Dual Inhibition of Canonical and Noncanonical NF-κB Pathways Demonstrates Significant Antitumor Activities in Multiple Myeloma

Claire Fabre1,2, Naoya Mimura1,2, Kathryn Bobb3, Sun-Young Kong1,5, Gülü Gorgun1,2, Diana Cirstea1,2, Yiguo Hu1,2, Jiro Minami1,2, Hiroto Ohguchi1,2, Jie Zhang4, Jeffrey Meshulam3, Ruben D. Carrasco2, Yu-Tzu Tai1,2, Paul G. Richardson1,2, Teru Hideshima1,2, and Kenneth C. Anderson1,2

Abstract

Purpose: NF-κB transcription factor plays a key role in the pathogenesis of multiple myeloma in the context of the bone marrow microenvironment. Both canonical and noncanonical pathways contribute to total NF-κB activity. Recent studies have shown a critical role for the noncanonical pathway: selective inhibitors of the canonical pathway present a limited activity, mutations of the noncanonical pathway are frequent, and bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of canonical NF-κB activity.

Experimental Design: Multiple myeloma cell lines, primary patient cells, and the human multiple myeloma xenograft murine model were used to examine the biologic impact of dual inhibition of both canonical and noncanonical NF-κB pathways.

Results: We show that PBS-1086 induces potent cytotoxicity in multiple myeloma cells but not in peripheral blood mononuclear cells. PBS-1086 overcomes the proliferative and antiapoptotic effects of the bone marrow milieu, associated with inhibition of NF-κB activity. Moreover, PBS-1086 strongly enhances the cytotoxicity of bortezomib in bortezomib-resistant multiple myeloma cell lines and patient multiple myeloma cells. PBS-1086 also inhibits osteoclastogenesis through an inhibition of RANK ligand (RANKL)–induced NF-κB activation. Finally, in a xenograft model of human multiple myeloma in the bone marrow milieu, PBS-1086 shows significant in vivo anti–multiple myeloma activity and prolongs host survival, associated with apoptosis and inhibition of both NF-κB pathways in tumor cells.

Conclusions: Our data show that PBS-1086 is a promising dual inhibitor of the canonical and noncanonical NF-κB pathways. Our preclinical study therefore provides the framework for clinical evaluation of PBS-1086 in combination with bortezomib for the treatment of multiple myeloma and related bone lesions. Clin Cancer Res; 18(17); 4669–81. ©2012 AACR.

Introduction

Multiple myeloma is a clonal proliferation of malignant plasma cells that accounts for 1% of all cancers and more than 10% of all hematologic malignancies. Pure osteolytic bone lesions are pathognomonic of multiple myeloma, while related bone lesions (bortezomib) have dramatically changed multiple myeloma prognosis by overcoming drug resistance to conventional treatments (2). However, resistance to bortezomib ultimately occurs, highlighting the urgent need for new therapeutic approaches (3).

NF-κB transcription factors play a key role in the pathogenesis of cancers, including multiple myeloma, by regulating genes involved in proliferation, survival, and drug resistance (4, 5). Constitutive NF-κB activity is present in human multiple myeloma cell lines and patient multiple myeloma cells (6, 7); moreover, adhesion of multiple myeloma cells to bone marrow stromal cells (BMSC) induces NF-κB–dependent cytokine (interleukin [IL]-6, TNF-α, IL1-β, SDF-1α, and B-cell activating factor [BAFF]) transcription and secretion by BMSCs, which in turn further activates NF-κB and thereby promotes multiple myeloma cell growth and survival (6, 8–10). NF-κB also modulates expression of antiapoptotic proteins and adhesion molecules such as ICAM-1 (CD54) and VCAM-1 (CD106) on both multiple myeloma and BMSCs, further enhancing

Authors' Affiliations: 1Jerome Lipper Multiple Myeloma Center, 2Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; 3Profectus BioSciences Inc.; 4rel-MD, Inc., Baltimore, Maryland; and 5Research Institute and Hospital, National Cancer Center, Korea

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Kenneth C. Anderson, Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215. Phone: 617-632-2144; Fax: 617-632-2140; E-mail: kenneth_anderson@dfci.harvard.edu

doi: 10.1158/1078-0432.CCR-12-0779

©2012 American Association for Cancer Research.
NF-κB transcription factor plays a key role in the pathogenesis of multiple myeloma in the context of the bone marrow microenvironment. Several studies validate NF-κB pathway as a promising therapeutic target in multiple myeloma, with both canonical and noncanonical pathways contributing to total NF-κB activity. However, selective inhibitors against these pathways have not yet been developed. The preclinical study presented here is designed to characterize the antitumor activity of PBS-1086, a dual inhibitor of NF-κB pathways, in vitro in multiple myeloma cell lines, patient multiple myeloma cells, and also in the presence of bone marrow stromal cells. We show that PBS-1086 induces potent selective cytotoxicity in multiple myeloma cells and overcomes the prosurvival advantage conferred by the bone marrow milieu. We show a synergistic cytotoxicity in bortezomib-resistant multiple myeloma cell lines and patient multiple myeloma cells, suggesting a potential clinical use of PBS-1086 in combination with bortezomib. In addition, our results indicate that PBS-1086 inhibits osteoclast activity, suggesting potential benefit in multiple myeloma–related bone disease. Finally, PBS-1086 shows significant antitumor activity in a human multiple myeloma xenograft murine model with improvement of overall survival. Our preclinical study provides the rationale for clinical evaluation of PBS-1086 in combination with bortezomib for the treatment of multiple myeloma.

