin V–APC (eBioscience, Inc.) or Annexin V–FITC according to the manufacturer’s instructions. Cells were first incubated with APC- or FITC-conjugated Annexin V for 15 minutes at room temperature, followed by DNA staining with either PI (eBioscience, Inc.) or 5 mol/L DAPI. Cells were then subjected to FACs analysis within less than 1 hour of harvesting. To measure intracellular superoxide and hydrogen peroxide by flow cytometry, cells were washed with PBS and incubated in growth medium containing 10 mol/L H2-DCFDA or 10 mol/L dihydroethidium (DHE) at 37°C for 30 minutes in the dark. Hydrogen peroxide oxidizes the H2-DCFDA probe to a green fluorescent DCFDA, and superoxide oxidizes the DHE probe to the red fluorescent hydroethidium. The mean fluorescent intensity was calculated relative to samples treated with DMSO.

**Western blot analysis**

All assays were performed as described previously (4). Antigens used include anti-NPM1 mouse monoclonal antibody (ab10530; Abcam), anti-NPM1 rabbit antibody (ab24412; Abcam), anti-p62 (#5114; Cell Signaling Technology), anti-GCLM (ab124827; Abcam), anti-NQO1 (ab2346; Abcam), anti-Nrf2 (H-300, sc-13032), anti-HO-1 (ab13248; Abcam), and anti-actin (A5441; Sigma).

**GSH and GSH-GSSG assay**

Reduced intracellular GSH levels were quantified using the GSH-Glo Glutathione Assay (Promega), and oxidized GSSG levels were measured using GSH/GSSG-Glo Assay (Promega). For each condition, 20,000 cells counted in triplicate and washed in cold PBS were incubated in white 96-well microplates. Reduced and oxidized GSH were measured according to the manufacturer’s instructions. The luminescence generated from luciferin was measured using a microplate reader with a 96-well format. Assays were performed in triplicate or greater.

**NADP/NADPH quantification**

Intracellular nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form, NADPH, were measured by colorimetric assay using the NADP/NADPH quantification Kit according to the manufacturer’s instructions (Sigma-Aldrich) using 1 × 10^5 cells for each assay per condition in triplicate.

**RNA isolation and qRT-PCR**

Real-time PCR analysis was performed using the SYBR Green PCR Master Mix (Applied Biosystems) and an Applied Biosystems 7900HT instrument (Applied Biosystems). See Supplementary Table S1 for specific primer sequences used in this study. For relative quantification of each mRNA, we calculated the relative quantity of each sample using relative standard curves. The normalized amount of target mRNA was calculated by dividing the amount of each target mRNA by the amount of GAPDH corresponding to each sample. The results are presented as the fold increase over control.

**Analysis of drug synergy**

The effects of combining ixazomib with SAHA were evaluated using the Chou–Talalay method to determine the combination index (CI) using the CalcuSyn Version 2.0 software (Biosoft). Each affected fraction (Fa) was calculated by comparing the absorbance values of drug-treated wells measured by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, inner salt (MTS) viability assay after 24-hour treatment at 37°C, as described above, to the absorbance of control wells. The drug concentration that induced Fa of 0.5 signifies a 50% reduction in absorbance and growth. Background absorbance was set at Fa = 1. Based on this approach, combinations were defined as synergistic (CI < 0.9), additive (CI, 0.9–1.1), or antagonistic (CI > 1.1). Very strong synergism is defined as CI < 0.1, whereas strong synergism is CI 0.1–0.3, synergism is CI 0.3–0.7, moderate synergism is CI 0.7–0.85, and slight synergism is CI 0.85–0.90.

**Statistical analysis**

Results are expressed as mean ± SD. Data shown reflect multiple independent biologic replicates, not technical replicates. Comparisons between untreated and treated cohorts or measurements at different time points were made by unpaired Student t test. A P value of less than 0.05 was considered to be statistically significant.

