Ibrutinib inhibits BTK-driven NF-κB p65 activity to overcome bortezomib-resistance in multiple myeloma

Megan Y Murray[1], Lyubov Zaitseva[1], Martin J Auger[2], Jenny I O Craig[3], David J MacEwan[4], Stuart A Rushworth[1*] and Kristian M Bowles[1,2*]

1. Department of Molecular Haematology, Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom.

2. Department of Haematology, Norfolk and Norwich University Hospitals NHS Foundation Trust, Colney Lane, Norwich, NR4 7UY, United Kingdom.

3. Department of Haematology, Addenbrooke's Hospital, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Cambridge, CB2 0QQ, United Kingdom.

4. Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, Ashton Street, Liverpool, L69 3GE, United Kingdom.

* SAR and KMB contributed equally

Running title: Combining ibrutinib and bortezomib to treat bortezomib-resistant MM

Key words: multiple myeloma, bortezomib, ibrutinib, NF-κB, drug-resistance, BTK

Abbreviations: MM – multiple myeloma, PI – proteasome inhibitor, NF-κB – nuclear factor-kappa B, BMSC – bone marrow stromal cells, BTK – Bruton's tyrosine kinase.

Corresponding Author: Prof Kristian Bowles, Department of Molecular Haematology, Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom. Email: k.bowles@uea.ac.uk

Conflicts of interest: The authors declare no competing financial interests.

Manuscript Information: Word count: approx. 6125, Figures: 5, Tables: 0.

Abstract

Multiple Myeloma (MM) is a haematologic malignancy characterised by the accumulation of clonal plasma cells in the bone marrow. Over the last 10-15 years the introduction of the proteasome-inhibitor bortezomib has improved MM prognosis, however relapse due to bortezomib-resistance is inevitable and the disease, at present, remains incurable. To model bortezomib-resistant MM we generated bortezomib-resistant MM cell lines (n=4) and utilised primary malignant plasma cells from patients relapsing after bortezomib treatment (n=6). We identified enhanced Bruton's tyrosine kinase (BTK) activity in bortezomib-resistant MM cells and found that inhibition of BTK, either pharmacologically with ibrutinib (0.5 μM) or via lenti-viral miRNA-targeted BTK interference, re-sensitised previously bortezomib-resistant MM cells to further bortezomib therapy at a physiologically relevant concentration (5 nM). Further analysis of pro-survival signalling revealed a role for the NF-κB p65 subunit in MM bortezomib-resistance, thus a combination of BTK and NF-κB p65 inhibition, either pharmacologically or via further lenti-viral miRNA NF-κB p65 interference, also restored sensitivity to bortezomib, significantly reducing cell viability (37.5±6.9 %, ANOVA $p \leq 0.001$). Accordingly, we propose the clinical evaluation of a bortezomib/ibrutinib combination therapy, including in patients resistant to single-agent bortezomib.

Introduction

Multiple Myeloma (MM) is characterised by the accumulation of clonal plasma cells in the bone marrow. The American Cancer Society estimates that there will be approximately 24,000 new cases diagnosed and 11,000 deaths from MM in 2014 in the US.{, 2014 #8628} Clinical manifestations of the disease occur as a consequence of the tumour bulk; including anaemia and fatigue, immune paresis leading to infection, renal failure, and osteolytic bone breakdown by activated osteoclasts, resulting in painful lytic bone destruction and hypercalcaemia.{Raab, 2009 #6283} An effective MM therapeutic succeeds, therefore, by 'de-bulking' the MM tumour mass, thus reducing the associated symptoms and subsequently maintaining remission.

The proteasome inhibitor (PI) bortezomib, approved for the treatment of MM by the FDA in 2003{Twombly, 2003 #8549}, is one such de-bulking agent that has greatly contributed to improved outcomes observed in MM.{Palumbo, 2011 #8529} Despite this advance, however, relapse following bortezomib therapy remains inevitable due to the emergence of bortezomib-resistant plasma cell sub-clones.{Kapoor, 2012 #8550} For example; recent 'whole genome' sequencing studies of MM patients confirmed a high level of genetic heterogeneity; occurring both between separate patients, and within patient samples before and after therapy{Chapman, 2011 #5683}, indicating the presence of a variety of genetically distinct plasma cell 'sub-clones'.{Egan, 2012 #8564;Keats, 2012 #8566} Different sub-clones further indicate the presence of numerous minor tumour initiating cell populations with complex and divergent evolutionary histories.{Magrangeas, 2013 #8666} The diversity and potential quiescence of these MM sub-clones can contribute to enhanced