Translational Relevance

Materials and Methods

Reagents

PBS-1086 was provided by Profectus BioSciences Inc. Bortezomib was obtained from Selleck Chemicals. Doxorubicin and z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) were obtained from Sigma-Aldrich. TNF-α, insulin-like growth factor-I (IGF-I), and recombinant IL-6 were purchased from R&D Systems.

Human multiple myeloma cell lines

Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL); RPMI-8226 and U266 were purchased from the American Type Culture Collection; doxorubicin (Dox)-resistant RPMI-Dox40 (Dox40) and melphalan-resistant RPMI-LR5 (LR5) cell lines were provided by Dr. William Dalton (Moffitt Cancer Center, Tampa, FL); KMS18 by the DSMZ; IL-6–dependent INA6 by Dr. Renate Burger (University of Kielh, Kiehl, Germany); and bortezomib-resistant IL-6–dependent cell line ANBL6-VRS and its parental counterpart ANBL6-wt by Dr. Robert Orlowski (MD Anderson Cancer Center, Houston, TX). All multiple myeloma cell lines were cultured in RPMI-1640 containing 10% FBS (Sigma Chemical Co.); 20% FBS for ANBL6). 2 μmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (from Gibco). INA6 and ANBL6 cell lines were cultured with IL-6 at 2.5 and 5 ng/mL, respectively.

Tumor cells and BMSCs from multiple myeloma patients

Blood samples from healthy volunteers were processed by Ficoll Hypaque (GE Healthcare) gradient to obtain peripheral blood mononuclear cells (PBMC) and stimulated by phytohemagglutinin. Tumor cells and BMSCs from patients with multiple myeloma were obtained from bone marrow microenvironment. Moreover, mutations of the noncanonical pathway in 20% of multiple myeloma are associated with resistance to steroids versus sensitivity to proteasome inhibitors.

To date, the canonical NF-κB pathway can be blocked by small-molecule inhibitors of IKKβ (e.g., PS-1145 and MLN120B), which inhibit multiple myeloma cell growth in vitro. However, in vivo anti–multiple myeloma activity of IKKβ inhibitors is limited because of the compensatory activation of the noncanonical pathway (7, 18). Moreover, bortezomib inhibits inducible NF-κB activity in multiple myeloma cells but unexpectedly enhances constitutive NF-κB activity via activation of the canonical pathway. Therefore, bortezomib-induced cytotoxicity cannot be fully attributed to inhibition of canonical NF-κB activity in multiple myeloma cells (19, 20). Because inhibition of both canonical and noncanonical pathways is required to efficiently block total NF-κB activity, we here characterize the antitumor activity of PBS-1086, an inhibitor of both canonical and noncanonical NF-κB pathways (21), in multiple myeloma.
Osteoclasts culture and differentiation assay

PBMCs were isolated as described above and cultured in α-MEM containing 10% fetal calf serum (FCS), 100 µM L-glutamine, 100 µg/mL streptomycin, 25 µg/mL macrophage colony-stimulating factor (M-CSF; R&D Systems), and 25 µg/mL RANKL (PeproTech). After 24 hours, the adherent population was reseeded in 96-well plates. After a culture period of 14 to 21 days, cells were stained for TRAP activity, using a leucocyte acid phosphatase kit (Sigma-Aldrich). Histologic micrographs were taken using a Leica DM200 microscope (aperture HC PLANs 10×, objective lenses: N PLAN 10×) and a SPOT/insight QE model camera with SPOT advanced acquisition software (Diagnostic Instruments). Mature osteoclasts were identified as large multinucleated (>2 nuclei) TRAP-positive cells and quantified by light microscopy.

Growth inhibition assay

The growth-inhibitory effect of PBS-1086 on multiple myeloma or osteoclast cell growth was assessed by measuring MTT (Sigma-Aldrich) dye absorbance. Cells were pulsed with MTT for 4 hours of culture, followed by isopropanol containing 0.04 N HCl. Absorbance was measured at 570/630 nm using a spectrophotometer.

Detection of cytokines

Bone serologic marker TRAP5b (tartrate-resistant acid phosphatase 5b; ref. 22) was measured by ELISA (TRAP5b: Quidel) in culture supernatants from osteoclast, with or without PBS-1086.