Results

Ixzomib preferentially induced apoptosis in AML cell lines expressing NPMc+

We have previously reported that AML cells expressing NPMc+ were sensitive to the proteasome inhibitor, bortezomib, and to ATO (4). To determine whether ixazomib had preclinical anti-leukemic activity similar to that seen with bortezomib, we first treated two human leukemia cell lines, OCI-AML3 (which expresses NPMc+) and K562 (which expresses WT NPM1), with ixazomib (Fig. 1A). OCI-AML3 cells treated with ixazomib were 22-fold more sensitive at 24 hours and 10-fold more sensitive at 48 hours as compared with K562 cells. Similar effects were seen with the MLN2238 compound in OCI-AML3 cells (Supplementary Fig. S1A). To determine whether these cytotoxic effects were specifically due to NPMc+ expression, we reduced the expression
Cytotoxicity of ixazomib for NPMc⁺ AML

We then assessed the induction of ROS after ixazomib treatment in AML cells at pharmacologically achievable doses (15). Ixazomib treatment of OCI-AML3 cells for 20 hours resulted in a highly significant, dose-dependent increase in the induction of superoxide compared with control (Fig. 2A and Supplementary Fig. S2A). Hydrogen peroxide levels did not increase during this period (Supplementary Fig. S2A and S2B). Superoxide induction was associated with apoptosis, which was abrogated by treatment with NPM1/NPMc⁺ depletion on apoptosis induced by treatment with ixazomib at 75 nmol/L and 150 nmol/L for 24 hours as measured by FACS assay. E, stable overexpression of GFP, WT NPM1, or NPMc⁺ in K562 cells as demonstrated by Western blot analysis. The expression of strep-tag II and flag dual tagged NPM1 (SF-NPM1) and NPMc⁺ (SF-NPMc⁺) was detected with strep-tag II antibody and N-terminal NPM1 antibody. F, effects of NPM1 and NPMc⁺ overexpression on ixazomib-induced cytotoxicity. K562 cells stably expressing GFP, WT NPM1, or NPMc⁺ were treated with increasing doses of ixazomib for 24 hours, followed by MTS cell viability assay. G, effects of NPM1 or NPMc⁺ overexpression on ixazomib-induced apoptosis in K562 cells. Graphs represent mean values ± SD of three biologic replicates. *, P ≤ 0.05; **, P ≤ 0.01; ***; P ≤ 0.001; ****, P ≤ 0.0001.

**Figure 1.**
Selective cytotoxicity of ixazomib for NPMc⁺-expressing cells. A, comparison of IC5₀ values of ixazomib for OCI-AML3 (IC5₀ values at 24 h: 65.9 ± 12.5 nmol/L; 48 h: 24.5 ± 10.8 nmol/L) and K562 cells (IC5₀ values at 24 h: 1462.9 ± 52.4 nmol/L; 48h: 245.2 ± 46.7 nmol/L). Viability was determined by MTS colorimetric assay after treatment with ixazomib for 24 or 48 hours. B, effect of inducible NPM1/NPMc⁺ shRNA expression on NPM1/NPMc⁺ expression after doxycycline (dox) treatment (1 μg/mL for 6 days). Blots were probed with antibodies specific for WT NPM1 (top) or NPMc⁺ (middle); actin served as a loading control (bottom). C, effects of NPM1/NPMc⁺ depletion on cell viability after treatment with ixazomib at pharmacologically achievable concentrations (15; Fig. 1C). Treatment with doxycycline for 6 days (doxycycline, d6) led to a significant 2-fold increase (P ≤ 0.04) in cell viability at almost every dose level of ixazomib as compared with OCI-AML3 cells in the absence of doxycycline and to cells expressing a control lentiviral shRNA. Ixazomib treatment resulted in the induction of apoptosis, as determined by FACS analysis of Annexin V–positive cells, whereas knockdown of NPM1/NPMc⁺ expression significantly reduced the extent of apoptosis at both 75 nmol/L and 150 nmol/L ixazomib (75 nmol/L, 2.5-fold, P = 0.0004; 150 nmol/L, 1.8-fold, P = 0.01; Fig. 1D). Conversely, stable overexpression of NPMc⁺ in K562 cells (Fig. 1E) resulted in significantly decreased cell viability (P = 0.04; Fig. 1F) and increased the induction of apoptosis (75 nmol/L, P = 9 × 10⁻⁵; 150 nmol/L, P = 0.01; 300 nmol/L, P = 0.0003) as compared with expression of GFP alone or of unmutated NPM1 (Fig. 1G and Supplementary Fig. S1B and S1C). These results strongly suggest that the expression of NPMc⁺ is directly associated with the preferential cytotoxicity of ixazomib in AML cells.

