Bedaquiline amplifies proteasome inhibitor efficacy and overcomes resistance in multiple myeloma

Michela Cumerlato,[1,2] Monica Maccagno,[1,2] María Labrador,[1,2] Beatrice Luciano,[1,2] Elisabetta Mereu,[1,2] Mariangela Porro,[1,2] Cecilia Bandini,[1,2] Mattia Furlan,[3] Domenico Maisano,[4] Mattia D'Agostino,[1,5] Alessandra Larocca,[1,5] Francesca Gay,[1,5] Annalaura Tamburrini,[2,6] Francesca Anselmi,[2,6] Paolo Ettore Porporato,[1,2] Salvatore Oliviero,[2,6] Benedetto Bruno,[1,5] Eugenio Morelli,[3,7] Nikhil Munshi[4] and Roberto Piva[1,2,5]

[1]Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy; [2]Molecular Biotechnology Center (MBC) "Guido Tarone", Turin, Italy; [3]Candiolo Cancer Institute, FPO-IRCCS, Turin, Italy; [4]Dana Farber Cancer Institute, Boston, MA, USA; [5]Città della Salute e della Scienza Hospital, Turin, Italy; [6]Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy and [7]Department of Oncology, University of Turin, Turin, Italy

Correspondence: R. Piva
roberto.piva@unito.it

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Abstract

Proteasome inhibitors (PI) are cornerstone therapies for multiple myeloma (MM), yet resistance remains a major barrier to durable responses. To identify druggable vulnerabilities that enhance PI efficacy, we performed a small-molecule chemical screen in the presence of carfilzomib (CFZ). We identified bedaquiline (BDQ), a Food and Drug Administration-approved antimycobacterial agent, as a potent synergistic partner. BDQ and its fumarate salt (BDQ-F) significantly amplified CFZ-induced cytotoxicity in PI-sensitive and PI-resistant MM cells, in AL amyloidosis and other B-cell malignancies, with minimal toxicity to normal cells. Mechanistic studies confirmed that BDQ specifically targets the ATP5F1C subunit of mitochondrial ATP synthase. The BDQ-CFZ combination triggered extensive apoptosis, exacerbating proteotoxic stress and proteasome-associated pathways. BDQ specifically enhanced CFZ's inhibition of the proteasome's chymotrypsin-like activity. Importantly, BDQ synergized with multiple proteasome and ubiquitin-activating enzyme inhibitors, but not with other standard MM agents, underscoring its selective interaction with the ubiquitin-proteasome system pathway. BDQ-CFZ co-treatment markedly reduced MM cell viability and tumor burden in patient-derived cells and zebrafish xenograft models. These findings support the therapeutic repurposing of BDQ to potentiate PI efficacy and overcome resistance in MM and related B-cell malignancies.

Introduction

Multiple myeloma (MM) is a malignancy of terminally differentiated B cells marked by clonal plasma cell expansion in the bone marrow and excess monoclonal immunoglobulin production.[1] It is the second most common hematologic cancer in high-income countries and is typically diagnosed between the ages of 60 and 70.[2] Standard frontline therapies combine proteasome inhibitors (PI), immunomodulatory agents, corticosteroids, and monoclonal antibodies targeting CD38.[3-5] Recently, chimeric antigen receptor T cells and bispecific T-cell engagers have shown remarkable efficacy in heavily pretreated patients with relapsed/refractory MM.[6,7] While these regimens have improved survival, MM remains incurable due to intrinsic and acquired mechanisms of resistance.[8,9] Identifying new vulnerabilities and combinatorial strategies to enhance efficacy while reducing toxicity is essential.[10] However, the extensive genetic and epigenetic heterogeneity of MM complicates treatment and fuels resistance.[11,12] To address this, omics-driven approaches and *ex vivo* functional screens are being increasingly used to identify actionable targets.[13] MM cells exhibit high proteostasis stress due to their immunoglobulin load, making them susceptible to PI.[14] Yet, metabolic and epigenetic reprogramming enable adaptation and drug resistance.[15,16] Targeting these compensatory pathways offers a rational strategy to resensitize cells to PI. We previously identified several PI synergistic interactors, including IDH2 and LSD1, as actionable nodes to restore PI sensitivity.[17-19] Here, we describe the Food and Drug Administration-approved ATP synthase inhibitor bedaquiline (BDQ)[20] as a novel synergistic partner of the PI carfilzomib (CFZ). BDQ-CFZ co-treatment

selectively induces proteotoxic stress and apoptosis in MM cells. Given BDQ's established safety and clinical availability, our findings support its repurposing to enhance PI efficacy and overcome resistance in MM and potentially other B-cell malignancies.

Methods

Detailed experimental procedures for cell culture conditions, reagents, cellular and biochemical assays, plasmids, virus production, RNA extraction, reverse transcription-quantitative polymerase chain reaction, western blotting, RNA sequencing, proteomics, and data analyses are included in the *Online Supplementary Material and Methods*.

Drug screening

Primary compound screening was performed using a selective small-molecule inhibitor library (L3500, Selleck Chemicals) consisting of 320 inhibitors targeting 123 key proteins involved in diverse signaling pathways. U-266 PI-resistant MM cells (U-266^{PIR}) were treated with each compound at four concentrations (10 μM, 1 μM, 100 nM, and 10 nM). After 2 hours (h), cells were exposed to either a sublethal dose of carfilzomib (CFZ; 20 nM) or dimethylsulfoxide (DMSO) as a vehicle control. Cell viability was measured at baseline (0 h) and 72 h after treatment using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), and results were collected in duplicate. Growth rate (GR) was calculated by normalizing the luminescent signal at 72 h to the baseline signal and further normalized to the DMSO-treated controls. Drug combination effects were quantified using the excess over Bliss model, calculated from GR values using the following formula: [1 – GR(combination)] – [1 – GR(CFZ)] – [1–GR(drug)] + [1–GR(CFZ)][1 – GR(drug)].[21] Compounds yielding excess over Bliss scores ≥0.4 at any concentration were considered potential hits and shortlisted for secondary validation in a panel of five MM cell lines (U-266^{PIR}, U-266, KMM-1, AMO-1, and KMS-28). Compounds that achieved excess over Bliss scores ≥0.4 in at least three cell lines were designated top synergistic candidates (TOP14) and are listed in *Online Supplementary Table S1*.

Samples from multiple myeloma patients and healthy donors

Peripheral blood mononuclear cells (PBMC) from healthy donors and bone marrow aspirates from MM patients were obtained from the local Blood Bank (Città della Salute e della Scienza Hospital, Turin, Italy). PBMC were isolated from buffy coats via Ficoll-Hypaque density gradient, resuspended in RPMI + 10% fetal bovine serum (FBS), and seeded at 5×10^5 cells/mL. Bone marrow mononuclear cells were isolated by Ficoll gradient centrifugation following Miltenyi's protocol. CD138$^+$ plasma cells were identified by flow cytometry using anti-CD138-APC (clone 44F9, Miltenyi), and samples with ≥10% CD138$^+$ cells were used for downstream assays. Cells were seeded at 2.5×10^5 cells/mL in RPMI + 10% FBS and treated as described in *Online Supplementary Table S2*.

Zebrafish xenograft assay

Approximately 250 RPMI-8226 cells stably expressing DsRed fluorescent protein were injected (2 nL) into the yolk sac of 48 hours post-fertilization (hpf) embryos with a manual microinjector (Eppendorf, Germany). Embryos were maintained at 28°C in embryo medium supplemented with 0.003% 1-phenyl-2-thiourea (PTU), 1 g/L glucose, and 5 mM L-glutamine. At 24 hours post-injection (hpi), xenograft-positive embryos were identified by fluorescence microscopy (ZEISS Axio Observer, 10× objective), randomly allocated into four treatment groups (DMSO; 2.5 nM CFZ; 10 μM BDQ; or their combination), and placed individually into 48-well plates. Tumor burden was assessed at 72 hpi by fluorescence microscopy and quantified using ImageJ as the fluorescence area normalized to the 24 hpi baseline. Adult zebrafish were euthanized with a tricaine overdose. All procedures were approved by the local ethics committee and the Italian Ministry of Health.

