Unlocking the therapeutic potential of selective CDK7 and BRD4 inhibition against multiple myeloma cell growth

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Received: March 31, 2024.
Accepted: July 15, 2024.
Early view: July 25, 2024.

https://doi.org/10.3324/haematol.2024.285491

Abstract

Multiple myeloma (MM) is a plasma cell malignancy that is considered incurable despite the recent therapeutic advances. Effective targeted therapies are, therefore, needed. Our previous studies proved that inhibiting CDK7 impairs the cell cycle and metabolic programs by disrupting E2F1 and MYC transcriptional activities, making it an appealing therapeutic target for MM. Given that CDK7 and BRD4 operate in two distinct regulatory axes in MM, we hypothesized that targeting these two complementary pathways simultaneously would lead to a deeper and more durable response. Indeed, combination therapy had superior activity against MM cell growth and viability, and induced apoptosis to a greater extent than did single-agent therapy in both cell lines and patients' cells. This synergistic activity was also observed in Waldenström macroglobulinemia (WM) cells and with other inhibitors of E2F1 activity. Dual inhibition effectively impaired the MYC and E2F transcriptional programs and MM tumor growth and progression in xenograft animal models, providing evidence for the potential of combination therapy as a therapeutic strategy in MM and WM.

Introduction

Multiple myeloma (MM) is characterized by aberrant cell cycle regulation and enhancer alterations.[1] Dysregulation of the cyclin-dependent kinase (CDK)–retinoblastoma (RB)–early 2 factor (E2F) axis, which is the core transcriptional machinery controlling cell cycle progression and timing and fidelity of genome replication, is universally observed, fostering uncontrolled malignant cell proliferation.[2-4]

Additionally, besides cell cycle regulators, transcription factors, chromatin-associated factors that mark DNA and histone proteins with heritable, chemical modifications and other essential components of the transcriptional apparatus, are frequently disrupted in MM, manifesting as a notable dependency, as indicated by meta-analyses of pan-cancer genome-wide CRISPR screening data.[5]

Bromodomains are protein interaction modules that specifically recognize ε-N-lysine acetylation motifs functioning as "readers" of the lysine acetylation state.[6] Dysfunction of bromodomain proteins has been linked to the development of several diseases, including cancer.[7] Bromodomain-containing protein 4 (BRD4) is a member of the bromodomain and extraterminal (BET) family of proteins characterized by two N-terminal bromodomains and an extraterminal (ET) domain, which is involved in many processes including transcription elongation and activation of genes involved in cell growth and cell cycle progression.[8] Many studies have been focused on the role of BRD4 in cancer, and BET inhibitors have shown significant activity against various types of tumors.[8] BRD4 positively correlates with disease progression in MM patients and MM cells are sensitive to treatment with the prototypic BRD4 inhibitor JQ1, which causes preferential loss of transcription at super-enhancer-associated genes and induces cell cycle arrest and a senescent phenotype.[9] Other BET bromodomain inhibitors showed significant efficacy in preclinical MM models.[9-12]

However, the emergence of intrinsic and acquired resistance to BRD4 inhibitors as well as their unwanted toxicities and side effects hampered their widespread clinical application.[13,14] These problems may potentially be overcome by combining BRD4 inhibitors with complementary therapies, enabling lower doses of BRD4 inhibitors and enhancing treatment specificity.[15]

In our previous investigation, we delineated distinct regulatory axes controlled by E2F1 and BRD4 in MM, with E2F predominately regulating growth/proliferation genes at active promoters and BRD4 mainly affecting enhancer-regulated tissue-specific genes.[16] Intriguingly, E2F1 genetic depletion potentiated the anti-MM effect of JQ1 *in vitro* and *in vivo*.[16,17] We recently uncovered CDK7, a kinase with a dual role in cell cycle progression and transcription,[16] as a major driver of E2F1 activity in MM.[17,18] Indeed, its suppression by the small molecule YKL-5-124[17,19] exerted therapeutic effects in MM by mitigating cell cycle CDK plasticity and perturbing the E2F1 transcriptional program in an Rb-dependent manner.[17]

Given that E2F1 and BRD4 operate in two distinct regulatory axes in MM, we hypothesized that simultaneously targeting E2F1 (via CDK7 or CDK4/6 small molecule inhibitors) and BRD4 (via BET inhibitors) would be more effective at reducing tumor growth compared to targeting only one or the other with a single agent. Indeed, the data reported in this study suggest that the combination therapy halts tumor cell growth and viability, offering a promising therapeutic strategy to improve outcomes for patients with MM while minimizing the risk of resistance and toxicity associated with the use of high doses of single agents.

