Synergistic Anti-Myeloma Activity of the Proteasome Inhibitor Marizomib and the IMiD® Immunomodulatory Drug Pomalidomide

Deepika Sharma Das¹, Arghya Ray¹, Yan Song¹, Paul Richardson¹, Mohit Trikha², Dharminder Chauhan¹,⁶,*, and Kenneth C. Anderson¹,⁶,*

¹LeBow Institute for Myeloma Therapeutics and Jerome Lipper Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA
²Triphase Accelerator, San Diego, CA

Abstract

The proteasome inhibitor bortezomib is an effective therapy for the treatment of relapsed and refractory multiple myeloma (RRMM); however, prolonged treatment can be associated with toxicity, peripheral neuropathy and drug resistance. Our earlier studies showed that the novel proteasome inhibitor marizomib is distinct from bortezomib in its chemical structure, mechanisms of action and effects on proteasomal activities, and that it can overcome bortezomib resistance. Pomalidomide, like lenalidomide, has potent immunomodulatory activity and has been approved by the US Food and Drug Administration for the treatment of RRMM. Here, we demonstrate that combining low concentrations of marizomib with pomalidomide induces synergistic anti-MM activity. Marizomib plus pomalidomide-induced apoptosis is associated with: 1) activation of caspase-8, caspase-9, caspase-3 and PARP cleavage; 2) downregulation of cereblon (CRBN), IRF4, MYC and MCL1; and 3) suppression of chymotrypsin-like, caspase-like, and trypsin-like proteasome activities. CRBN-siRNA attenuates marizomib plus pomalidomide-induced MM cells death. Furthermore, marizomib plus pomalidomide inhibits the migration of MM cells and tumour-associated angiogenesis, as well as overcomes cytoprotective effects of bone marrow microenvironment. In human MM xenograft model studies, the combination of marizomib and pomalidomide is well tolerated, inhibits tumour growth and prolongs survival. These preclinical studies provide the rationale for on-going clinical trials of combined marizomib and pomalidomide to improve outcome in patients with RRMM.

*Joint Senior authorship

Authors’ contributions: DSD performed the experiments, interpreted data and wrote the manuscript; YS and AR helped in animal studies; PR provided clinical samples; MT reviewed the manuscript; DC designed research, analysed data and wrote the manuscript; KCA analysed data and wrote the manuscript.

Conflict-of-interest disclosure MT is an employee of Triphase Accelerator; KCA is on Advisory board of Celgene, Onyx, Gilead, Sonofi Aventis and is a Scientific founder of Acetylon and Oncopep; DC is consultant to Triphase Accelerator. The remaining authors declare no competing financial interests.

Supplementary information is available online.
Keywords
Marizomib; Pomalidomide; Multiple myeloma; cancer and drug therapy

Introduction
The ubiquitin proteasome pathway (UPP) is a validated therapeutic target in multiple myeloma (MM), evidenced by the US Food and Drug Administration (FDA) approval of bortezomib (Velcade™) and carfilzomib (Kyprolis™). Even though bortezomib and carfilzomib therapies are major advances, they are associated with possible off-target toxicities and the development of drug-resistance. Our previous studies showed that the novel proteasome inhibitor marizomib (Feling, et al 2003) is distinct from bortezomib and triggers apoptosis even in MM cells resistant to bortezomib therapies. (Chauhan, et al 2005a) These preclinical data provided the basis for the on-going phase-1 clinical trials of marizomib in patients with relapsed/refractory MM (RRMM). (Potts, et al 2011, Richardson, et al 2011) In addition, we showed that the combination of marizomib with the immunomodulatory agent lenalidomide induces synergistic anti-MM activity. (Chauhan, et al 2010)

Pomalidomide, like lenalidomide, is a thalidomide analogue with potent immunomodulatory activity. Based on increased progression-free survival (Gras 2013, Richardson, et al 2013, Richardson, et al 2014), pomalidomide has been approved by the FDA for the treatment of patients with RRMM who have received at least two prior therapies, including lenalidomide and bortezomib, and who showed disease progression on or within 60 days of completion of the most recent therapy. (Gras 2013, Richardson, et al 2013, Richardson, et al 2014)

In the present study, we characterize the effects of the combination of marizomib and pomalidomide treatment against MM cell lines and primary patient cells resistant to conventional and novel therapies. Both in vitro models and in vivo MM xenograft models demonstrate that marizomib plus pomalidomide trigger synergistic anti-MM activity and overcome drug resistance. Our preclinical studies support the continuation of clinical trials of combined marizomib and pomalidomide to improve outcome in patients with RRMM.

Materials and methods

Cell culture and reagents
Human MM cell lines MM.1S, MM.1R, INA-6, ARP-1, RPMI-8226, DOX40, LR5, ANBL-6.WT (wild type), and ANBL-6-bortezomib-resistant (ANBL-6.BR), as well as peripheral blood mononuclear cells (PBMCs) from normal healthy donors, were cultured in RPMI-1640 medium supplemented with complete medium (10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) at 37°C and 5% CO₂. ANBL-6.WT and ANBL-6.BR cell lines were kindly provided by Dr. Robert Orlowski (MD Anderson Cancer Center, Texas). Bone marrow stromal cells (BMSCs) were cultured in
Dulbecco’s modified Eagle medium supplemented with complete medium. Patient CD138+ MM cells, BMSCs and plasmacytoid dendritic cells (pDC) were isolated and cultured as described previously.(Chauhan, et al. 2009) Informed consent was obtained from all patients, in accordance with an Institutional Review Board approved clinical protocol. Marizomib was obtained from Triphase Accelerator Corporation (San Diego, CA, USA), and pomalidomide was purchased from Selleck chemicals (Houston, TX, USA).