Detection of apoptosis by Annexin V/propidium iodide

Detection of apoptosis was done with the Annexin V-FITC double-positive cells late apoptotic, PI single-positive cells necrotic, and Annexin V-FITC single-positive cells were considered to be early apoptotic, PI/Annexin V-FITC double-positive cells late apoptotic.

DNA synthesis

DNA synthesis was measured by [³H]-thymidine ([³H]-TdR, Perkin-Elmer) uptake to evaluate growth of multiple myeloma cells adherent to BMSCs in BMSC-coated 96-well plates. Cells were pulsed with [³H]-TdR (0.5 µCi/well) during the last 8 hours of culture, harvested onto glass filters with an automatic cell harvester (Cambridge Technology), and counted using the Betaplate scintillation counter.
Figure 1. PBS-1086 inhibits both canonical and noncanonical NF-κB pathways. A, chemical structure of PBS-1086. B, MM.1S cells were cultured with PBS-1086 at the indicated doses (0.01, 0.1, 1, and 10 µmol/L) for 2 hours. NF-κB DNA binding activity in MM.1S nuclear extracts was assessed in vitro using TransAM NF-κB family transcription factor assay kit. NF-κB canonical activity included p65 (--; p50 (--; and c-Rel (--.--.--). NF-κB noncanonical activity included p52 (--; and RelB (--.--.--). C, MM.1S cells were cultured with PBS-1086 (5 µmol/L) at the indicated times (0.5–12 hours). NF-κB DNA binding activity in MM.1S nuclear extracts was measured by ELISA. NF-κB canonical activity included p65 (--; p50 (--; and c-Rel (--.--.--). NF-κB noncanonical activity included p52 (--; and RelB (--.--.--). Results are expressed as percentage inhibition from the quantity of NF-κB protein bound in the PBS-1086–treated relative to the maximum quantity bound in the control (CT). ELISA data shown are representative of 3 independent experiments. D, MM.1S cells were treated with PBS-1086 (0.5 and 5 µmol/L) for the indicated times (1 and 2 hours). NF-κB DNA binding activity in MM.1S nuclear extracts was measured by ELISA. NF-κB canonical activity included p65 (--; p50 (--; and c-Rel (--.--.--). NF-κB noncanonical activity included p52 (--; and RelB (--.--.--). Treatment with TNF-α (10 ng/mL) for 1 hour served as a positive control of NF-κB activity for both time points, 1 and 2 hours. The results of ELISA are expressed as relative absorbance. Data represent mean ± SD of 3 independent experiments. OD, optical density. E, MM.1S cells were cultured with PBS-1086 (1 µmol/L) for the indicated times (2 to 12 hours). Treatment with TNF-α (10 ng/mL) for 1 hour served as a positive control of increased p65, p50, and p52 nuclear translocation. Nuclear and cytoplasmic extracts were subjected to Western blotting using p50, p52, p65, GAPDH, and nucleolin antibodies. GAPDH and nucleolin were used as purity and loading controls for cytoplasmic and nuclear extracts, respectively. Blots are representative of 3 independent experiments. F, the densitometric analysis of scanned immunoblotting images was done with the NIH ImageJ Software. Results of nuclear (--; left) and cytoplasmic (--; right) protein expression are expressed as fold change relative to control.
Figure 2. PBS-1086 induces cytotoxicity and apoptosis in multiple myeloma cells. A, multiple myeloma cell lines MM.1S ( ), INA6 ( ), and KMS18 ( ) were cultured with PBS-1086 (0.08–2.5 µmol/L) for 48 hours (left). Multiple myeloma cell lines MM.1R ( ), Dox40 ( ), LRS ( ), RPMI 8226 ( ), and U266 ( ) were treated with PBS-1086 (1.25–40 µmol/L) for 48 hours (right). B, CD138⁺ multiple myeloma cells from 4 patients were treated with PBS-1086 at 0 ( ), 0.62 ( ), and 1.25 µmol/L ( ) for 48 hours. C, mononuclear cells isolated from 4 healthy donors and stimulated by phytohemagglutinin were cultured with PBS-1086 (0.15–10 µmol/L) for 48 hours. Cell viability was assessed by MTT assay of triplicate cultures, expressed as percentage of untreated control. Data represent mean ± SD viability. D, MM.1S cells were treated with PBS-1086 at the indicated doses (0.31–1.25 µmol/L) for 24 and 48 hours. Apoptotic cells were analyzed by flow cytometry using Annexin V/PI staining. Percentages of viable (AV⁺/PI⁻; ), early apoptotic (AV⁺/PI⁺; ), late apoptotic (AV⁻/PI⁻; ), and necrotic cells (AV⁻/PI⁺; ) are shown as a histogram. Data represent mean ± SD of 3 independent experiments. E, MM.1S cells were cultured with PBS-1086 (0.62 and 1.25 µmol/L) for 24 and 48 hours in the presence (■) or absence (□) of z-VAD (20 µmol/L). Cell viability was assessed by MTT assay of triplicate cultures, expressed as percentage of untreated control. Data represent mean ± SD viability. CT, control.
PLANS 10×/22, objective lenses: N PLAN 10×/C2 and a SPOT/insight QE model camera with SPOT advanced acquisition software (Diagnostic Instruments). The average pit resorption area is indicated as percentage of control (osteoclasts stimulated with M-CSF and RANKL).