Methods

Cells

Human MM cell lines (MM1s, JJN3, OPM2, LR5, ANBL6WT, H929, AMO1, SKMM1, XG1, KMS12BM and IM9) and Waldenström macroglobulinemia (WM) cell lines (BCWM1, MWCL1 and RPCIWM1) were cultured in RPMI-1640 (Gibco or KeyGEN BioTECH) supplemented with 10% fetal bovine serum (Gibco or Bio-channel), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin, with 2.5 ng/mL of interleukin-6 in ANBL6 and XG1 cells.

In accordance with the Declaration of Helsinki and under the oversight of the Ethics Consultation Service at Dana Farber Cancer Institute, primary MM cells and bone marrow mononuclear cells were isolated from bone marrow aspirates of myeloma patients using Ficoll-Hypaque density gradient sedimentation and anti-CD138 microbead separation. Primary WM cells were separated from bone marrow samples of WM patients with anti-CD19 microbeads. Peripheral blood mononuclear cells were isolated from healthy donors using Ficoll-hypaque density gradient sedimentation and activated with 20 μg/mL phytohemagglutinin (InvivoGen, #inh-phap). Bone marrow stromal cells were established from mononuclear cells isolated via Ficoll-Hypaque density gradient centrifugation from bone marrow specimens of MM patients, as previously described.[20,21]

Reagents

YKL-5-124 was a kind gift from Nathanael S. Gray. JQ1, palbociclib, ARV-825 and I-BET151 were purchased from MedChemexpress. Compounds were dissolved in dimethyl sulfoxide unless otherwise stated.

Cell viability, cell cycle and apoptosis assays

Cell viability was detected by CellTiter-Glo assay (Promega, G7572) or Cell Counting Kit-8 (VICMED). The cell cycle was measured by flow cytometric assay following staining with propidium iodide (BD Biosciences, 564907) and then analyzed by ModFit software. Apoptosis was evaluated by flow cytometric analysis following staining with APC annexin-V (BD Biosciences, 561012) and DAPI (BD Biosciences, 564907).

RNA sequencing

AMO1 cells were treated with YKL-5-124 (50 nM) or JQ1 (200 nM) alone or in combination for 24 hours. RNA was extracted using a RNeasy Plus Mini Kit (QIAGEN, #74136) according to the manufacturer's instructions, and then subjected to bulk RNA sequencing. RNA-sequencing analysis was performed using the HG19 ERCC human reference genome with HG19 ERCC gene annotations. Gene set enrichment analysis was performed via the computational platform of the Broad Institute with the Hallmark gene signature.

Murine xenograft model of human multiple myeloma or Waldenström macroglobulinemia

SCID mice were purchased from Charles River Laboratories. All animal studies were approved by and conducted according to the protocols of the Animal Ethics Committee of the Dana-Farber Cancer Institute. The mice were irradiated by 200 cGy and then inoculated subcutaneously in the right or left flank with 5×10^6 MM or WM cells. Tumor growth was measured in two dimensions by caliper, and volume was calculated using the formula: $V=0.5\times a\times b^2$, where "a" and "b" represent the length and width of the tumor. The body weight of the animal was measured every week throughout the study to monitor the toxicity of drugs.

Statistical analysis

All values are displayed as mean ± the standard deviation. The statistical significance of differences between experimental variables was analyzed using the Student *t* test, or analysis of variance for multiple comparisons, with Prism GraphPad software. The significance of the *P* value is $*P<0.05$, $**P<0.01$, and $***P<0.001$.

Results

Dual E2F1 and BRD4 inhibition leads to synergistic anti-myeloma effects

We evaluated the effect of dual inhibition of CDK7 and BET bromodomain proteins with low doses of YKL-5-124 and JQ1, respectively, in an extensive array of cell lines (N=11), to account for the molecular heterogeneity of MM cells and their varying response to therapy. We observed

superior activity of the combination against all MM cell lines tested compared to single perturbation alone (Figure 1A and *Online Supplementary Figure S1A*). Isobologram analysis revealed strong synergism of the combination as compared to single agents, with a combination index <1.0 at all tested doses (Figure 1B). On the other hand, peripheral blood mononuclear cells from healthy donors were less sensitive to the combination therapy (*Online Supplementary Figure S1B*). We also tested the effect of the combination in XG1 MM cells, which have intrinsic resistance to both YKL-5-124 and JQ1 and confirmed dual inhibition to be synthetically lethal in these cells (Figure 1C, D and *Online Supplementary Figure S1C*). These findings were validated with two additional BET inhibitors: ARV-825 and I-BET151 (Figure 1E, F).

