EZH2 inhibition overcomes immunomodulatory drug resistance in multiple myeloma via a cereblon-dependent pathway

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Abstract

Immunomodulatory agents (IMiD) and the next-generation cereblon (CRBN) E3 ligase modulators (CELMoD), targeting the IKZF1/IKZF3-IRF4-MYC axis, are effective therapies for multiple myeloma (MM) across all stages of disease. Resistance to treatment can be acquired following exposure, but a subset of patients has primary resistance, with both states necessitating the development of alternative treatment strategies. Enhancer of zeste homolog 2 (EZH2) has been shown to have increased expression at myeloma relapse and higher expression is associated with a shorter progression-free survival from diagnosis. EZH2 inhibitors have been studied as single agents in myeloma and in combination treatments to overcome drug resistance in other malignancies. In this study KMS-11 and RPMI-8226 myeloma cell lines were used as models of primary IMiD resistance, demonstrating persistent interferon regulatory factor 4 (IRF4) expression after IMiD/CELMoD exposure without loss of cell viability. The combination of tazemetostat, a Food and Drug Administration-approved EZH2 inhibitor, with IMiD/CELMoD significantly reduced IRF4 expression, induced apoptosis, and led to synergistic cell death in these resistant cell lines. Further investigations revealed that the synergistic effect of EZH2 inhibition appeared specific to IMiD/CELMoD, was CRBN-dependent and was rescued by IRF4 overexpression. Mechanistically, tazemetostat appeared to reduce IKZF1 binding to the *IRF4* promoter and super-enhancer, explaining how the combination with IMiD/CELMoD which also have this effect may reach the threshold required to suppress IRF4 expression and ultimately inhibit MM cell growth in resistant cell lines. Our findings highlight a potential strategy for treating MM patients with IMiD resistance.

Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by the clonal proliferation of plasma cells in the bone marrow, accounting for approximately 2% of cancers and about 10% of all hematologic malignancies. As the median age of onset is around 70 years there has been a 38% increase in incidence rates since the early 1990s as the population ages.[1] Immunomodulatory drugs (IMiD), such as lenalidomide and pomalidomide, have significantly improved patients' survival,[2] and the next-generation cereblon (CRBN) E3 ligase modulators (CELMoD), including iberdomide and mezigdomide, are currently in phase III clinical trials. IMiD/CELMoD act as molecular glues binding CRBN, a substrate receptor in the CRBN-CRL4 E3 ubiquitin ligase complex.[3,4] This interaction alters CRBN's substrate affinity, leading to the ubiquitination and degradation of two key B-cell transcription factors, IKZF1 (Ikaros) and IKZF3 (Aiolos) (Figure 1A).[4-6] This degradation leads to the downregulation of the IRF4-MYC axis, which is essential for myeloma pathogenesis, and so ultimately inhibits MM cell proliferation. Resistance to treatment can be acquired following exposure. The mechanisms underlying this acquired resistance are heterogeneous and include CRBN mutations and decreased CRBN expression in some but not all patients.[7] A subset of patients has primary or intrinsic resistance to these agents without prior exposure; around 25-40% of patients have primary resistance to lenalidomide used in early line therapy.[8,9] The mechanisms underlying this and to what extent acquired resistance may be reflective

of the selection of intrinsically resistant subclones rather than a *de novo* acquired state, are less well understood, but genetic alteration of CRBN or modified expression is unlikely to be responsible for intrinsic resistance.[10] Studying the underlying reasons for resistance and how it can be overcome may allow combination treatment approaches to mitigate this challenge.

Interferon regulatory factor 4 (IRF4) is a critical dependency in myeloma, and its inhibition leads to MM cell death.[11] The primary mechanisms by which IRF4 contributes to MM

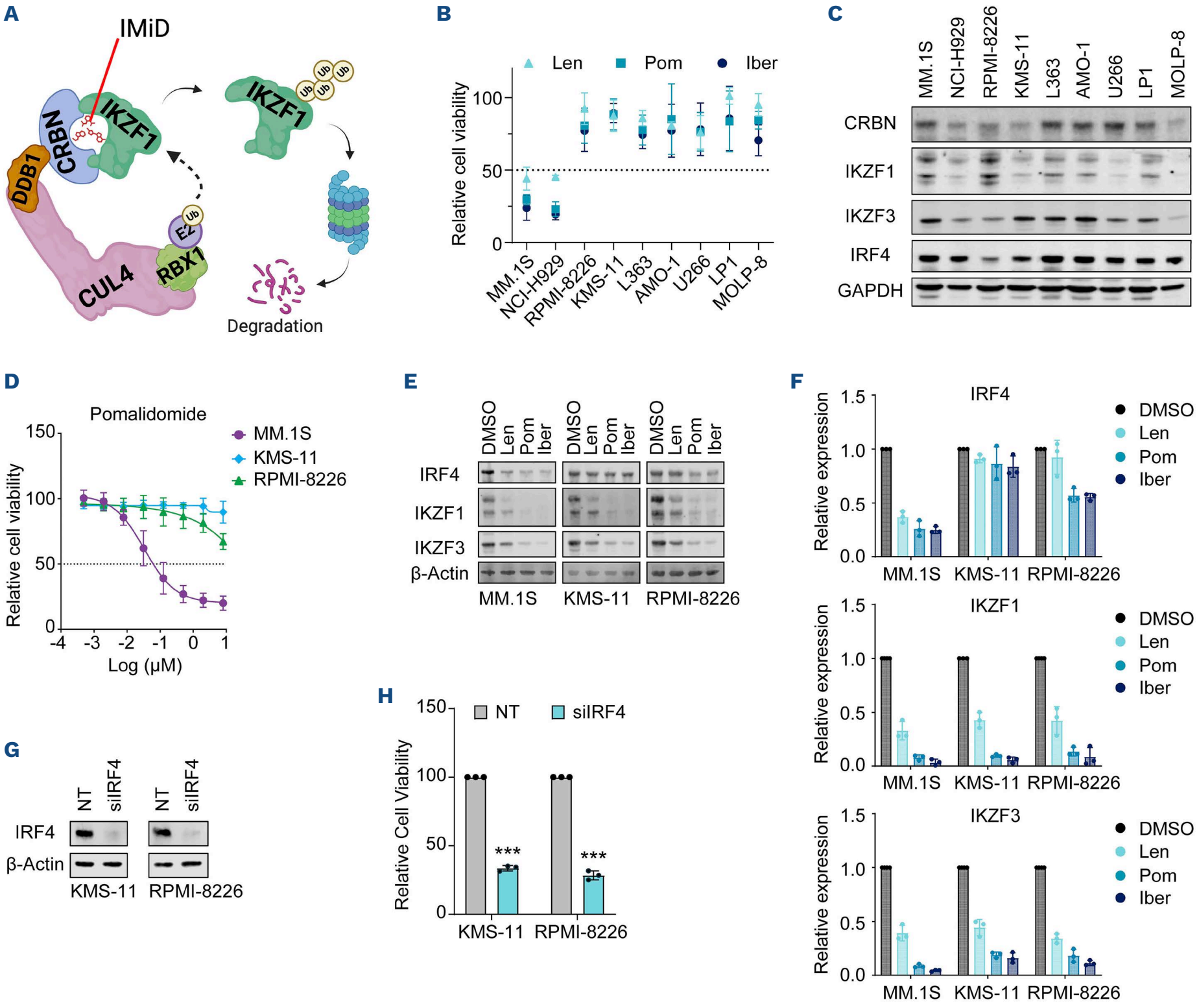


Figure 1. Resistance to immunomodulatory drugs and cereblon E3 ligase modulators is associated with persistent IRF4 expression. (A) Schematic of immunomodulatory drug (IMiD)-cereblon (CRBN)-mediated IKZF1/IKZF3 degradation. IMiD binding alters the substrate specificity of CRBN leading to the binding of IKZF1. This results in ubiquitin tagging which marks IKZF1 for degradation via the proteasome. Figure panel created in BioRender. Pawlyn, C. (2025) *https://BioRender.com/p91v715*. (B) Relative cell viability assessed at day 5 in multiple myeloma (MM) cell lines treated with the indicated IMiD/cereblon E3 ligase modulator (CELMoD) at the concentration of 5 μM. (C) Western blot analysis of indicated proteins in a panel of MM cell lines without treatment. (D) Dose-response curve assessed by a cell viability assay in MM cell lines treated with pomalidomide for 5 days (highest concentration 8 μM). Data shown are mean ± standard deviation (SD), N=3 biological replicates. (E) Western blot analysis of indicated proteins in MM cell lines treated with IMiD/CELMoD (10 μM) for 5 days. (F) Quantification of IRF4, IKZF1 and IKZF3 expression (normalized to actin) in (E). Data shown are mean ± SD, N=3 biological replicates. (G) Western blot analysis of IRF4 knockdown efficiency. (H) Relative cell viability was assessed at day 5 in the indicated MM cell lines treated with siIRF4. Data shown are mean ± SD, N=3 biological replicates. ***P<0.001. CRBN: cereblon; IKZF1: ikaros; DDB1: damage-specific DNA-binding protein 1; CUL4: cullin-4; RBX1: ring-box protein 1; Ub: ubiquitin. Len: lenalidomide; Pom: pomalidomide; Iber: iberdomide; IRF4: interferon regulatory factor 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DMSO: dimethylsulfoxide; NT: not transduced.