Cytotoxicity and apoptosis assays

Cell viability in MM cell lines, patient MM cells and normal PBMCs were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/water-soluble tetrazolium salt 1 (WST-1) assay. MM cell growth assessment in co-culture studies with BMSCs or pDCs were performed using bromodeoxyuridine (BrdU) cell proliferation kits as previously described.(Chauhan, et al. 2008a) Apoptosis was quantified using FACSCanto (BD Biosciences, San Jose, CA, USA). Caspase-8 and -9 fluorometric assay kits (ALX-850-222-K101 and ALX-850-224-K101, Enzo Life Sciences, Farmingdale, NY) were utilized to measure caspase-8 and caspase-9 enzymatic activity.

In vitro migration and capillary-like tube structure formation assays

The migration assay was performed using 24-well Transwell plates (Millipore, Billerica, MA, USA) in the presence of 10% fetal bovine serum, and migrating cells were quantified by measuring the fluorescence intensity, as previously described.(Chauhan, et al. 2010) Angiogenesis was determined in vitro by matrigel capillary-like tube structure formation assay, as previously described.(Chauhan, et al. 2008a) Human vascular endothelial cells (HUVECs)(American Type Culture Collection [ATCC], Manassas, VA, USA) were maintained in endothelial cell growth medium-2 supplemented with 5% FBS. After 3 passages, HUVEC viability was measured using Trypan blue exclusion assay; less than 5% of cell death was observed with single or combined agents.

Western blotting and proteasome activity assays

Immunoblot analysis was performed using antibodies (Abs) against poly ADP ribose polymerase (PARP) (BD Bioscience Pharmingen, San Diego, CA), cereblon (CRBN) (Novus Biologicals), caspase-8, caspase-9, caspase-3, IRF4, MYC (c-Myc), MCL1, HSP90AA1 (Hsp-90), HSPA1A (Hsp-70), HSPB1 (Hsp-27) or GAPDH (Cell Signaling, Beverly, MA). Blots were then developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). In vitro proteasome activity was performed using fluorogenic peptide substrates, as previously described.(Chauhan, et al 2008a)

Transfection assays

CRBN knockdown experiment was performed using CRBN-siRNA (Origene, Rockville, MD, USA). MM.1S cells were transfected with CRBN-siRNA or scrambled (scr)-siRNA using the cell line Nucleofactor Kit V solution (Amaxa Biosystems/Lonza, Allendale, NJ, USA), as per the manufacturer’s instructions. After cells were pre-treated with pomalidomide for 24 h, marizomib was added for an additional 24h, followed by analysis of cell viability by WST-1 assay.

Br J Haematol. Author manuscript; available in PMC 2016 December 01.
**Human plasmacytoma xenograft model**

The Animal Care and Use Committee of Dana Farber Cancer Institute approved all experiments involving animals. The subcutaneous MM.1S mouse xenograft model was generated as previously described (Chauhan, et al 2005a, Chauhan, et al 2008a, Chauhan, et al 2010) CB-17 SCID-mice (n=30; Taconic, Petersburgh, NY, USA) were subcutaneously inoculated with $5.0 \times 10^6$ MM.1S cells in 100 μl of serum-free RPMI 1640 medium. Tumour size was measured in two dimensions using calipers; tumour volume was calculated using the formula: $V = 0.5 \times a \times b^2$, where $a$ and $b$ are the long and short diameter of the tumour, respectively. Animals were sacrificed when their tumours reached 2 cm$^3$.

When tumours were measurable (~150 mm$^3$), approximately 3 weeks after MM-cell injection, mice (5 mice/group) were divided into random groups blindly and treated orally with vehicle alone, marizomib (0.15 mg/kg), pomalidomide (0.5 mg/kg or 2.5 mg/kg) or marizomib (0.15 mg/kg) plus pomalidomide (0.5 mg/kg or 2.5 mg/kg) for 24 days on a twice-weekly schedule for marizomib and on 4 consecutive days weekly for pomalidomide.

**Immunostaining**

Sections of tumours harvested from mice were stained with anti-Ki67, anti-Caspase-3, Factor VIII, or vascular endothelial growth factor receptor 1 (VEGFR1) Abs, as previously described (Chauhan, et al 2010). Immunostained tissues were imaged with a Zeiss AxioImager M1 Microscope (Zeiss, Oberkochen, Germany) (Chauhan, et al 2010).