**Murine xenograft model of human multiple myeloma**

CB17 SCID mice (6- to 8-week-old male) were purchased from Charles River Laboratories, Inc. All animal studies were conducted according to protocols approved by and conform to the relevant regulatory standards of the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute. Mice were injected subcutaneously with 5×10⁶ MM.1S cells in 100 µL FCS-free RPMI-1640 medium. When tumor was measurable, mice were assigned into 6 treatment groups: PBS-1086 (at 7.5 mg/kg), bortezomib, PBS-1086 (at 2.5 or 7.5 mg/kg) with bortezomib, vehicle (20% dimethyl sulfoxide/80% Cremophor), and control (100% saline). PBS-1086 was given intraperitoneally (i.p.) once daily, 5 d/wk for 4 weeks. Control and vehicle were administered i.p. with the same schedule. Bortezomib was given i.v. at 0.5 mg/kg twice a week for 4 weeks. Each group consisted of at least 8 tumor-bearing mice. Tumor volume was calculated from caliper measurements 3 times per week, using the following formula: 

\[ V = \frac{a^2 \times b}{2} \]

in which \(a\) and \(b\) are the long and short diameters of the tumor, respectively. Mice were euthanized when tumor volume reached 2 cm³. Survival was evaluated from the first day of treatment until death.

**In situ detection of apoptosis and immunohistochemistry**

Sections from harvested tumors were subjected to immunohistochemical (IHC) staining for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) for detection of apoptosis. Ki67 was also assessed by IHC staining to quantify proliferation.

**Statistical analysis**

Statistical significance was determined by sample t test and test for equal variance. The minimal level of significance was \(P < 0.05\). Overall survival was analyzed by Kaplan-Meier method.
Meier method. The Kaplan–Meier curves were constructed for each group using GraphPad Prism software, and the log-rank (Mantel–Cox) test was used to compare survival curves among groups. The combined effect of PBS-1086 with bortezomib was analyzed by isobologram analysis using the CompuSyn software program (ComboSyn, Inc).

**Results**

**PBS-1086 inhibits both canonical and noncanonical NF-κB pathways**

We first evaluated the effect of PBS-1086 (chemical structure in Fig. 1A) on NF-κB activity in multiple myeloma cells. PBS-1086 inhibited the binding of all Rel proteins to DNA. At 0.1 μmol/L PBS-1086, p65 and p50 were inhibited by 62% and 56%, compared with 28% and 22% inhibition of p52 and RelB (Fig. 1B). To evaluate the kinetics of NF-κB inhibition, MM.1S cells were treated with PBS-1086 for 0.5 to 12 hours. PBS-1086 inhibited NF-κB binding to DNA even after a short exposure, with potent inhibition of p65, p50 (90%–95%), p52 (63%), and RelB (79%; Fig. 1C). Dose- and time-dependent NF-κB inhibition after PBS-1086 treatment was also confirmed in MM.1S cells (Fig. 1D). Inhibition of NF-κB activity by PBS-1086 in additional

