

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

3 Megan Y Murray¹, Lyubov Zaitseva¹, Martin J Auger², Jenny I O Craig³, David J
4 MacEwan⁴, Stuart A Rushworth^{1*} and Kristian M Bowles^{1,2*}

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

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

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

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

15 * SAR and KMB contributed equally

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

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

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

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

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

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

25

26 **Abstract**

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

45

46 **Introduction**

47 Multiple Myeloma (MM) is characterised by the accumulation of clonal plasma cells
48 in the bone marrow. The American Cancer Society estimates that there will be
49 approximately 24,000 new cases diagnosed and 11,000 deaths from MM in 2014 in
50 the US.{, 2014 #8628} Clinical manifestations of the disease occur as a
51 consequence of the tumour bulk; including anaemia and fatigue, immune paresis
52 leading to infection, renal failure, and osteolytic bone breakdown by activated
53 osteoclasts, resulting in painful lytic bone destruction and hypercalcaemia.{Raab,
54 2009 #6283} An effective MM therapeutic succeeds, therefore, by 'de-bulking' the
55 MM tumour mass, thus reducing the associated symptoms and subsequently
56 maintaining remission.

57 The proteasome inhibitor (PI) bortezomib, approved for the treatment of MM by the
58 FDA in 2003{Twombly, 2003 #8549}, is one such de-bulking agent that has greatly
59 contributed to improved outcomes observed in MM.{Palumbo, 2011 #8529} Despite
60 this advance, however, relapse following bortezomib therapy remains inevitable due
61 to the emergence of bortezomib-resistant plasma cell sub-clones.{Kapoor, 2012
62 #8550} For example; recent 'whole genome' sequencing studies of MM patients
63 confirmed a high level of genetic heterogeneity; occurring both between separate
64 patients, and within patient samples before and after therapy{Chapman, 2011
65 #5683}, indicating the presence of a variety of genetically distinct plasma cell 'sub-
66 clones'.{Egan, 2012 #8564;Keats, 2012 #8566} Different sub-clones further indicate
67 the presence of numerous minor tumour initiating cell populations with complex and
68 divergent evolutionary histories.{Magrangeas, 2013 #8666} The diversity and
69 potential quiescence of these MM sub-clones can contribute to enhanced

70 tumorigenicity and an intrinsic resistance to therapy.{Chen, 2014 #8667} Therefore;
71 the eradication of the majority bortezomib-sensitive sub-clones may ultimately
72 promote the growth of pre-existing minority bortezomib-resistant sub-clones, limiting
73 the efficacy of single-agent bortezomib. This finding is further supported by the
74 clinical observation that approximately half of initially bortezomib-sensitive MM
75 patients are no longer able to respond to bortezomib once relapsed.{Lonial, 2011
76 #8554} This sub-clonal bortezomib-resistance has been attributed to a range of
77 mechanisms; including enhanced growth factor expression{Kuhn, 2012 #8558},
78 mutated proteasome subunits{Oerlemans, 2008 #5368}, deregulated plasma cell
79 maturation markers{Stessman, 2013 #8553}, and nuclear factor-kappa B (NF- κ B)
80 pathway 'addiction'{Annunziata, 2007 #5684} [for a more in-depth exploration of this
81 topic see Murray *et al.* 2014{Murray, 2014 #8633}]. Furthermore, pro-survival NF- κ B
82 signalling pathway members were also found to have a broader than anticipated
83 profile in MM whole genome sequencing data.{Chapman, 2011 #5683} These
84 findings are consistent with our previous studies into the role of NF- κ B signalling in
85 haematological malignancies{Rushworth, 2013 #6375;Murray, 2013
86 #8580;Rushworth, 2012 #6377}, suggesting that a greater understanding of the NF-
87 κ B signaling network in bortezomib-resistant MM may be central to achieving
88 therapeutic advances in this disease.

