Clinically relevant pulse-treatment generates bortezomib-resistant myeloma cell line that lacks proteasome mutations and is sensitive to Bcl-2 inhibitor venetoclax

Sondra L Downey-Kopyscinski
Dartmouth College
Sriraja Srinivasa
Auburn University
Alexei F Kisselev (AFK0006@auburn.edu)
Auburn University

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Abstract

Proteasome inhibitors bortezomib and carfilzomib are the backbone of treatments of multiple myeloma, which remains uncurable despite many recent advances. With many patients relapsing despite high initial response rates to proteasome inhibitor-containing regimens, it is critical to understand the process of acquired resistance. In vitro generated resistant cell lines are important tools in this process. The majority of previously developed bortezomib-resistant cell lines bear mutations in the proteasome PSMB5 sites, the prime target of bortezomib and carfilzomib, which are rarely observed in patients. Here we present a novel bortezomib-resistant derivative of KMS-12-BM multiple myeloma cell line, KMS-12-BM-BPR. Unlike previously published bortezomib-resistant cell lines, this sub-line was created using a clinically relevant twice-weekly pulse treatments with bortezomib instead of continuous incubation. It does not contain mutations in proteasome active sites and retains its sensitivity to carfilzomib. Reduced load on proteasome due to decreased protein synthesis appears to be the main cause of resistance. In addition, KMS-12-BM-BPR cells express less Mcl-1 than wild type and are more sensitive to Bcl-2 inhibitor venetoclax. Overall, this study demonstrates the feasibility of creating a proteasome-resistant myeloma cell lines by using clinically relevant pulse treatments and provides a novel model of acquired resistance.

Introduction

Proteasome inhibitors bortezomib (Btz), carfilzomib (Cfz) and ixazomib (Ixz) are one of the backbones in the treatment of multiple myeloma (MM). The proteasome is a large proteolytic complex which degrades abnormal and misfolded proteins and thus plays the key role in the protein quality control and maintenance of protein hemostasis in every mammalian cell. Multiple myeloma cells secrete large amounts of immunoglobulins, creating an enormous load on protein quality control machinery, making them exquisitely sensitive to proteasome inhibitors[1–5]. Btz partially inhibits proteasome by blocking its β5 sites, which are responsible for the chymotrypsin-like activity, and to a lesser extent, β1 sites, which are responsible for the caspase-like activity. Cfz is a more specific and potent inhibitor of the chymotrypsin-like activity[6]. Such partial inhibition causes build-up of abnormal proteins specifically in myeloma cells, ultimately leading to apoptosis.

While initial response rate of proteasome inhibitor-containing combinations approach 90%[7,8], patients eventually relapse and become resistant to all FDA-approved treatments. A better understanding of the mechanism of resistance to proteasome inhibition is necessary to improve treatment for resistant and refractory patients. Resistant cell lines created by the in vitro exposure to the low concentrations of the agent are important tools widely used to gain understanding of the mechanism of resistance. The majority of Btz-resistant MM cell lines developed by this approach display mutations in the ß5 sites[9–14], which reduce Btz binding. However, these mutations are rarely detected in relapsed patients[15–18], which raises questions about clinical relevance of these models. Most of these cell lines were created by continuously culturing cells in Btz, which does not accurately mimic the clinical pharmacokinetics of the drug that is administered twice weekly as a bolus[19,20]. Here we describe the development and initial

characterization of a novel Btz-resistant KMS-12-BM cell line that lacks mutations in the catalytic sites and was created by twice weekly one-hour pulse treatments cells with increasing concentrations of Btz.

Results

Creation of a resistant KMS-12-BM-BPR sub-line. To develop a Btz-resistant cell line using clinically relevant pulse-treatment, we treated KMS-12-BM cells twice weekly with 1hr pulses of Btz. As resistance developed, we increased the dose stepwise from 900nM to 7.2μM Btz (Fig. 1a) over the course of six months, until we derived a sub-line that was approximately 10-fold more resistant (Fig. 1b). We named it KMS-12-BM-BPR, where BPR stands for “bortezomib-pulse resistant”. For brevity, we will also refer to it as BPR. This sub-line did not require culturing in the presence of Btz to maintain resistance and grew at the same rate as the parental line (Fig. S1a). Thus, it is feasible to develop Btz-resistant cell lines using clinically relevant pulse treatments.