**Ixazomib induced superoxide-mediated cytotoxicity**

of NPM1/NPMc⁺ in OCI-AML3 cells using doxycycline-inducible shRNA expression (Fig. 1B) and determined the effect of NPM1/NPMc⁺ reduction on cell viability after treatment with ixazomib at pharmacologically achievable concentrations (ref. 15; Fig. 1C). Treatment with doxycycline for 6 days (doxycycline, d6) led to a significant 2-fold increase (P ≤ 0.04) in cell viability at almost every dose level of ixazomib as compared with OCI-AML3 cells in the absence of doxycycline and to cells expressing a control lentiviral shRNA. Ixazomib treatment resulted in the induction of apoptosis, as determined by FACS analysis of Annexin V–positive cells, whereas knockdown of NPM1/NPMc⁺ expression significantly reduced the extent of apoptosis at both 75 nmol/L and 150 nmol/L ixazomib (75 nmol/L, 2.5-fold, P = 0.0004; 150 nmol/L, 1.8-fold, P = 0.01; Fig. 1D). Conversely, stable overexpression of NPMc⁺ in K562 cells (Fig. 1E) resulted in significantly decreased cell viability (P = 0.04; Fig. 1F) and
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Figure 2.
Effect of ixazomib on superoxide generation in OCI-AML3 cells and reversal of apoptosis by NAC. A, superoxide generation as a function of ixazomib concentration after 20 hours, as assessed by DHE staining and expressed as mean absolute value. B, the extent of apoptosis was determined by Annexin V staining at the concentrations shown for 24 hours with and without NAC treatment. C, effects of NPM1/NPMc depletion on the Nrf2-antioxidant pathway response following ixazomib treatment. D, effects of NPMc overexpression on ixazomib-induced superoxide generation in K562 ectopically expressing GFP, WT NPM1, or NPMc. Cells were treated with increasing doses of ixazomib for 20 hours. E, effects of NAC on ixazomib-induced superoxide generation in K562 cells stably overexpressing NPMc. Cells were treated with increasing ixazomib doses for 20 hours with and without NAC treatment. F, effect of NAC on ixazomib-mediated induction of Nrf2 in K562 cells stably overexpressing NPMc. Graphs in A to B and D to E represent mean values ± SD of three biologic replicates. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

with NAC, a GSH precursor and ROS scavenger (Fig. 2B and Supplementary Fig. S2C and S2D).

We asked whether ixazomib induced NPMc+-associated oxidative stress response by examining the Nrf2-antioxidant response pathway. The induction of superoxide with ixazomib was accompanied by a marked upregulation of Nrf2 and of Nrf2-targeted antioxidant proteins, including p62 (both an Nrf2 target gene and an activator of Nrf2; ref. 17), NQO1, GCLM, and HO-1 in OCI-AML3 cells (Fig. 2C). Consistent with the reduction in ixazomib-induced cell death following depletion of NPMc+ (Fig. 1C and D), depletion of NPMc+ greatly attenuated the upregulation of the Nrf2-antioxidant pathway (Fig. 2C). Conversely, increasing doses of ixazomib resulted in a significant increase in superoxide in K562 cells ectopically expressing NPMc+, which was also abrogated with NAC treatment (Fig. 2D and E). To further dissect the possible contribution of proteasomal inhibition as opposed to oxidative stress on the induction of Nrf2 in response to ixazomib, we evaluated the effect of NAC on Nrf2 expression in AML cells ectopically expressing NPMc+. Ixazomib enhanced Nrf2 expression in a dose-dependent manner, and NAC pretreatment abrogated this effect (Fig. 2F), demonstrating that the Nrf2 response results primarily from ixazomib-induced oxidative stress. These results support the conclusion that superoxide generation is the major contributor to the cytotoxic effects of ixazomib treatment in AML cells expressing NPMc+.