tumorigenicity and an intrinsic resistance to therapy.{Chen, 2014 #8667} Therefore; the eradication of the majority bortezomib-sensitive sub-clones may ultimately promote the growth of pre-existing minority bortezomib-resistant sub-clones, limiting the efficacy of single-agent bortezomib. This finding is further supported by the clinical observation that approximately half of initially bortezomib-sensitive MM patients are no longer able to respond to bortezomib once relapsed.{Lonial, 2011 #8554} This sub-clonal bortezomib-resistance has been attributed to a range of mechanisms; including enhanced growth factor expression{Kuhn, 2012 #8558}, mutated proteasome subunits{Oerlemans, 2008 #5368}, deregulated plasma cell maturation markers{Stessman, 2013 #8553}, and nuclear factor-kappa B (NF-κB) pathway 'addiction'{Annunziata, 2007 #5684} [for a more in-depth exploration of this topic see Murray *et al.* 2014{Murray, 2014 #8633}]. Furthermore, pro-survival NF-κB signalling pathway members were also found to have a broader than anticipated profile in MM whole genome sequencing data.{Chapman, 2011 #5683} These findings are consistent with our previous studies into the role of NF-κB signalling in haematological malignancies{Rushworth, 2013 #6375;Murray, 2013 #8580;Rushworth, 2012 #6377}, suggesting that a greater understanding of the NF-κB signaling network in bortezomib-resistant MM may be central to achieving therapeutic advances in this disease.

In its primary mode of action bortezomib successfully inhibits 'inducible' NF-κB expression in MM cells, such as the expression stimulated by MM-bone marrow stromal cell (BMSC) interaction{Chauhan, 1996 #8481}, via its function as an inhibitor of the 20s proteasome β5 subunit.{Lu, 2013 #8629} Conversely, however, bortezomib also enhances 'constitutive' levels of NF-κB through activation of IKKβ, ultimately leading to NF-κB nuclear translocation and the transcription of multiple NF-

κB-induced genes, including Bruton's tyrosine kinase (BTK).{Yu, 2008 #8527} BTK, a non-receptor tyrosine kinase, is now known to be of key importance to a number of haematological malignancies, including MM{Tai, 2012 #8198}, chronic lymphocytic leukemia (CLL){O'Brien, 2012 #8167} and acute myeloid leukemia (AML).{Rushworth, 2014 #8589} The potential feedback mechanism between NF-κB and BTK signalling, whereby BTK also lies upstream of several NF-κB inducible signalling pathways{Khan, 2001 #8247;Jefferies, 2004 #8630}, provides a rationale for investigation of combined NF-κB and BTK inhibition in MM.

Previously we and others have shown *ex vivo* efficacy of BTK inhibition in MM.{Tai, 2012 #8198;Rushworth, 2013 #6375} Specifically, we have shown that the irreversible BTK inhibitor ibrutinib can enhance the action of bortezomib via repression of the NF-κB survival pathway in primary tissue, e.g., bone marrow-derived MM cells from treatment-naïve patients.{Rushworth, 2013 #6375} Despite these findings, however, early phase II clinical trial data for ibrutinib monotherapy in MM have so far proved disappointing{Pharmacyclics, 2012 #8523}, whilst the study of ibrutinib efficacy in patients with relapsed of refractory MM is currently recruiting (ClinicalTrials.gov Identifier:NCT01478581).

Taken together these data highlight the need to develop novel therapeutic strategies that can overcome bortezomib-resistance{Murray, 2014 #8633}, whilst still de-bulking the tumour and protecting the patient from related organ and tissue impairment.

MM clonal development and selection is impacted by the timing, order, and combinations of therapies received; however, all patients treated with bortezomib therapy are destined to relapse and become bortezomib-resistant. Here, we utilise *in*

vitro modelling to demonstrate BTK pro-survival pathway activity in bortezomib-resistant MM. These data provide justification for further assessment of greater patient numbers in the clinic, which will establish whether ibrutinib therapy can be used to overcome bortezomib-resistance in MM in practice.

Results

Generation and characterisation of bortezomib-resistant MM cells

To determine the importance of the BTK pro-survival pathway in bortezomib-resistance we first generated bortezomib-resistant cells *in vitro*. We cultured MM-derived B lymphocytic cell lines (H929, LP-1, RPMI-8226, and U266) in the continuous presence of bortezomib, increasing bortezomib concentration in stepwise increments up to 10 nM. Corresponding bortezomib-naïve MM cell lines were used as a control. Bortezomib-resistance in the cell lines was confirmed by a significant increase in IC_{50} log[bortezomib] (Figure 1 A) and enhanced cell viability following exposure to physiologically relevant{Papandreou, 2004 #8632} levels of bortezomib [5 nM (Figure 1 B)] when compared to control 'bortezomib-naïve' MM cells. In addition, we explored relative chymotrypsin-like proteasome activity in bortezomib-naïve and bortezomib-resistant MM cell lines and observed significantly higher levels of proteasome activity in bortezomib-resistant MM cells (Figure 1 C), further confirming their inability to respond to bortezomib's proteasome-inhibitor function.