**Statistical analysis**

Statistical significance of differences observed in drug-treated vs. control culture was determined using the Student’s $t$ test. Statistical significance of differences observed in tumour volumes in marizomib-, pomalidomide- or marizomib plus pomalidomide-treated mice was determined using a Student’s $t$ test. $P$ values <0.05 were considered statistically significant. Survival of mice was determined by GraphPad Prism software (GraphPad Software, La Jolla, CA). Isobologram analysis (Chou and Talalay 1984) was performed by using the CalcuSyn software program (Biosoft, Ferguson, MO, and Cambridge, United Kingdom). A combination index value < 1.0 indicates synergism, and values > 1.0 indicate antagonism.

**Results**

**Anti-MM activity of combined low concentrations of marizomib and pomalidomide in vitro**

Human MM-cell lines (MM.1S, INA-6, RPMI-8226, MM.1R, Dox-40, LR5, ANBL6.WT and ANBL6.BR) were pretreated with pomalidomide for 24h; marizomib was then added for an additional 24h, followed by assessment for cell viability using MTT assays. For the combination studies, we utilized marizomib and pomalidomide across a range of concentrations. An analysis of synergistic anti-MM activity using the method of Chou and Talalay (1984) demonstrated a significant decrease in viability of all cell lines in response to treatment with combined low concentrations of marizomib and pomalidomide compared with either agent alone (Fig 1A–1D, and Supplementary Fig 1A–1E). Isobologram analysis (Chou and Talalay 1984) confirmed synergistic anti-MM activity, with a
combination index of < 1.0 in all MM cell lines tested. Importantly, the combination of low concentrations of marizomib and pomalidomide overcame resistance to both conventional and novel anti-MM agents. For example, marizomib plus pomalidomide decreased viability of cell lines resistant to bortezomib (ANBL6.BR), dexamethasone (MM.1R), doxorubicin (Dox-40) and melphalan (LR5) (Fig 1, and Supplementary Fig 1). Additionally, synergistic anti-MM activity of combined marizomib and pomalidomide was also observed against TP53-null ARP-1 MM cells (Fig 1C).

To determine whether the combination of low concentrations of marizomib and pomalidomide similarly affected purified patient MM cells, we examined purified (CD138+) MM cells from 2 newly diagnosed (Patients 6 and 7) and 5 patients with MM refractory to multiple therapies (Fig 1E), including dexamethasone (Patient 1), bortezomib/dexamethasone (Patients 2, 4 and 5), and lenalidomide/bortezomib/dexamethasone (Patient 3). MM was considered refractory to a specific therapy when disease progressed on that therapy or relapsed within two months of discontinuing therapy. At the concentrations used, either agent was moderately active in cells isolated from Patients 1 and 2 and was weakly active or inactive in cells isolated from Patients 3–7. Importantly, a significant decrease in cell viability of all patient MM cells was noted after combination therapy as compared to either compound alone (p < 0.05 for all patients; Fig 1E). In contrast, marizomib (1.25 nM) plus pomalidomide (2.5 μM) did not significantly decrease the viability of normal PBMCs (Fig 1F), suggesting a favourable therapeutic index for this combination regimen.

**Combined low concentrations of marizomib and pomalidomide block migration of MM cells and angiogenesis**

Migration of MM cells and associated angiogenesis contributes to the progression of MM. (Giuliani, et al 2003, Podar, et al 2001, Vacca, et al 2003) To determine whether marizomib plus pomalidomide affects these processes, we utilized Transwell insert systems and *in vitro* tubule formation assays. Serum alone increased MM.1S cell migration; importantly, marizomib (1.25 nM) plus pomalidomide (2.5 μM) inhibited serum-dependent MM.1S cell migration, as evidenced by a marked decrease in the number of migrating crystal violet-stained cells (Fig 2A). Cells were >90% viable before and after performing the migration assay, excluding the possibility that inhibition of migration was due to cell death. Our findings show that marizomib plus pomalidomide blocked MM cell migration, which may prevent homing of MM cells to the BM.

To determine whether marizomib plus pomalidomide exhibits anti-angiogenic activity, we utilized *in vitro* capillary-like tube structure formation assays. Specifically, we performed matrigel capillary-like tube structure formation assays: HUVECs plated onto matrigel differentiate and form capillary-like tube structures reflecting *in vivo* neovascularization, a process dependent on cell-matrix interaction, cellular communication and cellular motility. Treatment of HUVECs with combined low concentrations of marizomib and pomalidomide, but not either agent alone, significantly decreased tubule formation (Fig 2B). HUVEC viability was assessed using the trypan blue exclusion assay and < 10% cell death was observed with either single agents or the combined treatment. These findings suggest that the combination of marizomib and pomalidomide block angiogenesis.
Combination of low concentrations of marizomib plus pomalidomide inhibits bone marrow stromal cells (BMSCs)- and plasmacytoid dendritic cells (pDCs)-induced growth of MM cells

The MM-host BM microenvironment confers growth, survival and drug resistance in MM cells. (Anderson 2007, Chauhan, et al 1996) Therefore, we next examined whether the combination of marizomib plus pomalidomide retains its anti-MM activity in the presence of the MM BM milieu. MM.1S cells were cultured with or without patient BMSCs in the presence or absence of marizomib, pomalidomide, or marizomib plus pomalidomide. Low concentrations of marizomib plus pomalidomide significantly inhibited BMSC-induced growth of MM.1S cells, as assessed by WST-1 assays (Fig 2C).

