Pharmacologic targeting of the p62 ZZ domain enhances both anti-tumor and bone-anabolic effects of bortezomib in multiple myeloma

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Title: Pharmacologic targeting of the p62 ZZ domain enhances both anti-tumor and bone-anabolic effects of bortezomib in multiple myeloma

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**Running Title:** Targeting p62 increases PIs efficacy in myeloma

**Keywords:** multiple myeloma, bone disease, bortezomib, necroptosis, p62

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Synthesis and purification of XRK3F2 was done by the Chemical Genomics Core Facility (CGCF) at Indiana University School of Medicine. Cell death analysis was assisted by the Flow Cytometry Resource Facility (FCRF) of the IU Simon Comprehensive Cancer Center. The CoMMpass data used in this study were generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiatives (https://research.themmrf.org and www.themmrf.org).

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Abstract

Multiple myeloma (MM) is a malignancy of plasma cells whose antibody secretion creates proteotoxic stress relieved by the N-end rule pathway, a proteolytic system that degrades N-arginylated proteins in the proteasome. When the proteasome is inhibited, protein cargo is alternatively targeted for autophagic degradation by binding to the ZZ-domain of p62/sequestosome-1. Here, we demonstrate that XRK3F2, a selective ligand for the ZZ-domain, dramatically improved two major responses to the proteasome inhibitor bortezomib by increasing: 1) killing of human MM cells by stimulating both bortezomib mediated apoptosis and necroptosis, a process regulated by p62; and 2) preservation of bone mass by stimulating osteoblasts differentiation and inhibiting osteoclastic bone destruction. Co-administration of bortezomib and XRK3F2 inhibited both branches of the bimodal N-end rule pathway exhibited synergistic anti-MM effects on MM cell lines and CD138+ cells from MM patients, and prevented stromal-mediated MM cell survival. In mice with established human MM, co-administration of bortezomib and XRK3F2 decreased tumor burden and prevented the progression of MM-induced osteolytic disease by inducing new bone formation more effectively than either single agent alone. The results suggest that p62-ZZ ligands enhance the anti-MM efficacy of proteasome inhibitors and can reduce MM morbidity and mortality by improving bone health.
Introduction

Multiple myeloma (MM) is the second most common hematological malignancy. It affects the elderly and causes skeletal destruction, leading to bone pain and disability.\textsuperscript{1,2} First-line therapies rely on several classes of drugs, including proteasome inhibitors (PIs) such as Bortezomib (Btz), which are the mainstay of MM therapy. However, the development of PIs resistance remains a major clinical problem that requires switching to different treatment regimens.\textsuperscript{3} Bone complications occur in 80\% of MM patients, leading to severe morbidity and increased mortality. Current treatments for multiple myeloma bone disease are limited to anti-resorptives, which neither inhibit tumor growth nor increase new bone.\textsuperscript{1} PIs stimulate new bone formation\textsuperscript{4-7}, but the effect is transient, allowing the persistence of bone lesions, which often do not heal even during remission.\textsuperscript{8} Thus, ways to increase the anti-MM efficacy of PIs are needed to improve therapeutic responses, disease-free survival, and bone health in MM patients.\textsuperscript{9}

MM cells secrete abundant monoclonal immunoglobulins, a process requiring assembly of light and heavy chains (HCs) during biosynthesis in the endoplasmic reticulum (ER), where HCs bind to heat shock protein (HSPA5, also known as GRP78 and BiP) until combined with light chains.\textsuperscript{10} HSPA5 cycles out of the ER with proteotoxic cargo (such as excess HC) and is N-terminally, N-Arginine modified.\textsuperscript{11} The N-end rule pathway\textsuperscript{12} then determines binding to the UBR1 ubiquitin ligase, which targets the cargo to the proteasome for degradation.\textsuperscript{13,14} Inhibition of the proteasome induces the alternative second N-end rule pathway by increasing the expression of p62 (SQSTM/sequestosome-1), a multidomain protein scaffold that regulates autophagy, NF\textsuperscript{κ}B signaling, necroptosis, and other pathways.\textsuperscript{15,16} We previously identified the ZZ-domain of p62 as an important regulator of both autophagy and signaling pathways in MM and bone cells.\textsuperscript{17-19} Ligand binding to the ZZ-domain triggers a conformational switch leading to
oligomerization and formation of a liquid phase-separated state that leads to autophagy, RIPK1 binding to the ZZ-domain, which regulates necroptotic cell death\textsuperscript{20}, and conformational changes of the ZZ and TBS domains, which affect TRAF6 and NF\textsubscript{κ}B signaling, important regulators of cell survival and bone cell activity.\textsuperscript{21-23} We developed a small molecule ligand of the ZZ-domain, XRK3F2, that decreased osteoclast formation and activity\textsuperscript{17} and reversed MM-epigenetic suppression of osteoblast differentiation.\textsuperscript{18} XRK3F2 as a single agent induced local new bone formation in MM-bearing mice but did not reduce tumor burden.\textsuperscript{17}

In this manuscript, we reasoned that XRK3F2 blocks only one of the two branches of the bimodal N-end rule degradation pathway and should be much more effective as an anti-MM agent when combined with a proteasome inhibitor. We report that XRK3F2 amplifies the response to Btz in MM cells by preventing Btz-induced p62 accumulation and triggering simultaneous induction of multiple death pathways. Further, mice with established MM treated with XRK3F2-Btz combination exhibit decreased tumor growth and reduced bone destruction at doses where single agents were ineffective. Collectively, our data show that XRK3F2 boosts both the anti-tumor and bone-anabolic effects of PIs and provides a strong rationale for developing a new therapeutic regimen based on co-administration of XRK3F2 and Btz to treat MM.
Methods

Antibodies and compounds are described in Supplementary Methods.

Human primary CD138+ and BMSC cell purification and MM cell lines

Patient studies were approved by the Indiana University School of Medicine IRB and CD138+ MM cells were isolated as previously described. Human MM cell lines were purchased from ATCC (Manassas, VA, USA) (MM.1S; NCI-H929; RPMI8226) or generously provided by Drs. Louis Stancato (U266), Kenneth Anderson (KMS-11), and Nicola Giuliani (JJN3) and cultured in RPMI with 10% FCS/1% P/S. Cell line authentication was routinely examined for proper morphology, population doubling, and paraprotein production. HS-5 human stromal cell lines were obtained from ATCC and were cultured in DMEM with 10% FCS/1% P/S. All cells were cultured under 37°C and 5% CO2 conditions.