Ethics approval and consent to participate

PBMC from healthy donors and bone marrow aspirates from MM patients were obtained from the local Blood Bank and the Hematology Unit at Città della Salute e della Scienza Hospital (Turin, Italy). Informed consent was obtained from all participants following the Declaration of Helsinki, and the study was approved by the local ethics committee (protocol N. 00143/2022). All animal experiments were conducted in compliance with institutional guidelines and approved by the Ethical Committee of the University of Turin.

Results

Bedaquiline synergistically enhances proteasome inhibitor efficacy in multiple myeloma and other hematologic malignancies through ATP5F1C modulation

To identify compounds that enhance the efficacy of CFZ in MM, we performed a functional screen of 320 small-molecule inhibitors in the MM PI-resistant cells U-266^{PIR} in the presence or absence of a sublethal dose of CFZ (Figure 1A). Top candidates were validated in five MM cell lines. BDQ and its fumarate form (BDQ-F), approved to treat multidrug-resistant tuberculosis, showed strong synergy with CFZ in five and three out of five lines, respectively (*Online Supplementary Table S1*), and were prioritized for further study. BDQ and BDQ-F strongly synergized with CFZ in almost all tested PI-resistant and PI-sensitive MM cell lines (Figure 1B, C; *Online Supplementary Figure S1A, B*). The combination also enhanced cytotoxicity in systemic

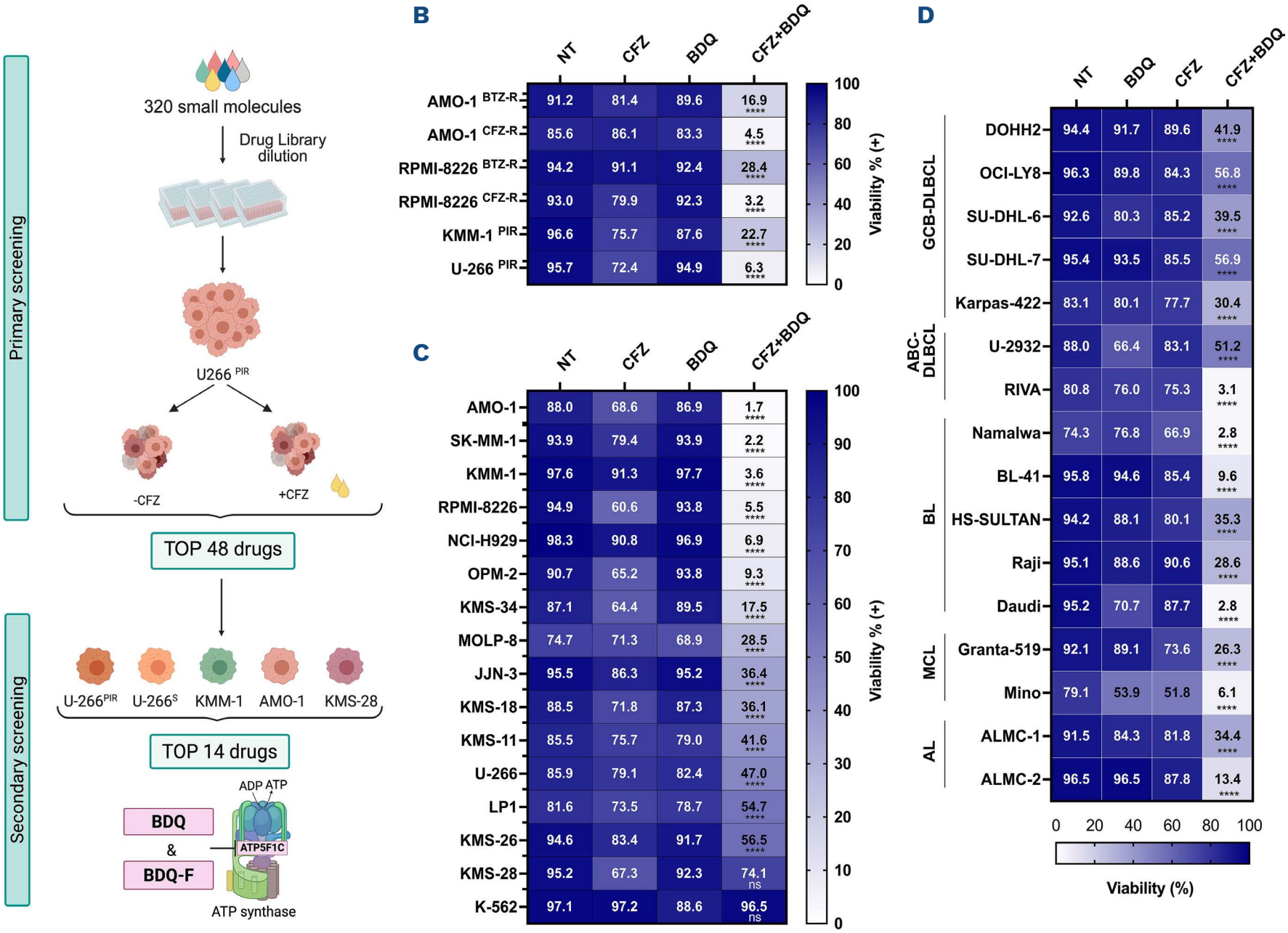


Figure 1. Bedaquiline synergistically enhances proteasome inhibitor efficacy in multiple myeloma and other hematologic malignancies. (A) Schematic overview of the functional drug screening strategy used to identify compounds that potentiate carfilzomib (CFZ) activity. A library of 320 small-molecule inhibitors was screened in U-266 proteasome inhibitor (PI)-resistant multiple myeloma (MM) cells, with or without sublethal doses of CFZ. Top hits were validated in a secondary screen across five MM cell lines, identifying bedaquiline (BDQ) and its fumarate salt (BDQ-F) as strong synergistic partners of CFZ. (B, C) Cell viability heatmaps showing the synergistic effects of BDQ and CFZ in PI-resistant (B) and PI-sensitive (C) MM cell lines. (D) Heatmap summarizing cell viability in systemic light chain amyloidosis and B-cell non-Hodgkin lymphoma cell lines, including germinal center B-cell–like and activated B-cell–like diffuse large B-cell lymphoma, Burkitt lymphoma, and mantle cell lymphoma, following treatment with BDQ, CFZ, or their combination. Data represent the mean of three independent experiments. Compound concentrations, detection methods, time points, and mean ± standard deviation values are provided in *Online Supplementary Tables S3* and *S4*. Statistical significance was determined using one-way analysis of variance (****P<0.0001). ABC: activated B-cell–like; AL: systemic light chain amyloidosis; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma; GCB: germinal center B-cell–like; MCL: mantle cell lymphoma; NHL: non-Hodgkin lymphoma; NT: not treated (i.e., dimethylsulfoxide vehicle control); PIR: proteasome inhibitor-resistant.

AL amyloidosis, mantle cell lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma (Figure 1D). Importantly, the BDQ-CFZ combination showed no synergistic toxicity in PBMC from healthy donors, immortalized B-lymphocytes (IST EBV TW6B), and bone marrow-derived stromal cells (HS-5) (Figure 2A-C).

To confirm that BDQ-CFZ synergy is mediated via ATP synthase inhibition, we tested the alternative oligomycin-derived ATP synthase inhibitor 1 (ATPSi). ATPSi-CFZ co-treatment significantly reduced the viability of AMO-1 and RPMI-8226 cells, including their PI-resistant derivatives (P<0.0001) (Figure 2D). Accordingly, ATP5F1C knockdown sensitized AMO-1 cells to CFZ (P<0.0001) (Figure 2E; *Online Supplementary Figure S2A, B*), Conversely, ATP5F1C overexpression mitigated BDQ-CFZ-induced cytotoxicity (P<0.0001) (Figure 2F; *Online Supplementary Figure S2C, D*). Collectively, these results demonstrate that BDQ and BDQ-F synergize with CFZ across diverse hematologic malignancies, selectively inducing cytotoxicity via modulation of ATP5F1C, without harming healthy cells.