![Figure 4.](image-url)
Figure 5. PBS-1086 inhibits osteoclast activity associated with inhibition of NF-κB. A, osteoclasts (OC) and MM.1S cells were cultured for 48 hours with PBS-1086 at 0, 0.31, 0.62, 1.25, 2.5, and 5 μmol/L. Cell viability was assessed by MTT assay of triplicate cultures, expressed as percentage of untreated control. Data represent mean ± SD viability. B, cell viability was assessed by thymidine uptake of quadruplicate cultures, expressed as percentage of untreated control. Data represent mean ± SD viability. C, mature osteoclasts were treated for 48 hours with PBS-1086 (0.31–5 μmol/L). Cells were stained for TRAP activity. Cell density was equal in all samples. TRAP-positive multinucleated osteoclasts after PBS-1086 treatment are quantitated as percentage of untreated control. For nontreated control as well as PBS-1086 (1.25 and 5 μmol/L)-treated cultures, the corresponding micrographs are shown (×10), with inserts at higher magnification. Data represent mean ± SD counts of 3 independent experiments. D, mature osteoclasts were treated for 48 hours with PBS-1086 (0.31–5 μmol/L). The presence of TRAP5b in the supernatants of culture was quantified by ELISA, expressed as percentage of untreated control. Data represent mean ± SD absorbance of triplicate experiments. E, primary human osteoclast precursors derived from 2 patients with multiple myeloma were seeded on calcium phosphate–coated plates. Cells were treated with PBS-1086 (0.31–5 μmol/L) in the presence of M-CSF (25 ng/mL) and RANKL (50 ng/mL) to mature osteoclasts. Osteoclasts not stimulated with M-CSF nor RANKL served as a negative control and failed to resorb. Osteoclasts stimulated with M-CSF and RANKL (positive control) resorbed calcium phosphate (>95%). The average pit resorption area with PBS-1086 was expressed as percentage of positive control. For nontreated control, as well as PBS-1086 (1.25 and 5 μmol/L)–treated cultures, the corresponding micrographs are shown (×10), with inserts at higher magnification. Data represent mean ± SD of 4 independent experiments. F, nuclear and cytoplasmic extracts from mature osteoclasts were cultured for 2 hours with PBS-1086 at 1 and 5 μmol/L. Control nontreated osteoclasts were only stimulated with RANKL and M-CSF. Nuclear extracts were subjected to Western blotting using p50, p52, and nucleolin antibodies. Cytoplasmic extracts were subjected to Western blotting using IκBα (Ser 32/36), p38, and α-tubulin antibodies. Nucleolin and α-tubulin were used as purity and loading controls for nuclear and cytoplasmic extracts, respectively. Blots are representative of 3 independent experiments. CT, control.
multiple myeloma cell lines is shown in Supplementary Fig. S1A (KMS18) and S1B (INA6). We examined the expression of p65, p50, and p52 in cytoplasmic and nuclear extracts from MM.1S cells treated with PBS-1086 by Western blotting (Fig. 1E). A time-dependent decrease in expression of NF-kB proteins was observed in nuclear extracts, whereas cytoplasmic protein remained stable (Fig. 1F), confirming that PBS-1086 acts through an inhibition of NF-kB translocation into the nucleus. Furthermore, PBS-1086 did not inhibit the phosphorylation and degradation of IkBα in multiple myeloma cells (Supplementary Fig. S2). Altogether, these data confirm that PBS-1086 blocks both canonical and noncanonical NF-kB pathways in multiple myeloma cell lines in a dose- and time-dependent manner.

**PBS-1086 induces cytotoxicity and apoptosis in multiple myeloma cells**

We next investigated the growth-inhibitory effect of PBS-1086 in vitro. Treatment of multiple myeloma cells with PBS-1086 for 48 hours induced a dose-dependent decrease in cell viability, with IC_{50} values of PBS-1086 ranging from 0.31 to 5 μmol/L (Fig. 2A). Cells vary in their baseline NF-kB activity (19) and included sensitive cells (MM.1S, INA6, and KMS18 with IC_{50} 0.31–0.62 μmol/L), intermediate sensitive cells (MM.1R, Dox40, and RPMI-8226 with IC_{50} 1.25–2.5 μmol/L), and resistant cells (LR5 and U266 with IC_{50} 2.5–5 μmol/L). However, growth-inhibitory effect of PBS-1086 was observed in multiple myeloma cell lines irrespective of their sensitivity or resistance to conventional treatments or their genetic background. A similar growth-inhibitory effect was observed in 4 patient multiple myeloma cells treated for 48 hours with PBS-1086 (Fig. 2B). Normal PBMCs from 4 healthy volunteers stimulated by phytohemagglutinin were treated for 48 hours with PBS-1086 and similarly analyzed for cytotoxicity. PBS-1086 showed only modest cytotoxicity on normal PBMCs, with a maximum viability loss of 10% at 1.25 μmol/L (Fig. 2C) and 1.25 μmol/L, indicating a higher sensitivity of tumor cells than PBMCs to PBS-1086. These results suggest that PBS-1086 potently inhibits the growth of multiple myeloma cell lines in a dose-dependent manner with a favorable therapeutic window. To analyze molecular mechanisms whereby PBS-1086 induces cytotoxicity in multiple myeloma cells, MM.1S cells treated with PBS-1086 were analyzed for apoptosis using Annexin V-PI staining. PBS-1086 significantly increased Annexin V (+)/PI(−) in MM.1S cells in a time- and dose-dependent manner (Fig. 2D). Moreover, PBS-1086 triggered cleavage of caspase-8, caspase-3/7, and PARP indicating that the intrinsic apoptotic pathway was predominantly activated by PBS-1086 (Fig. 2E). Conversely, pan-caspase inhibitor z-VAD-fmk markedly abrogated PBS-1086–induced apoptosis, confirming that PBS-1086–induced cytotoxicity is mediated, at least in part, via caspase-dependent apoptosis (Fig. 2F). Taken together, these results show that PBS-1086 induced apoptosis in multiple myeloma cells.