We also analysed primary human treatment-naïve MM patient samples (primary naïve; n=5) and primary human MM samples from patients that had initially responded to bortezomib but subsequently relapsed (primary relapsed; n=6). All primary naïve patient samples analysed (n=5/5) showed significantly reduced cell viability in response to bortezomib *in vitro*, whilst the majority (n=4/6) of primary relapsed samples showed no significant reduction in viability. The remaining (n=2/6) primary relapsed samples showed significantly decreased cell viability in response to bortezomib, in-keeping with the clinical observation that approximately half of

patients retreated with bortezomib will respond again {Taverna, 2012 #8634;Petrucci, 2013 #8635} (Figure 1 D).[30,31]

Basal BTK activity is enhanced in bortezomib-resistant MM cell lines and is resistant to inhibition with bortezomib

We have previously shown that the BTK pathway is active in MM.{Rushworth, 2013 #6375} Furthermore, others have shown that bortezomib can reduce the expression of BTK mRNA and protein via an NF-κB p65-dependent mechanism.{Yu, 2008 #8527} We therefore examined whether BTK expression and activity in bortezomib-resistant MM cell lines reflects a mechanistic change in the BTK pro-survival signalling pathway in response to bortezomib treatment. We first examined basal levels of BTK activity in bortezomib-naïve and bortezomib-resistant U266 MM cells by immunocytochemistry. Utilising antibodies against total BTK (BTK) and Y223-phosphorylated BTK (pBTK) we observed higher levels of pBTK in bortezomib-resistant cells [n ≥ 80] compared to bortezomib-naïve cells, suggesting a greater level of BTK activity in the resistant cells (Figure 2 A; left panel). Further quantitative analysis of the immunocytochemistry confirmed significantly enhanced basal pBTK levels in bortezomib-resistant MM cell lines (Figure 2 A; right panel [n≥20]). We next examined what effect this enhanced level of basal BTK activity has on bortezomib-resistant MM cell response to further bortezomib exposure. Following bortezomib treatment (4 h), we observed a significant decrease in relative BTK mRNA in the bortezomib-naïve but not bortezomib-resistant MM cell lines (Figure 2 B), suggesting BTK expression is not inhibited by bortezomib treatment in the bortezomib resistant cells.

To further investigate this difference in BTK mRNA expression and activity in bortezomib-resistant MM cells we examined activity of the BTK promoter, utilising a chemi-luminescent BTK promoter-luciferase reporter construct, specifically containing two NF-κB transcription factor binding sites [κB1 and κB2]; pGL4.BTK (Figure 2 C). Following 24 h bortezomib treatment bortezomib-naïve U266 MM cells transfected with pGL4.BTK showed significantly reduced BTK promoter activity (Figure 2 D). Conversely, there was no change in BTK promoter activity when bortezomib-resistant U266 MM cells were transfected with pGL4.BTK, and the level of BTK promoter activity in these cells remained significantly higher than in bortezomib-naïve cells exposed to bortezomib. These results demonstrate that the ability of bortezomib to repress BTK promoter activity is lost in bortezomib-resistant MM cells.

Pharmacological BTK inhibition with ibrutinib restores sensitivity to bortezomib in bortezomib-resistant MM cells

As BTK is emerging as an important regulator of downstream survival pathways in MM{Tai, 2012 #8198;Rushworth, 2013 #6375}, and further to our observations of enhanced BTK activity and expression in bortezomib-resistant MM cells (Figure 2), we investigated the functional effect of combined pharmacological BTK inhibition and bortezomib treatment in bortezomib-naïve and bortezomib-resistant MM cell lines and primary human MM samples. Ibrutinib 'pulse' treatment [0.5 μM/1 h] alone significantly reduced cell viability in bortezomib-resistant MM cells lines after 48 h (Figure 3 A). When combined with bortezomib exposure [5 nM/24 h], the ibrutinib 'pulse' also significantly reduced cell viability in bortezomib-resistant U266 MM cells below levels observed in bortezomib-resistant cells receiving bortezomib alone, and

bortezomib-naïve cells receiving the same combination therapy (Figure 3 B). Importantly, ibrutinib 'pulse' treatment followed by bortezomib exposure also dramatically reduced cell viability in primary relapsed MM samples [n=6], likewise below levels observed in primary relapsed samples receiving bortezomib alone, and primary naïve MM samples [n=5] also receiving the combination therapy (Figure 3 C).