Bedaquiline potentiates proteasome inhibition and specifically synergizes with proteasome inhibitors in multiple myeloma

To elucidate the basis of BDQ-CFZ synergy in MM, we first profiled early apoptotic and stress responses. Immunoblotting of RPMI-8226 and AMO-1 cells 16 h after treatment revealed robust activation of caspase-3, PARP cleavage, and increased phosphorylation of H2A.X and RPA32, indicating enhanced apoptosis and DNA damage (Figure 3A; *Online Supplementary Figure S3A*). BDQ–CFZ co-treatment significantly reduced oxygen consumption rate and increased levels of mitochondrial reactive oxygen species

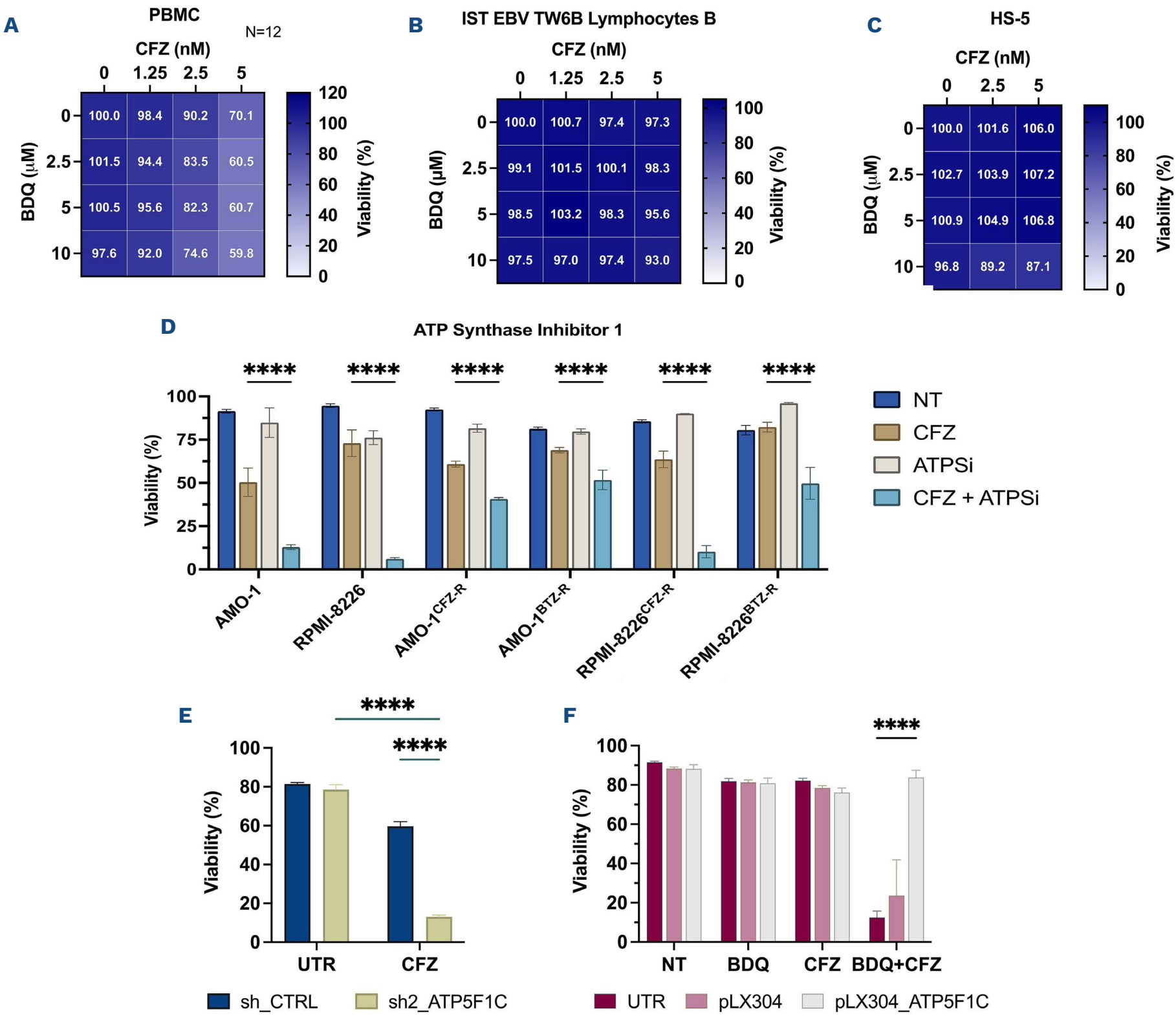


Figure 2. Bedaquiline-carfilzomib co-treatment selectively induces cytotoxicity in multiple myeloma cells via ATP5F1C modulation. (A-C) Dose-response matrices showing cell viability after co-treatment with bedaquiline (BDQ) and carfilzomib (CFZ) in normal human cell models. (A) Peripheral blood mononuclear cells (PBMC) from 12 healthy donors. (B) Epstein-Barr virus (EBV)-transformed B lymphocytes (IST EBV TW6B; N=3). (C) HS-5 bone marrow stromal cells (N=3). Viability was measured by propidium iodide staining (PBMC) or tetramethylrhodamine (TMRM) staining (IST EBV TW6B, HS-5) 96 hours after treatment. (D) Viability of proteasome inhibitor-sensitive (AMO-1, RPMI-8226) and proteasome inhibitor-resistant (AMO-1^{CFZ-R}, AMO-1^{BTZ-R}, RPMI-8226^{CFZ-R}, and RPMI-8226^{BTZ-R}) multiple myeloma cells treated with ATP synthase inhibitor, CFZ, or their combination. Viability was assessed 72 hours after treatment using TMRM staining and flow cytometry. (E) Viability of AMO-1 cells expressing sh_CTRL or sh2_ATP5F1C treated with 3 nM CFZ or vehicle (dimethylsulfoxide, DMSO), measured by TMRM staining 72 hours after treatment. (F) Viability of AMO-1 cells transduced with pLX304 or pLX304_ATP5F1C and treated with 2.5 μM BDQ, 2.5 nM CFZ, their combination, or vehicle (DMSO). Viability was assessed 6 days after treatment by TMRM staining and flow cytometry. Data represent the mean ± standard deviation from three independent experiments unless otherwise indicated. Statistical significance was assessed using one-way analysis of variance (****P<0.0001). ATPSi: ATP synthase inhibitor; CTRL: control; NT: not treated (i.e., dimethylsulfoxide vehicle control); UTR: untranslated region.