89 In its primary mode of action bortezomib successfully inhibits 'inducible' NF- κ B
90 expression in MM cells, such as the expression stimulated by MM-bone marrow
91 stromal cell (BMSC) interaction{Chauhan, 1996 #8481}, via its function as an
92 inhibitor of the 20s proteasome β 5 subunit.{Lu, 2013 #8629} Conversely, however,
93 bortezomib also enhances 'constitutive' levels of NF- κ B through activation of IKK β ,
94 ultimately leading to NF- κ B nuclear translocation and the transcription of multiple NF-

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

103 Previously we and others have shown *ex vivo* efficacy of BTK inhibition in MM.{Tai,
104 2012 #8198;Rushworth, 2013 #6375} Specifically, we have shown that the
105 irreversible BTK inhibitor ibrutinib can enhance the action of bortezomib via
106 repression of the NF- κ B survival pathway in primary tissue, e.g., bone marrow-
107 derived MM cells from treatment-naïve patients.{Rushworth, 2013 #6375} Despite
108 these findings, however, early phase II clinical trial data for ibrutinib monotherapy in
109 MM have so far proved disappointing{Pharmacocyclics, 2012 #8523}, whilst the study
110 of ibrutinib efficacy in patients with relapsed or refractory MM is currently recruiting
111 (ClinicalTrials.gov Identifier:NCT01478581).

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

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

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

123

124 **Results**

125 *Generation and characterisation of bortezomib-resistant MM cells*

126 To determine the importance of the BTK pro-survival pathway in bortezomib-
127 resistance we first generated bortezomib-resistant cells *in vitro*. We cultured MM-
128 derived B lymphocytic cell lines (H929, LP-1, RPMI-8226, and U266) in the
129 continuous presence of bortezomib, increasing bortezomib concentration in stepwise
130 increments up to 10 nM. Corresponding bortezomib-naïve MM cell lines were used
131 as a control. Bortezomib-resistance in the cell lines was confirmed by a significant
132 increase in IC₅₀ log[bortezomib] (Figure 1 A) and enhanced cell viability following
133 exposure to physiologically relevant{Papandreou, 2004 #8632} levels of bortezomib
134 [5 nM (Figure 1 B)] when compared to control 'bortezomib-naïve' MM cells. In
135 addition, we explored relative chymotrypsin-like proteasome activity in bortezomib-
136 naïve and bortezomib-resistant MM cell lines and observed significantly higher levels
137 of proteasome activity in bortezomib-resistant MM cells (Figure 1 C), further
138 confirming their inability to respond to bortezomib's proteasome-inhibitor function.

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

147 patients retreated with bortezomib will respond again {Taverna, 2012
148 #8634;Petrucci, 2013 #8635} (Figure 1 D).^{30,31}

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

151 We have previously shown that the BTK pathway is active in MM.{Rushworth, 2013
152 #6375} Furthermore, others have shown that bortezomib can reduce the expression
153 of BTK mRNA and protein via an NF- κ B p65-dependent mechanism.{Yu, 2008
154 #8527} We therefore examined whether BTK expression and activity in bortezomib-
155 resistant MM cell lines reflects a mechanistic change in the BTK pro-survival
156 signalling pathway in response to bortezomib treatment. We first examined basal
157 levels of BTK activity in bortezomib-naïve and bortezomib-resistant U266 MM cells
158 by immunocytochemistry. Utilising antibodies against total BTK (BTK) and Y223-
159 phosphorylated BTK (pBTK) we observed higher levels of pBTK in bortezomib-
160 resistant cells [$n \geq 80$] compared to bortezomib-naïve cells, suggesting a greater
161 level of BTK activity in the resistant cells (Figure 2 A; left panel). Further quantitative
162 analysis of the immunocytochemistry confirmed significantly enhanced basal pBTK
163 levels in bortezomib-resistant MM cell lines (Figure 2 A; right panel [$n \geq 20$]). We next
164 examined what effect this enhanced level of basal BTK activity has on bortezomib-
165 resistant MM cell response to further bortezomib exposure. Following bortezomib
166 treatment (4 h), we observed a significant decrease in relative BTK mRNA in the
167 bortezomib-naïve but not bortezomib-resistant MM cell lines (Figure 2 B), suggesting
168 BTK expression is not inhibited by bortezomib treatment in the bortezomib resistant
169 cells.

170 To further investigate this difference in BTK mRNA expression and activity in
171 bortezomib-resistant MM cells we examined activity of the BTK promoter, utilising a
172 chemi-luminescent BTK promoter-luciferase reporter construct, specifically
173 containing two NF- κ B transcription factor binding sites [κ B1 and κ B2]; pGL4.BTK
174 (Figure 2 C). Following 24 h bortezomib treatment bortezomib-naïve U266 MM cells
175 transfected with pGL4.BTK showed significantly reduced BTK promoter activity
176 (Figure 2 D). Conversely, there was no change in BTK promoter activity when
177 bortezomib-resistant U266 MM cells were transfected with pGL4.BTK, and the level
178 of BTK promoter activity in these cells remained significantly higher than in
179 bortezomib-naïve cells exposed to bortezomib. These results demonstrate that the
180 ability of bortezomib to repress BTK promoter activity is lost in bortezomib-resistant
181 MM cells.