pathogenesis are through the IRF4-MYC auto-regulatory oncogenic feedback loop and IRF4 self-regulatory feedback loop.[12] Therefore, targeting the IRF4-MYC axis presents significant challenges as compensatory mechanisms to maintain expression by transcriptional rewiring can arise.[13,14] Enhancer of zeste homolog 2 (EZH2), a catalytic subunit of the polycomb repressive complex 2 (PRC2), mediates histone H3 methylation at lysine 27 (H3K27me3), resulting in transcriptional repression.[15,16] Its role in cancer initiation, progression, and drug resistance has been extensively reviewed.[17] EZH2 is frequently overexpressed in MM, in which it is associated with poor prognosis.[18-20] In the DREAM Challenge analysis, one of the genes whose expression had the highest concordance with shorter progression-free survival was PHF19, which directly regulates the PRC2 complex by binding to H3K36me3, leading to activation of EZH1/2 and H3K27me3.[21] EZH2 itself was also highly correlated. At the DNA level, EZH2 mutations have been identified as novel drivers at relapse with significant enrichment in events, including increased frequency of new mutations and increased cancer cell fraction of previously identified mutations.[22]

Preclinical studies have shown that EZH2 inhibition can suppress oncogenes and restore tumor suppressor gene expression in MM models.[19,23,24] Tazemetostat, the first-in-class EZH2 inhibitor, is approved by the Food and Drug Administration (FDA) for the treatment of follicular lymphoma and epithelioid sarcoma. The combination of an EZH2 inhibitor with other drugs may yield synergistic effects by simultaneously targeting multiple pathways involved in the pathogenesis of MM.[25,26] The combination of 5-azacytidine and an EZH2 inhibitor was shown to restore sensitivity to IMiD in MM cell lines with acquired resistance.[27] In the same study, a suggestion that EZH2 inhibition alone may lead to sensitization to lenalidomide and pomalidomide was raised but not further explored. No studies to date have fully investigated the efficacy of a combination of tazemetostat with IMiD/CELMoD in overcoming primary resistance to IMiD/CELMoD in MM, or explored the underlying mechanism.

Here, we investigated the role of EZH2 inhibition in overcoming primary IMiD/CELMoD resistance using MM cell line models. Our findings provide new insights into the interplay between epigenetic regulation and IMiD/CELMoD sensitivity and suggest a novel approach to treating resistant MM.

Methods

Cell culture and generation of stable cell lines

Human multiple myeloma cell lines, AMO-1, NCI-H929, L363, LP1, MM.1S, MOLP-8, U266, RPMI-8226 and KMS-11 were cultured in RPMI-1640 (61870, Gibco) supplemented with 10% fetal bovine serum (A5256801, Gibco) and 50 U/mL penicillin/streptomycin (15140122, Gibco) in a humidified 37°C incubator with 5% CO2. To generate CRBN knockout (KO) cells, six sgRNA sequences (TAAACAGACATGGCCGGCGA, GTCCTGCTGATCTCCTTCGC, ATATGCCTATCGAGAAGAAC, ATAGTACCTAGGTGCTGATA, CGCACCATACTGACTTCTTG, AAAATCCTGTTCTTCTCGAT) targeting CRBN exons 1-4 were cloned into pSpCas9-BB-2A-GFP (PX458, Addgene #48138)[28] and plasmids were pooled. Plasmids were transfected into the parental KMS-11 cell line using the Nucleofector kit V (Lonza) and Amaxa Nucleofector Technology (Lonza) with G-016 pulsing parameters. Transfected cells were maintained for 48 h prior to isolating green fluorescence protein (GFP)-positive cells via fluorescence activated cell sorting (SH800 Cell Sorter, Sony) and single-cell seeding to generate clonal cell lines. Successful KO was validated after cell line recovery by real-time quantitative polymerase chain reaction (RT-qPCR) (TaqMan Gene expression probes Hs01020593_m1, Hs00372266_m1, Hs00372271_m1, Thermo Fisher Scientific), as well as immunoblotting (CRBN, D8H3S, CST). Re-expression of CRBN mutations (or wild-type CRBN) was performed in KMS-11 and MM.1S cell lines as previously described.[29]

Codon-optimized IRF4 (from gBlocks [IDT]) was cloned into pLenti-CMV-GFP-hygro (656-4, Addgene, #17446) between XbaI and BamHI and was lentivirally transduced into KMS-11 to generate IRF4-GFP overexpressing cells. Cells were selected by hygromycin (250 µg/mL) and sorted by flow cytometry based on GFP expression. The success of viral transduction was validated by detecting codon-optimized *IRF4* mRNA using RT-qPCR (exoIRF4 #1F, ACGATCTCAGTTGGACATAAGTG; #1R, GCTCGGATAAGGTGTAGTCATC; #2F, TCTTAGTGAGCTCCAAGCATTC; #2R, TGATATGTGCTCGGGAAGGTCA), and western blotting.

Cell viability assay

Cell viability assays were employed to evaluate cell viability affected by the EZH2 inhibitor or IMiD as single reagents or in combination. Briefly, if treated with a single reagent, cells were seeded and treated in a 96-well plate and incubated for 120 h. For drug combinations, cells were primed in dimethylsulfoxide (DMSO) or tazemetostat for 120 h, followed by reseeding into a 96-well plate with continued exposure to DMSO/tazemetostat and IMiD/CELMoD at different concentrations for a further 120 h. For siRNA, cells were treated with siRNA in a 12-well plate for 96 h and were then reseeded in a 96-well plate and incubated for 72 h. Cell viability was then measured by CellTiter-Blue (G8081, Promega) according to the manufacturer's instructions. Relative cell viability was calculated by normalizing each treatment condition to its respective controls except where otherwise indicated. A synergy score for drug combination was calculated using SynergyFinder+.[30]

Proteomics

KMS-11 cells were incubated in 0.25 µM tazemetostat or DMSO for 10 days and then in combination with 8 µM po-

malidomide or DMSO for 24 h. Proteomics analysis was performed as previously described[31] with further details provided in the *Online Supplementary Methods*. Differentially expressed proteins were identified using the DEP package[32] in R (4.4.1). $|\log_2$fold change$|$ >0.5 and adjusted *P* values (*P*.adj) <0.05 were used to determine statistical significance.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.30. The Student *t* test (unpaired, two-tailed) was performed to determine significance when comparing data from different groups.

Further methods

Methods for siRNA studies, western blotting, RNA analysis, apoptosis assays, chromatin immunoprecipitation and quantitative polymerase chain reaction (ChIP-qPCR) and proteomics are described in the *Online Supplementary Methods*. This work followed research integrity and ethical standards appropriate to a laboratory study in the UK.

Results

Persistent IRF4 expression is associated with primary resistance to Immunomodulatory drugs and cereblon E3 ligase modulators

To assess IMiD/CELMoD resistance, a panel of MM cell lines representing the inherent molecular heterogeneity of disease were treated with lenalidomide, pomalidomide or iberdomide at various concentrations for 5, days and the half maximal inhibitory concentration (IC_{50}) was calculated (*Online Supplementary Table S1*). MM.1S and NCI-H929 demonstrated the greatest sensitivity to IMiD/CELMoD, as evidenced by reduced cell viability at day 5 compared to other cell lines, while RPMI-8226 and KMS-11 exhibited the greatest resistance (Figure 1B). No clear correlation was observed between resistance and the expression of proteins involved in the CRBN pathway (Figure 1C) or with molecular subtype of disease (*Online Supplementary Table S2*) (adapted from Keats laboratory[33]). Consequently, RPMI-8226 and KMS-11 were selected as the resistant cell line models for further study (Figure 1D).