Our recent studies have identified an integral role of pDCs in MM pathogenesis. (Chauhan, et al 2009, Ray, et al 2015, Ray, et al 2014) The functional significance of increased numbers of pDCs in the MM BM is evidenced by our observations that pDCs are relatively resistant to novel and conventional therapies, protect tumour cells from therapy-induced cytotoxicity, promote tumour growth and survival as well as suppress immune responses. (Chauhan, et al 2009, Ray, et al 2015, Ray, et al 2014) We therefore examined the effect of marizomib plus pomalidomide on pDC-induced MM cell growth. MM.1S cells (5 x 10^4 cells/200 μl) and patient pDCs (1 x 10^4 cells/200 μl) were cultured either alone or together. Cells were pre-treated with pomalidomide for 24 h; marizomib was then added for an additional 24 h; and growth was measured using a WST-1 assay. A significant inhibition of pDC-induced MM.1S cell growth was observed in response to treatment with marizomib plus pomalidomide (Fig 2D). Together, our data suggests that the combination of marizomib and pomalidomide has the ability to overcome BMSC- or pDC-mediated MM cell growth and drug resistance.

Mechanisms mediating the anti-MM activity of marizomib plus pomalidomide

As shown in Fig 1, the combination of low concentrations of marizomib plus pomalidomide decreased the viability of MM cell lines and patient cells. This decrease in viability was due to induction of apoptosis, as determined by Annexin V/propidium iodide staining and flow cytometry (Supplementary Fig 2). Moreover, marizomib plus pomalidomide-induced apoptosis in MM cell lines (MM.1S, RPMI-8226, Dox-40) was associated with PARP cleavage (Fig 3A) and the activation of caspase-3, caspase-8 and caspase-9 (Fig 3B). Biochemical inhibitors of caspase-3, caspase-8, caspase-9 or pan-caspases abrogated marizomib plus pomalidomide-induced cytotoxicity (Fig 3C). Of note, blockade of either caspase-8 or caspase-9 led to a similar degree of inhibition of marizomib plus pomalidomide-induced apoptosis.

Effects of marizomib plus pomalidomide on chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) proteasome activities

The primary target of proteasome inhibitors is the proteasome, which mediates degradation of proteins via three (CT-L, T-L and C-L) proteolytically active sites. (Arendt and Hochstrasser 1997, Heinemeyer, et al 1997) Therefore, we next examined the effect of combined marizomib and pomalidomide on CT-L, C-L, and T-L activity. The combination of low concentrations of marizomib (1.25 nM) and pomalidomide (2.5 μM) induced a
marked inhibition of all three proteasomal activities versus either agent alone at these concentrations (Fig 4A), suggesting that pomalidomide enhances the ability of marizomib to target all three proteasome activities.

**Effect of marizomib plus pomalidomide on cereblon and associated downstream signaling**

The primary target of pomalidomide is the E3 ubiquitin ligase, cereblon (CRBN). (Lu, et al 2014) Immunoblot analysis of CRBN expression in MM cells and normal healthy donor PBMCs showed significantly higher CRBN expression in MM cell lines than in normal PBMCs (Fig 4B). We therefore examined the functional role of CRBN during marizomib plus pomalidomide-induced cytotoxicity using loss-of-function studies. The functional specificity of CRBN-siRNA was confirmed by a marked decrease in CRBN levels (Fig 4C, immunoblot). Importantly, transfection of CRBN-siRNA, but not negative-control (scrambled) siRNA significantly inhibited marizomib plus pomalidomide-induced apoptosis in MM.1S cells (Fig 4C; p < 0.005). We found no statistically significant difference upon treatment with marizomib alone in either CRBN siRNA or scrambled siRNA transfected cells. Non-transfected cells showed results similar to those noted in scrambled siRNA transfected cells (data not shown). These findings suggest that marizomib plus pomalidomide-triggered apoptosis in MM cells is mediated, at least in part, via CRBN.

CRBN is linked to the activation of the downstream IRF4/MYC signalling axis. (Thakurta, et al 2014) IRF4 is a survival factor for MM cells, and MYC is a direct target of IRF4 in activated B cells and MM cells. Conversely, IRF4 is also a direct target of MYC transactivation, generating an autoregulatory circuit in MM cells. (Shaffer, et al 2008) Importantly, MYC coordinates cellular growth, metabolism and proliferation. (Holien, et al 2012) In this study, treatment of MM.1S and RPMI-8226 cells with marizomib plus pomalidomide led to reduction of CRBN, IRF4 and MYC protein expression (Fig 4D and Supplementary Fig 3). NF-κB (NFKB1) is another key mediator of MM cell survival. (Chauhan, et al 1996) Interestingly, lenalidomide inhibits IRF4, which in turn downregulates NF-κB in a CRBN-dependent manner. (Zhang, et al 2013) In our study, treatment of MM cells with low doses of marizomib and pomalidomide also blocks NF-κB activity (Supplementary Fig 4). Together, these findings suggest that marizomib plus pomalidomide-induced apoptosis in MM cells is associated with downregulation of the CRBN/IRF4/MYC signalling axis.