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

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

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

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

202 Although originally developed as an inhibitor of BTK, ibrutinib has more recently
203 been shown to have 'off-target' effects, inhibiting multiple members of the TEC
204 kinase family, including interleukin-2-inducible T-cell kinase (ITK).{Dubovsky, 2013
205 #8624} Therefore, to ensure our results were not due to potential off-target inhibitor
206 activity, we evaluated bortezomib-resistant and bortezomib-naïve U266 MM cell line
207 response to lenti-viral-mediated BTK repression. Specifically, we generated artificial
208 **and exogenous** miRNA sequences **specifically** targeting the BTK transcript [miRBTK]
209 and utilised lenti-viral infection to achieve semi-stable BTK mRNA knockdown [as
210 described previously{Rushworth, 2014 #8589}] in both bortezomib-naïve and
211 bortezomib-resistant U266 MM cells (Figure 4 A). Furthermore, the introduction of
212 miRBTK in conjunction with bortezomib treatment (5 nM/24 h) confirmed that
213 bortezomib-resistant U266 MM cells experienced significantly reduced cell viability
214 compared to bortezomib-naïve cells also infected with miRBTK and exposed to
215 bortezomib, and compared to bortezomib-resistant cells infected with a non-targeting
216 control miRNA [miRNEG] (Figure 4 B). This strongly suggests that the results
217 observed following ibrutinib and bortezomib combination therapy in bortezomib-

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

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

222 We have previously shown that BTK inhibition reduces NF-κB p65 activity in
223 bortezomib-naïve MM cells and primary naïve patient samples.{Rushworth, 2013
224 #6375} To determine the role of NF-κB p65 in the regulation of BTK activity in
225 bortezomib-resistant MM cells we initially examined basal levels of sub-cellular NF-
226 κB p65 protein distribution in bortezomib-naïve and bortezomib-resistant U266 MM
227 cells by immunocytochemistry. Utilising an antibody against the NF-κB p65 subunit
228 we observed higher levels p65 in the nucleus of bortezomib-resistant cells compared
229 to bortezomib-naïve cells [n≥20], suggesting a greater level of NF-κB p65
230 transcription factor binding, and therefore NF-κB signalling in the bortezomib-
231 resistant cells (Figure 5 A; inset). Further analysis of nuclear vs. cytoplasmic NF-κB
232 p65 revealed higher basal levels of nuclear localisation in bortezomib-resistant
233 compared to bortezomib-naïve U266 MM cells; both by quantitative analysis (Figure
234 5 A) and western immunoblot (Figure 5 B). Additionally, analysis of sub-cellular NF-
235 κB p65 localisation in response to ibrutinib ‘pulse’ treatment combined with
236 bortezomib exposure [5 nM/4 h] revealed significant repression of NF-κB p65 nuclear
237 localisation in bortezomib-resistant and bortezomib-naïve U266 MM cells compared
238 to those treated with bortezomib alone (Figure 5 C). Importantly, bortezomib-
239 resistant cells showed no significant reduction in NF-κB p65 nuclear localisation in
240 response to bortezomib treatment alone compared to bortezomib-naïve cells,
241 potentially suggesting a level of constitutive NF-κB p65 activity in these cells.

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

253

254 Discussion

255 The proteasome inhibitor bortezomib is licensed to treat newly diagnosed and
256 relapsed MM in the clinic. Single agent bortezomib has a response rate of
257 approximately 30% but, when used in combination with chemotherapy and/or
258 corticosteroids, response rates range from approximately 60% to over 90%
259 depending on the regimen.{Murray, 2014 #8633} Despite this relative success,
260 however, clinical relapse following bortezomib therapy presently remains inevitable
261 and resistance to further bortezomib treatment is common{Taverna, 2012
262 #8634;Petrucci, 2013 #8635}, not only as a consequence of, but also further driving
263 the selection and emergence of drug-resistant clones.{Egan, 2012 #8564;Keats,
264 2012 #8566} An improved understanding of the mechanisms underlying bortezomib-
265 resistance is, therefore, vital for the progressive development of novel pharmacologic
266 strategies to overcome the clinical phenomenon of bortezomib-resistance. In this
267 study we have explored bortezomib-resistance in MM *in vitro*, utilising bortezomib-
268 resistant MM cell lines generated in the laboratory, and primary patient samples from
269 both treatment naïve patients and patients that have relapsed following bortezomib
270 therapy. Despite a the limitsof the sample size, this data provides thefoundation for
271 future *in vivo* assessment of ibrutinib treatment of patients with bortezomib-relapsed
272 MM in a larger numbers of patients in the clinic.