KMS-12-BPR cells line does not have mutations and does not overexpress proteasomes. We tested whether the BPR cells have a mutation in or around Btz binding pocket of the PSMB5 (β5c) subunit, which reduced Btz affinity to its prime target, as in the majority of previously developed Btz-resistant cells[9–11]. We isolated genomic DNA from both cells and Sanger-sequenced the exomes of PSMB5, PSMB6 (β1c), and PSMB7 (β2c) genes that encode catalytic subunits of the constitutive proteasome, and the PSMB8 (β5i), PSMB9 (β1i), PSMB10 (β2i) genes that encode catalytic subunits of lymphoid tissue specific immunoproteasome, which are expressed in the myeloma cells[21–23]. We did not find any specific mutations in the resistant cells. Some of previously reported resistant cells overexpressed the β5 subunits[9], resulting in an increase in proteasome activity. Although we observed a small increase in the chymotrypsin-like activity in the resistant cells, it was not significant (Fig. 1c).

Consistent with a lack of mutations, 1hr pulse treatment with Btz caused similar inhibition of ß5 (i.e., combined activity of β5c and β5i) and ß1 (i.e., combined activity of β1c and β1i) activities in the parental cells and the resistant subclone (Fig. 1d). Although there were small differences in inhibition of β5 activity at sub-toxic Btz concentrations, inhibition was the same in cells treated with 1μM Btz, which causes largest differences in viability. Thus, resistance in the BPR sub-line is not due to decreased affinity of Btz to the active sites. These data also indicate that the drug is still penetrating the cell and, most likely, is not being transported out of the cell at an increased rate.

Faster recovery of the proteasome activity after pulse treatment could contribute to resistance. We measured the proteasome activity of the cells over time after a pulse treatment with 1μM Btz, which causes maximal differences in sensitivity (Fig. 1d). The resistant cells have a slightly faster recovery of the β5 activity, but not the β1 activity. 20% of β5 activity recovered 4-6h after treatment in resistant cells (Fig. 1e), which is before the onset of apoptosis (Fig. 1f) raising the possibility that differences in recovery rates may contribute to resistance.

To determine whether different recovery rates translate into differences in protein degradation, we measured degradation of long-lived proteins in a pulse-chase experiment and accumulation of undegraded K48-linked ubiquitylated proteins, most of which are derived from undegraded nascent polypeptides[24]. After 1hr treatment, both approaches revealed a modest difference between sensitive and resistant cell lines, with resistant cells being slightly less susceptible for inhibition (Fig. 1g). Despite modest initial difference, sensitive cells accumulated substantially more ubiquitylated proteins over time. The difference was notable at 6hr and preceded the onset of apoptosis, which was detected only at 12hr (Fig. 1h), and therefore is more likely to be the cause and not the consequence of cell death. The decreased accumulation of ubiquitylated proteins in resistant cells could be caused by activation of deubiquitylating enzymes. However, we did not detect any activation of deubiquitylating enzymes using the ubiquitin-methyl ester activity-based probe[25] (Fig. S1b). Therefore, difference in the degradation rates is the most likely cause of resistance. How can similar occupancy of active sites by Btz lead to differences in protein degradation rates that appear to increase over time?

Sensitivity of myeloma cells to proteasome inhibitors is defined by the ratio of the load on the proteasome, i.e. amount of proteins degraded, to proteasome capacity, i.e. amount of active proteasomes[1–5]. Cells with lower load have many idling proteasomes and can maintain rates of protein degradation when proteasomes are partially inhibited (e.g., β5 sites are inhibited but two other sites are functional) simply by engaging idling proteasomes. Increasing load-to-capacity will engage idling proteasome and reduce spare capacity. Under this condition, even partial inhibition of individual active sites will result in the inhibition of protein degradation, resulting in accumulation of ubiquitylated proteins at lower concentrations of Btz, ultimately leading to higher Btz sensitivity. Since proteasome activity in the resistant cells was similar (Fig. 1c), increased capacity cannot account for resistance, suggesting that lower load is the major cause of resistance. The majority of ubiquitylated proteins that accumulate upon treatment of cells with proteasome inhibitor are derived from nascent polypeptides[24], therefore higher rates of protein synthesis should result in the higher load on the proteasome. We used puromycin incorporation assay[26] to measure rates of protein synthesis, and found that resistant cells incorporate substantially less puromycin that the sensitive one indicating slower protein synthesis rates (Fig. 1h). Thus, Btz resistance in KMS-12-BM-BPR cells is caused by decreased load on the proteasome due to lower protein synthesis rate.