**PBS-1086 overcomes the proliferative and antiapoptotic effects of BMSCs associated with inhibition of NF-κB**

Because IL-6 and IGF-I are major growth and/or survival factors in the bone marrow milieu (8, 23–28), we examined their effect on PBS-1086–induced apoptosis. Even with exogenous IL-6 or IGF-I, PBS-1086 induced cytotoxicity (Fig. 3A and B). In a dose-dependent manner, PBS-1086 also inhibited growth of MM.1S cells cocultured with BMSCs (Fig. 3C). We next investigated whether PBS-1086 could inhibit NF-kB inducible activity in the bone marrow microenvironment. Co-culture of MM.1S cells with BMSCs activated NF-kB, predominantly via the canonical pathway, which was significantly inhibited by PBS-1086 (Fig. 3D). We also investigated the effect of PBS-1086 on BMSCs. Importantly, PBS-1086 inhibited both canonical and noncanonical NF-kB pathways in BMSCs. Altogether, these results show that PBS-1086 targets not only multiple myeloma cells but also the bone marrow microenvironment to overcome the proliferative and antiapoptotic effects of BMSCs.

**PBS-1086 enhances cytotoxicity of bortezomib in bortezomib-resistant multiple myeloma cells**

We next evaluated the combination of PBS-1086 with bortezomib in bortezomib-resistant multiple myeloma cell lines. Combining PBS-1086 with bortezomib triggered synergistic cytotoxicity against Dox40 (Fig. 4A and Supplementary Table S3) and ANBL6-VR5 (Fig. 4B and Supplementary Table S3) cells. For example, PBS-1086 (0.31 μmol/L) and bortezomib (20 nmol/L) triggered 8% and 35% cytotoxicity, whereas PBS-1086 with bortezomib at the same concentrations induced 52% cytotoxicity (Fig. 4B). In bortezomib-resistant patient multiple myeloma cells, combining PBS-1086 with bortezomib markedly decreased multiple myeloma cell viability (Fig. 4C), suggesting that PBS-1086 overcomes bortezomib resistance (Supplementary Fig. S4). We further investigated the effect of PBS-1086/bortezomib combination on NF-kB activity in bortezomib-resistant ANBL6-VR5 cells. The binding activity of Rel proteins was similar in bortezomib-treated and control cells (Fig. 4D). Moreover, bortezomib-induced activation of canonical NF-kB pathway was inhibited by PBS-1086 (Fig. 4D and E). No overexpression in hsp27, which has been associated with bortezomib resistance (ref. 29; data not shown), nor change in p38, an upstream effector of hsp27 (30, 31), was observed in ANBL6-VR5 cells after PBS-1086 treatment (Supplementary Fig. S4).

**PBS-1086 inhibits osteoclast activity associated with inhibition of NF-κB**

We also investigated the effect of PBS-1086 on osteoclasts. PBS-1086 did not induce cytotoxicity in osteoclasts, as evidenced by both MTT and 3H-thymidine uptake (Fig. 5A and B). To examine the effect of PBS-1086 on osteoclast differentiation, mature osteoclasts were treated with PBS-1086, followed by TRAP assay. PBS-1086 inhibited osteoclast differentiation in a dose-dependent manner.
(Fig. 5C). To evaluate the effect of PBS-1086 on osteoclast activity, we quantified TRAP5b in the culture supernatants of mature osteoclasts treated with PBS-1086: 15% and 25% reduction in TRAP5b activity was observed at 1.25 and 5 μmol/L, respectively (Fig. 5D). Functional osteoclast activity resorbing bone substrate was also assessed using calcium phosphate–coated plates. PBS-1086 inhibited the bone resorption activity of mature osteoclasts in a dose-dependent manner, with 75% and 100% decrease in pit area at 1.25 and 5 μmol/L PBS-1086, respectively (Fig. 5E). Finally, we investigated whether the inhibitory effect of PBS-1086 on osteoclastogenesis was due to inhibition of NF-κB. Mature osteoclasts exhibited high baseline p50 expression in the nucleus (Fig. 5F), suggesting that NF-κB activity in mature osteoclasts is maintained via the canonical pathway. Importantly, PBS-1086 inhibited both p50 and p52 NF-κB in a dose-dependent manner. No change of p38MAPK, JNK, and ERK, or other pathways mediating osteoclastogenesis (32, 33), was observed (Supplementary Fig. S5). Taken together, our results indicate that PBS-1086 inhibits RANKL-induced osteoclastogenesis, associated with inhibition of NF-κB.