We next examined whether the combination of marizomib and pomalidomide overcomes mechanism(s) that confer survival and drug-resistance in MM cells. For example, the anti-apoptotic protein MCL1 confers drug resistance in MM cells (Fan, et al 2014), and we found a marked decrease in MCL1 levels in marizomib and pomalidomide-treated cells versus cells treated with either agent alone (Fig 4D). In addition, prior studies link heat shock proteins (HSPB1, HSPA1A, HSP90AA1) with drug resistance. (Chauhan, et al 2005b, Chauhan, et al 2003, Fribley, et al 2004, Landowski, et al 2005, Obeng, et al 2006) Importantly, we found that the combination of low concentrations of marizomib and pomalidomide did not induce HSPB1, HSPA1A or HSP90AA1 protein levels (Fig 4E). Given the role of MCL1 and heat shock proteins in the development of drug-resistance, our
data suggest that combined low doses of marizomib and pomalidomide may result in less frequent occurrence of drug resistance in MM.

**Combined marizomib and pomalidomide inhibit human MM cell growth in vivo and prolongs survival in the MM.1S MM xenograft mouse model**

Having defined the efficacy of combined marizomib and pomalidomide in targeting MM cells in vitro, we next validated these findings in vivo using our murine xenograft model of human MM. (Chauhan, et al 2008a, Chauhan, et al 2010) This model has been useful in validating the novel anti-MM therapies bortezomib and lenalidomide, leading to their translation to clinical trials and FDA approval for the treatment of MM. A marked reduction in tumour growth, as well as an increased prolongation of survival, was observed in marizomib plus pomalidomide-treated mice versus mice receiving either agent alone (Fig 5A and 5B). These data demonstrate the effectiveness of the anti-tumour activity of marizomib plus pomalidomide in vivo. The combination of marizomib and pomalidomide treatment was well tolerated, as evidenced by the lack of weight loss even after 3 weeks of treatment (Fig 5C). Blood chemistry profiles of marizomib plus pomalidomide-treated mice showed that haemoglobin, bilirubin and creatinine levels were not statistically significant altered (Fig 5D). Together, these findings suggest that combining marizomib and pomalidomide markedly reduced tumour growth and is well tolerated in vivo.

We next examined the apoptotic activity of this drug combination in vivo using immunostaining for caspase-3 activation and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of tumours harvested from mice treated with vehicle alone, marizomib, pomalidomide, or marizomib plus pomalidomide. The combination of marizomib plus pomalidomide increased the number of cleaved caspase-3- and TUNEL-positive apoptotic tumour cells compared to treatment with vehicle or either agent alone (Fig 6A and 6B). Furthermore, a decrease in the proliferation marker Ki67 was also noted in tumours excised from mice receiving marizomib plus pomalidomide (Fig 6C). In concert with our in vitro data (Fig 4D), tumours from mice treated with marizomib plus pomalidomide showed decreased MCL1 and IRF4 levels (Fig 6D).

MM cell growth is associated with angiogenesis, and vascular endothelial growth factor (VEGF) plays a role in this process. (Anderson 2012) Our in vitro data showed that the combination of marizomib and pomalidomide exerts anti-angiogenic activity (Fig 2A and 2B). To determine whether marizomib and pomalidomide trigger anti-angiogenic activity in vivo, we evaluated tumours harvested from mice by immunostaining using two distinct markers of angiogenesis, Factor VIII and VEGFR1. As seen in Fig 6E, low doses of marizomib or pomalidomide alone triggered a very modest decrease in the number of Factor VIII-positive cells compared to sections from control-treated tumours, whereas combination treatment decreased the number of Factor VIII-positive cells. Similarly, marizomib plus pomalidomide markedly decreased the number of VEGFR1-positive cells (Fig 6F). These data suggest that marizomib plus pomalidomide inhibits tumour-associated angiogenic activity.
Discussion

We show that the combination of low concentrations of marizomib and pomalidomide decreased viability of MM cell lines and primary patient tumour cells, without affecting normal PBMC viability. Our data show the anti-MM activity of marizomib plus pomalidomide against MM cell lines, including those sensitive and resistant to conventional and novel therapies, as well as representing distinct cytogenetic profiles. (Bergsagel, et al 1996, Bergsagel and Kuehl 2005) For example, we studied isogenic cell lines dexamethasone-sensitive MM.1S (Greenstein, et al 2003) and dexamethasone-resistant MM.1R with t(14;16) translocation and MAF (c-maf) overexpression; INA-6, an interleukin 6–dependent cell line with NRAS activating mutation; and RPMI-8266 with TP53, KRAS and EGFR mutations. (Avet-Loiseau, et al 2007, Bergsagel, et al 1996, Bergsagel and Kuehl 2005, Burger, et al 2001, Davies, et al 2003, Greenstein, et al 2003) Although combined treatment with marizomib and pomalidomide decreased viability in all MM cell lines, the 50% inhibitory concentration of this combination regimen was different for each cell line, which may be due to their distinct genetic heterogeneity and/or drug-resistance characteristics.

We observed maximal synergistic anti-MM activity of combined marizomib and pomalidomide when MM cells were pretreated with pomalidomide for 24 h followed by addition of marizomib for another 24 h versus the simultaneous addition of these agents (data not shown). It is likely that pomalidomide induces distinct apoptotic signalling versus marizomib and/or upregulates proteasome load in MM cells, which together primes the cells to undergo robust cell death in response to subsequent treatment with even low concentrations of marizomib.