Cfz and marizomib (Mzb), but not Ixz overcome Btz resistance. We next tested if other proteasome inhibitors could overcome Btz resistance in KMS-12-BM-BPR line (Fig. 2). We observed that Cfz, which is approved by the FDA for the treatment of multiple myeloma, and marizomib (Mzb, salinosporamide A, MPI-0052), a natural product undergoing clinical trials in multiple myeloma and glioblastoma, can overcome resistance (Fig. 2a). However, Ixz, an orally bioavailable FDA-approved analogue of Btz, did not. Unlike Cfz and Mzb, Btz and Ixz do not inhibit proteasome β2 sites, responsible for the trypsin-like activity. Although all four inhibitors blocked β5 with similar potency, we found that decrease of viability in Cfz and Mzb-treated cells coincided with co-inhibition of ß1 and ß2 sites (Fig. 2b). In comparison, Ixa and Btz

inhibit ß2 activity at higher concentrations than ß1 (Fig. 2b and 2c). Thus, it appears that inhibition of β2 sites is critical to overcoming Btz-resistance of KMS-12-BM BPR cells.

Increasing proteasome inhibition by targeting the β2 subunits can overcome Btz resistance. The ability of inhibitors that co-inhibit β2 sites to overcome Btz resistance is consistent with an expectation of the load-to-capacity hypothesis that resistant cells, which are able to withstand partial proteasome inhibition due to their low load-to-capacity ratio, will succumb to proteasome inhibitors that decrease proteasome capacity by blocking additional active sites. We previously found that β2 specific inhibitor LU-102 is a potent sensitizer of myeloma and solid tumor cells to Btz and Cfz, and that it overcomes acquired resistance to these agents[23,27–29]. To confirm the importance of ß2 sites for Btz resistance in the BPR line, we tested whether LU-102 restores Btz sensitivity. We found that specific blocking of β2 sites by LU-102 had no effect on cell viability of parental and resistant cells (Fig. 3a). When 1hr Btz treatment was followed by a subsequent treatment with sub-toxic, β2 specific concentrations of LU-102, both cell lines were sensitized to Btz (Fig. 3b). However, sensitization was much stronger in resistant cells. Co-treatment with LU-102 sensitized resistant cells to a wider range of Btz concentrations. The effect of LU-102 on viability of resistant cells was most noticeable at 1μM Btz, where addition of LU-102 restored the Btz sensitivity of KMS-12-BM-BPR cells to the level of the wild type. At this concentration, co-treatment with LU-102 accelerated accumulation of ubiquitylated proteins (Fig. 3c), which confirms that LU-102 acts through inhibition of proteasome and rules out contribution of autophagy to resistance. Thus, inhibition of ß2 sites overcomes resistance to Btz in KMS-12-BM-BPR cells.

KMS-12-BM-BPR cells are highly sensitive to Bcl-2 inhibition. We noticed that the resistant cells consistently turned phenol-red containing media orange and yellow (indicative of a pH change) at a faster rate than their parental cells and confirmed increased production of lactate (Fig. 4a), which may indicate reduced mitochondrial respiration. KMS-12-BM cells bear t(11;14) translocation. According to the literature, myeloma cells bearing t(11;14) translocation have lower oxygen consumption rates than myeloma cells without this translocation[30], and are more sensitive to Bcl-2 inhibitor ABT-199 (venetoclax)[31,32]. KMS-12-BM, however, is not the most venetoclax-sensitive myeloma line[31]. Therefore, we decided to investigate whether KMS-12-BM-BPR are more sensitive to Bcl-2 inhibitors than the parental cells.

We observed an increased sensitivity of the resistant cells to the dual Bcl-2/Bcl-X_L inhibitor, ABT-737 (Fig. 4b) and to Bcl-2 specific inhibitor ABT-199 (venetoclax, Fig. 4C) but not to the BCL-X_L specific inhibitor A-1155463[33] (Fig. 4c). When a 1hr Btz treatment was followed by ABT-199, the combination was synergistic in both cell lines (Fig. 4d). Sensitivity of myeloma cells to venetoclax depends on Bcl-2:Mcl-1 and Bcl-2:Bcl-X_L expression ratios[32]. Therefore, to determine the mechanism of increased Bcl-2 dependence, we examined expression of Bcl-2 members and found that Mcl-1 expression was dramatically reduced in the resistant cells, but Bcl-2 was not overexpressed in the resistant cells (Fig. 4e). Bcl-X_L expression was low in both lines (Fig. 4e). We conclude that increased Bcl-2:Mcl-1 ratio due to decreased expression of Mcl-1 sensitizes KMS-12-BM-BPR cells to venetoclax.