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

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

287 The oral BTK inhibitor ibrutinib, currently licenced for use in CLL and
288 MCL{McDermott, 2014 #8636;Cameron, 2014 #8637}, has shown promising clinical
289 activity and a favourable side-effect profile in a range of B cell malignancies.{Byrd,
290 2013 #8638;Wang, 2013 #8477;Advani, 2013 #8640;O'Brien, 2014 #8641} Ibrutinib
291 has also been found to reduce BTK expression in MM{Yu, 2008 #8527} and enhance
292 the cytotoxicity of bortezomib in bortezomib-naïve primary malignant plasma cells
293 and MM cell lines.{Rushworth, 2013 #6375} By inhibiting BTK with ibrutinib 'pulse'
294 treatment we were also able to restore a measurable cytotoxic response to
295 bortezomib in bortezomib-resistant MM cell lines and primary relapsed MM cells.
296 Importantly, although ibrutinib is known to target other kinases, we were able to
297 reproduce these results with highly specific artificial BTK-targeting microRNA,
298 miRBTK, introduced by lenti-virus. This suggests that it is BTK inhibition which is
299 restoring bortezomib activity in bortezomib-resistant MM cells and not an off target
300 effect of ibrutinib.

301 Multiple mechanisms of action of bortezomib have been described{Chauhan, 1996
302 #8481}{Murray, 2014 #8633}, including the inhibition of BTK expression driven via
303 repression of *inducible* NF-κB transcription factor translocation to the
304 nucleus.{Rushworth, 2013 #6375;Yu, 2008 #8527;Dasmahapatra, 2013 #8642} We
305 found enhanced nuclear localisation of NF-κB p65 in the bortezomib-resistant cells.
306 This is in-keeping with previous reports that bortezomib-resistance in MM is
307 conveyed through the NF-κB signalling network. For example; constitutive
308 expression of NF-κB is frequently seen in bortezomib refractory primary patient
309 samples.{Markovina, 2008 #8524} Furthermore, the acquisition of bortezomib-
310 resistance can follow up-regulation of heat shock protein (HSP)90 and HSP27,
311 which, in their action as ubiquitin chaperones, facilitate the activation of NF-κB in
312 MM.{Mitsiades, 2002 #8562;Navas, 2006 #8563} Active pBTK is also known to be
313 essential for NF-κB activation and B cell survival.{Davis, 2010 #8526} Thus, there
314 exists a positive auto-regulatory feedback loop that stimulates transcription of BTK
315 via two functionally competent NF-κB p65 sites in the BTK promoter.{Yu, 2008
316 #8527} Here we show that bortezomib-resistance in MM is associated with higher
317 basal nuclear NF-κB p65 in bortezomib-resistant MM cells, which, in turn, is
318 associated with a greater reliance on pBTK for survival. These observations suggest
319 that BTK inhibition by ibrutinib may be useful in the clinical treatment of bortezomib-
320 resistant MM.

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

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

330 **Materials and Methods**

331 *Materials*

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

335 *Cell culture*

336 MM cell lines (H929, LP-1, RPMI-8228, and U266) were cultured in a humidified
337 atmosphere at 37°C and 5% (v/v) CO₂ in RPMI-1640 media +L-Glutamine (Gibco
338 Life Technologies) supplemented with 10 % (v/v) foetal bovine serum ([FBS];
339 Biosera). Bortezomib-resistant cells were cultured as above but supplemented with
340 20 % (v/v) FBS. Bortezomib-resistance was selected for by dose escalation of a
341 once-weekly bortezomib treatment, through 0.5, 1, 2.5 and 5 nM, until cells could
342 tolerate 10 nM bortezomib (10-15 weeks; confirmed by luminescent cell viability
343 assay). Each concentration was maintained for approximately 2-3 weeks to allow for
344 proliferation of bortezomib-resistant sub-clones. Build-up of cell debris due to the
345 high level of cell death was removed by centrifugation at 300g for 5 minutes before
346 gently removing the supernatant and re-suspending the pellet.