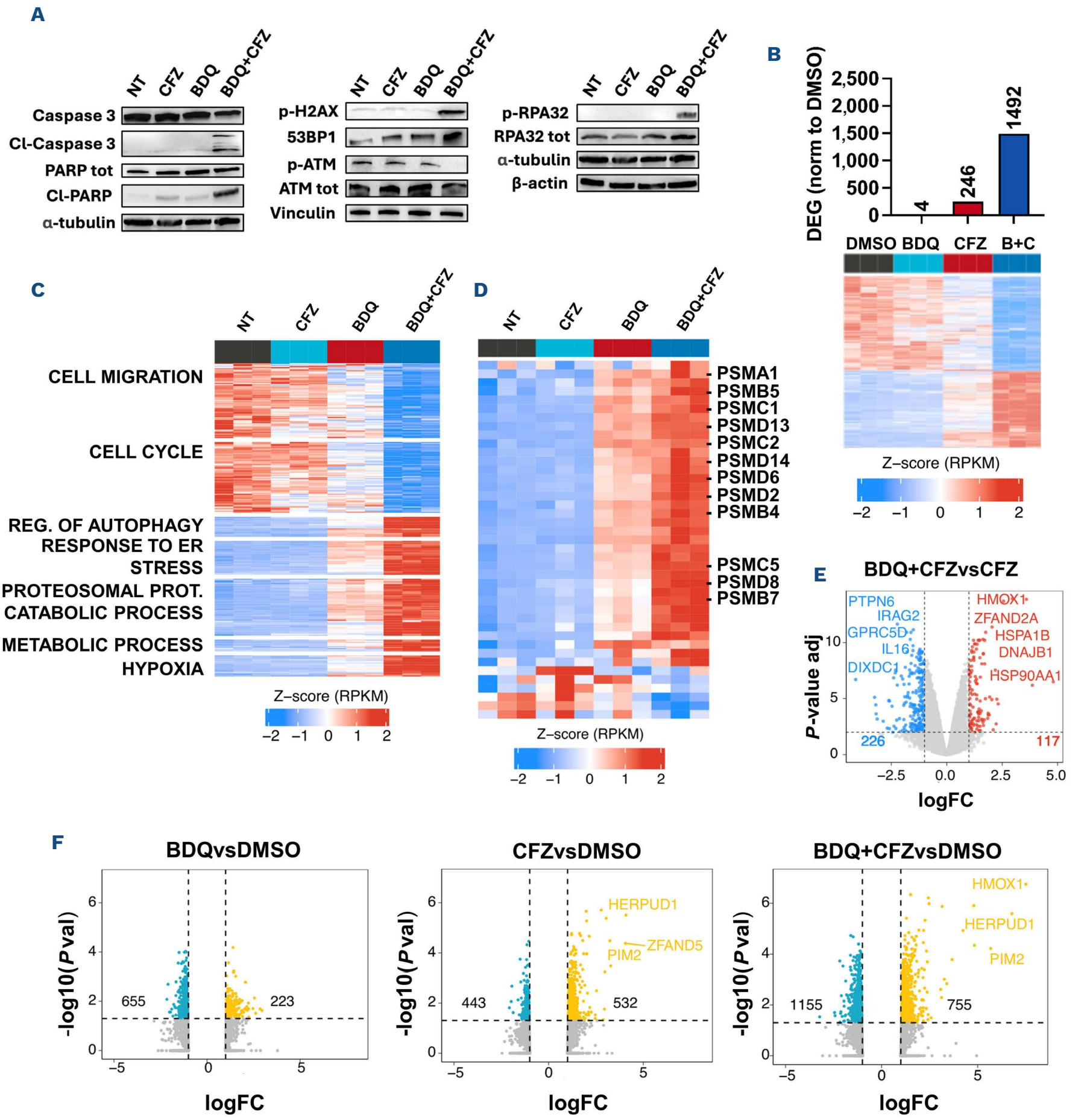


Figure 3. Bedaquiline-carfilzomib co-treatment triggers global transcriptional reprogramming and proteotoxic stress in multiple myeloma cells. (A) Immunoblot analysis of apoptotic and DNA damage markers in AMO-1 cells treated for 16 hours with bedaquiline (BDQ) (5 μM), carfilzomib (CFZ) (2.5 nM), their combination (BDQ-CFZ), or left untreated. α-Tubulin and β-actin were used as loading controls. (B) RNA sequencing analysis of AMO-1 cells reveals extensive transcriptional remodeling following BDQ-CFZ treatment. Bar plot: number of differentially expressed genes (DEG) relative to untreated controls. Heatmap: hierarchical clustering of DEG across treatment groups. (C) Gene ontology enrichment analysis of DEG in BDQ-CFZ-treated cells, identifying upregulation of pathways involved in autophagy, endoplasmic reticulum stress, hypoxia, proteasomal degradation, ATP metabolism, and oxidative phosphorylation. (D) Heatmap of Kyoto Encyclopedia of Genes and Genomes (KEGG)-defined proteasome gene expression showing transcriptional upregulation of proteasome components in BDQ-CFZ-treated cells. (E) Volcano plot of transcriptional changes comparing BDQ-CFZ *versus* CFZ treatment. Red: significantly upregulated genes; light blue: significantly downregulated genes; gray: non-significant. (F) Volcano plots of label-free quantitative proteomics comparing protein expression changes in AMO-1 cells treated with BDQ, CFZ, or their combination (BDQ-CFZ). Yellow: upregulated; light blue: downregulated. B+C: bedaquiline plus carfilzomib; DMSO: dimethylsulfoxide; ER: endoplasmic reticulum; FC: fold-change; NT: not treated (i.e., DMSO vehicle control); RPKM: reads per kilobase million.

compared to those after single treatments (*Online Supplementary Figure S2E, F*). In MM cells, BDQ exposure alone decreased oxidative phosphorylation; however, ATP5F1C protein abundance was not detectably altered following BDQ treatment (*Online Supplementary Figure S2G*).[22] Transcriptomic profiling of AMO-1 cells 12 h after treatment, prior to detectable viability loss (*Online Supplementary Figure S4A-C*), revealed 246 differentially expressed genes in response to CFZ, whereas BDQ alone had minimal transcriptional effects. Strikingly, BDQ-CFZ co-treatment led to 1,492 differentially expressed genes (Figure 3B). These included upregulation of autophagy, proteasome catabolism, endoplasmic reticulum stress, and oxidative phosphorylation pathways, alongside downregulation of cell cycle and migration-related genes (Figure 3C). Gene set enrichment analysis further demonstrated coordinated activation of stress and proteostasis-related programs, including unfolded protein response, ubiquitin–proteasome–associated processes, and mitochondrial stress pathways such as mitophagy (*Online Supplementary Figures S5* and *S6*). Cell-cycle–related programs were also perturbed, consistent with measurable alterations in cell-cycle distribution observed in MM cells (*Online Supplementary Figures S3D, E* and *S4G, H*). Notably, BDQ enhanced CFZ-induced expression of proteasome subunits (Figure 3D), consistent with previously described compensatory feedback mechanisms.[23] Independent real-time quantitative polymerase chain reaction analysis confirmed several of the most prominently upregulated and downregulated genes (Figure 3E; *Online Supplementary Figure S4D-F*). Complementary label-free quantitative proteomics further substantiated the synergistic impact of BDQ-CFZ treatment, revealing a marked increase in upregulated proteins compared to single-agent exposure (Figure 3F). Integrated analysis of transcriptomic and proteomic data highlighted convergent activation of cellular stress-response pathways (*Online Supplementary Figure S7*).

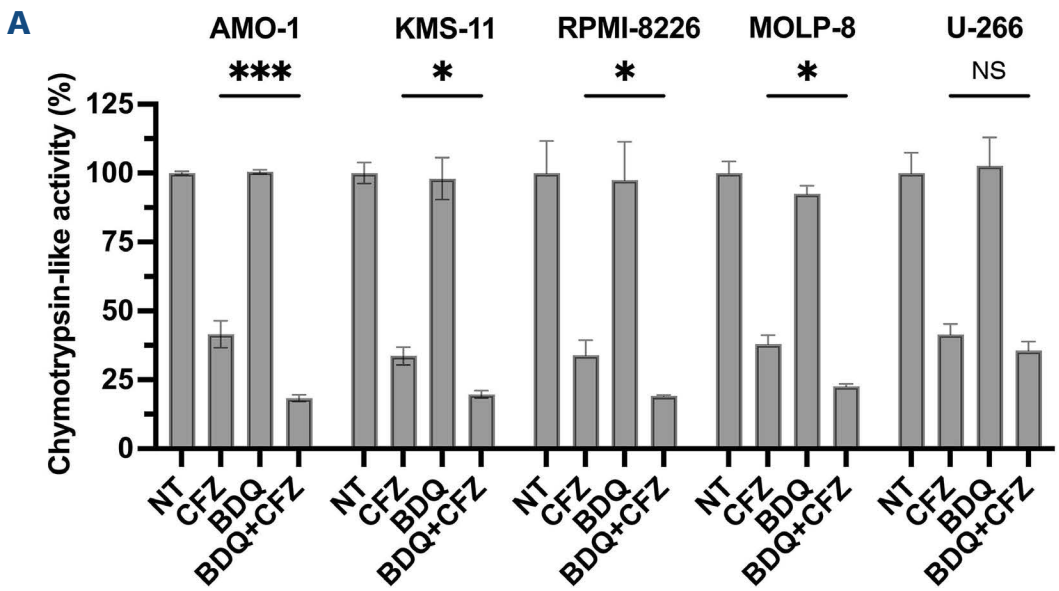
Importantly, we observed that BDQ potentiated CFZ-mediated chymotrypsin-like proteasome inhibition across MM cell lines, but not in low-responder U-266 cells (Figure 4A). This enhancement was selective to chymotrypsin-like activity, as caspase-like and trypsin-like activities remained largely unaffected (*Online Supplementary Figure S8A, B*). Proteasome blockade was confirmed in AMO-1 and AMO-$1^{BTZ\text{-}R}$ cells by expressing a short-lived green fluorescence protein (GFP) fusion protein (Ub-G76V-GFP), showing an increased GFP signal following BDQ-CFZ treatment, before cell death commitment (Figure 4B, C; *Online Supplementary Figure S8C-E*). BDQ synergized with other PI, including bortezomib, ixazomib, and marizomib, as well as the ubiquitin-activating enzyme inhibitor TAK-243 across a broad range of PI-sensitive and PI-resistant MM cell lines (Figure 4E-H; *Online Supplementary Figure S9*). However, no synergy was observed with standard agents (e.g., dexamethasone, venetoclax, lenalidomide, melphalan, or doxorubicin), underscoring the specificity of BDQ's interaction with drugs targeting the ubiquitin-proteasome system (*Online Supplementary Figure S10*). Together, these findings reveal that BDQ-CFZ co-treatment induces broad transcriptional and proteomic stress responses, selectively enhancing proteasome inhibition and cytotoxicity in MM cells.