The primary action of IMiD/CELMoD is degradation of IKZF1/IKZF3 and subsequent downregulation of IRF4 expression via the CRBN pathway. To investigate this effect, MM.1S, KMS-11, and RPMI-8226 were treated with lenalidomide, pomalidomide and iberdomide. The results demonstrated that all three drugs led to IKZF1/IKZF3 degradation (Figure 1E, F). However, significant IRF4 reduction was only observed in MM.1S, a sensitive cell line, not in KMS-11 and RPMI-8226. To confirm that IRF4 remained a key vulnerability in the IMiD-resistant state,[12] KMS-11 and RPMI-8226 were treated with siIRF4 for 5 days, after which cell viability was measured (Figure 1G, H). Depletion of IRF4 significantly reduced cell viability in these cell lines, indicating that IRF4 was essential for KMS-11 and RPMI-8226 cell growth and suggesting its persistent expression contributed to primary IMiD/CELMoD resistance.

EZH2 inhibition overcomes resistance to immunomodulatory drugs and cereblon E3 ligase modulators

To investigate the effect of combining epigenetic modification by an EZH2 inhibitor and IMiD/CELMoD on resistant cell lines, the optimal concentration of tazemetostat was identified that demonstrated an effect on target activity (reduction in H3K27me3) while maintaining cell survival (without significant single-agent activity), based on the dose-response curve of tazemetostat (*Online Supplementary Figure S1*). Cells were initially primed with tazemetostat for 5 days and then treated with pomalidomide or iberdomide for an additional 5 days in the continued presence of tazemetostat (Figure 2A). The results showed that tazemetostat and the IMiD/CELMoD combination significantly reduced cell viability in resistant cell lines compared to cells treated with IMiD/CELMoD monotherapy (Figure 2B), suggesting that EZH2 inhibition could overcome IMiD/CELMoD resistance. Apoptosis was significantly enhanced in KMS-11 and RPMI-8226 cell lines with the combination treatment compared to pomalidomide monotherapy, at both day 5 and day 3 following pomalidomide exposure (Figure 2C, D; *Online Supplementary Figures S2* and *S3*).

EZH2 inhibition is synergistic with immunomodulatory drugs and cereblon E3 ligase modulators

To explore whether the combined effect of EZH2 inhibition with different IMiD/CELMoD exceeded the sum of their individual effects, synergy scores were calculated using the Bliss model in SynergyFinder+.[30] A synergy score above 10 is considered indicative of a synergistic drug combination. Synergy scores of the tazemetostat-pomalidomide combination were 8.43, 10.76 and 13.51 in MM.1S, KMS-11 and RPMI-8226, respectively (Figure 3A), suggesting that the drug combination was synergistic in resistant MM cell lines. The synergy of tazemetostat with other IMiD/CELMoD was further investigated in KMS-11. Lenalidomide, which has the lowest CRBN binding potency, showed no synergy (1.08) with tazemetostat (Figure 3B). Iberdomide and mezigdomide, which have stronger CRBN binding potency, exhibited higher synergy scores (15.66 and 34.53, respectively) (Figure 3B). These results suggested that increased CRBN binding potency correlates with enhanced synergy with tazemetostat.

The synergy of the EZH2 inhibitor with other drugs used in MM therapy was also evaluated. Dexamethasone demonstrated synergy with tazemetostat (Bliss score 16.14), but all other tested drugs (cyclophosphamide, melphalan, bortezomib and doxorubicin) did not (*Online Supplementary*