Aberrant GSH homeostasis in AML cells expressing NPMc+
The regulation of redox homeostasis is critical to cell survival (18). Notably, GCLM, one of the subunits of glutamate cysteine ligase (19) directly involved in GSH biosynthesis and homeostasis, was upregulated by NPMc+ depletion (Fig. 2C). We hypothesized that reduced intracellular GSH might contribute to the increased susceptibility of NPMc+-expressing AML cells to ixazomib-induced apoptosis. To directly evaluate GSH pathway activity in AML cells expressing NPMc+, we quantitated intracellular GSH in OCI-AML3 cells after treatment with ixazomib (150 nmol/L) in the presence and absence of NAC (Fig. 3A). Ixazomib treatment depleted intracellular GSH levels over time. Pretreatment with
NAC elevated GSH levels and completely prevented the GSH depletion caused by ixazomib. This finding is concordant with the decrease in apoptosis induced by NAC. To determine whether ixazomib increases oxidized GSH (GSSG) in OCI-AML3 cells, thereby decreasing the ratio of reduced to oxidized GSH (GSH/GSSG) as an index of oxidative stress, we measured levels of GSH and GSSG directly in cell-culture wells. As predicted, the GSH to GSSG ratio remained similar at 18, 20, and 22 hours in control cells, but progressively decreased after ixazomib treatment (Fig. 3B). The alteration in the GSH to GSSG ratio (Supplementary Fig. S3A) correlated with the significant reduction in GSH (P = 0.001; Supplementary Fig. S3B) and the increase in GSSG (P = 0.02; Supplementary Fig. S3C). To test whether the reduction in GSH results directly from NPMc expression, we again depleted NPM1/NPMc with doxycycline and measured GSH with increasing ixazomib concentrations. As shown in Fig. 3C, reducing the expression of NPM1/NPMc led to a significant increase in GSH and markedly attenuated the ixazomib-induced depletion of GSH. NPMc reduction also resulted in a dramatic decrease in the NADP+/NADPH ratio (Fig. 3D). In addition, NPMc expression in K562 cells resulted in a significant reduction of GSH levels (P = 0.005; Fig. 3E). These data support the conclusion that NPMc expression reduces intracellular GSH and renders AML cells more sensitive to ROS-inducing drugs.

Cytotoxicity of ixazomib for primary AML cells

We also compared the induction of apoptosis by ixazomib in primary AML cells with and without the NPMc mutation. The patient characteristics and molecular phenotypes are shown in Supplementary Table S2. Viably frozen primary AML samples that were Ficoll-separated and stored in liquid nitrogen were thawed and sorted for PI-negative leukemic blasts and cultured for 24 hours with ixazomib. Baseline viability after sorting and short-term culture ranged from 85% to 99% (representative viability FACS plot; Supplementary Fig. S4A). Consistent with the results obtained from AML cell lines, cells expressing NPMc in both the de novo and relapsed AML cases were significantly more sensitive to ixazomib than were the AML samples without this mutation (Fig. 4A and B). The presence of common cooperating mutations (20), identified by clinical laboratory improvement amendments (CLIA)-approved genetic testing, did not appear to affect sensitivity to ixazomib in de novo NPMc AML. Although the number of
cases is small, these samples included 1 patient with a concomitant IDH2 mutation, 1 patient with concomitant FLT3-ITD, IDH1, NRAS, and DNTM3A mutations, and 1 patient with concomitant FLT3-ITD mutation. Further, there was a 5-fold lower level of GSH in cells expressing NPMc+ as compared with those that did not (P = 0.0057; Fig. 4C).

We next evaluated the expression of major antioxidant regulators in the Nrf2-antioxidant pathway in the same set of primary AML samples by qPCR (Supplementary Fig. S4B and Supplementary Table S1). Although not significant given the small numbers and variability among samples, there was a slight trend toward downregulation in the expression of genes in the Nrf2-antioxidant pathway (including NRF2, NQO1, GCLC, and GCLM) in NPMc+-expressing AML cells in both the de novo and relapsed setting compared with AML cells expressing unmutated NPM1.

Ixazomib has in vivo biologic activity in patients with NPMc+ AML.

Peripheral blood and bone marrow samples collected prospectively were available from only 3 of 4 total patients with relapsed NPMc+ AML who received single-agent oral ixazomib as participants of an IRB-approved research protocol. Superoxide levels increased in the peripheral blasts of 2 patients following treatment (Fig. 5A), and none had an increase in hydrogen peroxide (Supplementary Fig. S5A). Patient AML3 had a marked reduction in the peripheral blast count following two cycles of therapy (Supplementary Fig. S5B) and was the only one of the three with markedly reduced levels of intracellular GSH prior to treatment (Fig. 5B). Unfortunately, this patient was unable to continue treatment due to an unrelated illness. Patients AML1 and AML2, both of whom had higher levels of pretreatment intracellular GSH (Fig. 5B), had progressive disease following a single cycle of ixazomib and were taken off study.