Discussion

With the creation of KMS-12-BM-BPR cell line, we demonstrate that it is possible to isolate proteasome inhibitor resistant myeloma cells using a clinically relevant pulse treatment of established myeloma cell lines with Btz. Several properties of this line indicate that this approach generates a clinically relevant model. Like the majority of clinical cases, and in contrast to the majority of Btz-resistant cell lines described in the literature, the KMS-12-BM-BPR line does not have mutations in the active sites. Decreased immunoglobulin production as the result of de-differentiation was described in myeloma-resistant patient samples[34], and we found dramatically decreased protein synthesis rate in the KMS-12-BM-BPR line. De-differentiated myeloma cells express B-cell markers, and it was recently reported that expression B-cell markers correlates with venetoclax sensitivity in myeloma cells[31]. In this regard, decreased protein synthesis corelates with increased sensitivity of KMS-12-BM-BPR cells to venetoclax. Our results also correlate with a previously reported ability of venetoclax to overcome Btz resistance and synergize with Btz in a fraction of patients bearing t(11;14) translocation[35,36]. Similar to venetoclax responders in the clinical studies[35], the KMS-12-BM-BPR line had higher Bcl-2 to Mcl-1 ratio. This ability of Bcl-2 inhibitors to overcome Btz resistance has not been described in the in vitro generated cell lines, however none of them bear the t(4;11) translocation which sensitizes myeloma cells to Bcl-2 inhibitors.

Several previous studies have reported metabolic adaptations in Btz-resistant cells, and some of Btz-resistant cells with increased mitochondrial metabolism are also sensitive to Bcl-2 inhibitors[37]. Three studies have found that mitochondrial metabolism and oxidative phosphorylation are up-regulated in cell lines[37–39] and in myeloma patient cells[18]. On the other hand, Bajpai et al found that venetoclax-sensitive myeloma cells bearing t(11;14) translocation have lower oxygen consumption rates than myeloma cells that do not bear this translocation[30]. Increase in lactate production was reported in Btz-resistant RPMI-8226 cell line, in addition to higher activity of pentose phosphate and serine biosynthesis pathways[40]. Our KMS-12-BM-BPR appears to resemble RPMI-8226 cells in this regard. Exploring metabolic changes in the resistant cell was not the goal of our study, and it remains to be determined whether increased production of lactate is required to maintain resistant phenotype or is a by-product of resistance development.

Cfz overcomes resistance to Btz as it does in many Btz-refractory patients. We found that Cfz and Mzb, which produce stronger inhibition of β2 sites than Btz, overcome resistance, and that co-treatment with β2-specific inhibitor also overcomes resistance. On the other hand, KMS-12-BM-BPR cells were cross-resistant to Ixz, which does not inhibit β2 sites. This observation is also in agreement with our previous findings that co-inhibition of β2 sites overcomes Btz resistance in primary cells from myeloma patients[28], and that combined β2 and β5 inhibition produces stronger anti-neoplastic effect than combined β1 and β5 inhibition[27]. This data is also in agreement with clinical observations that efficacy of Cfz in MM seems to correlate with the co-inhibition of β2i sites[41].

In summary, we have demonstrated that it is feasible to generate resistant cells by a clinically relevant pulse exposure to bortezomib, and have generated a model bortezomib-resistant cell line that bears a t(11;14) translocation.

Material And Methods

Cell Lines. KMS-12-BM cell line was provided by Takemi Otsuki (Kawasaki Medical School, Japan). All cell culture was preformed using RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), penicillin (100ug/mL), streptomycin (100 units/mL) and anti-mycoplasma antibiotic Plasmocin (1.5ug/mL, Invivogen, San Diego, CA). KMS-12-BM-BPR subline was generated by repeated exposure of KMS-12-BM to a 1hr-pulses of increasing concentration of Btz, ranging from 0.9-7.2 μM over a period of 6 months as illustrated on Fig. 1a. The line was then maintained in a regular media in the absence of Btz without loss of resistance. The parental and the resistant lines were authenticated by STR profiling.