Cell viability and apoptosis/necroptosis assays

MM cells were incubated with suboptimal concentrations of XRK3F2 and Btz (below their 24h IC50s; Supplementary Table 1) alone or in combination for 24h ± inhibitors of necroptosis, apoptosis, or autophagy. Cell viability was quantified by alamarBlue® Cell Viability assays from ThermosFisher Scientific (Waltham, MA, USA), per the manufacturer’s protocol. The combined effects of XRK3F2 and Btz on MM cells were evaluated for synergism vs. additivity by combination index (CI) analysis. A CI less than 1.0 indicates synergism and a CI of 1 indicates additive activity. Lactate dehydrogenase (LDH Cytotoxicity Assay Kit, Thermo Scientific Pierce, 88953) and human high mobility group B (HMGB1, NBP2-62766, Novusbio, Centennial, CO) protein were assayed as markers of necroptosis in cell supernatant and serum, respectively.
Mouse model of human MM.

Immune-deficient 6-8 wk-old Fox Chase SCID (C.B.-17 SCID/SCID) mice Charles River Laboratories (Wilmington, NC) were injected intratibially with $1 \times 10^5$ human JJN3 myeloma cells in 20 μl of PBS. Mice were handled in accordance with the Guide for the Care and Use of Laboratory Animals, under a protocol approved by the Indiana University School of Medicine IACUC. After three weeks mice were treated with either XRK3F2 (27mg/kg, 5 times per week), Btz (0.25mg/kg/2xweek), XRK3F2-Btz (27mg/kg/5xweek plus 0.25mg/kg/2xweek), or vehicle intraperitoneally (IP) for two additional weeks when the mice were euthanized. The sample size was calculated based on a previous study.\textsuperscript{17} Details are in Supplementary Methods.

Bioinformatic analyses of publicly available datasets

Gene expression data for RIPK3, RIPK1, SQSTM1 (p62), and MLKL were obtained from the MMRF Researcher Gateway using version IA18 and analyzed as described in Supplementary Methods.

Statistical analysis

One or two-way analysis of variance was performed to determine differences between experimental groups. \textit{Post hoc} comparisons were accomplished via Tukey’s and Bonferroni’s tests, with statistical significance set a priori at $p \leq 0.05$. All statistics were performed using GraphPad Prism 9.3.1, and data are presented as means ± standard deviation. Isobologram analysis was performed using the CalcuSyn software program (Biosoft).
Results

**XRK3F2 increases the anti-MM efficacy of Btz in vitro and in vivo.**

To examine whether targeting p62 potentiates the anti-MM effects of PIs, we tested the effects of the small molecule ligand of the p62 ZZ-domain, XRK3F2, on MM cell viability as a single agent and in combination with Btz. Both agents were used at concentrations lower than their IC50s (**Supplementary Table 1**). Human MM cells with WT p53 (MM.1S and NCI-H929; **Fig. 1A** and **Supplementary Fig. 1A**) were more sensitive to Btz than those with mutant p53 (JJN3, RPMI-8226, U266; **Fig. 1B** and **Supplementary Fig. 1B and 1C**) or lacking p53 (KMS-11; **Fig. 1C**). Combined treatment significantly reduced MM cell viability compared to either drug alone, independent of p53 status. To determine the cause of the decreased MM cell viability, we examined apoptosis in MM1.S cells after 6h treatment. Btz alone or combined with XRK3F2 caused significant apoptosis compared to control (**Supplementary Fig. 1D**). The combination increased the percentage of Annexin V/propidium iodide double-positive cells compared to Btz alone, suggesting increased necrotic cell death. Chou-Talalay analysis showed that XRK3F2-Btz combination had synergistic anti-MM activity against all MM cell lines, with CI values <1. Similar results were obtained in CD138+ cells from 4 relapsed MM patients (**Fig. 1D**). Because the tumor microenvironment (TME) dictates MM cell responses to therapy, we next tested if XRK3F2 overcomes the pro-survival effects of stromal cells on MM cells treated with Btz. Co-culture with stromal cells protected JJN3 MM cells from Btz-induced apoptosis (**Fig 1E**). In contrast, co-administration of XRK3F2 hampered this protection and resulted in apoptotic levels similar to those in MM cells treated with Btz and cultured alone.
Further, we tested the *in vivo* anti-MM efficacy of single-agent vs. co-administration of XRK3F2 and Btz in an established xenograft mouse model of human MM (*Fig. 1F*).\(^\text{27}\) Three weeks after, JNN3-injected mice, exhibited detectable serum levels of the tumor biomarker human κ light chain, compared to saline-injected mice, indicative of active tumor growth. After 2 weeks, mice bearing MM treated with vehicle exhibited an 8-fold increase in tumor growth. Similar tumor progression was observed in mice receiving single agents. In contrast, the combination therapy reduced tumor burden by 50% compared to vehicle-treated mice and 24% compared to Btz only (*Fig. 1F* and *Supplementary Fig. 2A*). Dying tumor cells release high-mobility group box 1 protein (HMGB1). Treatment with low doses of XRK3F2 alone or combined with Btz, but not Btz alone, significantly increased serum human HMGB1 (8-fold and 13-fold increase, respectively, *Supplementary Fig. 2B*). None of the groups lost weight indicating limited toxicity of single agent and combination treatments (*Supplementary Fig. 2C*). Together, these *in vitro* and *in vivo* results support that XRK3F2-Btz combination decreases tumor growth by activating multiple MM cell death pathways.

**XRK3F2 blocks the activation of NFκB and autophagic-survival mechanisms in MM cells.**

Among the multi-domain functions of p62, regulation of NFκB signaling plays a pivotal role in promoting tumor growth and tumor-TME crosstalk to establish a favorable microenvironment for tumor progression.\(^\text{28}\) Additionally, NFκB is the main coordinator of TNF-α signaling, a well-established pro-survival and proliferation factor for MM cells and osteoclasts found in the MM bone marrow microenvironment.\(^\text{29-31}\) In MM, Btz increase p62 levels and activate NFκB by down-regulating p65-inhibitor IkBα.\(^\text{32}\) XRK3F2 treatment prevented NFκB activation triggered by Btz, as shown by a time-dependent reduction in IkBα (*Supplementary Fig. 3A*). Moreover, XRK3F2 inhibited TNFα-induced IkBα
and NFκB\textsuperscript{p65} phosphorylation (Supplementary Fig. 3B) and prevented nuclear translocation of NFκB\textsuperscript{p65} (Supplementary Fig. 3C) after 5 and 30 minutes respectively, thereby counteracting the pro-survival effects of NFκB activation caused by Btz. These results suggest that XRK3F2 may prevent the NF-kB-dependent pro-tumorigenic effect of Btz, an effect needed especially in relapsed, Btz-resistant MM patients.