The level of expression of proteins in the Nrf2 antioxidant pathway, including p62, was analyzed in the leukemic blasts of each patient by Western blot. The AML3 sample had dramatically reduced expression of Nrf2, NQO1, GCLM, and p62 compared with the AML1 and AML2 samples (Fig. 5C). Consistent with these results, AML3 cells had a significant decrease in NRF2, NQO1, and GCLC mRNA expression (Fig. 5D) compared with the two
nonresponders, AML1 and AML2 (Fig. 5E; Supplementary Fig. S5C). The expression of GCLM mRNA was unexpectedly elevated in AML3 cells (Fig. 5D), despite the low level of protein expression (Fig. 5C). There was upregulation of NRF2 mRNA as well as of its downstream effectors (with the exception of GCLM) in AML3 cells after the first dose of ixazomib (Fig. 5D). In contrast, the AML1 and AML2 samples demonstrated a decrease in the expression of these genes (Fig. 5E and Supplementary Fig. S5C), suggesting that increased antioxidant capacity might prevent the induction of NRF2 and related genes in these cells. Although these data are limited due to very small patient numbers, they are supportive of the in vitro data and suggest that the therapeutic response to ixazomib and, by extension, to other proteasome inhibitors may depend on a reduced Nrf2-mediated antioxidant response.

Synergistic effects of ixazomib with a histone deacetylase inhibitor in AML cells expressing NPMc

Single-agent SAHA (Vorinostat), a histone deacetylase (HDAC) inhibitor with modest single-agent antileukemic activity (21), has also been shown to induce ROS as a mode of cytotoxicity in AML models (22), with evidence for synergistic cytotoxicity in combination with bortezomib (23–25). We therefore asked whether SAHA in combination with ixazomib would augment superoxide induction and enhance cytotoxicity. Analysis of the data obtained using the Chou and Talalay method (26) showed that low concentrations of ixazomib and SAHA were strongly synergistic (Fig. 6A). To determine whether synergy was dependent on NPMc+ expression, OCI-AML3 cells in which NPM1/NPMc+ expression had been reduced by 6 days of exposure to doxycycline were treated with ixazomib and SAHA, alone and in combination. Knockdown of NPM1/NPMc+ attenuated apoptosis induced by each drug alone, as well as by the combination (Fig. 6B). The combination of the two drugs produced more superoxide than either agent alone (Fig. 6C).

Similar effects were obtained when TSA, another histone deacetylase inhibitor, was substituted for SAHA (Supplementary Fig. S6A–S6C). The combination of TSA and ixazomib resulted in enhanced apoptosis and cytotoxicity that was markedly reduced by NAC. Superoxide was similarly induced by these drugs alone and in combination (Supplementary Fig. S6B). Comparable results were also obtained with bortezomib and SAHA, again demonstrating dependence on NPMc+ expression (Supplementary Fig. S6D and S6E). These results in aggregate strongly suggest that a subset of AML cells that have reduced antioxidant capacities

Figure 5.
Effects of ixazomib treatment on cells from patients with relapsed NPMc+ AML. Leukemic samples were obtained from 3 patients with relapsed NPMc+ AML before and after ixazomib therapy (AML1, AML2, and AML3). AML3 is labeled with S to indicate sensitive to ixazomib treatment. AML1 and AML2 are labeled with R to indicate resistance to ixazomib treatment. A, comparison of mean absolute superoxide generation levels in leukemic blasts of AML3 versus the nonresponders (AML1 and AML2) before and after first dose of ixazomib collected at 22 hours. B, baseline intracellular GSH levels in leukemic blasts in AML3 versus nonresponders (AML1 and AML2). C, comparison of expression of Nrf2 and Nrf2-regulated antioxidant proteins in patients AML1, AML2, and AML3. D and E, mRNA expression of Nrf2 and downstream genes in patients AML1 (R) and AML3 (S) before (solid bars) and 22 hours after first dose of ixazomib (striped bars). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
may preferentially benefit from the combination of ixazomib with an HDAC inhibitor.