To determine whether the combination of marizomib and pomalidomide overcomes bortezomib resistance, we used previously characterized (Kuhn, et al 2009) bortezomib-sensitive (ANBL-6.WT) and -resistant (ANBL-6.BR) MM cell lines. We found significant anti-MM activity of marizomib plus pomalidomide against ANBL-6.BR, confirming the ability of this regimen to overcome bortezomib-resistance. Importantly, we observed similar responses to marizomib plus pomalidomide in patient MM cells resistant to therapies including bortezomib, lenalidomide or Dex. Moreover, the combination of marizomib and pomalidomide overcomes the MM cell growth advantage conferred by the MM-host BM microenvironment.

Mechanistic analysis showed that anti-MM activity of marizomib plus pomalidomide is associated with the activation of the caspase cascade, inhibition of proteasomal activities, and downregulation of both CRBN/IRF4/MYC signalling axis and MCL1. Studies using biochemical inhibitors showed that blockade of either caspase-8 or caspase-9 led to a similar degree of inhibition of marizomib plus pomalidomide-induced apoptosis. Of note, our earlier study showed that the combination of marizomib with the immunomodulatory drug lenalidomide relies more on caspase-8 activity than caspase-9 for inducing cell death (Chauhan, et al 2010). Nonetheless, our data suggest that marizomib plus pomalidomide-induced MM cell apoptosis is dependent, at least in part, on both caspase-8- and caspase-9-mediated signalling pathways.
Previous studies showed that marizomib inhibits CT-L, C-L and T-L proteasome activities. (Chauhan, et al 2008b, Chauhan, et al 2005a) Our prior study also showed that lenalidomide enhances marizomib-induced inhibition of proteasome function (Chauhan, et al 2010). In an analogous manner, we found that the combined low concentrations of marizomib and pomalidomide block all three proteasomal activities versus either agent alone at these low concentrations. The mechanism whereby pomalidamide enhances the ability of marizomib to potently inhibit proteasome activity remains to be defined. Given the immunomodulatory characteristics of pomalidomide, it is likely that pomalidomide affects immunoproteasome activities (CT-Li, C-Li and T-Li), which in turn may alter function of constitutive proteasome activities. This notion is supported by earlier findings that proteasome active sites allosterically regulate each other: for example, occupancy of C-L sites triggers the T-L activity of proteasomes in a compensatory manner. (Kisselev, et al 2003) Indeed, additional loss-of-function studies are required to delineate the functional role of each proteasome subunit.

Another possible mechanism mediating the marizomib plus pomalidomide-induced blockade of proteasome activity is the involvement of E3 ligase CRBN within the ubiquitin proteasome pathway. CRBN is a direct protein target of pomalidomide. (Ito, et al 2010, Lu, et al 2014) A recent study showed that CRBN modulates proteasome activity by binding to the 20S core proteasome subunit beta type 4. (Lee, et al 2012) It is therefore possible that CRBN may similarly affect the β5, β2 or β1 proteasome subunits to modulate overall proteasome activity. Our genetic studies using CRBN-siRNA suggest that marizomib plus pomalidomide-induced cell death is mediated, at least in part, by CRBN. A further evidence for the involvement of CRBN is noted by a marked decrease in CRBN associated signalling via IRF4 and MYC. Together, these data suggest that marizomib plus pomalidomide-induced apoptosis is associated with a marked inhibition of all three proteasome activities, as well as CRBN-associated downstream signalling via IRF4/MYC.

In addition to activating pro-apoptotic signaling, the combination of marizomib and pomalidomide also downregulates proteins associated with survival and drug resistance. For example, MCL1 is a pro-survival member of the BCL2 family, which plays a crucial role in MM pathogenesis and drug resistance. (Fan, et al 2014) We found that low concentrations of marizomib plus pomalidomide reduced the levels of anti-apoptotic protein MCL1. Similarly, prior studies have linked bortezomib-induced apoptosis with the upregulation of heat shock proteins, which confer drug resistance in MM. (Chauhan, et al 2005b, Mitsiades, et al 2002, Obeng, et al 2006) In this context, our data shows that marizomib plus pomalidomide combination therapy does not induce HSP90AA1, HSPA1A or HSPB1. These data suggest that drug resistance may be less frequent in patients receiving this combined low dose regimen.

In vitro data showing anti-MM activity of marizomib plus pomalidomide was validated in vivo using the human MM.1S xenograft mouse model. A marked reduction in tumour progression and prolongation of survival was observed in marizomib plus pomalidomide-treated mice versus mice receiving either agent alone. Moreover, marizomib plus pomalidomide treatment was well tolerated, because differences in body weight and overall appearance were not observed. The remarkable anti-MM and anti-angiogenic activity of
marizomib plus pomalidomide in vivo was confirmed by IHC analysis of tumours harvested from control- and combination-treated mice using molecular markers of apoptosis (caspase-3 cleavage, TUNEL staining), proliferation (Ki67), and associated angiogenesis (Factor VIII and VEGFR1). Tumour lysates showed decreased MCL1 and IRF4 levels after marizomib plus pomalidomide treatment. Therefore, these findings indicate a dual effect of combining marizomib plus pomalidomide: increased apoptosis and decreased MM-cell proliferation.