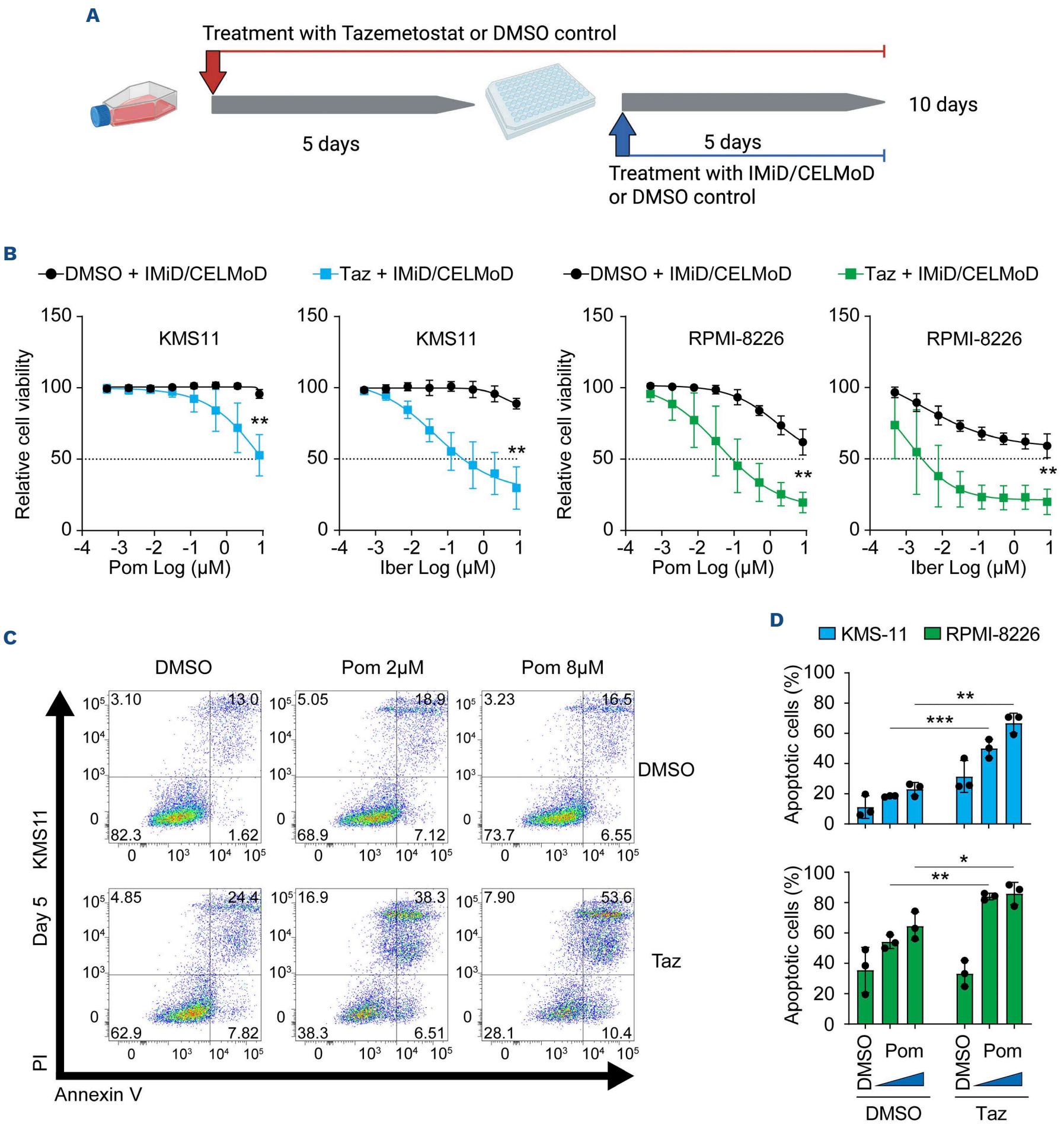
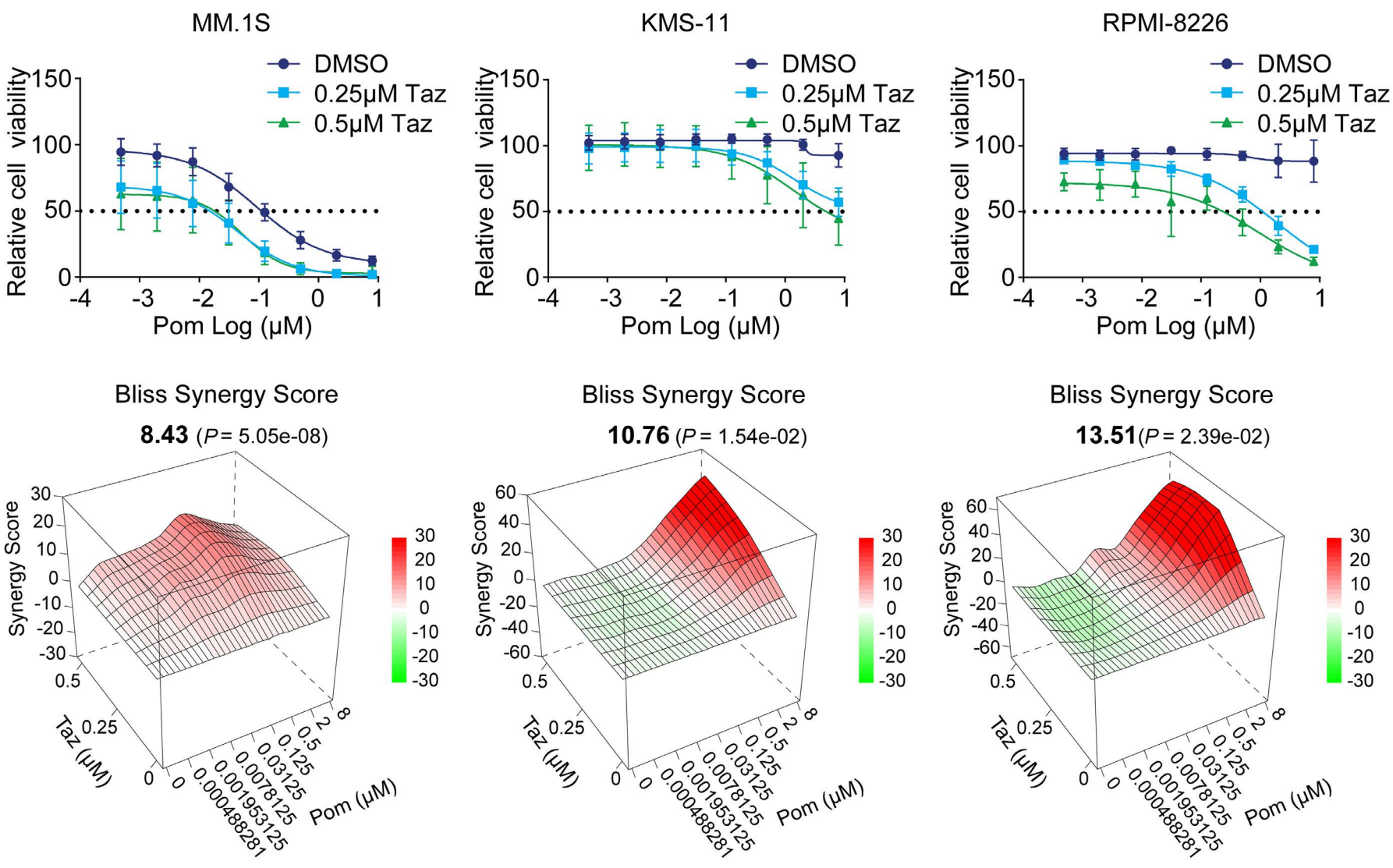
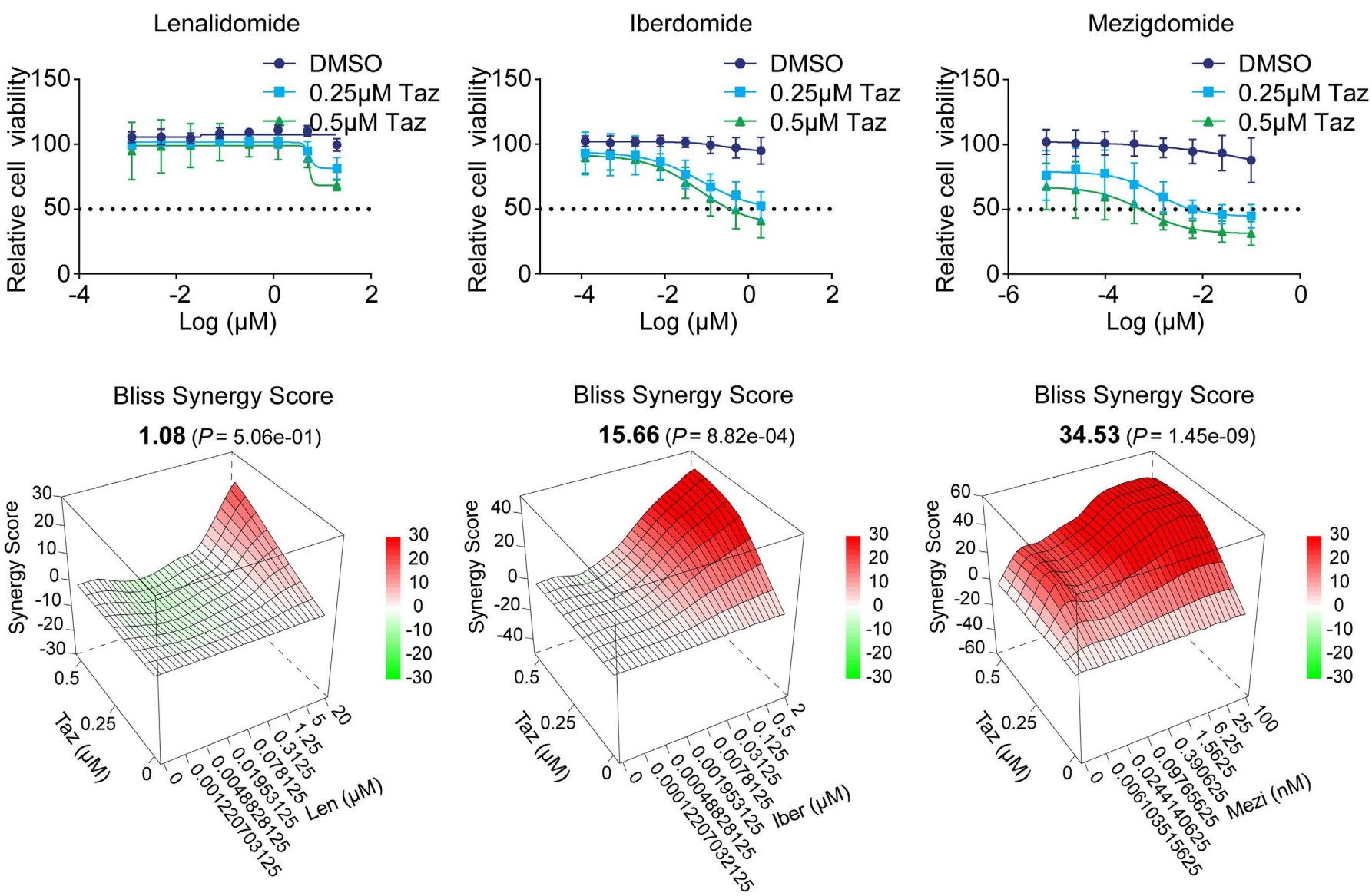


Figure 2. EZH2 inhibition overcomes resistance to Immunomodulatory drugs and cereblon E3 ligase modulators, leading to cell death via apoptosis. (A) Schematic of combination treatment with tazemetostat and immunomodulatory drugs (IMiD)/cereblon E3 ligase modulators (CELMoD). Cells were first primed in tazemetostat (0.25 μM and 0.125 μM for KMS-11 and RPMI-8226, respectively) or dimethylsulfoxide (DMSO) at equivalent concentration for 5 days and then seeded into a 96-well plate for IMiD/CELMoD treatment for a further 5 days with the ongoing presence of tazemetostat/DMSO. Figure panel created in BioRender. Pawlyn, C. (2025) *https://BioRender.com/yng6r6g*. (B) Dose-response curve assessed by cell viability assay in multiple myeloma cell lines treated with drug combinations as per (A). Pomalidomide/iberdomide highest concentration 8 μM. Data shown are mean ± standard deviation (SD), N≥3 biological replicates. ***P*<0.01. (C) Representative plot of annexin V/propidium iodide staining for detecting apoptosis in KMS-11-primed and pomalidomide-treated cells as per (A). The equivalent plot for RPMI-8226 is shown in *Online Supplementary Figure S2* and at day 3 of pomalidomide treatment in *Online Supplementary Figure S3*. (D) Quantification of apoptotic induction in KMS-11 and RPMI-8226. Data shown are mean ± SD, N=3 biological replicates. For statistical comparison the drug combination was compared to pomalidomide monotherapy. **P*<0.05; ***P*<0.01; ****P*<0.001. Taz: tazemetostat; Pom: pomalidomide; PI: propidium iodide.