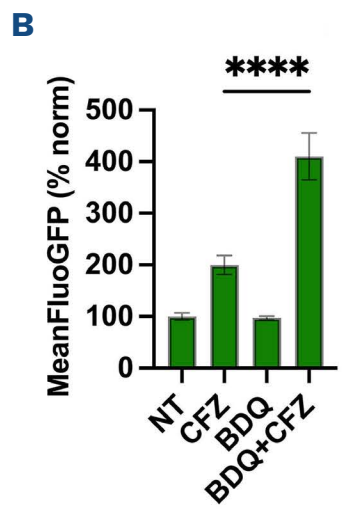
Bedaquiline and carfilzomib co-treatment potentiates proteasome inhibition in patient-derived and *in vivo* models

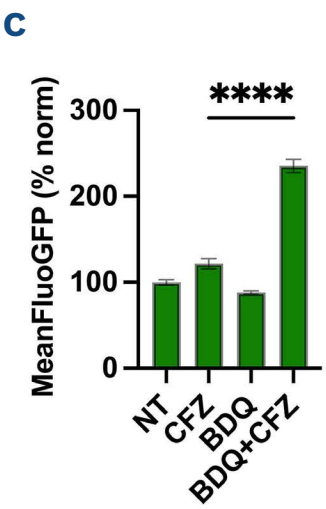
To assess the translational potential of BDQ-CFZ, we tested its efficacy in two- and three-dimensional co-culture systems with HS-5 stromal cells, mimicking the bone marrow niche. The combination significantly increased MM cell death over time in both settings, while sparing stromal cells (Figure 5A, B; *Online Supplementary Figure S11*), demonstrating selective activity even in protective microenvironments. Public gene expression data highlighted that ATP5F1C is upregulated in patients with newly diagnosed or relapsed/refractory MM compared with healthy donors, and more highly expressed in relapsed/refractory MM than in monoclonal gammopathy of undetermined significance (Figure 5C). Analysis of the CoMMpass IA21 cohort (N=631) showed that high ATP5F1C expression (top 30% of the distribution) was significantly associated with inferior progression-free survival and overall survival (log-rank adjusted $P=9.2\times10^{-4}$ and $P=4.7\times10^{-4}$, respectively) (Figure 5D, E). In multivariable Cox proportional hazards models adjusting for age and the Consensus Genomic Staging high-risk classification, as recently defined by the International Myeloma Society and the International Myeloma Working Group,[24] ATP5F1C expression retained independent prognostic significance for both progression-free survival and overall survival (*Online Supplementary Figure S12*A). In contrast, ATP5F1A and ATP5F1B expression did not show comparable associations with survival outcomes in the same cohort (*Online Supplementary Figure S12B-E*). *Ex vivo* treatment of $CD138^+$ cells from 13 MM patients' bone marrow samples showed that BDQ significantly enhanced CFZ-induced cytotoxicity, regardless of cytogenetic background or treatment history ($P<0.01$) (Figure 5F; *Online Supplementary Table S2*). Finally, in a zebrafish xenograft model, BDQ-CFZ reduced $DsRed^+$ tumor burden (Figure 5G), confirming *in vivo* efficacy. These findings support the safety, specificity, and clinical relevance of BDQ-CFZ therapy for MM, and highlight ATP5F1C as a potential biomarker for therapeutic response.

Discussion

The present study demonstrates that the ATP synthase inhibitor BDQ, along with its fumarate salt form (BDQ-F), exhibits consistent and robust synergy with agents targeting the ubiquitin-proteasome system across various MM models, as well as in amyloidosis and diverse B-cell







D

AMO-1

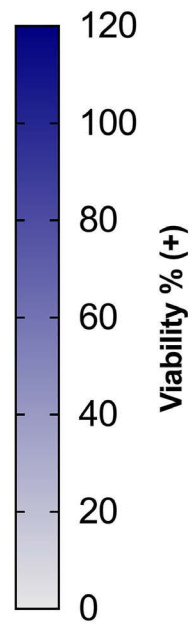
		0	1.5nM	2nM	2.5nM	5nM	10nM
CFZ	NT	100.0	83.3	86.9	80.7	57.6	15.3
	+BDQ	91.3	102.0	55.9	28.2	6.4	6.8

		0	1.5nM	2nM	2.5nM	5nM	10nM
BTZ	NT	100.0	92.8	81.9	23.1	1.4	6.1
	+BDQ	91.3	67.8	59.8	21.2	5.2	5.9

		0	1.5nM	2nM	5nM	10nM	20nM
IXA	NT	100.0	88.6	76.8	57.3	8.7	0.5
	+BDQ	91.3	62.2	37.7	30.7	10.2	0.1

		0	1.5nM	2nM	2.5nM	5nM	10nM
MAR	NT	100.0	95.1	84.6	73.4	24.9	10.0
	+BDQ	91.3	74.9	46.2	46.8	13.1	9.9

		0	25nM	50nM	100nM	200nM	400nM
TAK	NT	100.0	87.7	85.7	72.6	56.8	21.1
	+BDQ	96.5	78.9	61.1	18.7	4.7	1.6



E

AMO-1	BS
BTZ	5.5
IXA	21.1
MAR	14.4
TAK	29.5
CFZ	19.3

F

AMO-1 CFZ-R

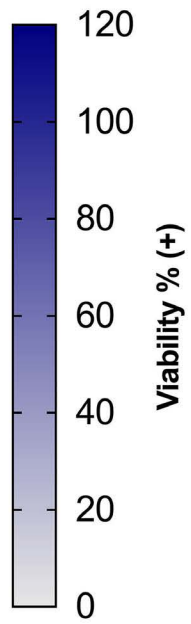
		0	50nM	100nM	200nM	400nM	800nM
CFZ	NT	100.0	91.2	71.7	22.7	19.8	3.2
	+BDQ	111.7	15.2	6.7	2.5	6.5	1.6

		0	2.5nM	5nM	10nM	20nM	40nM
BTZ	NT	100.0	111.3	112.5	94.9	36.6	6.4
	+BDQ	93.2	95.5	84.3	40.7	6.3	1.2

		0	12.5nM	25nM	50nM	75nM	100nM
IXA	NT	100.0	90.5	77.1	41.3	22.3	8.6
	+BDQ	111.7	83.2	74.3	33.4	14.8	6.7

		0	1.25nM	2.5nM	5nM	10nM	20nM
MAR	NT	100.0	84.7	67.7	16.5	4.2	1.0
	+BDQ	73.6	72.4	52.6	12.8	4.4	1.4

		0	25nM	50nM	100nM	200nM	400nM
TAK	NT	100.0	103.2	76.2	93.9	25.0	6.2
	+BDQ	111.7	93.4	46.0	11.4	4.9	12.9