Inhibitors, Viability, and Cell-based Activity Assays. Btz and Cfz were purchased from LC laboratories. Ixz was provided by Millennium Pharmaceuticals. Mzb was the kind gift of Dr. Bradley Moore. LU-102 was synthesized as previously described and provided by Dr. Herman Overkleeft[29]. All inhibitors were dissolved in DMSO and then further diluted in tissue culture media. Viable cells were assessed with Alamar Blue mitochondrial dye conversion assay as described[42]. Caspase 3/7 activity was determined using Apo-One Homogeneous Caspase-3/7 Assay (Promega). Proteasome activity in cultures was measured using Proteasome Glo assay (Promega) that contains site-specific luminogenic substrates Suc-LLVY-amino-luciferin (aLuc, β5 sites), Z-nLPnLD-aLuc (β1 sites), and Boc-LRR-aLuc (β2 sites). Experiments were performed as in the past studies that established the specificity of these regents in MM cells[42,43]. Activity in extracts was measured using a standard Suc-LLVY-4-amico-methylcoumarin (amc) substrate as described[44].

Western blotting. Frozen cell pellets were lysed in 50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, and 0.5% CHAPS. Protein content of lysates was determined by Pierce 660nm protein assay reagent (ThermoFisher Scientific) and used to normalize gel loading. Lysates were run NuPAGE gels (Invitrogen) using either MOPS or MES running buffer before being transferred to a PDVF membrane, which was then blocked with Odyssey Blocking Buffer (LiCor), or 5% fat free milk. Following antibodies were used: anti-K48-linkage Specific Polyubiquitin (D9D5, Cell Signaling, #8081), anti-actin (Abcam ACTN05(C4), or Cell Signaling 8H10D10 Cat #3700), anti-Bcl-X_L (Cell Signaling #2764), anti-Bcl-2 (Dako, # M0877), and anti-Mcl-1 (BD Pharmigen, # 559027), anti- Puromycin (clone 12D10, EMD Millipore cat #MABE343), HRP-conjugated anti-mouse IgG (Cell Signaling #7074), HRP-conjugated anti-rabbit IgG (Cell Signaling #7076), IRDye800CW labelled anti-mouse-IgG (Licor, #926-68070), Alexa680-labeled anti-rabbit-IgG (Invitrogen, #A21076). Bands were revealed using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) followed by imaging using a CCD camera (GelDoc (Bio-Rad) or Azure c600 instruments) or on Odyssey scanner (LiCOR) using auto-exposure settings.

Inhibition of Protein Degradation. To measure the breakdown of long-lived proteins, cultures of MM cells ($1x10^6$ cells/ml) were pulse-labeled with 30 μCi/mL ^{3}H-Leucine overnight and washed 3 times with warm chase media containing Leucine at 2.5x normal concentration (1 mM) to remove unincorporated ^{3}H-Leucine. Each suspension culture was then incubated in the chase media for 1hr to allow for degradation of short-lived proteins. Treatment with Btz (1 μM) and Z-Leu_3-epoxyketone (ZL3ek) was performed during this chase. ZL3ek was used at concentration that completely inhibit all three proteasomal activities to determine the background dues to non-proteasomal protein degradation. After additional wash in a chase media to remove excess of inhibitors, cells were cultured in fresh chase media for 1 hours when degradation was stopped by mixing cultures with 1/10 volume of ice-cold 100% trichloroacetic acid (TCA). After 25 min incubation on ice, precipitated undegraded proteins were separated from TCA-soluble degradation products by centrifugation for 15 min at 20,000 g at +4^{o}C. Pellets of TCA-precipitated proteins were washed twice with ice-cold acetone, air-dried, dissolved in 100% TCA, and counted in scintillation plate reader alongside with supernatants from the 10% TCA precipitation step. Fraction of proteins degraded was calculated by dividing total dpm in TCA supernatant by a sum of total counts in TCA supernatant and total counts in the TCA pellet. Three technical replicates were averaged, and background (e.g., degradation in the presence of ZL3ek) was subtracted to determine proteasomal degradation and inhibition in the presence of 1 μM Btz was calculated.