Collectively, our preclinical studies demonstrate potent in vitro and in vivo anti-MM activity of marizomib plus pomalidomide at low doses that are well tolerated in a human plasmacytoma xenograft mouse model. These findings provide the rationale for the continuation of clinical trials of low-dose combination marizomib plus pomalidomide therapy to enhance cytotoxicity, overcome drug resistance, reduce side effects, and improve patient outcome in MM.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

**Grant Support:** This investigation was supported by National Institutes of Health Specialized Programs of Research Excellence (SPORE) grant PS0100707, PO1-CA078378, and RO1 CA050947. K.C.A. is an American Cancer Society Clinical Research Professor.

**References**

Burger R, Guenther A, Bakker F, Schmalzing M, Bernand S, Baum W, Duerr B, Hocke GM, Steininger H, Gebhart E, Gramatzki M. Gp130 and ras mediated signaling in human plasma cell line

*Br J Haematol*. Author manuscript; available in PMC 2016 December 01.


Br J Haematol. Author manuscript; available in PMC 2016 December 01.


Ray A, Das DS, Song Y, PGR, Chauhan DKCA. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cells interactions with T cells, natural killer cells, and multiple myeloma cells. Leukemia. 2015 (In press). 10.1038/leu.2015.11


## Key points

1. Combining low concentrations of marizomib and pomalidomide triggers synergistic cytotoxicity in MM cells and overcomes bortezomib-resistance.

2. Combination of marizomib with pomalidomide represents a promising novel therapy in patients with relapsed and refractory MM.
Figure 1. Combination of low doses of marizomib and pomalidomide induces synergistic cell death in MM cell lines, patient MM cells but not normal PBMCs

(A–D) MM-cell lines (MM.1S, ANBL6.BR, ARP-1, and MM.1R) were pretreated with or without pomalidomide (POM) for 24 h; marizomib was then added for an additional 24 h, followed by assessment for cell viability using the MTT assay. The experiments with single agents and respective combinations were carried out simultaneously. Isobologram analysis shows the synergistic cytotoxic effect of marizomib and pomalidomide. The graph (right panel) is derived from the values given in the table (left panel). A combination index (CI) <1 indicates synergy. (E) Purified patient MM cells (CD138-positive) were pretreated with pomalidomide for 24 h; marizomib was then added for an additional 24 h, followed by cell death analysis using CellTiter-Glo assay. Data are mean ± SE of triplicate samples (p < 0.05 for all patient samples, for single agent vs combination treated samples). (F) Peripheral blood mononuclear cells (PBMCs) from healthy donors were treated (as in panel A) with indicated concentrations of marizomib, pomalidomide or marizomib plus pomalidomide, and then analysed for viability using the WST-1 assay. Data are mean ± SE (n=5). Fa = fraction of cells affected.
Figure 2. Combined low doses of marizomib and pomalidomide block migration, tubule formation, and cytoprotective effects of BMSCs and pDCs

(A) Migration assay: MM.1S cells were pretreated with pomalidomide for 12h, and then marizomib was added for an additional 6h; cells were more than 90% viable at this time point. The cells were washed and cultured in serum-free medium. After 2h incubation, cells were plated on a fibronectin-coated polycarbonate membrane in the upper chamber of transwell inserts and exposed for 4h to serum-containing medium in the lower chamber. Cells migrating to the bottom face of the membrane were fixed with 90% ethanol and stained with crystal violet. A total of 3 randomly selected fields were examined for cells that
had migrated from the top to the bottom chambers. (Left panel) Image is representative of 2 experiments with similar results. (Right panel) The bar graph represents quantification of migrated cells. Data are mean ± SE (p < 0.05). (B) Human vascular endothelial cells were cultured in the presence or absence of combined low doses of marizomib plus pomalidomide for 48 h, and then assessed for in vitro angiogenesis using matrigel capillary-like tube structure formation assays (Left panel). Image is representative from 3 experiments with similar results. In vitro angiogenesis is reflected by capillary tube branch formation (dark brown). (Right panel) The bar graph represents quantification of capillary-like tube structure formation in response to indicated agents: Branch points in several random view fields/well were counted, values were averaged, and statistically significant differences were measured using Student’s t test. (C–D) MM.1S cells were cultured in bone marrow stromal cells (BMSC)- or plasmacytoid dendritic cells (pDC)-coated or uncoated wells with control medium, marizomib, pomalidomide or marizomib plus pomalidomide. Cell proliferation was assessed by Brdu colorimetric assay. Data are mean ± SE (n=3; p < 0.05, for control vs combination treated samples).
Figure 3. Marizomib plus pomalidomide-induced apoptosis in MM cells is associated with activation of caspases
(A) MM.1S, RPMI-8226 or Dox-40 cells were pre-treated with or without pomalidomide for 24 h, and then marizomib was added for an additional 24 h; protein lysates were subjected to immunoblot analysis with anti-PARP, anti-caspase-3, anti-caspase-8, anti-caspase-9 or anti-GAPDH FL, full length; CF, cleaved fragment. Blots shown are representative of 3 independent experiments. (B) MM.1S cells were pre-treated with or without pomalidomide for 24 h, and then marizomib was added for an additional 24 h; protein lysates were subjected to measurement of caspase-8 or caspase-9 enzymatic activity using fluorometric
kit. Data are mean ± SE (n=3; p < 0.05, for single agent vs combination treated samples) (C) MM.1S cells were treated with indicated agents (as in panel ‘A’) in the presence or absence of biochemical inhibitors of caspase-3, caspase-8, caspase-9, or pan-caspase and then analyzed for apoptosis using WST-1 assay. Data are mean ± SE (n=3; p < 0.05, for control vs combination treated samples).
Figure 4. Mechanisms mediating anti-MM activity of marizomib plus pomalidomide