BTK inhibition via lenti-viral miRNA targeting also restores sensitivity to bortezomib in bortezomib-resistant MM cells

Although originally developed as an inhibitor of BTK, ibrutinib has more recently been shown to have 'off-target' effects, inhibiting multiple members of the TEC kinase family, including interleukin-2-inducible T-cell kinase (ITK).{Dubovsky, 2013 #8624} Therefore, to ensure our results were not due to potential off-target inhibitor activity, we evaluated bortezomib-resistant and bortezomib-naïve U266 MM cell line response to lenti-viral-mediated BTK repression. Specifically, we generated artificial and exogenous miRNA sequences specifically targeting the BTK transcript [miRBTK] and utilised lenti-viral infection to achieve semi-stable BTK mRNA knockdown [as described previously{Rushworth, 2014 #8589}] in both bortezomib-naïve and bortezomib-resistant U266 MM cells (Figure 4 A). Furthermore, the introduction of miRBTK in conjunction with bortezomib treatment (5 nM/24 h) confirmed that bortezomib-resistant U266 MM cells experienced significantly reduced cell viability compared to bortezomib-naïve cells also infected with miRBTK and exposed to bortezomib, and compared to bortezomib-resistant cells infected with a non-targeting control miRNA [miRNEG] (Figure 4 B). This strongly suggests that the results observed following ibrutinib and bortezomib combination therapy in bortezomib-

resistant cells are due to ibrutinib-driven irreversible BTK inhibition, rather than any off target ibrutinib effects.

Bortezomib-resistance is driven by enhanced NF-κB p65 activity and can be reversed by BTK inhibition

We have previously shown that BTK inhibition reduces NF-κB p65 activity in bortezomib-naïve MM cells and primary naïve patient samples.{Rushworth, 2013 #6375} To determine the role of NF-κB p65 in the regulation of BTK activity in bortezomib-resistant MM cells we initially examined basal levels of sub-cellular NF-κB p65 protein distribution in bortezomib-naïve and bortezomib-resistant U266 MM cells by immunocytochemistry. Utilising an antibody against the NF-κB p65 subunit we observed higher levels p65 in the nucleus of bortezomib-resistant cells compared to bortezomib-naïve cells [n≥20], suggesting a greater level of NF-κB p65 transcription factor binding, and therefore NF-κB signalling in the bortezomib-resistant cells (Figure 5 A; inset). Further analysis of nuclear vs. cytoplasmic NF-κB p65 revealed higher basal levels of nuclear localisation in bortezomib-resistant compared to bortezomib-naïve U266 MM cells; both by quantitative analysis (Figure 5 A) and western immunoblot (Figure 5 B). Additionally, analysis of sub-cellular NF-κB p65 localisation in response to ibrutinib 'pulse' treatment combined with bortezomib exposure [5 nM/4 h] revealed significant repression of NF-κB p65 nuclear localisation in bortezomib-resistant and bortezomib-naïve U266 MM cells compared to those treated with bortezomib alone (Figure 5 C). Importantly, bortezomib-resistant cells showed no significant reduction in NF-κB p65 nuclear localisation in response to bortezomib treatment alone compared to bortezomib-naïve cells, potentially suggesting a level of constitutive NF-κB p65 activity in these cells.

To confirm the functional relevance of enhanced basal nuclear NF-κB p65 localisation in bortezomib-resistant MM cells, and to confirm that any response to bortezomib observed was specifically due to its ability to repress *inducible* NF-κB p65 activity, we generated artificial and exogenous miRNA sequences specifically targeting NF-κB p65 (miRp65) and utilised lenti-viral infection to achieve semi-stable p65 mRNA knockdown in bortezomib-naïve and bortezomib-resistant U266 MM cells (Figure 5 D). Introduction of miRp65, in combination with ibrutinib 'pulse' treatment, significantly reduced cell viability in bortezomib-resistant U266 MM cells compared to bortezomib-naïve cells exposed to the same treatment, and compared to bortezomib-resistant cells infected with a non-targeting control miRNEG or ibrutinib 'pulse' treatment alone (Figure 5 E).

Discussion

The proteasome inhibitor bortezomib is licensed to treat newly diagnosed and relapsed MM in the clinic. Single agent bortezomib has a response rate of approximately 30% but, when used in combination with chemotherapy and/or corticosteroids, response rates range from approximately 60% to over 90% depending on the regimen.{Murray, 2014 #8633} Despite this relative success, however, clinical relapse following bortezomib therapy presently remains inevitable and resistance to further bortezomib treatment is common{Taverna, 2012 #8634;Petrucci, 2013 #8635}, not only as a consequence of, but also further driving the selection and emergence of drug-resistant clones.{Egan, 2012 #8564;Keats, 2012 #8566} An improved understanding of the mechanisms underlying bortezomib-resistance is, therefore, vital for the progressive development of novel pharmacologic strategies to overcome the clinical phenomenon of bortezomib-resistance. In this study we have explored bortezomib-resistance in MM *in vitro,* utilising bortezomib-resistant MM cell lines generated in the laboratory, and primary patient samples from both treatment naïve patients and patients that have relapsed following bortezomib therapy. Despite a the limitsof the sample size, this data provides thefoundation for future *in vivo* assessment of ibrutinib treatment of patients with bortezomib-relapsed MM in a larger numbers of patients in the clinic.