A



B



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Figure 3. Tazemetostat is synergistic with immunomodulatory drugs and cereblon E3 ligase modulators in resistant cell lines. (A, B) Dose-response curve assessed by cell viability assay of the indicated MM cell lines treated with multi-dose tazemetostat-pomalidomide drug combinations (A) and the KMS-11 cell line treated with multi-dose tazemetostat and immunomodulatory drug/cereblon E3 ligase modulators (B). Cells were primed in the indicated concentration of tazemetostat or dimethylsulfoxide (DMSO) for 5 days as in Figure 2A and then with the combination treatment for 5 days. The highest concentration of pomalidomide used in the dose-response assay was 8 μM. Data shown are mean ± standard deviation, N>3 biological replicates. Relative cell viability was calculated by normalizing each treatment condition to the global DMSO control to ensure comparability across treatments and enable synergy score calculation. The mean cell viability for each concentration combination was used to calculate the Bliss synergy score. Taz: tazemetostat; Pom: pomalidomide; Len: lenalidomide; Mezi: mezigdomide.

Figure S4). Taken together, our results suggested that the tazemetostat-IMiD/CELMoD combinations were synergistic in resistant cell lines and this synergy was specific to IMiD/CELMoD (and dexamethasone).

EZH2 inhibition overcomes resistance to Immunomodulatory drugs and cereblon E3 ligase modulators in a cereblon-dependent manner

The association between increased CRBN binding potency and enhanced synergy with tazemetostat suggested a key role for CRBN-related degradation. One hypothesis considered was that tazemetostat may alter the neo-substrate degradation profile induced by IMiD. To explore this, proteomic analysis was performed and differentially expressed proteins between conditions were compared. Known neo-substrates, such as IKZF1, IKZF3 and ZFP91,[34] were downregulated in cells treated with pomalidomide alone ($|Log_2FC|>0.5$, *P*.adj<0.05) (Figure 4A left). This pattern was not caused by tazemetostat alone (Figure 4A mid-left), or altered by the addition of tazemetostat (Figure 4A mid-right), suggesting no direct alteration of neo-substrate degradation was induced. However, the combination of tazemetostat and pomalidomide resulted in 43 significantly downregulated proteins and 130 significantly upregulated proteins compared to those in control cells (Figure 4B). Enriched pathways associated with downregulated proteins included ferroptosis, pyrimidine metabolism and p53 signaling; enriched pathways associated with upregulated proteins included cytoskeleton in muscle cells, proteoglycans in cancer and arrhythmogenic right ventricular cardiomyopathy (*Online Supplementary Figure S5*). IRF4 was downregulated only in the cells treated with the tazemetostat-pomalidomide combination, but not in cells treated with tazemetostat alone or pomalidomide alone (Figure 4A, B). Western blot analysis further confirmed that only the tazemetostat-pomalidomide combination reduced IRF4 expression in resistant cell lines (Figure 4C).

An isogenic KMS-11 cell line with CRBN KO was generated to investigate the role of CRBN in the effect of the tazemetostat-IMiD/CELMoD combination (*Online Supplementary Figure S6*). Cell viability assays demonstrated that the KO completely abrogated the effect of the combination both in terms of cell viability and decreased IRF4 expression (Figure 4D-F), indicating that CRBN expression is critical for the combination's effect. Supporting this, experiments in the MM.1S cell line generated with acquired resistance to Iberdomide, with low but not absent CRBN expression, responded to the tazemetostat combination but only with CELMoD and not the less potent IMiD (*Online Supplementary Figure S7*) and to a greater extent with mezigdomide than with iberdomide. Resistant cell lines with no detectable CRBN expression behaved like the CRBN KO cells. Further experiments were conducted in cell lines engineered to express key CRBN mutations in the CRBN KO background, to simulate another IMiD resistance mechanism previously identified in patients. Re-expression of wild-type (WT) CRBN resulted in synergy between tazemetostat and pomalidomide or iberdomide (*Online Supplementary Figure S8*) while mutations known to completely abrogate CRBN function through mutation of the thalidomide binding domain (e.g., p.W386A) showed no significant synergy, further highlighting the key role of functional CRBN in the synergistic effect. Consistent with our previous findings, tazemetostat appeared to act synergistically with iberdomide but not pomalidomide in the presence of the p.C326G mutation.[29]

IRF4 overexpression partially rescues the synergistic effect of the combination of tazemetostat with immunomodulatory drugs and cereblon E3 ligase modulators

The key role of IRF4 in the CRBN-dependent synergy demonstrated between tazemetostat and IMiD/CELMoD was explored further. GFP-tagged IRF4 (codon-optimized) was overexpressed in the KMS-11 cell line, and the overexpression of exogenous IRF4 was confirmed by qPCR and western blotting (*Online Supplementary Figure S9*). In this model the expression of exogenous IRF4 was driven by a cytomegalovirus promoter, rather than the native IKZF1/IKZF3 binding promoter and the effect of the tazemetostat-IMiD/CELMoD combination on altered IRF4 expression was ameliorated as the result of IRF4 self-regulatory function (Figure 5A, B). In addition, compared to the control cell line, exogenous expression of IRF4 partially rescued the MYC reduction (Figure 5C) and, therefore, the effect of the tazemetostat-IMiD/CELMoD combination on cell proliferation (Figure 5D). These results indicated that the synergy between tazemetostat and IMiD/CELMoD was, at least partially, mediated by IRF4 downregulation.