**PBS-1086 inhibits tumor growth in a murine xenograft model of human multiple myeloma**

Finally, we investigated the effect of PBS-1086 in combination with bortezomib on MM.1S cell growth in a murine xenograft model of human multiple myeloma cells. Mice were injected subcutaneously with MM.1S cells that exhibit both canonical and noncanonical NF-κB pathways. In combination with bortezomib, PBS-1086 significantly inhibited tumor growth versus control (2.5 mg/kg, \( P = 0.00039 \) and 7.5 mg/kg, \( P = 0.00084 \); Fig. 6A). Tumor growth was also significantly reduced in PBS-1086 with bortezomib versus bortezomib alone (2.5 mg/kg, \( P = 0.0133 \) and 7.5 mg/kg, \( P = 0.0121 \)) treated cohorts. Importantly, treatment with PBS-1086 and bortezomib prolonged overall survival versus control (2.5 mg/kg, \( P = 0.00039 \) and 7.5 mg/kg, \( P = 0.0057 \)) treated cohorts. Overall survival was also significantly increased in PBS-1086 and bortezomib groups versus bortezomib alone (2.5 mg/kg, \( P = 0.0151 \) and 7.5 mg/kg, \( P = 0.0307 \); Fig. 6B). With a median follow-up of 140 days (range, 28–170), 50% mice receiving PBS-1086 7.5 mg/kg and bortezomib were alive with no detectable tumor. Overall, treatment with PBS-1086, alone or in combination, was well tolerated, with no significant body weight changes compared with control group (Fig. 6C). IHC staining for NF-κB on excised tumor showed time-dependent inhibition of both canonical and noncanonical NF-κB pathways, with significantly decreased expression of p65, p50, and p52 in the nucleus in tumor cells harvested from PBS-1086–treated mice (data not shown). PBS-1086 also induced time-dependent apoptosis (TUNEL staining) and decrease in proliferation (Ki67 staining) in vivo (data not shown). Altogether, our in vivo study shows that inhibition of NF-κB by PBS-1086 is associated with significant in vivo anti–multiple myeloma activity and improved overall survival.
Dual Pathway Inhibition of NF-κB in Myeloma

Discussion

NF-κB transcription factors play a critical role in the pathogenesis of multiple myeloma because constitutive NF-κB activation promotes cell growth, survival, and drug resistance (6). NF-κB is involved in the intimate crosstalk among multiple myeloma cells, the bone marrow microenvironment, and the bone matrix (34). Consequently, targeting direct NF-κB is a promising treatment strategy in multiple myeloma. The importance of NF-κB noncanonical pathway in multiple myeloma has been confirmed both by the frequency of mutations affecting this pathway (15, 16, 18) and the limited activity of IKKβ inhibitors of the canonical pathway (18). Therefore, inhibition of both canonical and noncanonical NF-κB pathways is necessary to achieve complete blockade of NF-κB activity. In the present study, we investigated the effect of PBS-1086, a dual NF-κB inhibitor. PBS-1086 binds to Rel proteins to form covalent bonds with cysteine 38 in RelA (p65), cysteine 144 in RelB, or cysteine 67 in c-Rel to inhibit binding of Rel to the κB sites in DNA (35).

We first confirmed that PBS-1086 strongly inhibits both canonical and noncanonical NF-κB pathways in multiple myeloma cells. MTT evaluation in multiple myeloma cell lines and patient CD138 + multiple myeloma cells shows a selective effect on multiple myeloma, with a favorable therapeutic index. PBS-1086 induces apoptosis in multiple myeloma cells through activation of the intrinsic apoptotic pathway. Partial reversibility of cell killing in the presence of a pan-caspase inhibitor z-VAD-fmk suggests that PBS-1086 might also trigger signaling pathways other than caspases. PBS-1086 induces synergistic cytotoxicity in combination with bortezomib in bortezomib-resistant multiple myeloma cells and in patient multiple myeloma cells refractory to bortezomib. Overexpression of hsp27 confers bortezomib resistance (29); however, this mechanism was not observed in ANBL6-VR5 cell line. We did not determine whether PBS-1086 might interfere with additional mechanisms contributing to bortezomib resistance, such as mutations of proteasome subunits or increased activity of the aggresome pathway (36, 37). In combination with bortezomib, PBS-1086 induced potent anti-multiple myeloma activity in vivo in a murine xenograft model of human multiple myeloma, with significant tumor growth reduction. Importantly, significantly prolonged overall survival was observed in PBS-1086/bortezomib combination treatment groups. IHC analysis of harvested tumors has shown that PBS-1086 anti-multiple myeloma activity in vivo was associated with dual inhibition of NF-κB pathway in tumor cells. Altogether, our results suggest a broad clinical applicability of PBS-1086 to overcome bortezomib resistance not only in multiple myeloma but also in other hematologic malignancies and solid tumors (38).