We and others have previously demonstrated activity of the BTK survival pathway in treatment-naïve MM cells.{Tai, 2012 #6376;Rushworth, 2013 #6375} Furthermore, bortezomib has been reported to inhibit total BTK expression in a treatment-naive B lymphocyte cell line.{Yu, 2008 #8527} Here, utilising *in vitro* models of post-bortezomib relapsed MM, we describe a role for BTK activity in bortezomib-

resistance, demonstrating higher basal levels of active pBTK protein in bortezomib-resistant MM cell lines and a failure of these cells to respond to bortezomib in terms of BTK mRNA and BTK promoter activity repression. In particular, the higher basal BTK activity and altered BTK promoter activity in bortezomib-resistant cells would be consistent with process of clonal selection, driven by constant exposure to bortezomib.{Keats, 2012 #8566} This identifies BTK as a candidate therapeutic target in bortezomib-resistant MM. In addition, this may also be relevant to bortezomib-naïve MM, in which potentially bortezomib-resistant sub-clones as yet form a minority population within of the tumour 'bulk'.

The oral BTK inhibitor ibrutinib, currently licenced for use in CLL and MCL{McDermott, 2014 #8636;Cameron, 2014 #8637}, has shown promising clinical activity and a favourable side-effect profile in a range of B cell malignancies.{Byrd, 2013 #8638;Wang, 2013 #8477;Advani, 2013 #8640;O'Brien, 2014 #8641} Ibrutinib has also been found to reduce BTK expression in MM{Yu, 2008 #8527} and enhance the cytotoxicity of bortezomib in bortezomib-naïve primary malignant plasma cells and MM cell lines.{Rushworth, 2013 #6375} By inhibiting BTK with ibrutinib 'pulse' treatment we were also able to restore a measurable cytotoxic response to bortezomib in bortezomib-resistant MM cell lines and primary relapsed MM cells. Importantly, although ibrutinib is known to target other kinases, we were able to reproduce these results with highly specific artificial BTK-targeting microRNA, miRBTK, introduced by lenti-virus. This suggests that it is BTK inhibition which is restoring bortezomib activity in bortezomib-resistant MM cells and not an off target effect of ibrutinib.

Multiple mechanisms of action of bortezomib have been described{Chauhan, 1996 #8481}·{Murray, 2014 #8633}, including the inhibition of BTK expression driven via repression of *inducible* NF-κB transcription factor translocation to the nucleus.{Rushworth, 2013 #6375;Yu, 2008 #8527;Dasmahapatra, 2013 #8642} We found enhanced nuclear localisation of NF-κB p65 in the bortezomib-resistant cells. This is in-keeping with previous reports that bortezomib-resistance in MM is conveyed through the NF-κB signalling network. For example; constitutive expression of NF-κB is frequently seen in bortezomib refractory primary patient samples.{Markovina, 2008 #8524} Furthermore, the acquisition of bortezomib-resistance can follow up-regulation of heat shock protein (HSP)90 and HSP27, which, in their action as ubiquitin chaperones, facilitate the activation of NF-κB in MM.{Mitsiades, 2002 #8562;Navas, 2006 #8563} Active pBTK is also known to be essential for NF-κB activation and B cell survival.{Davis, 2010 #8526} Thus, there exists a positive auto-regulatory feedback loop that stimulates transcription of BTK via two functionally competent NF-κB p65 sites in the BTK promoter.{Yu, 2008 #8527} Here we show that bortezomib-resistance in MM is associated with higher basal nuclear NF-κB p65 in bortezomib-resistant MM cells, which, in turn, is associated with a greater reliance on pBTK for survival. These observations suggest that BTK inhibition by ibrutinib may be useful in the clinical treatment of bortezomib-resistant MM.

Taken together, these results show that bortezomib-resistance observed in the clinic occurs, at least in part, because bortezomib-resistant MM cells lose the capacity to be influenced by the inhibition of BTK-driven NF-κB p65, and the consequent NF-κB p65-driven auto-regulation of BTK. Accordingly, introduction of ibrutinib to the treatment regimens of bortezomib-resistant MM patients may lead to enhanced cell

death of the bortezomib-resistant sub-clones through inhibition of this BTK/NF-κB p65 signalling axis. Here we provide a biologic and molecular rationale for the clinical evaluation of bortezomib and ibrutinib combination therapy in post-bortezomib relapsed MM, including patients that have previously been refractory to bortezomib.