G

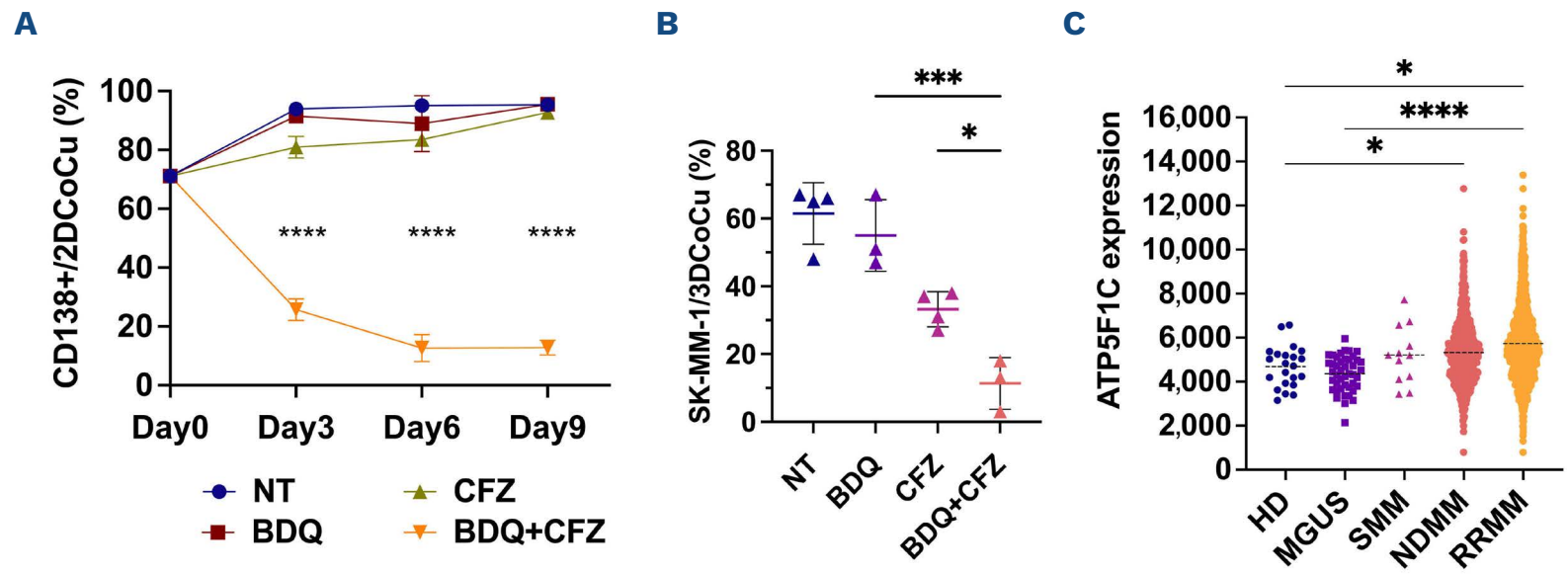
AMO-1^{CFZR}	BS
BTZ	21.8
IXA	10.6
MAR	- 3.5
TAK	34.3
CFZ	49.6

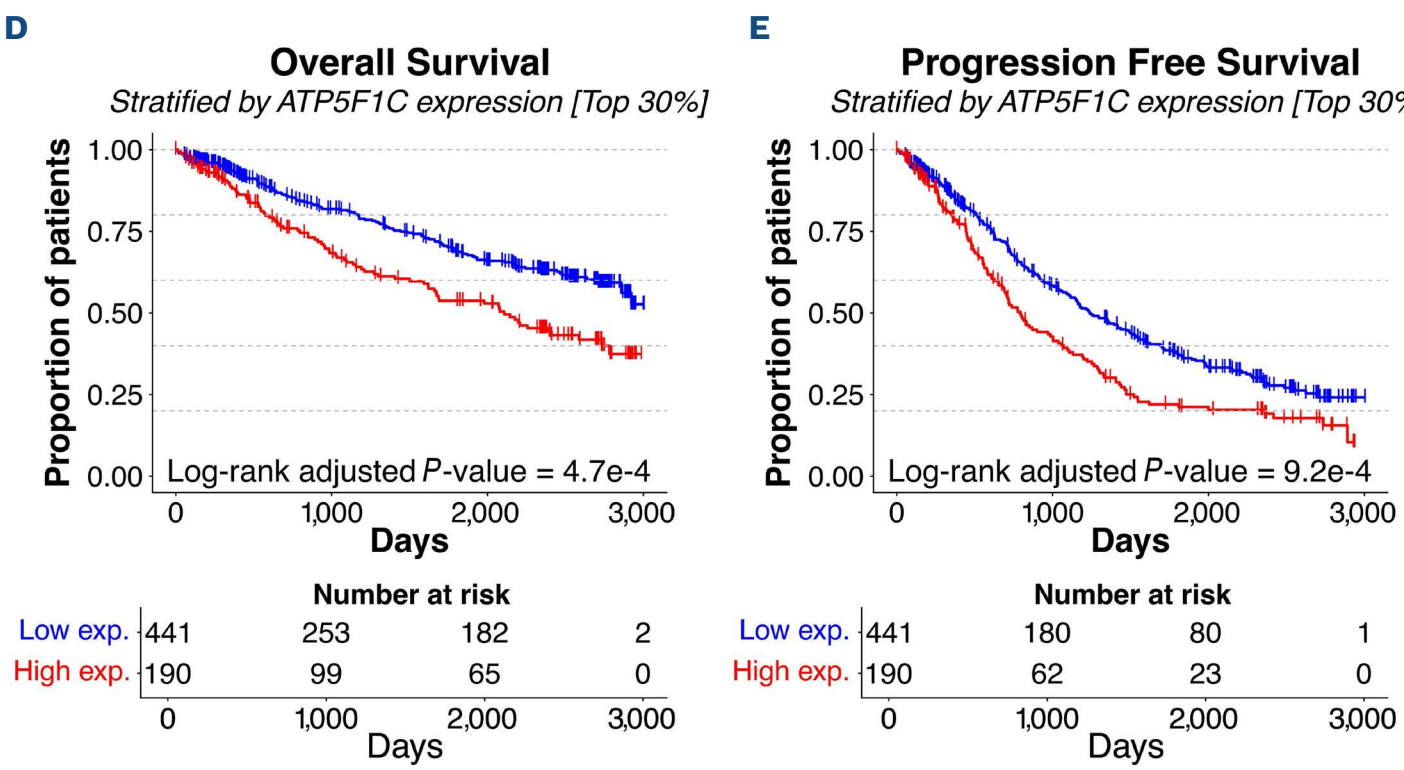
Figure 4. Bedaquiline potentiates proteasome inhibition and selectively synergizes with proteasome-targeting agents in multiple myeloma. (A) Chymotrypsin-like proteasome activity was measured 5 hours after treatment with bedaquiline (BDQ) (5 μM or 10 μM for U-266), carfilzomib (CFZ) (2.5 nM), or their combination in a panel of multiple myeloma cell lines (AMO-1, KMS-11, RPMI-8226, MOLP-8, and U-266). BDQ significantly enhanced CFZ-mediated proteasome inhibition in all cell lines except U-266, which showed reduced sensitivity to the combination. (B, C) Quantification of proteasome inhibition using a Ub-G76V-GFP reporter assay in AMO-1 and AMO-1^{BTZ-R} cells. Flow cytometry analysis showed increased accumulation of green fluorescent pro-

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tein upon BDQ-CFZ treatment, indicating enhanced proteasome inhibition. Fluorescence intensity was normalized to untreated controls and represents the average of three independent experiments (mean ± standard deviation). Statistical analysis confirmed significant synergy in both parental and resistant cells (****P<0.0001, one-way analysis of variance). (D, F) Heatmaps representing cell viability of AMO-1 (D) and AMO-1^{CFZ-R} (F) cell lines treated with increasing concentrations of CFZ, bortezomib, ixazomib, marizomib, and TAK-243 in the presence or absence of 10 μM BDQ. (E, F) The tables indicate the Bliss score for AMO-1 (E) and AMO-1^{CFZ-R} (G) for each compound tested in the presence of BDQ. Strong synergistic combination scores are highlighted in green, mild synergistic scores in yellow, and non-synergistic ones in red. Cell viability was measured with ATPLite 72 hours after treatment. BS: Bliss score; BTZ: bortezomib; IXA: ixazomib; MAR: marizomib; NT: not treated (i.e., dimethylsulfoxide vehicle control); TAK: TAK-243.