Btz increases p62 levels by inducing de novo p62 expression and preventing its degradation. In agreement with the previous studies\textsuperscript{33}, we found that Btz increased levels of p62 mRNA 8-fold and protein (Fig. 2D and Supplementary Fig. 2D) independent of autophagy since changes in LC3I-LC3II conversion were not found in Btz-treated cells. Btz-induced p62 mRNA expression was reduced by XRK3F2. Ligands binding to p62-ZZ stimulate p62 oligomerization and autophagy.\textsuperscript{34} XRK3F2 alone or in combination with Btz increased LC3I-LC3II conversion in MM.1S, indicating induction of autophagy (Fig. 2D left panel). Pretreatment of MM cells with bafilomycin A1 (Baf), which disrupts autphagic flux, further increased LC3II/LC3I ratio and p62 protein levels compared to XRK3F2 alone or XRK3F2-Btz combination (Fig. 2D right panel). Furthermore, autophagy blockade by Baf treatment further amplified the negative effects of XRK3F2 and Btz on MM cell viability (Fig. 2A and Supplementary Fig. 4A), indicating that the autophagy-induced by targeting p62-ZZ domain in MM cells was not the pathway responsible for MM cell death.

**XRK3F2 and Btz combination treatment activates multiple cell death pathways in MM cells.**

We previously reported that high doses of XRK3F2 triggered caspase 3 cleavage in MM.1S cells.\textsuperscript{17} Here, we found that in MM.1S cells, co-administration of 5µM XRK3F2 and 3nM Btz strongly activated caspase-8 and caspase-3 (Fig. 2E left panel). In contrast, we only detected
partial activation of caspases by Btz alone and no activation by XRK3F2 at the concentration used. To test if the increased cell death seen with the combination therapy was due to apoptosis, we used the pan-caspase inhibitor Q-VD-OPh (QVD) and the caspase-3 inhibitor Z-DEVD-FMK (Z-DEV). QVD and Z-DEV completely blocked Btz-induced cleavage of caspases 3 and 8 (Fig. 2E and 2F right panels). In addition, QVD or Z-DEV fully prevented the decrease in cell viability induced by Btz (Fig. 2B and 2C; Supplementary Fig. 4B and 4C) but enhanced the anti-MM effects of XRK3F2. QVD and Z-DEV only partially blocked caspase 8 and 3 cleavage by XRK3F2-Btz and partially reduced the effects of the combination on MM cell viability. These findings suggest that XRK3F2-Btz combination inhibits MM viability by activating both caspase-dependent and independent-cell death pathways.

**XRK3F2 increases sensitivity to PIs by induction of necroptosis.**

In addition to binding ligand proteins for autophagic degradation, the p62-ZZ domain is also a scaffold for necroptosome formation by binding RIPK1, which forms a complex with RIPK3 and mixed lineage kinase domain-like effector (MLKL). Inhibition of RIPK1 binding using necrostatin-1 (Nec-1) prevented cell death induced by XRK3F2, but not by Btz, in MM cell lines (Fig. 3A and Supplementary Fig. 4D) and CD138+ cells from MM patients (Fig. 3B) by preventing the loss of plasma membrane integrity, measured by the LDH release after 16h culture (Fig. 3C). Nec-1 also prevented cell death induced by XRK3F2-Btz combination, inhibiting both necroptosis and RIPK1-dependent apoptosis. Nec-1 did not affect basal autophagy (Supplementary Fig. 4E), suggesting that RIPK1 kinase activity is not required to suppress autophagy. Upon treatment with XRK3F2, RIPK3 co-immunoprecipitated with RIPK1, and the association was increased by inhibition of apoptosis (Fig. 3D top). XRK3F2 stimulated MLKL phosphorylation at S358 and consequent cell death in MM cells (Fig. 3D bottom).
Inhibiting RIPK3 or MLKL phosphorylation with GSK872 (RIPK3 kinase inhibitor) or necrosulfonamide (NSA, an MLKL inhibitor), blocked XRK3F2-induced cell death induced (Fig. 3E). Taken together, these results suggest that XRK3F2 treatment leads to induction of necroptosis and synergizes with Btz-mediated apoptosis increasing MM cell death via concurrent activation of multiple death pathways (Fig. 3F).

**p62 and RIPK3 expression levels inversely correlated with MM disease progression.**

To investigate the significance of p62, RIPK1, RIPK3, and MLKL expression in MM patients, publicly available datasets containing normal, monoclonal gammopathy of unknown significance (MGUS), high-risk smoldering MM (SMM), and newly diagnosed MM patients\textsuperscript{35} were analyzed. We found that p62 (SQSTM1) expression increased at the mRNA level across disease stages with active MM plasma cells (PCs) expressing higher p62 levels compared to premalignant MGUS PC (p = 0.0092, Fig. 4A). Further, we observed expression of SQSTM1 in MM cells regardless of ISS disease stage (Supplementary Fig. 5). RIPK1, RIPK3 and MLKL mRNA were all expressed in MM cells albeit RIPK3 and MLKL mRNA levels were significantly reduced in MM patients with active disease compared to MGUS (p = 0.0206 for RIPK3; p = 0.022 for MLKL) and healthy PCs (p = 0.0097 for RIPK3; p = 0.0336 MLKL; Fig. 4C and 4D). No significant differences were observed for RIPK1 (Fig. 4B). Further, we confirmed these findings in immunoblots from primary CD138+ selected cells from MM patients and MM cell lines (Fig. 4E and Supplementary Fig. 6). We observed that p62 was strongly expressed at the protein level in the majority of primary MM cells (10/11 MM patients samples Fig. 4E). RIPK3 and MLKL proteins were detected in 100% (11/11) or 82% (9/11) of the primary CD138+ cells from MM patients although their level varied. These data suggest that p62 upregulation and RIPK3/MLKL downregulation correlate with MM disease progression.
Low RIPK3 expression at diagnoses correlates with reduced survival and response to Bortezomib-based therapies in MM patients.

To evaluate the impact of the expression of RIPK3, MLKL, and p62 on clinical outcomes for MM patients, we employed the MMRF-CoMMpass (IA18) dataset. Newly diagnosed MM patients (NDMM) with low expression RIPK3 exhibited lower overall survival (OS) (Fig. 5A, p=0.00084) and progression-free survival (PFS) (p=0.013) than those with higher expression. We observed similar OS and PFS trends in NDMM patients with low MLKL expression or high p62 expression; however, the results did not reach statistical significance (Supplementary Fig. 7A and 7B).