**Discussion**

Ixazomib is an orally administered inhibitor of the proteasomal degradation pathway that is in advanced clinical trials for multiple myeloma. This study was undertaken to evaluate the antileukemic potential of ixazomib in AML cells expressing NPMc\(^{+}\) and to elucidate the molecular mechanisms underlying the preferential cytotoxicity for this leukemic subset. Although we have previously observed that AML cells expressing NPMc\(^{+}\) are sensitive to ROS-inducing drugs, including bortezomib and ATO (4), the mechanism by which NPMc\(^{+}\) expression accounts for that sensitivity was not clear. The present studies show that ixazomib, like bortezomib (4), induces ROS and has some antileukemic activity in human AML cells. The specificity for cells expressing NPMc\(^{+}\) was made clear by expressing exogenous NPMc\(^{+}\) in K562 cells that do not have the NPMc\(^{+}\) mutation and by depleting NPMc\(^{+}\) in OCI-AML3 cells. The dependence of cytotoxicity on ROS induction was supported by the selective generation of superoxide in NPMc\(^{+}\) cells and by the abrogation of cytotoxicity by preincubation with NAC.

A coordinated expression of both detoxifying and antioxidant enzymes is necessary to counteract oxidative stress in order to maintain cellular redox homeostasis. The antioxidant response is largely mediated by the transcription factor Nrf2 that interacts with the antioxidant responsive element (ARE) found in the promoters of specific effector genes (27). The stress sensor protein Keap1 tightly regulates Nrf2 by forming a complex that is targeted for degradation by the 26S proteasome (28). Oxidative stress inactivates Keap1 and prevents the degradation of Nrf2, resulting in its accumulation and nuclear translocation (29). Proteasome inhibitors increase Nrf2 both by inhibiting its Keap1-mediated...
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degradation (27, 28) and by inactivating Keap1 through ROS generation (29–32). Our present study clearly demonstrated that ixazomib-induced upregulation of the Nrf2-antioxidant pathway in NPMc⁺ expressing leukemic cells is primarily due to oxidative inactivation of Keap1, as NAC treatment largely abrogated the ixazomib-induced upregulation of Nrf2-antioxidant pathway. Thus, treatment with ixazomib would be expected to increase Nrf2, the antioxidant response proteins, and GSH levels. Our results, necessarily limited to 3 patients, suggest that leukemic cells with inherently low basal antioxidant capacity may be particularly susceptible to ixazomib-induced cytotoxicity due to an insufficient antioxidant response to ROS generation. Elevated levels of Nrf2 (33) and of other proteins in the antioxidant pathways (34) have been associated with chemoresistance in AML, further supporting the conclusion that identification of AML subsets with low basal antioxidant capacity and low intracellular GSH may be predictive of responses, not only to proteasome inhibitors but also to drugs, such as daunorubicin, that are frequently used in AML treatment. The underlying mechanism leading to differential regulation of NRF2 and Nrf2-target genes in AML cells is not clear. Acquired mutations in the Nrf2 and KEAP1 pathways have been seen in other malignancies (35, 36), but similar mutations have not been detected in AML (37). Constitutive activation of NF-kappaB, which has been detected in primary AML specimens (38), also results in the upregulation of Nrf2 (37). A third possibility is altered epigenetic regulation of Nrf2 itself. Epigenetic silencing of antioxidant genes, including ARE-regulated Nrf2, has been previously reported in AML (39). Which, if any, of these explanations pertain to NPMc⁺ effects on the redox system remains to be explored.

There is evidence that HDAC inhibitors as single agents induce both oxidative stress in AML (22) and the activation of redox-sensitive transcription factors that interact with the ARE (40). In multiple myeloma, the combination of proteasome inhibitors and HDAC inhibitors has demonstrated clinical benefit (41). In AML, treatment with HDAC inhibitors alone has only modest clinical efficacy (21, 42). In this study, treatment of OCI-AML3 leukemic cells with single-agent HDAC inhibitors also demonstrated little effect. However, OCI-AML3 cells treated with ixazomib or bortezomib and an HDAC inhibitor resulted in strong synergy and in the induction of higher levels of superoxide than did either agent alone. Both treatment with NAC and the depletion of NPM1/NPMc⁺ markedly rescued these cells from apoptosis. Indeed, the combination of ixazomib plus SAHA was synergistic in cells at doses that were lower than the individual MTD for each agent. Thus, the combination of ixazomib and SAHA was more effective than either agent alone and may overcome the resistance to ROS-inducing drugs, such as proteasome inhibitors. Future studies are needed to understand the role of NPMc⁺ in regulating the Nrf2-antioxidant pathway and in assessing GSH as a predictor of response to ROS-inducing drugs in patients with relapsed disease.

References

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