malignancies. BDQ is an orally bioavailable antimicrobial agent originally developed to inhibit ATP synthase in *Mycobacterium tuberculosis*, and has revolutionized the therapeutic landscape for multidrug-resistant tuberculosis.[25] In addition, BDQ can inhibit the γ-subunit of human mitochondrial ATP synthase, ATP5F1C.[26] Importantly, ATP5F1C plays an essential role in mitochondrial ATP production and has been linked to increased tumor aggressiveness and adverse clinical outcomes.[22,27] Mechanistic validation using both genetic knockdown and pharmacological inhibition of ATP5F1C phenocopied BDQ-CFZ cytotoxicity, while overexpression conferred resistance, thereby confirming ATP5F1C as the key functional target. Transcriptomic and proteomic analyses revealed that BDQ co-treatment with CFZ amplified stress-response pathways, inducing DNA damage, apoptosis, and proteotoxic stress. Although BDQ–CFZ induced measurable alterations in cell-cycle distribution, these effects were not uniform across MM models and did not reflect a consistent cytostatic arrest. Instead, they likely represent context-dependent consequences of stress-induced apoptosis. Importantly, BDQ potentiated CFZ-mediated inhibition of chymotrypsin-like





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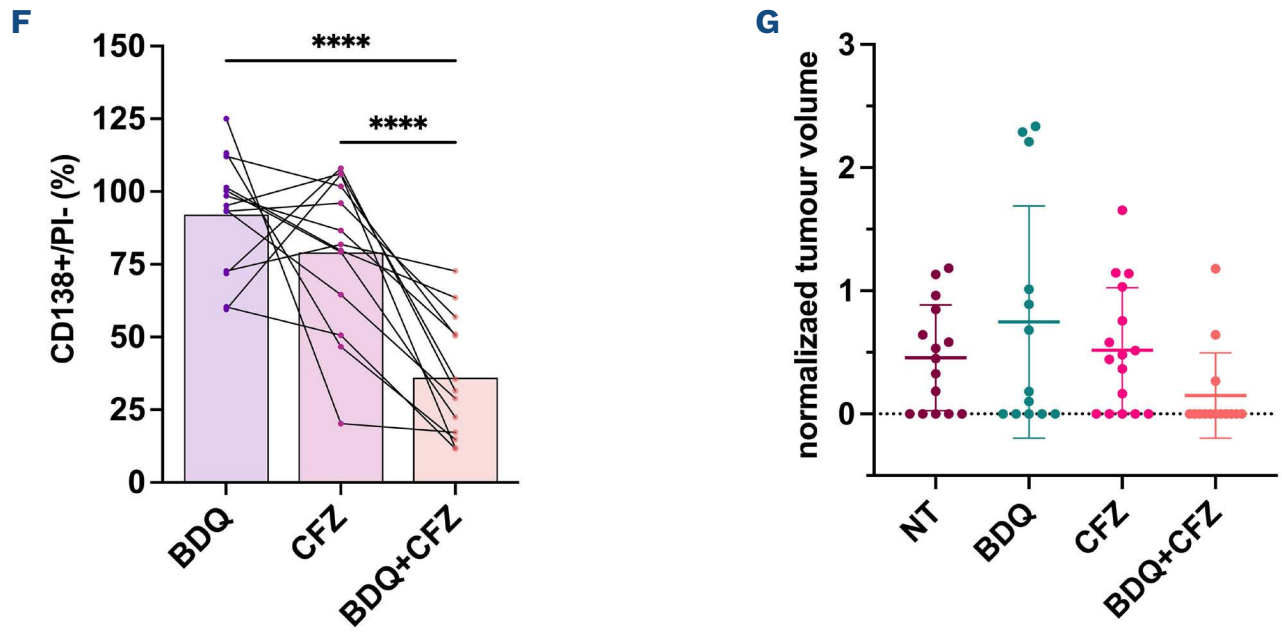


Figure 5. The combination of bedaquiline and carfilzomib enhances multiple myeloma cell death in patient-derived samples and *in vivo* models. (A, B) Co-treatment with bedaquiline (BDQ) and carfilzomib (CFZ) potentiates multiple myeloma (MM) cell death in bone marrow–mimicking microenvironments. (A) AMO-1 cells were cultured in two-dimensions with GFP$^+$ HS-5 stromal cells and treated with dimethylsulfoxide (DMSO), BDQ (5 μM), CFZ (2.5 nM), or their combination at day 0 and day 3. Cell viability was assessed by flow cytometry using propidium iodide exclusion, followed by identification of myeloma cells based on CD138 expression. Data are expressed as the percentage of viable MM cells within the total live cell population at the indicated timepoints (left). Statistical analysis: two-way analysis of variance (N=2-3). (B) In a three-dimensional co-culture system, GFP$^+$ HS-5 stromal cells and tRFP$^+$ SK-MM1 MM cells were seeded into scaffolds and treated with CFZ (6 nM), BDQ (10 μM), or their combination. MM cell viability was quantified by flow cytometry 6 days after treatment by gating on live cells and identifying MM cells based on fluorescent labeling. BDQ-CFZ co-treatment significantly reduced the viability of MM cells compared to single agents (Tukey multiple comparisons test, P=0.0242; N=3-4). *P<0.05; **P<0.01; ***P<0.001. (C) ATP5F1C expression increases with disease progression across healthy donors, and patients with monoclonal gammopathy of undetermined significance, smoldering MM, newly diagnosed MM and relapsed/refractory MM (data from GSE2658, GSE5900, GSE31161). (D, E) High ATP5F1C expression is associated with inferior overall survival and progression-free survival in MM patients from the CoMMpass study (IA21, N=631). Patients were stratified according to ATP5F1C expression (top 30% *vs.* remaining patients). Statistical significance was assessed using the log-rank test with correction for multiple testing. (F) BDQ enhances CFZ-induced cytotoxicity in primary MM cells. Bone marrow mononuclear cells from 13 MM patients were treated with CFZ, BDQ, or the combination for 96 hours. Viability was assessed by flow cytometry using propidium iodide and CD138 staining and normalized to DMSO controls. Drug concentrations for each patient are listed in *Online Supplementary Table S5*. (G) *In vivo* validation using a zebrafish xenograft model. RPMI-8226 cells (tRFP$^+$) were injected into zebrafish embryos and treated with BDQ, CFZ, or their combination. Tumor burden at 72 hours post injection (hpi) was normalized to tumor area at 24 hpi (DMSO: N=15; CFZ: N=16; BDQ: N=13; BDQ-CFZ: N=14). 2DCoCu: two-dimensional co-culture; 3DCoCu: three-dimensional co-culture; exp: expression; HD: healthy donors; MGUS: monoclonal gammopathy of undetermined significance; NDMM: newly diagnosed MM; NT: not treated (i.e., DMSO vehicle control); PI: propidium iodide; RRMM: relapsed/refractory MM; SMM: smoldering MM.

proteasome activity. This suggests that BDQ, by impairing ATP production and disrupting oxidative phosphorylation, exacerbates proteotoxic stress induced by proteasome inhibition. While BDQ has been reported to reduce ATP5F1C protein abundance in metastatic breast cancer models,[22] we did not detect changes in ATP5F1C protein levels in MM cells despite impaired mitochondrial respiration, suggesting functional modulation of ATP synthase activity rather than altered protein abundance. In addition to these stress-related programs, we observed down-modulation of GPRC5D expression following BDQ–CFZ treatment. Given the ongoing clinical development of GPRC5D-targeting immunotherapies, modulation of antigen expression may be relevant when considering treatment sequencing or combination strategies.[28,29] However, the biological functions and regulatory pathways of GPRC5D in MM remain incompletely defined, and its role in proliferation or drug resistance has not been fully elucidated.[29] Further studies will be required to clarify the functional and therapeutic implications of GPRC5D modulation. While HS5 stromal cells enabled reproducible assessment of BDQ–CFZ activity in a bone marrow–mimicking setting, evaluation of this combination in primary bone marrow–derived stromal co-cultures will be important to further delineate its impact within a patient-specific microenvironment.

Beyond the mechanistic evidence, survival analyses in the CoMMpass cohort further supported the clinical significance of ATP5F1C. The independent prognostic impact of ATP5F1C expression, even after adjustment for age and contemporary high-risk cytogenetic classification, underscores the clinical relevance of mitochondrial ATP synthase activity in the biology of MM. These findings suggest that elevated ATP5F1C expression captures a component of disease aggressiveness not fully explained by established genomic risk features. Notably, the lack of comparable prognostic significance for ATP5F1A and ATP5F1B further supports the

specific relevance of the γ subunit in MM progression.