Materials and Methods

Materials

All reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO), unless stated otherwise. All MM/B-lymphoblast cell lines were obtained from the European Collection of Cell Cultures (ECCC).

Cell culture

MM cell lines (H929, LP-1, RPMI-8228, and U266) were cultured in a humidified atmosphere at 37°C and 5% (v/v) CO_2 in RPMI-1640 media +L-Glutamine (Gibco Life Technologies) supplemented with 10 % (v/v) foetal bovine serum ([FBS]; Biosera). Bortezomib-resistant cells were cultured as above but supplemented with 20 % (v/v) FBS. Bortezomib-resistance was selected for by dose escalation of a once-weekly bortezomib treatment, through 0.5, 1, 2.5 and 5 nM, until cells could tolerate 10 nM bortezomib (10-15 weeks; confirmed by luminescent cell viability assay). Each concentration was maintained for approximately 2-3 weeks to allow for proliferation of bortezomib-resistant sub-clones. Build-up of cell debris due to the high level of cell death was removed by centrifugation at 300*g* for 5 minutes before gently removing the supernatant and re-suspending the pellet.

Primary MM cells were obtained under local ethical approval (LREC ref. 07/H0310/146) and were isolated from the bone marrow aspirates of MM patients, as described previously.{Rushworth, 2013 #6375} Primary MM cells from bortezomib-relapsed patients were not exposed to further bortezomib treatment until experimentation.

Ibrutinib 'pulse' treatment

To mimic *in vivo* pharmacokinetics of rapid adsorption and elimination of the irreversible BTK inhibitor ibrutinib{Byrd, 2013 #8478}, we employed a method of 'pulse' exposure to physiologically relevant levels of ibrutinib *in vitro*, as described previously.{Honigberg, 2010 #8631} Briefly, cells were exposed to 500 nM ibrutinib for 1 h before washing, followed by any additional drug treatment or assay in fresh media.

Luminescent cell viability assay and chymotrypsin-like proteasome activity assay

$1x10^5$ MM cells were treated as indicated. Relative cell viability was assayed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), as per the manufacturer's instructions. Proteasome activity was measured using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay (Promega), as per the manufacturer's instructions. Luminescence was measured with the FLUOstar Omega Microplate Reader (BMGLabtech) and calculated as relative to untreated control samples.

Quantification of protein activity and subcellular localisation by immunocytochemistry

Relative activity of phosphorylated versus total BTK (pBTK vs. BTK), and subcellular distribution of nuclear vs. cytoplasmic NF-κB p65 (nuc. vs. cyt. p65) were analysed by immunocytochemistry. Briefly, 2×10^4 U266 MM cells were fixed onto microscope slides using the CytoSpin 4 Cytocentrifuge system (Thermo Fisher Scientific) and 4 % (w/v) paraformaldehyde (PFA). Fixed cells were incubated with rabbit anti-human primary mAbs against pBTK (Tyr223) and goat anti-human primary mAbs against total BTK, or goat anti-human primary mAbs against NF-κB p65 (Cell Signalling

Technology). Cells were then incubated with Alexa Fluor® goat anti-rabbit and rabbit anti-goat IgG (H+L) secondary antibodies (Molecular Probes Life Technologies), or rabbit anti-goat IgG alone, respectively. Nuclei were localised by 4',6-diamidino-2-phenylindole (DAPI) staining. After mounting, cells were viewed using the Axio Imager.D2 microscope and Axiovision software (Carl Zeiss Microscopy).

Image analyses and quantification of pBTK vs. BTK, and nuc. vs. cyt. p65 staining intensity was performed using ImageJ 1.46 software (National Institute of Health) and plugins, as described previously.{Rushworth, 2014 #8589} Briefly, a threshold of staining intensity was applied and the area above the threshold (pixles^2) was measured (n≥80 [BTK] and n≥20 [NF-κB] individual cells). Staining intensity (pixles^2) was converted to a percentage as follows: pBTK/total BTK (%), or, nuclear/[cytoplasmic + nuclear NF-κB p65] (%).

Western Immunoblotting

Protein fractions of equal total protein concentration were extracted from U266 MM cells using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. SDS/PAGE and western immunoblot analyses were performed as described previously{Rushworth, 2010 #5374}. ECL detection using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and subsequent quantification were performed with the ChemiDoc-It2 Imaging System (UVP).