Tazemetostat reduces IKZF1 enrichment within the IRF4 promoter and enhancer

Given that EZH2 inhibition epigenetically modifies chromatin

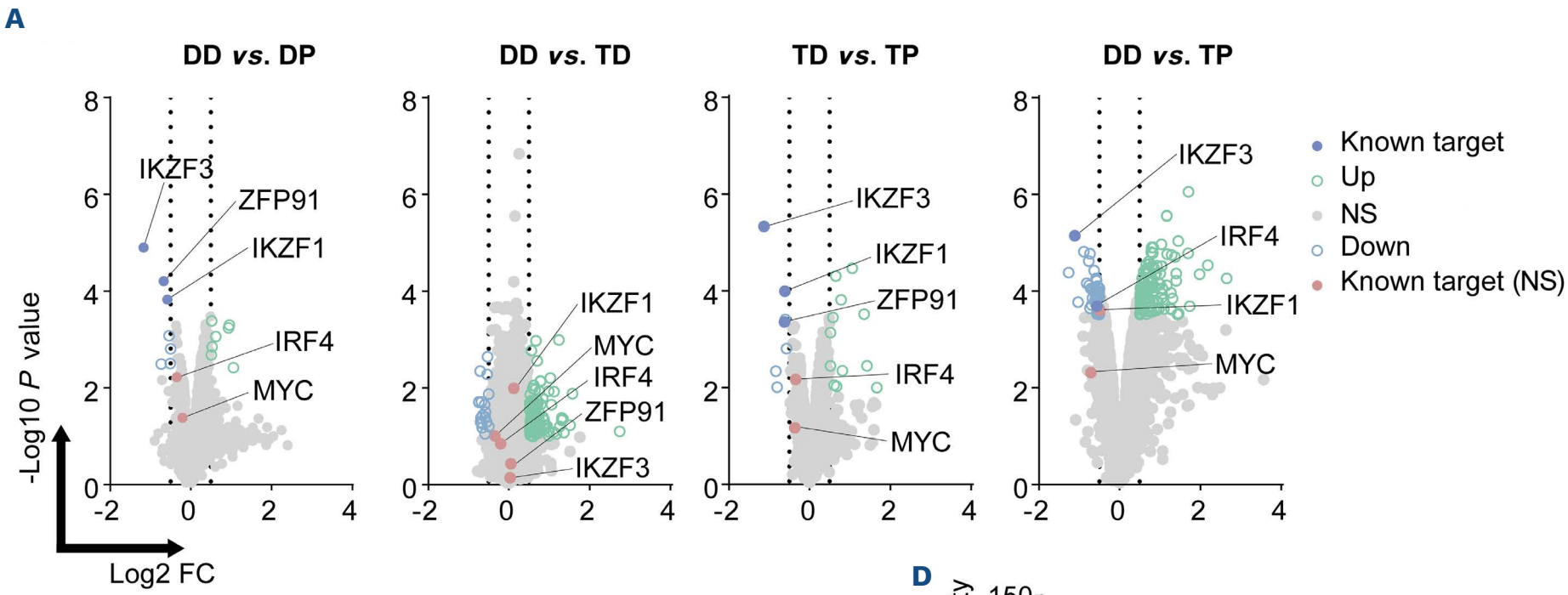


Figure 4. Tazemetostat overcomes resistance to immunomodulatory drugs and cereblon E3 ligase modulators in a cereblon-dependent manner. (A) Volcano plot for proteomic analysis in KMS-11 cells treated with tazemetostat-pomalidomide drug combinations. From left to right: dimethylsulfoxide (DMSO)+DMSO (DD) *versus* DMSO+pomalidomide (DP), differentially expressed proteins (DEP) with addition of pomalidomide without tazemetostat priming; DMSO+DMSO (DD) *versus* tazemetostat+DMSO (TD), DEP with addition of tazemetostat; DMSO+pomalidomide (DP) *versus* tazemetostat+pomalidomide (TP), DEP with addition of pomalidomide in the context of tazemetostat; DMSO+DMSO (DD) *versus* tazemetostat+pomalidomide (TP), DEP with drug combinations compared to the DMSO control; *P* and *P*.adj values were calculated by the DEP package[34] in R (4.4.1). The plot shows the $\log_{10}P$ value (y axis) against $\log_2$FC (x axis). The significant DEP with *P*.adj <0.05 are highlighted (blue = downregulated, green

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= upregulated). The data associated with this figure are shared in an *Online Supplementary Appendix*. (B) Venn plot of downregulated proteins (DRP, top) and upregulated proteins (URP, bottom) when comparing DP to DD (DRP/URP in pomalidomide) or TP to DD (DRP/URP in combination). (C) Western blot analysis of proteins in multiple myeloma cell lines treated with tazemetostat-pomalidomide drug combinations. Cells were primed in 0.25 μM tazemetostat for 10 days and treated with 0.5, 2 or 8 μM pomalidomide for KMS-11, and 2 or 8 μM pomalidomide for RPMI-8226 for 24 h. (D) Dose-response curve assessed by cell viability assays for drug combinations in KMS-11 cells with cereblon (CRBN) knockout. Cells were primed in the indicated concentration of tazemetostat or DMSO for 5 days and then exposed to pomalidomide/iberdomide as in Figure 2A. The highest concentration of pomalidomide was 8 μM. Data shown are mean ± standard deviation (SD), N=3 biological replicates. **P*<0.05; ****P*<0.001. (E, F) Western blot analysis of indicated proteins for the combination in the KMS-11 cell line with CRBN knockout. Cells were primed in 0.25 μM tazemetostat for 10 days and treated with 8 μM pomalidomide/iberdomide for 24 h. Data shown are mean ± SD, N≥3 biological replicates. **P*<0.05, ***P*<0.01. NS: not significant; FC: fold change; Taz: tazemetostat; Pom: pomalidomide; CTL: control; KO: knockout; Iber: iberdomide.

and gene transcription, the impact of the drug combination on *IRF4* mRNA levels was analyzed. IMiD/CELMoD treatment alone significantly reduced *IRF4* mRNA expression in the sensitive cell line MM.1S, whereas it had a limited effect on *IRF4* expression in resistant cell lines (Figure 6A). Combined with the protein data (Figure 1E, F), these findings suggested that *IRF4* mRNA expression was not transcriptionally regulated by IMiD-CRBN-mediated IKZF1/IKZF3 degradation in the resistant setting. Expression of *IRF4* mRNA was, however, reduced by the combination treatment and this reduction was CRBN-dependent (Figure 6B), suggesting that tazemetostat might epigenetically modify the resistant cell lines leading to restoration of transcriptional regulation of *IRF4* mRNA expression by IKZF1/IKZF3.

To further investigate, *IRF4* mRNA expression and its epigenetic regulation were examined in cells treated with tazemetostat alone. Tazemetostat had no significant impact on reducing *IRF4* mRNA and protein expression in KMS-11 cells, but significantly reduced *IRF4* mRNA and protein expression in RPMI-8226 cells (Figure 6C, D). These findings were consistent with the cell viability data, which indicated that the RPMI-8226 cell line was somewhat sensitive at higher concentrations to tazemetostat alone (*Online Supplementary Figure S1*). ChIP-qPCR analysis in KMS-11 showed that tazemetostat significantly reduced H3K27me3 enrichment at the *IRF4* promoter (Figure 6E). This finding suggested that tazemetostat altered chromatin compaction at the *IRF4* promoter, which might be expected to increase gene expression. However, the results showed either unchanged or reduced *IRF4* mRNA expression in KMS-11 and RPMI-8226, respectively (Figure 6C, D). Since IKZF1/IKZF3 are key transcriptional factors for *IRF4* known to bind at both the promoter and upstream super-enhancer (SE) region within *DUSP22*,[14,35-37] ChIP-qPCR to assess IKZF1 binding to both these sites was performed. Three pairs of primers were used to detect different regions of the *IRF4* promoter and three at the SE (Figure 6F, G). For the promotor, primer #1 detected a known IKZF1 binding region,[35] while primers #2 and #3 targeted putative binding sites predicted by JASPAR.[38] For the enhancer, primers SE1-3 targeted peaks of H3K27ac within the SE. The results showed an apparent reduction in IKZF1 enrichment at the *IRF4* promoter and enhancer, with greater binding and reduction in binding seen at the enhancer (Figure 6F, G).

These results suggested that the synergistic tazemetostat-IMiD/CELMoD combination significantly reduced IRF4 transcription by resensitizing cells to IMiD-CRBN-mediated IKZF1/IKZF3 degradation. Mechanistically, tazemetostat reduced IKZF1 binding to the IRF4 promoter. Based on this, we propose a model for how the tazemetostat-IMiD/CELMoD combination could overcome drug resistance (Figure 6H). Alteration of IKZF1/IKZF3 at the *IRF4* promotor and/or enhancer regions by either tazemetostat or IMID/CELMoD as single agents is insufficient to alter *IRF4* expression enough to result in cell death. In contrast, exposure to EZH2 inhibition modulates transcriptional control of *IRF4* expression to an extent that IMID/CELMoD-mediated degradation of IKZF1/IKZF3 leads to reduced IRF4 expression, resulting in the synergistic effect and cell death.

Discussion

Despite significant improvements in outcomes for patients with MM over the last few decades, precipitated by novel therapies including IMiD, therapy resistance remains an ongoing challenge. Newer agents, including the more potent CELMoD, are demonstrating efficacy in relapsed refractory disease, but patients continue to inevitably relapse and so approaches to address the resistant state are needed to improve outcomes for patients.

In this study, we demonstrate that KMS-11 and RPMI-8226 myeloma cell lines exhibit resistance to IMID/CELMoD with persistent *IRF4* expression despite expression of functional CRBN and degradation of IKZF1 and IKZF3. Reducing *IRF4* expression has been shown to inhibit the proliferation of MM cells and multiple effective anti-myeloma therapeutics have this downstream result.[12,39] We demonstrate IRF4 dependency is maintained in KMS-11 and RPMI-8226 cell lines with depletion of IRF4 significantly reducing cell viability. This supports the findings of other studies recently reported that suggest transcriptional plasticity as an underlying route to IMiD resistance[13,14] with studies showing that c-FOS/c-JUN, BATF or ETV4 could substitute IKZF1/IKZF3 to maintain *IRF4* expression and confer resistance.[13,14,40] Epigenetic aberrations are closely associated with the