The observed synergy highlights a therapeutic opportunity to exploit metabolic vulnerabilities to overcome PI resistance. By acting through energy stress pathways, BDQ may circumvent canonical resistance mechanisms and sensitize cells to ubiquitin-proteasome system-targeted therapy. Although zebrafish models provide robust *in vivo* proof-of-concept validation, further evaluation of BDQ–CFZ activity in murine xenograft systems will be important to define its pharmacological and translational potential in mammalian contexts. Importantly, BDQ's clinical approval supports translational development; however, its preferential inhibition of mycobacterial ATP synthase at submicromolar concentrations[24] raises important pharmacological considerations. The effective *in vivo* dosing required to achieve sustained inhibition of mitochondrial ATP synthase in cancer cells remains to be defined, and more selective inhibitors targeting the γ subunit of mitochondrial ATP synthase should be developed. It also remains to be determined whether inhibition of other subunits (e.g., α or β) would confer comparable antitumor synergy. In addition, ATP depletion may disrupt broader mitochondrial functions or intersect with the tricarboxylic acid cycle, contributing to cytotoxicity. Dissecting these downstream consequences will be essential to fully elucidate the mechanism of action and to rationally design future combination regimens.

In summary, this study supports a therapeutic strategy combining proteasomal and metabolic stress to restore drug sensitivity in MM. The clinical availability of BDQ offers a translational opportunity, while future work should refine target specificity, optimize pharmacological parameters, and explore this approach in other malignancies characterized by proteotoxic stress.

Disclosures

No conflicts of interest to disclose.

Contributions

MC carried out most of the experiments and contributed to the interpretation of biological data together with EMe, ML, MP, and MM. EMe performed drug screening experiments and analysis. BL studied bedaquiline combinations with proteasome inhibitors. AT, MF, and FA performed RNA-sequencing experiments and bioinformatics analyses. SO supervised bioinformatics analyses. PEP supervised biochemical assays. AL, MD'A, FG, and BB provided clinically annotated multiple myeloma samples. MC, MM, and CB performed zebrafish xenograft experiments. EMo and NM participated in the experimental design and interpretation of biological data. RP designed the studies and supervised the project. MC and RP wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Data sharing statement

RNA-sequencing and proteomics datasets generated during this study are available from the corresponding author upon reasonable request. Processed data supporting the findings of this study are included in the article and its Online Supplementary Material.

References

1. Kumar SK, Rajkumar V, Kyle RA, et al. Multiple myeloma. Nat Rev Dis Primers. 2017;3(1):17046.
2. van de Donk N, Pawlyn C, Yong KL. Multiple myeloma. Lancet. 2021;397(10272):410-427.
3. Costa LJ, Gay F, Landgren O, et al. Evolution of frontline treatment for multiple myeloma: clinical investigation of quadruplets containing carfilzomib and anti-CD38 monoclonal antibodies. Ann Hematol. 2025;104(3):1329-1351.
4. Kumar SK, Callander NS, Adekola K, et al. NCCN guidelines(R) insights: multiple myeloma, version 1.2025. J Natl Compr Canc Netw. 2025;23(5):132-140.
5. Sonneveld P, Dimopoulos MA, Boccadoro M, et al. Daratumumab, bortezomib, lenalidomide, and dexamethasone for multiple myeloma. N Engl J Med. 2024;390(4):301-313.
6. Jurczyszyn A, Bator M, Vesole DH, et al. New therapies in multiple myeloma: benefits and limitations. Pol Arch Intern Med. 2025;135(4):16994.
7. Neri P, Leblay N, Lee H, et al. Just scratching the surface: novel treatment approaches for multiple myeloma targeting cell membrane proteins. Nat Rev Clin Oncol. 2024;21(8):590-609.
8. Gay F, Marchetti E, Bertuglia G. Multiple myeloma unpacked. Hematol Oncol. 2025;43(Suppl 2):e70067.

9. Mitsiades CS. Proteasome inhibitors in multiple myeloma: biological insights on mechanisms of action or resistance informed by functional genomics. Hematol Oncol Clin North Am. 2024;38(2):321-336.
10. Setton J, Zinda M, Riaz N, et al. Synthetic lethality in cancer therapeutics: the next generation. Cancer Discov. 2021;11(7):1626-1635.
11. Manier S, Salem KZ, Park J, et al. Genomic complexity of multiple myeloma and its clinical implications. Nat Rev Clin Oncol. 2017;14(2):100-113.
12. Maura F, Rajanna AR, Ziccheddu B, et al. Genomic classification and individualized prognosis in multiple myeloma. J Clin Oncol. 2024;42(11):1229-1240.
13. Kropivsek K, Kachel P, Goetze S, et al. Ex vivo drug response heterogeneity reveals personalized therapeutic strategies for patients with multiple myeloma. Nat Cancer. 2023;4(5):734-753.
14. Fricker LD. Proteasome inhibitor drugs. Annu Rev Pharmacol Toxicol. 2020;60:457-476.
15. Milan E, Gulla A. Editorial: Proteomic and metabolic reprogramming in myeloma cells within the tumor microenvironment. Front Oncol. 2023;13:1264740.
16. Renatino Canevarolo R, Sudalagunta PR, Meads MB, et al. Epigenetic plasticity drives carcinogenesis and multi-therapy resistance in multiple myeloma. Research Square. Apr 14, 2025. https://doi.org/10.21203/rs.3.rs-6306816/v1 [preprint, not peer-reviewed].
17. Bandini C, Mereu E, Paradzik T, et al. Lysin (K)-specific demethylase 1 inhibition enhances proteasome inhibitor response and overcomes drug resistance in multiple myeloma. Exp Hematol Oncol. 2023;12(1):71.
18. Bergaggio E, Riganti C, Garaffo G, et al. IDH2 inhibition enhances proteasome inhibitor responsiveness in hematological malignancies. Blood. 2019;133(2):156-167.
19. Mereu E, Abbo D, Paradzik T, et al. Euchromatic histone lysine methyltransferase 2 inhibition enhances carfilzomib sensitivity and overcomes drug resistance in multiple myeloma cell lines. Cancers (Basel). 2023;15(8):2199.
20. Zhang Y, Lai Y, Zhou S, et al. Inhibition of M. tuberculosis and human ATP synthase by BDQ and TBAJ-587. Nature. 2024;631(8020):409-414.
21. Niepel M, Hafner M, Duan Q, et al. Common and cell-type specific responses to anti-cancer drugs revealed by high throughput transcript profiling. Nat Commun. 2017;8(1):1186.
22. Fiorillo M, Scatena C, Naccarato AG, Sotgia F, Lisanti MP. Bedaquiline, an FDA-approved drug, inhibits mitochondrial ATP production and metastasis in vivo, by targeting the gamma subunit (ATP5F1C) of the ATP synthase. Cell Death Differ. 2021;28(9):2797-2817.
23. Meiners S, Heyken D, Weller A, et al. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. J Biol Chem. 2003;278(24):21517-21525.
24. Avet-Loiseau H, Davies FE, Samur MK, et al. International Myeloma Society/International Myeloma Working Group Consensus Recommendations on the definition of high-risk multiple myeloma. J Clin Oncol. 2025;43(24):2739-2751.
25. Guglielmetti L, Khan U, Velasquez GE, et al. Oral regimens for rifampin-resistant, fluoroquinolone-susceptible tuberculosis. N Engl J Med. 2025;392(5):468-482.
26. Luo M, Zhou W, Patel H, et al. Bedaquiline inhibits the yeast and human mitochondrial ATP synthases. Commun Biol. 2020;3(1):452.
27. Wu X, Li F, Wang X, et al. Antibiotic bedaquiline effectively targets growth, survival and tumor angiogenesis of lung cancer through suppressing energy metabolism. Biochem Biophys Res Commun. 2018;495(1):267-272.
28. Lee H, Ahn S, Gonzales GA, et al. Multimodal antigenic escape to GPRC5D-targeted T cell engagers in multiple myeloma. Nat Med. 2026;32(3):964-977.
29. Wang X, Cui Y, Wang Y, Fang B. Emerging role of G protein-coupled receptor class C group 5 member D-directed immunotherapy in multiple myeloma: advances, resistance and combination strategies. Br J Haematol. 2025;207(5):1765-1778.