347 Primary MM cells were obtained under local ethical approval (LREC ref.
348 07/H0310/146) and were isolated from the bone marrow aspirates of MM patients, as
349 described previously.^{Rushworth, 2013 #6375} Primary MM cells from bortezomib-
350 relapsed patients were not exposed to further bortezomib treatment until
351 experimentation.

352 *Ibrutinib 'pulse' treatment*

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

359 *Luminescent cell viability assay and chymotrypsin-like proteasome activity assay*

360 1×10^5 MM cells were treated as indicated. Relative cell viability was assayed using
361 the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega), as per the
362 manufacturer's instructions. Proteasome activity was measured using the
363 Proteasome-Glo[™] Chymotrypsin-Like Cell-Based Assay (Promega), as per the
364 manufacturer's instructions. Luminescence was measured with the FLUOstar
365 Omega Microplate Reader (BMGLabtech) and calculated as relative to untreated
366 control samples.

367 *Quantification of protein activity and subcellular localisation by immunocytochemistry*

368 Relative activity of phosphorylated versus total BTK (pBTK vs. BTK), and subcellular
369 distribution of nuclear vs. cytoplasmic NF- κ B p65 (nuc. vs. cyt. p65) were analysed
370 by immunocytochemistry. Briefly, 2×10^4 U266 MM cells were fixed onto microscope
371 slides using the CytoSpin 4 Cytocentrifuge system (Thermo Fisher Scientific) and 4
372 % (w/v) paraformaldehyde (PFA). Fixed cells were incubated with rabbit anti-human
373 primary mAbs against pBTK (Tyr223) and goat anti-human primary mAbs against
374 total BTK, or goat anti-human primary mAbs against NF- κ B p65 (Cell Signalling

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

380 Image analyses and quantification of pBTK vs. BTK, and nuc. vs. cyt. p65 staining
381 intensity was performed using ImageJ 1.46 software (National Institute of Health)
382 and plugins, as described previously.{Rushworth, 2014 #8589} Briefly, a threshold of
383 staining intensity was applied and the area above the threshold (pixels²) was
384 measured (n≥80 [BTK] and n≥20 [NF-κB] individual cells). Staining intensity (pixels²)
385 was converted to a percentage as follows: pBTK/total BTK (%), or,
386 nuclear/[cytoplasmic + nuclear NF-κB p65] (%).

387 *Western Immunoblotting*

388 Protein fractions of equal total protein concentration were extracted from U266 MM
389 cells using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher
390 Scientific) according to the manufacturer's instructions. SDS/PAGE and western
391 immunoblot analyses were performed as described previously{Rushworth, 2010
392 #5374}. ECL detection using Pierce ECL Western Blotting Substrate (Thermo Fisher
393 Scientific) and subsequent quantification were performed with the ChemiDoc-It2
394 Imaging System (UVP).

395 *RNA extraction and quantitative real time-PCR*

396 Total RNA was extracted from 1 x 10⁶ U266 MM cells using Total RNA Lysis Solution
397 (Applied Biosystems-Life Technologies) according to the manufacturer's instructions.

398 Reverse transcription of total RNA was performed using the GeneAmp® Gold RNA
399 PCR Core Kit (Applied Biosystems-Life Technologies). Resultant cDNA was
400 analysed by quantitative real-time PCR (qRT-PCR) using LightCycler® 480 SYBR
401 Green I Master mix (Roche) and qRT-PCR primers for GAPDH [forward 5'-
402 ACCAGCCTCAAGATCATCAGC-3' and reverse 5'-TGCTAAGCAGTTGGTGGTGC-
403 3'], BTK [forward 5'-CACACAGGTGAACTCCAGAAAG-3' and reverse 5'-
404 AGAGATACTGCCCATCGATCCAGA-3'] and NF-κB p65 [forward 5'-
405 ACCGCTGCATCCACAGTT-3' and reverse 5'-GGATGCGCTGACTGATAGC-3']
406 (Invitrogen-Life Technologies), on the LightCycler® 480 Real-Time PCR system
407 (Roche), as previously described.{Murray, 2013 #8580} Gene expression was
408 analysed using the comparative cycle threshold algorithm ($\Delta\Delta C_T$); mRNA expression
409 was standardised against GAPDH expression.