Next, we examined if clinical responses to Btz-based therapies correlate with RIPK3 or p62 expression. Among patients receiving Btz-based therapies, those with lower expression of RIPK3 exhibited worse OS (Fig. 5B, p=0.00032) and PFS (p=0.017) than those with higher RIPK3 expression. Similar, non-significant trends were observed in Btz-treated patients with low MLKL expression (Supplementary Fig. 7C). In contrast, OS was better in MM patients receiving Btz-based therapy with lower p62 expression (Fig. 5C, p=0.013); although PFS was not affected. Collectively, these results highlight the relevance of the N-rule pathway in clinical responses in MM patients and suggest that modulating RIPK3 and MLKL activities with XRK3F2 can be exploited to improve clinical outcomes in MM patients.

XRK3F2 and Btz combination prevents bone destruction and stimulates bone formation in a human xenograft mouse model of established multiple myeloma disease.

MM patients frequently have severe osteolysis leading to increased morbidity and mortality. Whilst PIs have been shown to transiently increase bone formation, MM-induced lesions rarely repair. We previously showed that XRK3F2 as a single agent induced cortical bone formation in
a syngeneic mouse model of MM. Here, we investigated the effects of XRK3F2 in combination with Btz on an immunodeficient mouse model of human MM bone disease. Three weeks after, JNJ3-injected mice showed overt osteolytic lesions in the injected tibias, indicative of established bone disease. No lesions were observed in saline-injected mice. Bone destruction was observed in mice treated with XRK3F2 or Btz alone, while the combination significantly preserved bone mass and reduced the number of osteolytic lesions compared with vehicle or single agent-treated mice (Fig. 6A). Both Btz alone and XRK3F2-Btz combination decreased the serum levels of the bone resorption marker CTX compared to vehicle-treated mice. However, only the combination therapy significantly restored serum levels of bone formation marker P1NP to level observed in tumor naïve mice (Fig. 6B). JNJ3-injected mice had ~38% decreased cortical BV/TV (bone volume/total volume), measured at the fibular-tibia junction, compared to saline-injected animals. XRK3F2-Btz co-administration mitigated the progression of bone disease by preserving cortical BV/TV (Fig. 6C and 6D). We also performed bone architectural analysis of contralateral tibiae of mice receiving XRK3F2-Btz combination treatment. These bones displayed increased trabecular BV/TV (50%), thickness, Tb.Th (7%), number, Tb.N (45%), and decreased spacing, Tb.Sp (19%), compared to non-MM bearing mice that received vehicle (Fig. 7A). Next, we determined the effects of XRK3F2 on osteoblast differentiation and bone-forming activity. Treatment of pre-osteoblastic MC3T3-MC4 cells with XRK3F2-Btz combination increased Runx2, Osterix, and ATF4 mRNA expression levels compared to vehicle or single treatment (Fig. 7B). The combination, but not the single agents, also blocked the inhibitory effects of TNFα on osteoblast differentiation by restoring Runx2 mRNA expression (Fig. 7C). XRK3F2-Btz combination but not Btz alone, significantly increased matrix production and mineral deposition of primary bone marrow stromal cells, without affecting viability (Fig.
These findings support that XRK3F2 in combination with Btz stimulates bone formation by promoting osteoblast differentiation and activity.

Discussion

MM cells subjected to sustained proteasomal inhibition, as occurs during Btz-therapy, rely on p62-mediated autophagic degradation to reduce the proteotoxic load derived from excessive immunoglobulin (Ig) heavy chain synthesis.\textsuperscript{33, 36} Excess Ig heavy chain bound to HSPA5 is proteotoxic and induces all three ER stress response regulators: IRE1\textsubscript{α}, ATF6\textsubscript{α}, and PERK.\textsuperscript{10} HSPA5, with its bound cargo, is exported from the ER to the cytoplasm\textsuperscript{11}, where it provides a bimodal degron for cellular elimination.\textsuperscript{15} N-Arg bearing substrates are eliminated by either the proteasome following UBR1-catalyzed ubiquitylation or autophagy following binding to the ZZ domain of p62. In the presence of proteasomal inhibition, N-terminally arginylated peptides bind to the p62 ZZ-domain with micromolar affinity and trigger p62 oligomerization\textsuperscript{37} and autophagy.\textsuperscript{22} Thus, in the presence of PIs, p62 provides an alternate pathway for cargo degradation.\textsuperscript{15} We developed XRK3F2 by molecular modeling of the ZZ-domain\textsuperscript{17}, and Cha-Molstad et al.\textsuperscript{37} next showed that XRK3F2 binds to the arginyl-peptide binding site and triggers p62 oligomerization. XRK3F2 functions as a ligand rather than an inhibitor of p62, triggering ineffectual autophagy in the absence of cargo. Thus, the combination of a PI, such as Btz, with XRK3F2 blocks both proteolytic pathways for N-end rule degradation, allowing cytoplasmic accumulation of proteotoxic cargo, such as Ig heavy chain bound to HSPA5 and subjects MM cells to uncontrolled proteotoxic stress and consequent cell death. Here we demonstrate in vitro
and *in vivo* that co-administration of suboptimal concentrations of Btz and XRK3F2 synergistically killed MM cells.

XRK3F2-Btz combination-induced cell death not only was incompletely prevented by blocking apoptosis or autophagy but was increased when caspase activation was prevented. These findings are in line with recent evidence showing that apoptosis and necroptosis are tightly connected and can cross-regulate each other.\(^3\) Several recent studies suggested that autophagy is implicated in caspase-independent cell death.\(^3\) The significant reduction in cell death induced upon RIP1 kinase inhibition supports necroptosis as a second mechanism of cell death induced by the XRK3F2-Btz combination in both MM cell lines and primary CD138+ MM cells. Furthermore, selective pharmacological inhibition of MLKL and RIP3 kinase prevented XRK3F2-induced MM cell death, implying that the viability rescue seen by necroptosis inhibition was not due to off-target effects of Nec1.\(^4\) Several studies have shown that triggering necroptosis in cancer cells can increase chemotherapeutic drug responses (reviewed in\(^4\)). Consistent with the relevant role of this pathway in MM, we found that p62 expression was higher, while RIPK3 and MLKL expression levels were lower, in MM patients compared to MGUS patients, and downregulation of RIPK3 correlated with poor clinical outcomes and responses to Btz-based therapies. These results are consistent with the increase in p62 expression and loss expression or mutation of components of the necrosome found in other malignancies, and their correlation with poor prognosis.\(^4\) TME plays a central role in MM onset and progression and interactions between MM and cells of the BM transform the marrow into an ideal niche for the migration, proliferation, and survival of MM cells.\(^4\) Adhesion to stromal cells and release of cytokines from them have been shown to decrease the efficacy of chemotherapy on MM cells by activating pro-survival pathways. We
previously showed that XRK3F2 prevented coculture-induced TNFα upregulation in both 5TGM1 cells and BMSCs following co-culture and in BMSC, these effects required p62.17 Here, although we cannot exclude the contribution of XRK3F2-mediated changes in TNFα production by BMSC, we show that XRK3F2-Btz combination similarly increased MM cell death regardless of the presence or absence of stromal cells. These results suggest that, in the context of Btz therapy, targeting p62, bypasses the pro-survival and chemoprotective effects of stromal cells for MM cells, making them more susceptible to the pro-apoptotic actions of Btz.