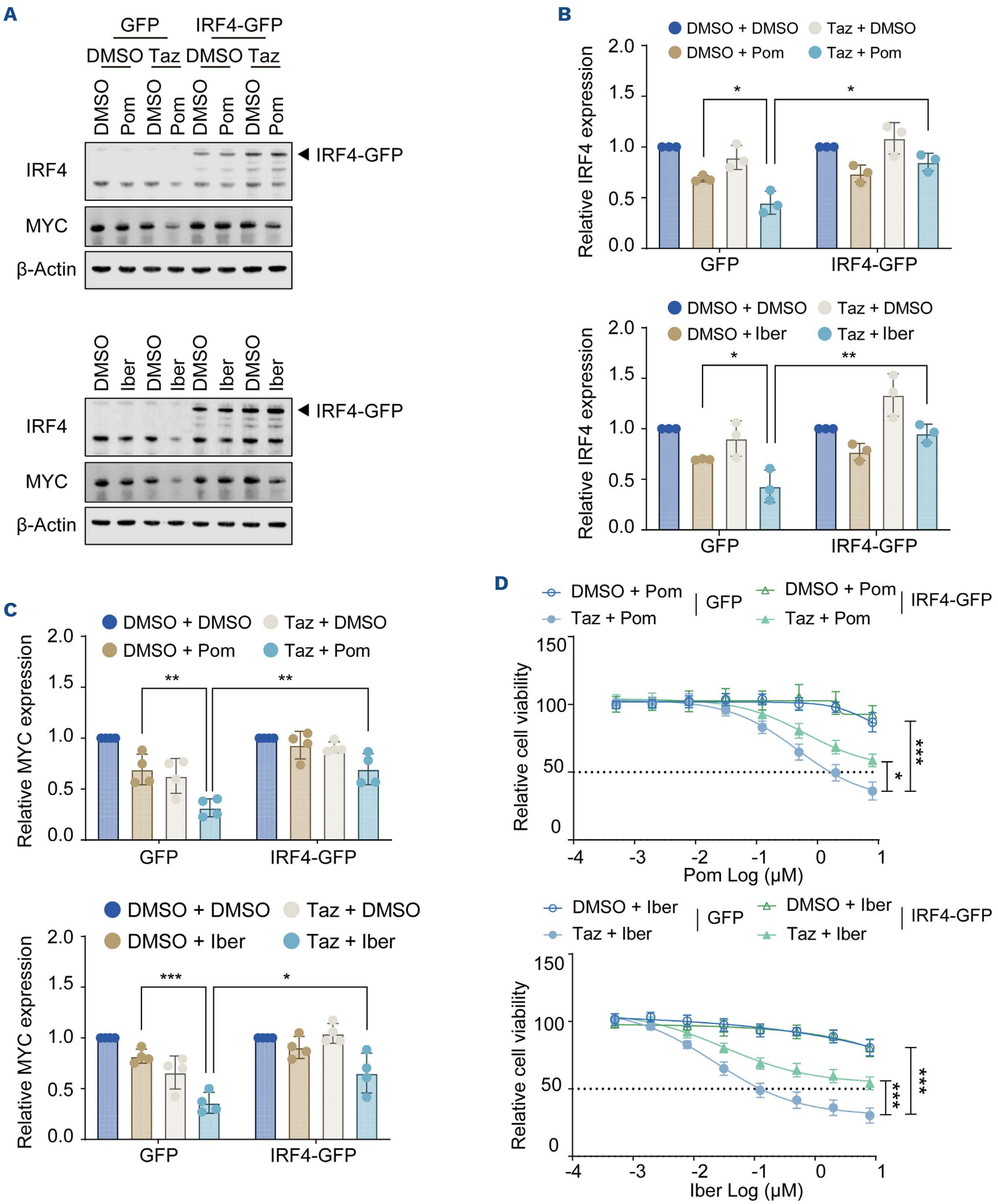
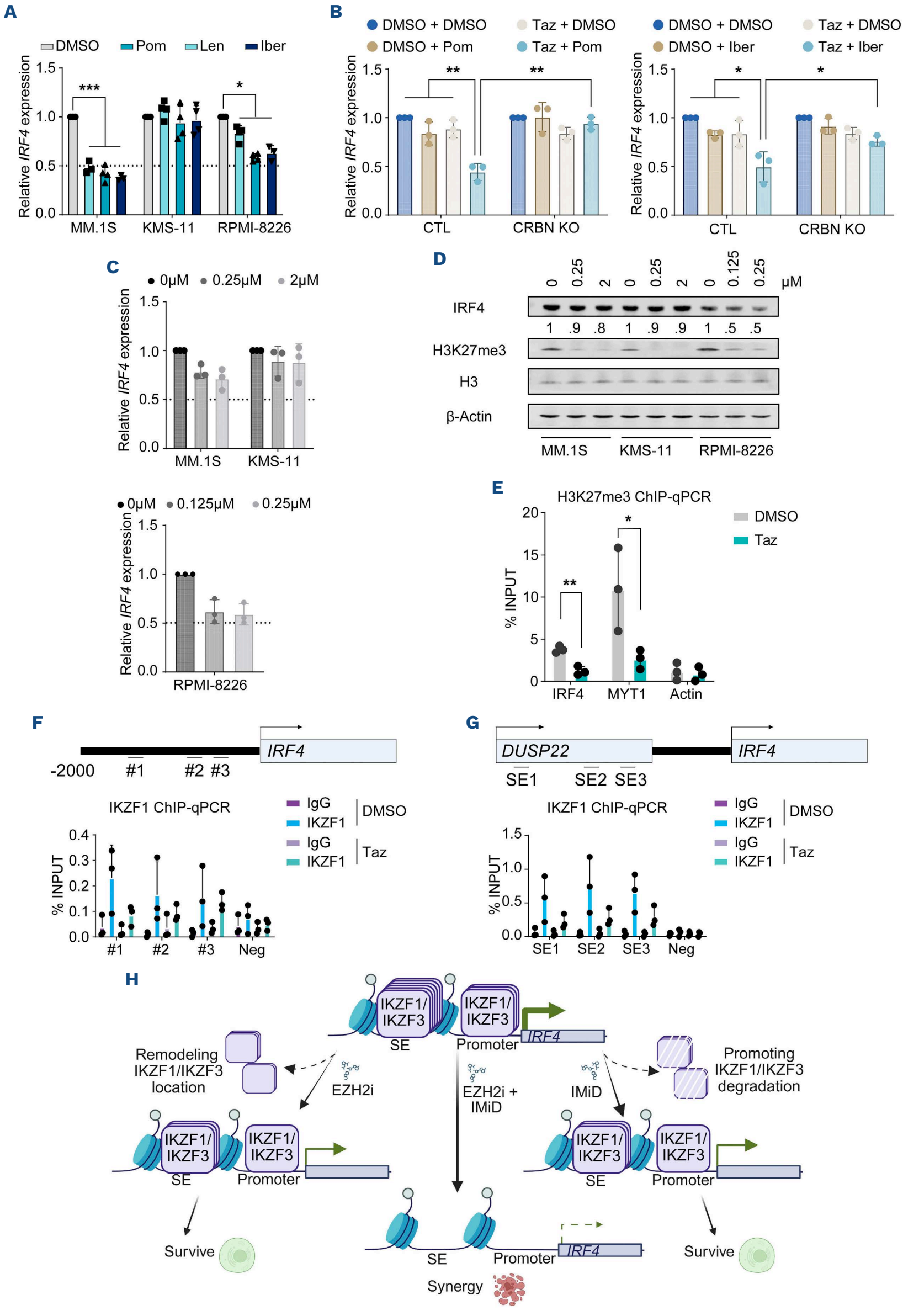


Figure 5. Tazemetostat overcomes resistance to immunomodulatory drugs and cereblon E3 ligase modulators in an IRF4-dependent manner. (A-C) Western blot analysis of interferon regulatory factor 4 (IRF4) and MYC expression for the drug combinations in the KMS-11 cell line overexpressing IRF4-GFP. Cells were primed in 0.25 μM tazemetostat for 10 days and treated with 8 μM pomalidomide/iberdomide for 24 h. Data shown in (B) and (C) are quantified from blots, normalized to actin; mean ± standard deviation (SD), N≥3 biological replicates. **P*<0.05, ***P*<0.01. (D) Dose-response curve assessed by cell viability assay for drug combinations in KMS-11 with IRF4 overexpression. Cells were primed in the indicated concentration of tazemetostat or dimethylsulfoxide for 5 days and then exposed to pomalidomide/iberdomide as in Figure 2A. Highest concentration of pomalidomide/iberdomide was 8 μM. Data shown are mean ± SD, N≥3 biological replicates. **P*<0.05; ****P*<0.001. GFP: green fluorescence protein; DMSO: dimethylsulfoxide; Taz: tazemetostat; Pom: pomalidomide; Iber: iberdomide.