410 *Lenti-virus construction and infection*

411 Lenti-virus containing **artificial** exogenous microRNA (miRNA) sequences targeting
412 human NF-κB p65 (miR-p65 [5'-TACGTTTCTCCTCAATCCGGT-3']), BTK (miR-BTK
413 [5'-TTCAGTGGACTCTTCACCTCT-3']) or a control/scrambled sequence (miR-NEG),
414 and an EmGFP-pre-miRNA encoding fragment, were constructed and produced as
415 described previously.{Rushworth, 2014 #8589} Briefly, artificial miRNA targets were
416 identified and designed to interact with the relevant exon using Block-iT RNAi
417 designer software (Invitrogen). This ensures high specificity of miR-p65 and miR-
418 BTK, with no 3'UTR interaction, unlike endogenous miRNAs.

419 5×10^5 U266 MM cells were infected and transduced with each lenti-virus (MOI: 15),
420 in serum-free medium with 8 µg/mL Polybrene™. Following infection and before
421 experimentation, transduced cells were analysed for target knock-down and

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

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

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

433 U266 MM cells were co-transfected with a total of 1 μ g DNA, composed of the
434 pGL4.BTK promoter-luciferase reporter construct and a pRL-TK *Renilla* Luciferase
435 Control Reporter Vector (Promega) using FuGENE[®] HD Transfection Reagent
436 (Promega) and incubated for 24 h at 37°C and 5% (v/v) CO₂ before any further
437 treatment.

438 *Chemi-luminescent luciferase reporter assay*

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

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

446

447

448 **References**

449

450

451 **Figures**

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

462

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

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

480

481 **Figure 3. BTK inhibition enhances sensitivity to bortezomib in bortezomib-**
482 **naïve and bortezomib-resistant MM cells.** (A) Relative cell viability of bortezomib-
483 naïve and bortezomib-resistant MM cell lines 48 h post-ibrutinib 'pulse' treatment. (B)
484 Relative cell viability of bortezomib-naïve and bortezomib-resistant MM cells post-
485 ibrutinib 'pulse' treatment in combination with bortezomib (5 nM/24 h). (C) Relative
486 cell viability of primary naïve and primary relapsed MM patient samples post *in vitro*
487 ibrutinib 'pulse' treatment in combination with bortezomib (5 nM/24 h). Statistical
488 significance between treatments was calculated by Student's *t* test; * indicates $p \leq$
489 0.05. Statistical significance between cohorts was calculated by ANOVA; # indicates
490 $p \leq 0.01$.

491

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

500 calculated by Student's *t* test; * indicates $p \leq 0.05$. Statistical significance between
501 cohorts was calculated by ANOVA; # indicates $p \leq 0.01$.

502

503 **Figure 5. Bortezomib-resistance is driven by enhanced NF- κ B p65 activity and**

504 **can be reversed by BTK inhibition.** (A) Quantification of staining intensity of

505 nuclear p65/total NF- κ B p65 (%) in bortezomib-naïve and bortezomib-resistant U266

506 MM cells. Box and whisker indicates the mean \pm SEM ($n \geq 20$). Statistical

507 significance between cohorts was calculated by ANOVA; # indicates $p \leq 0.01$. Inset

508 shows example immunocytochemistry for NF- κ B p65. Dotted line indicates nucleus

509 as determined by DAPI staining. (B) Western immunoblot of bortezomib-naïve [N]

510 and bortezomib-resistant [R] U266 nuclear [Nuc.] and cytoplasmic [Cyt.] cell

511 fractions. GAPDH and Histone 3 [H3] show equal loading for cyt. and nuc. fractions,

512 respectively. (C) Quantification of staining intensity of nuclear NF- κ B p65/total NF- κ B

513 p65 (%) in bortezomib-naïve and bortezomib-resistant U266 MM cells. Box and

514 whisker indicates the mean \pm SEM ($n \geq 20$). Statistical significance between cohorts

515 was calculated by ANOVA; # indicates $p \leq 0.05$. (D) qRT-PCR analysis of basal NF-

516 κ B p65 mRNA expression in bortezomib-naïve and bortezomib-resistant MM U266

517 cells infected with lenti-viral miRNA constructs targeting NF- κ B p65 (miRp65)

518 transcription relative to GAPDH. (E) Relative cell viability of bortezomib-naïve and

519 bortezomib-resistant MM U266 cells infected with lenti-viral miRp65 24 h following

520 ibrutinib 'pulse' treatment. Values indicate the mean \pm SEM from 3 independent

521 experiments. Statistical significance between treatments was calculated by Student's

522 *t* test; * indicates $p \leq 0.05$. Statistical significance between cohorts was calculated by

523 ANOVA; # indicates $p \leq 0.01$.