Puromycin incorporation to measure protein synthesis. The rate of protein synthesis in parental and BPR cells was compared using puromycin incorporation assay[26]. Puromycin (10 ug/ml) was added to cell culture media for 25 minutes, after which cells were harvested, lysed, run on 10% NuPAGE gels using MES buffer, transferred to PVDF membrane, and analyzed by western using puromycin antibody.

Data analysis. All values shown on the graphs indicate means±S.E.M of several biological replicates, the exact number of which (n) is indicated in the caption. GraphPad Prism was used for graphing and analysis.

Declarations

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Author contribution statement

SLDK and AFK conceived the project, designed experiments, analyzed data and wrote the paper. SLDK and SS performed experiments. All authors critically revised the manuscript and approved the final version.

Data and Materials availability

KMS-12-BM-BPR cell lines are available from the corresponding offer upon request. This study did not generate any large datasets, or any novel DNA sequence data.

Additional Information

AFK is a founder and chief scientific officer of InhiProt LLC. Other authors do not report any conflicts.

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Figures

Figure 1

Development and characterization of Btz-resistant KMS-12-BM-BPR cells. a. The timeline of the creation of the KMS-12-BM-BPR cells by 1hr pulse treatment with increasing concentrations of Btz. **b.** Viability of resistant and parental cell was compared by the Alamar Blue mitochondrial dye conversion assay 48hr after 1hr pulse-treatment of cells, n=3. **c.** b5 activity in lysates of untreated cells was measured with Suc-LLVY-amc, n=3**. d.** Inhibition of proteasome in cells was measured immediately after 1hr Btz treatment using Proteasome Glo assay, n=2. **e.** Recovery of activity after 1hr pulse treatment with 1μM Btz was determined by Proteasome Glo, n=3. **f.** The parental KMS-12-BM cells were treated with 1μM Btz for 1hr, then cultured in a drug free media, and assayed for Caspase 3/7 activity. **g.** Accumulation of K48-linked ubiquitinated proteins at different times after 1hr treatment with 1 mM Btz. Inhibition of proteasomal degradation of long-lived protein was measured in cells treated with 1 mM Btz and compared with mock-treated controls; n=3. See Fig. 3c for a biological replicate. **h.** Incorporation of chain terminator puromycin in the nascent polypeptide chain was analyzed by western. Biological replicates are shown. Uncropped images are presented on Fig. S2a.

Figure 2

Cfz and Mzb but no Ixz overcome resistance to Btz. **a.** Cells were treated for 1hr, and Alamar Blue assay was performed 48hr later. **b-c.** Inhibition of each active site was measured with Proteasome-Glo immediately after 1hr treatment, and overlayed on viability data from Fig. 2a (b) or Fig. 1b (c); n=2.

Figure 3

LU-102 overcomes resistance in BPR cells. a. Effect of a single agent LU-102 treatments. Cells were treated continuously with LU-102 for 48hrs before viability was determined using Alamar Blue; in a parallel experiment, activity was measured with Proteasome Glo 2hr after treatment; n=2. **b.** Cells were treated with Btz for 1hr when it was replaced with 3 μM LU-102 for 48hr, when Alamar Blue assay was performed, n=2-3. **c.** Cells were treated with 1μM Btz for 1hr and then allowed to recover in media in the presence or absence of 3μM LU-102. Cell lysates were analyzed by westerns. Uncropped images are presented on Fig. S2b.

Figure 4

KMS-12-BM-PPR are more sensitive to BCL-2 inhibition that the parental cells. a. Lactate production was measured over time and normalized for cell count. **b. C**ells were treated continuously for 48hr with ABT-737 before being assayed for viability with Alamar Blue (n=3). **c.** BPR cells are more sensitive to a Bcl-2-specific inhibitor ABT-199, but not Bcl-X_L specific inhibitor. Left, cells were treated for 48hr with ABT-199 (left) or Bcl-X_L inhibitor (A-1155463) before viability was determined by Alamar Blue (n=2-3). **d.** Cells were treated with Btz for 1hr and then with ABT-199 for 47hr before viability was determined by Alamar Blue (n=2-4). Numbers on the graph are combination indexes (CI). **e.** Expression of Bcl-2 family member proteins was determined by westerns following 1hr pulse treatment with Btz and overnight recovery. Uncropped images are presented on Fig. S2c.

Supplementary Files

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