The combination also inhibited the pro-tumoral activation of NFκB and TNFα, crucial factors for the survival and progression of MM and bone cell activity, through additional activities of multiple domains of the p62 scaffold protein.

Most patients with MM suffer skeletal complications characterized by osteolytic bone destruction and inhibited bone formation1, 46 which may persist unhealed during years of remission. Although Btz decreased tumor burden, Btz-treated mice still presented extensive bone lesions. These could be due to the aggressiveness of the mouse model of established bone disease, and/or the suboptimal dose of Btz (0.25 mg/kg, twice a week) used. Nonetheless, even at higher doses, other groups showed that Btz reduces MM burden but was insufficient to reduce osteolytic lesions as a single agent.9, 47 Importantly, while PIs can promote transient bone formation, they do not effectively inhibit osteoclasts as single agents.7

We previously found that XRK3F2 inhibited osteoclasts, stimulated new bone formation, and increased osteoblast Runx2.17, 18 Here, we found that the combination of XRK3F2 with Btz preserved bone mass in an aggressive mouse model of established human MM bone disease. Further, the combination therapy increased bone mass after only two weeks in the contralateral, tumor-free leg. The anabolic action of combination therapy is supported by increased expression
of genes associated with mineralization (Runx2, Osterix, and ATF4) and overcoming the suppression of Runx2 by TNFα in osteoblasts.

The detailed mechanism of the positive effects on bone by XRK3F2-Btz remains to be clarified. Activation of ER stress, while killing MM cells, increases osteoblast activity. This activity may involve the XBP1 splicing branch of the tripartite ER stress response, which we have shown is important in both MM and bone cells. 48 XBP1 can affect Btz sensitivity independently of HSPA5, although HSPA5 plays an important role in MM 49 and stimulates osteoblastic mineralization.50 Our observations are compatible with both HSPA5- and XBP1-dependent mechanisms, which merit further research.

Overall, XRK3F2 provides a multifunctional supplement to proteasome inhibition for treating MM. It synergizes with Btz to kill MM cells by activating necroptosis, while positively suppressing bone destruction with actions on ER stress and proteolytic degradation pathways. XRK3F2 represents a first-in-class ligand for the ZZ-domain of p62, a clinically important multifunctional scaffold protein. XRK3F2 interferes with autophagy and NFκB signaling and activates necroptosis, identifying the ZZ-domain as an important, druggable target for the improved treatment of myeloma bone disease.
REFERENCES


Figure Legends

Fig. 1. Combination of low doses of XRK3F2 and Bortezomib synergistically increases MM cell death in vitro and inhibits tumor growth in vivo. A MM.1S; B, JJN3; C, KMS-11 and D, primary CD138+ cells were treated for 24h with XRK3F2 (5µM), Btz (3nM), or combined XRK3F2-Btz (5µM/3nM). MM cell viability was evaluated by alamarBlue assay and is reported as percent vs. DMSO vehicle control. CI of less than 1 indicates synergy. MM cell death was evaluated by (D) Trypan Blue uptake assays or by Annexin V/propidium iodide (PI) staining MM:HS5 cell-to-cell co-cultures after 24h of treatment (E). Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone by one-way ANOVA with post hoc Tukey’s correction. F In vivo experimental design (10^5 JJN3 MM cells injected intratibially). Serum levels of the JJN3 tumor biomarker human kappa light chain after in vivo treatment with XRK3F2 (27mg/kg/5xweek), Btz (0.25mg/kg/2xweek) or XRK3F2-Btz combination. Data are presented as box & whiskers plots where each dot represents a mouse n=7-10/group. #p<0.05 vs 3 weeks; *p<0.05 vs JJN3-vehicle and ^p<0.05 vs JJN3-XRK3F2 or Btz alone by one-way ANOVA with post hoc Tukey’s correction. The horizontal dotted line indicates the mean value for vehicle-treated mice bearing JJN3 tumors.

Fig. 2. XRK3F2 plus Bortezomib combination activates multiple death pathways and overcomes apoptosis resistance in MM. MM.1S cells treated with XRK3F2 (5µM), Btz (3nM), or combined XRK3F2-Btz (5µM/3nM) for 24h in the presence or absence of autophagy inhibitor Bafilomycin A1 (Baf, 40nM) (A and D), Pan Caspase OPH Inhibitor Q-VD (QVD, 20 µM) (B and E), or Caspase-3 Inhibitor Z-DEVD-FMK (Z-DEV, 20 µM) (C and F). Cell viability was evaluated using alamarBlue assays and is reported as percent vs. DMSO vehicle control (A-C). Analysis of autophagic flux and apoptosis were assessed by immunoblotting by LC3I-LC3II conversion (D) and for cleavage of caspase 8 and 3 (E and F). Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone and □ p<0.05 vs control vs Baf/QVD/ZDEV culture by two-way ANOVA with post hoc Bonferroni’s correction.