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Figure 6. Tazemetostat reduces IKZF1 enrichment within the IRF4 promoter. (A) Quantitative polymerase chain reaction (qPCR) analysis of *IRF4* mRNA expression in cell lines treated with different immunomodulatory drugs and cereblon E3 ligase modulators (IMiD/CELMoD). Data shown are mean ± standard deviation (SD), N≥3 biological replicates. **P*<0.05; ****P*<0.001. (B) qPCR analysis of *IRF4* mRNA expression in KMS-11 treated with drug combinations. Cells were primed in 0.25 μM tazemetostat for 10 days and treated with 8 μM pomalidomide/iberdomide for 24 h. Data shown are mean ± SD, N≥3 biological replicates. **P*<0.05; ***P*<0.01. (C, D) qPCR and western blot analysis of IRF4 mRNA expression in cell lines treated with the indicated concentrations of tazemetostat for 5 days. Data shown are mean ± SD, N=3 biological replicates. (E) Chromatin immunoprecipitation (ChIP)-qPCR analysis of H3K27me3 enrichment within the *IRF4* promoter. MYT1 served as a positive control; Actin served as a negative control. Data shown are mean ± SD, N=3 biological replicates. **P*<0.05; ***P*<0.01. (F) ChIP-qPCR analysis of IKZF1 enrichment within the *IRF4* promoter. Data shown are mean ± SD, N=3 biological replicates. (G) ChIP-qPCR analysis of IKZF1 enrichment within the *DUSP22* super-enhancer. Data shown are mean ± SD, N=3 biological replicates. (H) Proposed model of the synergistic effect of tazemetostat-IMiD/CELMoD combination in reducing IRF4 expression in the resistant cell line. Created in BioRender. Pawlyn, C. (2025) *https://BioRender.com/n0eekfj* DMSO: dimethylsulfoxide; Pom: pomalidomide; Len: lenalidomide; Iber: iberdomide; Taz: tazemetostat; CTL: control; KO: knockout; IKZF1: Ikaros; IKZF3: aiolos; SE: superenhancer; EZH2i: enhancer of zeste homolog 2 inhibitor; IRF4: interferon regulatory factor 4.

pathogenesis of MM and are considered as a mechanism of drug (including IMiD) resistance.[41-43] Two key recent studies highlight the role of EZH2 in myeloma treatment resistance and relapse,[21,22] but the effects of EZH2 inhibition in overcoming this have not been well explored. Our results show that tazemetostat-IMiD/CELMoD drug combinations led to a reduction of IRF4 expression and significantly induced cell death via apoptosis, compared to IMiD/CELMoD monotherapy in the intrinsically resistant cell lines. Determining the synergy score between tazemetostat and each of the IMiD/CELMoD agents, we observed that synergy increased with the potency of CRBN binding. This finding suggested that the synergy effect of drug combinations is CRBN-dependent which we confirmed using a knock-out model. Furthermore, our results demonstrate that tazemetostat does not exhibit synergy with the majority of conventional drugs used in MM treatment (with the exception of dexamethasone), suggesting that the synergy is largely specific to IMiD/CELMoD. Our proteomic analysis and subsequent validation demonstrated that only the drug combination significantly reduced IRF4 expression and led to cell death which could be partially rescued by IRF4 overexpression.

A recent study reported the use of 5-azacytidine and/or tazemetostat to restore sensitivity to IMiD in models of acquired resistance (to lenalidomide and pomalidomide) and concluded that this effect was not mediated by increasing CRBN expression.[27] This is consistent with our findings; their acquired resistant cells maintained low level (not absent) CRBN expression. We also expanded further on their findings in acquired resistance by analyzing the effect of the combination in CELMoD (iberdomide)-resistant cell lines with very low CRBN expression.

Our study focuses on mechanisms of intrinsic or primary resistance to IMiD/CELMoD as the cell lines studied were derived from patients prior to IMiD/CELMoD introduction to the clinic. Primary resistance remains an important clinical problem in itself and may also reflect the mechanism by which a subclone of cells leads to relapse in patients following therapy, or acquired resistance. It is currently not clear what proportion of patients at relapse have mechanisms similar to those of primary resistance *versus* those associated with genetic alteration or transcriptional downregulation seen only following exposure i.e., acquired resistance after treatment. Here we demonstrate that CRBN expression is required for EZH2 inhibitor and IMiD/CELMoD synergy but even low levels of CRBN may be sufficient, especially with the more potent CELMoD, such as iberdomide and mezigdomide, meaning this approach may be relevant for both states. Additionally, we explored the impact of the synergy of EZH2 inhibition and IMiD/CELMoD in the presence of mutations of CRBN, which highlighted the need for functional CRBN (without mutations that completely abrogate E3 ligase activity) for synergy to occur. Mutations that led to resistance to IMiD to a greater extent than to CELMoD in our previous work also demonstrated synergy between tazemetostat and CELMoD but not IMiD.

Exploring the mechanism of synergy between tazemetostat and IMiD/CELMoD we identified a reduction in H3K27me3 at the *IRF4* promotor with tazemetostat, consistent with inhibition of the EZH2 methyltransferase. As H3K27me3 is generally considered a transcriptional repressor it might be expected for this to result in increased *IRF4* expression; however, we did not identify a significant change in *IRF4* expression with tazemetostat alone either for RNA (Figure 6C) or the protein (Figure 4A mid-left and Figure 6D). This is consistent with previous studies of a single-agent EZH2 inhibitor in myeloma[23,26] in which, at higher concentrations than used in our study, *IRF4* expression was either unchanged or reduced. We therefore explored the binding of the key IRF4 transcription factors IKZF1 at the *IRF4* promotor and enhancer regions. Given our finding of a reduction in focal IKZF1 binding at the *IRF4* promotor and SE following EZH2 inhibition, we propose a model to explain the synergy observed in our study (Figure 6H). In the resistant state, control of *IRF4* expression is altered such that the reduction in IKZF1/IKZF3 binding at *IRF4* induced by either tazemetostat or IMID/CELMoD as single agents is insufficient to reduce *IRF4* expression. However, the combination of tazemetostat and IMiD/CELMoD works simultaneously to effectively reduce *IRF4* expression, leading to cell death. Our results provide *in-vitro* evidence to support the study of the combination of tazemetostat with IMiD/CELMoD in

relapsed/refractory myeloma and suggest that the more potent CELMoD, iberdomide or mezigdomide, would be the better partner, with these compounds demonstrating the greatest synergy score in cells with good CRBN expression while also demonstrating synergy in low CRBN states and in the presence of some CRBN mutations. Interestingly, our combinatorial work also demonstrated synergy between tazemetostat and dexamethasone, supporting the addition of the steroid in combination. The advanced clinical development of both tazemetostat (FDA-approved) and mezigdomide (in late-phase clinical trials) means our findings should be rapidly translatable to the clinic. Independently, a phase I/II (NCT05372354) trial is currently underway to evaluate the optimal dose of mezigdomide for just this combination (mezigdomide, tazemetostat and dexamethasone) with preliminary results suggesting the optimal dose is 1 mg.[44] Initial results from the study, in a very heavily pretreated group of patients (median 5 prior lines of therapy, range 3-14), have reported responses in around two-thirds of patients at the optimal dose, with responses seen even in aggressive extramedullary disease states. A phase II dose-expansion study is planned to further refine patients' response and tolerability.

In summary we provide a strong preclinical rationale to support further exploration of EZH2 inhibitor and IMiD/CELMoD combinations in myeloma patients.

Disclosures

The Institute of Cancer Research has a commercial interest in the development of compounds targeting CRL4-CRBN E3-ubiquitin ligases. CP has received honoraria from Celgene/BMS (a manufacturer of IMiD/CELMoD) for advisory boards, educational talks and participation in data monitoring committees. CP has also received honoraria for advisory boards and/or educational talks from AbbVie, Amgen, Takeda, Sanofi, iTEOS, Pfizer, Menarini Stemline, Johnson and Johnson and Opna Bio. The other authors have no conflicts of interest to disclose.

Contributions

YL, AB, and CP conceived all aspects of the project and designed the analysis. YL, AB, YC, SMa, SB, SMo and ML conducted wet-lab experiments. YL, AB and YC analyzed data. NC provided advice and primers for ChIP. ZK, TR and JC conducted and/or supervised the proteomics experiment. YL and CP drafted the paper. All authors contributed to critically revising the paper and approved the final submitted version.

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Data-sharing statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD057933.

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