Several studies have emphasized the role of the bone marrow microenvironment in promoting drug resistance, showing the need for anti-multiple myeloma agents to target not only multiple myeloma cells but also BMSCs (39). In the bone marrow milieu, TNF-α is secreted by multiple myeloma cells and induces NF-κB-dependent expression of adhesion molecules on both multiple myeloma cells and BMSCs, further increasing cell adhesion (11). Enhanced binding in turn confers resistance to apoptosis and triggers NF-κB-dependent secretion of cytokines (11). Our data show that PBS-1086 inhibits both constitutive and inducible NF-κB activity in multiple myeloma cells and in BMSCs. Our laboratory has previously shown that bortezomib inhibits TNF-α–induced NF-κB activation (18). However, some multiple myeloma cells have constitutive activation of NF-κB through proteasome inhibitor–resistant pathways, leading to bortezomib resistance due to both constitutive and inducible NF-κB activities (40). Our data show, unlike bortezomib, that PBS-1086 might be efficacious even when proteasome inhibitor–resistant pathways are activated. Moreover, during multiple myeloma progression, mutations of the NF-κB pathway lead to decreased dependence on extrinsic signals from the bone marrow microenvironment (41). Importantly, our data further suggest that PBS-1086 might prevent multiple myeloma progression by inhibition of TNF-α–induced NF-κB activation. Finally, we did not show any significant inhibitory effect of PBS-1086 on IL-6 secretion within the bone marrow milieu, even though NF-κB is involved in the transcriptional regulation of IL-6 expression in multiple myeloma cells (8). Recently, some authors have reported constitutive expression of IL-6 regulated by several transcription factors besides NF-κB, with no requirement for NF-κB binding activity to DNA (42, 43). The effect of PBS-1086, at least on paracrine IL-6 secretion, requires further investigation.

The role of NF-κB signaling in bone pathogenesis is primarily via RANKL/RANK-induced activation of NF-κB pathway (12). Mice deficient in both p50 and p52 subunits, which are deficient in total NF-κB activity, develop severe osteopetrosis due to the absence of osteoclast formation (44–46). In contrast, deletion of either p50 or p52 causes no detectable bone phenotype, with intact osteoclast formation (47). Dominant-negative mutant IKKβ leads to inhibition of NF-κB canonical pathway and results in decreased osteoclast differentiation (48). Here, we show that PBS-1086 inhibits both the differentiation and function of osteoclast by inhibition of both canonical and noncanonical NF-κB pathways. The inhibitory effect on bone resorption was more potent than on osteoclast differentiation, consistent with dual inhibition of NF-κB and more potent inhibition of the canonical pathway. As RANKL/RANK binding can also activate other signaling pathways, including p38/MAPK (32), JNK, and ERK (49), we confirmed that the inhibitory effect of PBS-1086 on osteoclastogenesis was specific to NF-κB inhibition. Bortezomib inhibits osteoclastogenesis through different pathways dependent on osteoclast differentiation status, with inhibition of p38/MAPK at early stages and inhibition of other pathways including NF-κB at later stages (49, 50). Therefore, we cannot exclude that PBS-1086 might also indirectly affect NF-κB–independent pathways at earlier stages of osteoclastogenesis in vivo.
Importantly, besides its direct cytotoxicity on multiple myeloma cells, PBS-1086 exerts an indirect inhibitory effect on the bone marrow milieu and bone matrix, thereby disrupting tumor–bone marrow milieu interactions, which contribute to multiple myeloma progression.

In conclusion, our study shows that PBS-1086 is a promising dual inhibitor of the canonical and noncanonical NF-κB pathways. Besides its potent and selective cytotoxicity on multiple myeloma cells, PBS-1086 also targets the bone marrow microenvironment and overcomes the proliferative and antiapoptotic effects of BMSCs, associated with an inhibition of TNF-α–inducible NF-κB activation. In addition, we show that PBS-1086 is synergistic with bortezomib against bortezomib-resistant multiple myeloma cells and patient multiple myeloma cells refractory to bortezomib. Finally, PBS-1086 inhibits RANKL-induced osteoclastogenesis, associated with an inhibition of NF-κB pathway. Our data therefore provide the framework for clinical evaluation of PBS-1086 in combination with bortezomib for the treatment of multiple myeloma and related bone lesions.

Disclosure of Potential Conflicts of Interest
K. Bobb, J. Zhang, and J. Meshulam (as the VP and COO) are employees of Profectus and rel=Md, which produce PBS-1086. P.G. Richardson is a consultant/advisory board member for Millennium, Celgene, and Johnson & Johnson. T. Hideshima is a consultant/advisory board member for Acetylton Pharmaceuticals. K.C. Anderson has ownership interest (including patents) in Acetylton as the Scientific Founder and is a consultant/advisory board member for Celgene, Millennium, Bristol Myers Squibb, Onyx, and Merck. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments
The authors thank Dhammir Chauhan, Caitriona M. Gianni, and Loredana Santoro for helpful suggestions.

References
Dual Inhibition of Canonical and Noncanonical NF-κB Pathways Demonstrates Significant Antitumor Activities in Multiple Myeloma

Claire Fabre, Naoya Mimura, Kathryn Bobb, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0779

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/07/18/1078-0432.CCR-12-0779.DC1

Cited articles
This article cites 50 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/17/4669.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/18/17/4669.full.html#related-urls

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, contact the AACR Publications Department at permissions@aacr.org.