RNA extraction and quantitative real time-PCR

Total RNA was extracted from 1 x 10^6 U266 MM cells using Total RNA Lysis Solution (Applied Biosystems-Life Technologies) according to the manufacturer's instructions.

Reverse transcription of total RNA was performed using the GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems-Life Technologies). Resultant cDNA was analysed by quantitative real-time PCR (qRT-PCR) using LightCycler® 480 SYBR Green I Master mix (Roche) and qRT-PCR primers for GAPDH [forward 5′-ACCAGCCTCAAGATCATCAGC-3′ and reverse 5′-TGCTAAGCAGTTGGTGGTGC-3′], BTK [forward 5′-CACACAGGTGAACTCCAGAAAG-3' and reverse 5'-AGAGATACTGCCCATCGATCCAGA-3'] and NF-κB p65 [forward 5′-ACCGCTGCATCCACAGTT-3′ and reverse 5′-GGATGCGCTGACTGATAGC-3′] (Invitrogen-Life Technologies), on the LightCycler® 480 Real-Time PCR system (Roche), as previously described.{Murray, 2013 #8580} Gene expression was analysed using the comparative cycle threshold algorithm ($\Delta\Delta C_T$); mRNA expression was standardised against GAPDH expression.

Lenti-virus construction and infection

Lenti-virus containing artificial exogenous microRNA (miRNA) sequences targeting human NF-κB p65 (miR-p65 [5′-TACGTTTCTCCTCAATCCGGT-3′]), BTK (miR-BTK [5′-TTCACTGGACTCTTCACCTCT-3′]) or a control/scrambled sequence (miR-NEG), and an EmGFP–pre-miRNA encoding fragment, were constructed and produced as described previously.{Rushworth, 2014 #8589} Briefly, artificial miRNA targets were identified and designed to interact with the relevant exon using Block-iT RNAi designer software (Invitrogen). This ensures high specificity of miR-p65 and miR-BTK, with no 3'UTR interaction, unlike endogenous miRNAs.

5 x 10^5 U266 MM cells were infected and transduced with each lenti-virus (MOI: 15), in serum-free medium with 8 μg/mL Polybrene™. Following infection and before experimentation, transduced cells were analysed for target knock-down and

efficiency by qRT-PCR for BTK and NF-κB p65, and flow cytometry to detect GFP using the Accuri-C6 flow cytometer (BD Biosciences).

Generation and transfection of the wild-type human BTK promoter-luciferase reporter construct

To generate the wild-type human BTK promoter-luciferase reporter construct (pGL4.BTK), the BTK promoter region, including two innate κB binding sites, was amplified from genomic DNA via PCR with specific forward [5′-TATCTCGAGGAAGAAAAGAGCCTGGGCA-3′] and reverse [5′-ATAAGATCTGTCTTTTTTTCTTCTCAGCAGCA-3′] primers. The amplified fragment was cloned into the Xho I/Bgl II site of the Promoterless Firefly Luciferase 'pGL4.11*[luc2P]*' Vector, according to the manufacturers' instructions (Promega).

U266 MM cells were co-transfected with a total of 1 µg DNA, composed of the pGL4.BTK promoter-luciferase reporter construct and a pRL-TK *Renilla* Luciferase Control Reporter Vector (Promega) using FuGENE® HD Transfection Reagent (Promega) and incubated for 24 h at 37°C and 5% (v/v) CO_2 before any further treatment.

Chemi-luminescent luciferase reporter assay

Firefly and *Renilla* promoter-luciferase reporter activity was analysed and quantified sequentially in a single sample with the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions, and the EnVision 2103 Multilabel Plate Reader (Perkin Elmer).

Financial Support: This work was funded by NIHR Flexibility and Sustainability Funding, the Big C Appeal, the Association for International Cancer Research, and the Humane Research Trust.

448 **References**

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Figures

Figure 1. Characterisation of bortezomib-resistant MM cell lines, and primary relapsed MM patient samples. (A) The IC_{50} Log[bortezomib] (nM) of bortezomib-naïve and bortezomib-resistant MM cell lines was analysed by luminescent cell viability assay. (B) Relative cell viability (5 nM/48 h) and (C) chymotrypsin-like proteasome activity in bortezomib-naïve and bortezomib-resistant MM cell lines in response to (5 nM/4 h) bortezomib. (D) Relative cell viability of primary naïve and primary relapsed MM patient samples in response to bortezomib (5 nM/24 h). Statistical significance between treatments was calculated by Student's *t* test; * indicates $p \leq 0.05$. Statistical significance between cohorts was calculated by ANOVA; # indicates $p \leq 0.05$.