Fig. 3. XRK3F2 induces caspase-independent necroptotic cell death. A MM.1S and B CD138+ human MM cells were treated with XRK3F2 (5µM), Btz (3nM), or combined XRK3F2-Btz (5µM/3nM) for 24h in the presence or absence of RIP1 kinase inhibitor Necrostatin-1 (Nec-1, 60µM) and viability assayed by alamarBlue assay and is reported as percent vs. DMSO vehicle control. C MM.1S cells were treated with 0, 5 or 10µM XRK3F2 for 16 hours ± 20µM Z-DEV or 60µM Nec-1 followed by LDH release analysis. D MM.1S cells were treated with 5 or 10 µM XRK3F2 for 4h hours ± 20µM Z-DEV and RIPK1-RIPK3 binding assessed by immunoprecipitation using anti-RIPK1 antibody or IgG2a control (top panel). MM.1S cells were treated with 10µM XRK3F2 for 2, 4, and 6h, and MLKL phosphorylation at Ser 358 was assessed by immunoblotting (bottom panel). E MM.1S cells were treated with 0, 5 or 10µM XRK3F2 for 24 hours in the presence or absence of RIP3 kinase inhibitor GSK872 (GSK, 3 µM), MLKL inhibitor necrosulfonamide (NSA, 1 µM) and 60µM Nec-1. Cell viability was evaluated by alamarBlue and reported as percent vs. DMSO vehicle control. Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone, and □ p<0.05 vs control vs Nec-1/GSK/NSA culture by two-way ANOVA with post hoc Bonferroni’s correction. F Schematic representation of XRK3F2-Btz combination mechanisms of action.
Fig. 4. High p62 and low RIPK3 expression in patients correlates with disease progression. Transcriptome analysis of the GDS4968 data set showing the mRNA expression levels in of A p62, B RIPK1, C RIPK3 and D MLKL in CD138+ primary cells of MM patients in different stages of the disease monoclonal gammapathy of undetermined significance (MGUS), smoldering multiple myeloma (MM), or MM and normal plasma cells (N). E p62, RIPK3, and MLKL protein expression levels were evaluated by immunoblotting in cell lysates of primary CD138+ plasma cells from MM patients (#1,6,13 newly diagnosed; #2 autologous SCT; #3,5,10,14 progressive disease; #4,12 refractory; #11 relapsed; Supplementary Table 2) using VCP/p97 as loading control.

Fig. 5. Low RIPK3 correlates with reduced survival and response to Bortezomib-based treatment in MM patients. Impact of gene expression on overall survival (OS) and progression-free survival (PFS) in patients from the MMRF CoMMpass cohort. A OS (left panel) and PFS (days, right panel) in patients with low and high RIPK3 expression. B OS (left panel) and OS (days, right panel) in response to Btz-based therapies in patients with high and low RIPK3 expression. C OS (left panel) and PFS (days, right panel) in response to Btz-based therapies in patients with high and low p62 expression.

Fig. 6. XRK3F2 plus Bortezomib combination decreases osteolysis and protects from bone disease progression in mice with established MMBD. A Representative X-rays images of tibiae 3 weeks after JNJ3 cell inoculation and 5 weeks after treatment with vehicle (0.01ml/g in 15% hydroxyl propyl β-cyclodextrin in saline/daily); XRK3F2 (27mg/kg/5xweek); Btz (0.25mg/kg/2xweek) and XRK3F2-Btz (27mg/kg/5xweek plus 0.25mg/kg/2xweek, respectively) for 2 weeks. B Bone resorption (CTX, left panel) and bone formation (P1NP, right panel) markers were evaluated by ELISA assay. C Representative reconstructed micro-CT images and D analysis of bone microarchitecture of cortical bone of the distal tibia (Ct. BV/TV cortical bone volume/ tissue volume; Ct. BV cortical bone volume). Data are presented as box & whiskers plots where each dot represents a mouse n=7-10/group. *p<0.05 vs saline injected mice; *p<0.05 vs JNJ3-vehicle and ^p<0.05 vs JNJ3-XRK3F2 or Btz alone by one-way ANOVA. The horizontal dotted line indicates the mean value for vehicle-treated mice bearing JNJ3 tumors.

Fig. 7. XRK3F2 treatment increases the bone anabolic effects of Bortezomib in mice Analysis of bone microarchitecture of trabecular bone of the contralateral proximal tibia of mice treated with vehicle (0.01ml/g in 15% hydroxyl propyl β-cyclodextrin in saline/daily); XRK3F2 (27mg/kg/5xweek); Btz (0.25mg/kg/2xweek) and XRK3F2-Btz (27mg/kg/5xweek plus 0.25mg/kg/2xweek, respectively) for 2 weeks. A trabecular BV/TV, trabecular thickness, Tb.Th; trabecular number, Tb.N; trabecular separation, Tb.Sp. mRNA expression of B Runx2; Osterix; Atf4 MC3T3-MC4 clones treated for 48h with XRK3F2 (100nM), Btz (2nM), or combined XRK3F2-Btz (100nM/2nM). C mRNA expression of Runx2 in MC3T3-MC4 pre-exposed to 100ng/ml of TNFα and treated as above and expressed as fold change versus vehicle-treated control. D Alizarin red staining and viability of primary BMSC cultured treated with XRK3F2 (100nM), Btz (2nM), or combined XRK3F2/Btz (100nM/2nM) for 28 days or 48h respectively. Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone by one-way ANOVA.
**A**

3 weeks

5 weeks

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**Bone formation**

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**Ct. BV**

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Supplementary Fig. 1. Combination of low doses of XRK3F2 and Bortezomib synergistically increases MM cell death in vitro. A NCI-H929, B RPMI-8226, C U266, and D MM1.S cells were treated for 24h (or 6h (D)) with XRK3F2 (5µM), Btz (3nM), or combined XRK3F2-Btz (5µM/3nM). MM cell viability was evaluated by alamarBlue assay and is reported as percent vs. DMSO vehicle control. CI of less than 1 indicates synergy. MM cell death was evaluated by (D) Annexin V/propidium iodide (PI) staining in monoculture after 6h of treatment. Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone by one-way ANOVA with post hoc Tukey's correction.
Supplementary Fig. 2. XRK3F2 plus Bortezomib combination suppresses human JJN3 MM cell growth in vivo.
Serum level of A JJN3 tumor biomarker human Kappa light chain and B human high-mobility group box 1 protein (HMGB1) and C body weight after in vivo treatment with XRK3F2 (27mg/kg/5xweek), Btz (0.25mg/kg/2xweek) or XRK3F2-Btz combination (27mg/kg/5xweek plus 0.25mg/kg/2xweek, respectively) n=7-10 mice/group. Data are presented as box & whiskers plots where each dot represents a mouse. *p<0.05 vs JJN3-vehicle and ^p<0.05 vs JJN3-XRK3F2 or Btz alone by one-way ANOVA with post hoc Dunnet’s correction. The horizontal dotted line indicates the mean value for vehicle-treated mice bearing JJN3 tumors. D Quantitative RT-PCR analysis of p62 mRNA in MM.1S cells treated with XRK3F2 (5µM), Btz (3nM), or combined XRK3F2-Btz (5µM/3nM) for 12 hours. Data are presented as bars, means±SD (n=4-6/group). *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone by one-way ANOVA with post hoc Tukey’s correction.
Supplementary Fig 3. XRK3F2 blocks Bortezomib-induced activation of NFκB.