(A) MM.1S cells were pretreated with or without pomalidomide for 12 h, and then marizomib was added for an additional 12 h; cells were harvested and cytosolic extracts were then analysed for chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) proteasome activities. Results are represented as percentage inhibition in proteasome activities in drug-treated versus vehicle control. Data are mean ± SE (n=3; p < 0.05, for single agent vs combination treated samples) (B) Total protein lysates from the indicated MM cell lines and normal healthy donor PBMCs were subjected to immunoblot analysis with anti-CRBN or anti-GAPDH Abs. (C) MM.1S cells were transfected with siRNA-Das et al. Page 22
CRBN or scr-siRNA for 24h. Transfected cells were pretreated with or without pomalidomide for 24h; marizomib was then added for an additional 24h, followed by analysis for apoptosis using WST-1 assay. Data are mean ± SE (n=3; p < 0.05, for single agent vs combination treated samples) Immunoblot shows CRBN expression in cells transfected with scr-siRNA or CRBN-siRNA. (D and E) MM.1S cells were treated with indicated agents (as treated in panel ‘A’); protein lysates were subjected to immunoblot analysis with anti-CRBN, anti-IRF4, anti-MYC, anti-MCL1, anti-HSP90AA1, anti-HSPA1A, anti-HSPB1 or anti-GAPDH Abs.
Figure 5. Combination of low doses of marizomib and pomalidomide inhibits human plasmacytoma growth in CB-17 SCID mice

(A) Mice bearing human MM.1S MM tumours (n=5/group) were treated with vehicle, marizomib (0.15 mg/kg; orally), pomalidomide (0.5 mg/kg or 2.5 mg/kg; orally), or marizomib plus pomalidomide (orally) at the indicated doses for 24 days on a twice-weekly schedule for marizomib and for 4 consecutive days weekly for pomalidomide. Bars represent mean ± SE. (B) Kaplan-Meier plots showing survival for mice treated with marizomib, pomalidomide, or marizomib plus pomalidomide at the indicated doses. Marizomib plus pomalidomide-treated mice show significantly increased survival (p < 0.05, Logrank test for trend) compared with the untreated group. (C) Vehicle-treated control mice,
as well as mice in the marizomib, pomalidomide, or marizomib plus pomalidomide-treated cohorts, were weighed every week. The average changes in body weight are shown. (D) Mice were treated with vehicle, marizomib, pomalidomide, or marizomib plus pomalidomide (as in panel ‘A’) for 24 days; blood samples were then obtained and subjected to analysis for serum bilirubin, hemoglobin, and creatinine levels using Quantichrom Creatinine, Bilirubin, and Haemoglobin Assay kit (BioAssay Systems, Hayward, CA, USA).
Figure 6. Effect of marizomib plus pomalidomide on apoptosis, MCL1, IRF4 and neovascularization in vivo in xenografted MM tumours

(A and B) Apoptotic cells in sections of tumours harvested from vehicle control-, marizomib-, pomalidomide-, or marizomib (0.15 mg/kg) plus pomalidomide (2.5 mg/kg)-treated mice were identified by immunostaining for activated caspase-3 (green cells) or TUNEL. Tumour sections were obtained on day 24. Images were obtained with a Leica SP5X laser scanning confocal microscope (40x magnification). (C) Tumours harvested from mice (as in panel ‘A’) were immunostained with Ki67 Abs (40x magnification). (D) Tumour lysates from control and drug-treated mice were subjected to immunoblot analysis using anti-MCL1, anti-IRF4, or anti-GAPDH Abs. Lanes 1 to 6 represent lysates of tumours...
harvested from mice receiving the following treatments: lane 1, vehicle alone (control); lane 2, marizomib (0.15 mg/kg); lane 3, pomalidomide (0.5 mg/kg); lane 4, pomalidomide (2.5 mg/kg); lane 5, marizomib (0.15 mg/kg) plus pomalidomide (2.5 mg/kg); and lane 6, marizomib (0.15 mg/kg) plus pomalidomide (0.5 mg/kg). (E and F) **Tumours harvested from mice** (as in panel ‘A’) were immunostained with Factor VIII or VEGFR1 Abs. Images were obtained with a Leica SP5X laser scanning confocal microscope (40x magnification). Photographs (A, B, C, E, and F) shown are representative of similar observations in 2 mice receiving the same treatment.