Figure 2. Enhanced BTK activity in bortezomib-resistant MM cell lines is resistant to inhibition with bortezomib. (A) Representative immunocytochemistry of basal levels of total and phosphorylated BTK (pBTK) in bortezomib-naïve and bortezomib-resistant U266 MM cells with DAPI nuclear staining [left panel] and subsequent quantification of basal pBTK/total BTK (%) staining intensity [right panel]. Box and whisker indicates the mean ± SEM ($n \geq 80$). (B) qRT-PCR analysis of BTK mRNA following bortezomib (5 nM/4 h) exposure in bortezomib-naïve and bortezomib-resistant U266 MM cells relative to GAPDH. Values indicate the mean ± SEM from 3 independent experiments. (C) Representation of wild type pGL4.BTK promoter-luciferase vector [including κB binding sites] transfected into bortezomib-naïve and bortezomib-resistant U266 MM cells. (D) Luciferase activity of pGL4.BTK promoter-luciferase vectors in bortezomib-naïve and bortezomib-resistant U266 MM cells following bortezomib (5 nM/24 h) exposure, normalised by co-transfection with

pRL-TK *Renilla* Luciferase Reporter Vectors. Values indicate the mean ± SEM from 3 independent experiments. Statistical significance between treatments was calculated by Student's *t* test; * indicates $p \leq 0.05$. Statistical significance between cohorts was calculated by ANOVA; # indicates $p \leq 0.01$.

Figure 3. BTK inhibition enhances sensitivity to bortezomib in bortezomib-naïve and bortezomib-resistant MM cells. (A) Relative cell viability of bortezomib-naïve and bortezomib-resistant MM cell lines 48 h post-ibrutinib 'pulse' treatment. (B) Relative cell viability of bortezomib-naïve and bortezomib-resistant MM cells post-ibrutinib 'pulse' treatment in combination with bortezomib (5 nM/24 h). (C) Relative cell viability of primary naïve and primary relapsed MM patient samples post *in vitro* ibrutinib 'pulse' treatment in combination with bortezomib (5 nM/24 h). Statistical significance between treatments was calculated by Student's *t* test; * indicates $p \leq 0.05$. Statistical significance between cohorts was calculated by ANOVA; # indicates $p \leq 0.01$.

Figure 4. BTK inhibition via lenti-viral miRNA targeting enhances sensitivity to bortezomib in bortezomib-naïve and bortezomib-resistant MM cells. (A) qRT-PCR analysis of basal BTK mRNA expression in bortezomib-naïve and bortezomib-resistant MM U266 cells infected with lenti-viral miRNA constructs targeting BTK (miRBTK) transcription relative to GAPDH. (B) Relative cell viability of bortezomib-naïve and bortezomib-resistant MM U266 cells infected with lenti-viral miRBTK before and after bortezomib treatment (5 nM/24 h). Values indicate the mean ± SEM from 3 independent experiments. Statistical significance between treatments was

calculated by Student's *t* test; * indicates p ≤ 0.05. Statistical significance between cohorts was calculated by ANOVA; # indicates p ≤ 0.01.

Figure 5. Bortezomib-resistance is driven by enhanced NF-κB p65 activity and can be reversed by BTK inhibition. (A) Quantification of staining intensity of nuclear p65/total NF-κB p65 (%) in bortezomib-naïve and bortezomib-resistant U266 MM cells. Box and whisker indicates the mean ± SEM (n ≥ 20). Statistical significance between cohorts was calculated by ANOVA; # indicates p ≤ 0.01. Inset shows example immunocytochemistry for NF-κB p65. Dotted line indicates nucleus as determined by DAPI staining. (B) Western immunoblot of bortezomib-naïve [N] and bortezomib-resistant [R] U266 nuclear [Nuc.] and cytoplasmic [Cyt.] cell fractions. GAPDH and Histone 3 [H3] show equal loading for cyt. and nuc. fractions, respectively. (C) Quantification of staining intensity of nuclear NF-κB p65/total NF-κB p65 (%) in bortezomib-naïve and bortezomib-resistant U266 MM cells. Box and whisker indicates the mean ± SEM (n ≥ 20). Statistical significance between cohorts was calculated by ANOVA; # indicates p ≤ 0.05. (D) qRT-PCR analysis of basal NF-κB p65 mRNA expression in bortezomib-naïve and bortezomib-resistant MM U266 cells infected with lenti-viral miRNA constructs targeting NF-κB p65 (miRp65) transcription relative to GAPDH. (E) Relative cell viability of bortezomib-naïve and bortezomib-resistant MM U266 cells infected with lenti-viral miRp65 24 h following ibrutinib 'pulse' treatment. Values indicate the mean ± SEM from 3 independent experiments. Statistical significance between treatments was calculated by Student's t test; * indicates p ≤ 0.05. Statistical significance between cohorts was calculated by ANOVA; # indicates p ≤ 0.01.