A MM.1S cells were treated with Btz (10nM) in the presence or absence of XRK3F2 (10µM) for the indicated time points after which IkBα expression levels were determined by immunoblot analysis. B and C MM.1S cells were pre-treated with either DMSO or XRK3F2 (10 µM) for 2h and then stimulated with TNFα (10ng/ml) for either 5, 15 minutes (B) or 30 and 60 minutes (C) after which expression levels of phosphorylated IkBα and NFkBp65 (B) or nuclear translocation of NFkBp65 (C) were determined by immunoblot analysis.
Supplementary Fig. 4. XRK3F2 plus Bortezomib combination activates multiple death pathways and overcomes apoptosis resistance in JJN3. JJN3 cells were treated with 5µM XRK3F2, 3nM Btz, or XRK3F2-Btz combination (5µM/3nM) for 24h hours in the presence or absence of Baf (40nM) (A), QVD (20µM) (B), Z-DEV (20µM) (C) Nec-1 (60µM) (D) or Baf (40nM) plus Nec-1 € Cell viability was evaluated using alamarBlue assays. Data are presented as bars, means±SD (n=4-6/group), and expressed as % of viable cells normalized versus vehicle-treated control. *p<0.05 vs vehicle, ^p<0.05 vs XRK3F2 or Btz alone, and † p<0.05 vs control vs Baf/QVD/ZDEV culture by two-way ANOVA with post hoc Bonferroni’s correction.
Supplementary Fig 5. p62 is highly expressed in MM patients CD138+ cells independent of the disease stages.
Supplementary Fig 6. p62, RIPK3 and MLKL expression in a panel of MM cell lines.
Supplementary Fig 7. Impact of gene expression on overall survival (OS) and progression-free survival (PFS) in MM patients. OS (left panel) and PFS (days, right panel) in patients with low and high A p62 mRNA expression or B MLKL mRNA expression. C OS (left panel) and OS (days, right panel) in response to Btz-based therapies in patients with high and low MLKL expression using MMRF CoMMpass (IA15) dataset.
Supplementary Methods, Tables and References

Chemicals. Cell culture media, penicillin and streptomycin were from Invitrogen. AlamarBlue® Cell Viability and Trypan blue assay kits were from ThermosFisher Scientific (Waltham, MA, USA). Caspase-3 Inhibitor Z-DEVD-FMK (#FMK004) and Pan Caspase Inhibitor Q-VD (#OPH001) were from R&D Systems (Minneapolis, MN); GSK’872 (#64920) and necrosulfonamide (#5025) were from Tocris Bioscience (Minneapolis, MN); Necrostatin-1 (#BML-AP309) was from Enzo Lifesciences (Farmingdale, NY); Bafilomycin A1 (#B1793) was from Sigma-Aldrich (Saint Louis, MO, USA). Bortezomib (Btz) (#S1013) was from Selleck Chemicals (Houston, TX). XRK3F2 was synthesized by a published route (1) and purified to greater than 97% purity by HPLC by the Chemical Genomics Core Facility at the Indiana University School of Medicine. F2 indicates the two fluorine atoms to increase stability in vivo added to the parental XRK3, which Cha-Molstad et al. (2) refer to as XIE62-1004.

Antibodies. Anti-SQSTM1/p62 (#ab155686), VCP (#ab11433) and human p-MLKL (#187,091) were from Abcam (Cambridge, MA); Cleaved Caspase-3 (#9664), Caspase-3 (#9662), Cleaved Caspase-8 (#9748), Caspase-8 (#4790), p-IκBα (#2859), IκBα (#9242), p-NFκB p65 (#3033) were from Cell Signaling Technology (Danvers, MA). Anti-RIP1 clone 38/RIP (#610459) was from BD Biosciences (Franklin Lakes, NJ). Anti-RIP3 (#2283) was from ProSci (Poway, CA). Anti-MLKL (#MABC604) was from Merck Millipore (Burlington, MA). Anti-LC3B (#L7543), β-actin (#A5441) and α-tubulin (#T9026) as well as propidium iodide were from Sigma-Aldrich. Anti-NFκB p65 (#sc-8008), anti-RIP3 (sc-374639) were from Santa Cruz Biotechnology (Dallas, TX).

Apoptosis/Necroptosis. Apoptosis/Necroptosis ratio was assessed by 1) Quantitative Assessment by Flow using TACS Annexin V-FITC Apoptosis Detection Kit (#4830-250, R&D Systems) and flow cytometry detection (Fortessa flow cytometer, Becton Dickinson) as previously described.
Post-acquisition analysis of the gated cell subsets was performed using FlowJo software (Tree Star, OR). 2) Release of the enzyme lactate dehydrogenase (LDH) using Pierce LDH Cytotoxicity Assay Kit (#88953) as previously described (4).

**Cell-to-cell co-cultures.** Direct MM:HS5 cell-to-cell co-cultures were established by adding MM cells on top of HS5 cells in a 1:5 (HS5:MM) ratio. Co-cultures were treated with Btz (3nM) every 24h. In these co-cultures, MM cells were stained with the fluorescent cell-tracker DiI following the manufacturer's recommendations. Apoptosis in DiD+ MM cells was assayed by flow cytometry using the Annexin V apoptosis Detection kit (BD Biosciences) following the manufacturer's recommendations. Samples were analyzed in a BD FACSCalibur (UAMS Core Facility for Flow cytometry) within 1h. At least 10,000 cells were used for each group, and the data was analyzed by FlowJo software to detect different cell populations.

**Immunoprecipitation and Western blotting.** Cell pellets were lysed with IP lysis buffer containing 25 mM TRIS-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, supplemented with proteinase inhibitor cocktail (#P8340, MilliporeSigma). Five hundred µg of lysates incubated with 2 µg anti-RIP1 (Clone 38/RIP; BD Bioscience) or control (mouse IgG2a) antibodies overnight at 4°C. Protein/antibody complexes were precipitated by protein A/G magnetic beads (Invitrogen) for 4h. Denatured protein complexes were separated by 10% SDS-PAGE gel electrophoresis and transferred to PVDF membrane. The immune complexes were subject to western blotting using anti-RIP1 (Clone 38/RIP; BD Bioscience) and anti-RIP3 (Prosci.Inc) primary antibodies and specific HRP-linked secondary antibodies, followed by visualization with enhanced chemiluminescence kit (Thermo Scientific). For the detection of phospho-MLKL, cell lysates were prepared in %SDS hot lysis buffer. Lysates were boiled (10 min; 95°C), sonicated (40kW, 3 seconds, intervals 3 seconds, 25-30 times) and the supernatant...
obtained after centrifugation (17000g; 10 minutes) was subject to protein concentration followed
by western blotting. For regular western blotting, protein lysates were extracted in RIPA lysis
buffer (#sc-24948; SCB) supplemented with protease inhibitors cocktail (Millipore Sigma). Equal
amounts of proteins, as determined by bicinchoninic acid assay protein analysis (Pierce), were
separated on Any kD™ SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) and transferred
onto PVDF membranes. For the detection of immune complexes, the membranes were incubated
with various primary antibodies and specific HRP-linked secondary antibodies, which were
detected using the enhanced chemiluminescence kit (Thermo Scientific). The immune complexes
were quantified by densitometry using ImageJ software after normalization to specific loading
controls.

**Assessment of autophagy.** During autophagy, the microtubule-associated protein 1A/1B-light
chain 3 (LC3) is converted from LC3-I to the lipidated form LC3-II. Densitometric determination
of the LC3-I/LC3-II ratio from Western blots provides and index of autophagic flux.

**Mouse model of human MM.** Immunodeficient 6-8 wk-old female SCID-CB17 mice (Fox Chase
SCID beige CB17.Cg-PrkdcscidLystbg-J/Crl congenics) were purchased from Charles River and
acclimated. Forty mice injected intratibially (IT) with 1x10⁵ human JJN3 myeloma cells in 20μl
of PBS and an additional five with PBS alone. After three weeks, mouse sera was assayed by
ELISA for human κ light chain to confirm tumor engraftment and limbs x-rayed by Faxitron under
isoflurane inhalation anesthesia for presence of osteolytic bone lesions. Engrafted mice were
randomized to four groups, then treated for two weeks with vehicle, XRK3F2, Btz or
XRK3F2+Btz. The target sample size of 8 mice was calculated based on our previous study (5).
All treatments were 100μL of 15% hydroxylpropyl-β-cyclodextrin in saline, which was also used
for delivery of XRK3F3 (insoluble in water or ethanol) at 27mg/kg, given IP daily Monday-Friday.
Bortezomib was freshly diluted in the same vehicle and injected subcutaneously twice a week for two weeks at 0.25 mg/kg. Serum was collected at euthanasia under isoflurane anesthesia and assayed by ELISA for human kappa light chain as marker of tumor burden. After preliminary analysis of each treatment group, mice showing light chain values greater than two standard deviations from the mean were excluded from the final analyses, leaving 7 or 8 mice per group.

**Bone analyses** Animal legs were analyzed by Faxitron X-ray, followed by removal of surrounding muscle and formalin fixation. Microcomputed x-ray tomography (μCT) scanning was performed to measure morphological indices of distal regions of tibiae (6). Images were acquired using a Bruker Skyscan 1176 with the following parameters: pixel size = 9 μm; peak tube potential = 50 kV; X-ray intensity = 500 μA; 0.3° rotation step. Raw images were reconstructed using SkyScan reconstruction software and analyzed using Skyscan CT Analyser software (CTAn; Bruker). Cortical bone of the injected legs was analyzed between 1 and 2 mm from the tibia-fibula junction using a threshold of 160–255. Trabecular bone of the contralateral leg was analyzed between 0.5 and 1.5 mm under the tibial proximal growth plate using a threshold of 80–255. After tomography, bones were decalcified in EDTA and embedded for routine histology.

**ELISA.** Human kappa light chain (E88-115, Bethyl Laboratories, Inc., Montgomery,TX), as a marker of myeloma tumor burden, and human high mobility group box 1 protein (HMGB1, # E88-115, Bethyl Laboratories), as a marker of myeloma cell death (7) were determined in serum using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions. Two markers of bone metabolism, mouse N-terminal propeptide of type I procollagen (P1NP, AC-33F1, a formation marker) and C-terminal telopeptides of type I collagen (CTX, AC-06F1, a marker of osteolysis), were likewise determined in serum by ELISAs.
Bioinformatic analyses of publicly available datasets. Gene expression data for RIPK3, RIPK1, SQSTM1 (p62), and MLKL were obtained from the MMRF Researcher Gateway using version IA18. Salmon count data were imported into R and normalized using DESeq2 (8). The optimal cutpoint for high and low gene expression groups was determined for each of the genes using the survminer (9) package in R, based on either progression-free survival (PFS) or overall survival (OS). The default method of the maximally selected rank statistics from the maxstat package was used for the optimal cutpoint algorithm selection method. The PFS and OS of bortezomib-treated and non-bortezomib-treated patients were compared within the context of high vs low expression of RIPK3, RIPK1, SQSTM1 (p62), and MLKL, based on the optimal cutpoint. Kaplan-Meier survival curves were generated using the survival and survminer packages in R using the ggsurvplot function.

Real-time RT-PCR (qPCR). Total mRNA was extracted using RNeasy (QIAGEN, Germantown MD) per the manufacturer’s protocol and reverse-transcribed using High-capacity cDNA reverse transcription kit (Applied Biosystem) on a T100 Thermal Cycler (Bio-Rad Laboratories). Quantitative PCR was performed on an CFX96 Real-Time System (Bio-Rad Laboratories) using a SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories) and cDNA equivalent to 40 ng RNA in a 10 µl reaction according to the manufacturer’s instructions. The DNA sequences of primers used for qPCR are listed in Supplemental Table S3. Relative expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method, with 18S rRNA used as a housekeeping gene.
Supplementary Table 1: MM cell lines IC50

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Supplementary Table 2: Main characteristics of the US patient cohort

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<td>Melph/Pred</td>
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<td>Lena/Dexa/Carfil/Poma/Dara/Cyc</td>
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<tr>
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<td>IgG lambda MM</td>
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Supplementary Table 3: Sequences of qPCR primers used for amplification of human (A) and mouse (B) mRNA

A. Human

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<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
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<tr>
<td>SQSTM1/p62</td>
<td>Forward</td>
<td>CGGCTGATTGAGTCCCTCTC</td>
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<td>Reverse</td>
<td>GCCGCTCCGATGTCATAGTT</td>
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B. Mouse

<table>
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<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGAAGCTTTGCTGACACGGT</td>
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<tr>
<td>Osterix</td>
<td>Forward</td>
<td>AGAGGTTCACTCGCTCTGACGA</td>
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<td>Reverse</td>
<td>TTGCTCAAGTGTCGCTCTCTG</td>
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<tr>
<td>ATF4</td>
<td>Forward</td>
<td>TCGGCCCCAACCTTATGACC</td>
</tr>
<tr>
<td></td>
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REFERENCES