Consistent with YKL-5-124 being a CDK7 inhibitor, we observed reduced phosphorylation of CDK4 and increased activation of RB with both YKL-5-124 single agent and combination therapy (Figure 2A, B), this latter causing early cell cycle arrest, with a higher percentage of cells in the G1 phase than with either agent alone (Figure 2C) and a moderate increase in apoptotic cell death and cleavage of caspase 3 (Figure 2D and *Online Supplementary Figure S1D*). In addition, we tested the combination of JQ1 with the Food and Drug Administration-approved CDK4/6 inhibitor palbociclib, confirming its greater anti-MM effect in this context than when administered as a single drug (*Online Supplementary Figure S1E*). The combination of low doses of JQ1 and palbociclib was indeed synergistic, with isobolographic analysis revealing a strong synergism in most MM cell lines (*Online Supplementary Figure S1F*). This synergism between BRD4 and cell cycle inhibitors was also observed in other cancer contexts.[22-24]

We next assessed whether this combination regimen could be applicable to other hematologic malignancies, such as WM. We observed that WM cells are more sensitive to the inhibition of CDK7 than to BET (*Online Supplementary Figure S2A*). Indeed, CDK7 inhibition robustly reduced the viability of WM cell lines (N=3) and primary WM cells in a dose- and time-dependent manner (*Online Supplementary Figure S2B*), impairing E2F, MYC and cell cycle–associated gene set signatures, as observed in MM cells (*Online Supplementary Figure S2C*). Moreover, combination treatment effectively killed WM cell lines and primary cells from patients, although the effect was greater in MM cells (*Online Supplementary Figure S2D, E*).

Combination therapy alters the tumor cell transcriptome

To gain deeper insight into the molecular basis of the observed drug synergism, we performed gene expression profiling after 24 hours of treatment with 200 nM JQ1 and 50 nM YKL-5-124 in the AMO1 cell line. RNA-sequencing analysis of cells treated with a low dose of YKL-5-124, a low dose of JQ1, or a combination showed that, in general, combination therapy had a more profound impact on the transcriptome than single-agent therapy (Figure 3A). Canonical pathway analysis revealed that the upregulated pathways were enriched in pathways for autophagy and senescence, whereas cell cycle regulation, glycolysis, the unfolded protein response, and cholesterol biosynthesis were enriched among the downregulated pathways (Figure 3B). Moreover, analysis of the predicted transcriptional regulators found that FOXM1, BRD4, E2F and MYC were among the inhibited regulators while CDKN1A(p21), TP53 and RB were among the activated regulators (Figure 3C).

In line with this result, we observed a pronounced decrease in the expression of MYC upon combination therapy (Figure 3D). Moreover, dual targeting synergistically decreased the expression of additional genes important to the MM oncogenic state, including MYBL2 (b-Myb) (Figure 3E). MYBL2 is a cell-cycle and proliferation gene frequently overexpressed in several cancers: here, we found MYBL2 to be a dependency in a panel of MM cell lines (*Online Supplementary Figure S3A*), with its high expression associated with poor outcome in datasets of MM patients (*Online Supplementary Figure S3B, C*).

CDK7 inhibition synergizes with JQ1 against multiple myeloma cells *ex vivo* and *in vivo*

We finally tested the therapeutic potential of the various drug combinations. We first confirmed the strong synergism observed in primary MM cells, where a combination index <1.0 was found at all tested doses, with the effect being considerably less significant in peripheral blood mononuclear cells derived from MM patients (Figure 4A). This was also confirmed in primary cells treated with a combination of JQ1 and palbociclib (*Online Supplementary Figure S4A, B*). We next evaluated the effect of the combination in the context of the bone marrow milieu. The combination of low doses of YKL-5-124 and JQ1 had a striking effect in inhibiting the proliferation of MM cells cultured for 24 hours with supernatant from bone marrow stromal cells derived from MM patients (Figure 4B). Similarly, the combination regimen had a significant impact on the viability of primary $CD138^+$ MM cells cultured in the presence of their bone marrow microenvironment, while $CD138^-$ bone marrow cells derived from the same patient were unaffected (Figure 4C). Altogether these data suggest a favorable therapeutic index.

The synergistic effects of combining YKL-5-124 with JQ1 *in vitro* prompted us to assess the efficacy of this combination in two *in vivo* models of MM. SCID mice were injected subcutaneously with MM1s or H929 cells. When tumors reached 100 mm^3, the mice were randomized to vehicle or a low dose of JQ1 (50 mg/kg) or YKL-5-124 (2.5 mg/kg) or combination treatment for 2 weeks. Combination therapy resulted in smaller tumors compared to those in the animals treated with the single agents (Figure 4D and *Online Supplementary Figure S4C*), without evident toxicity or weight loss (Figure 4E). This pattern was also

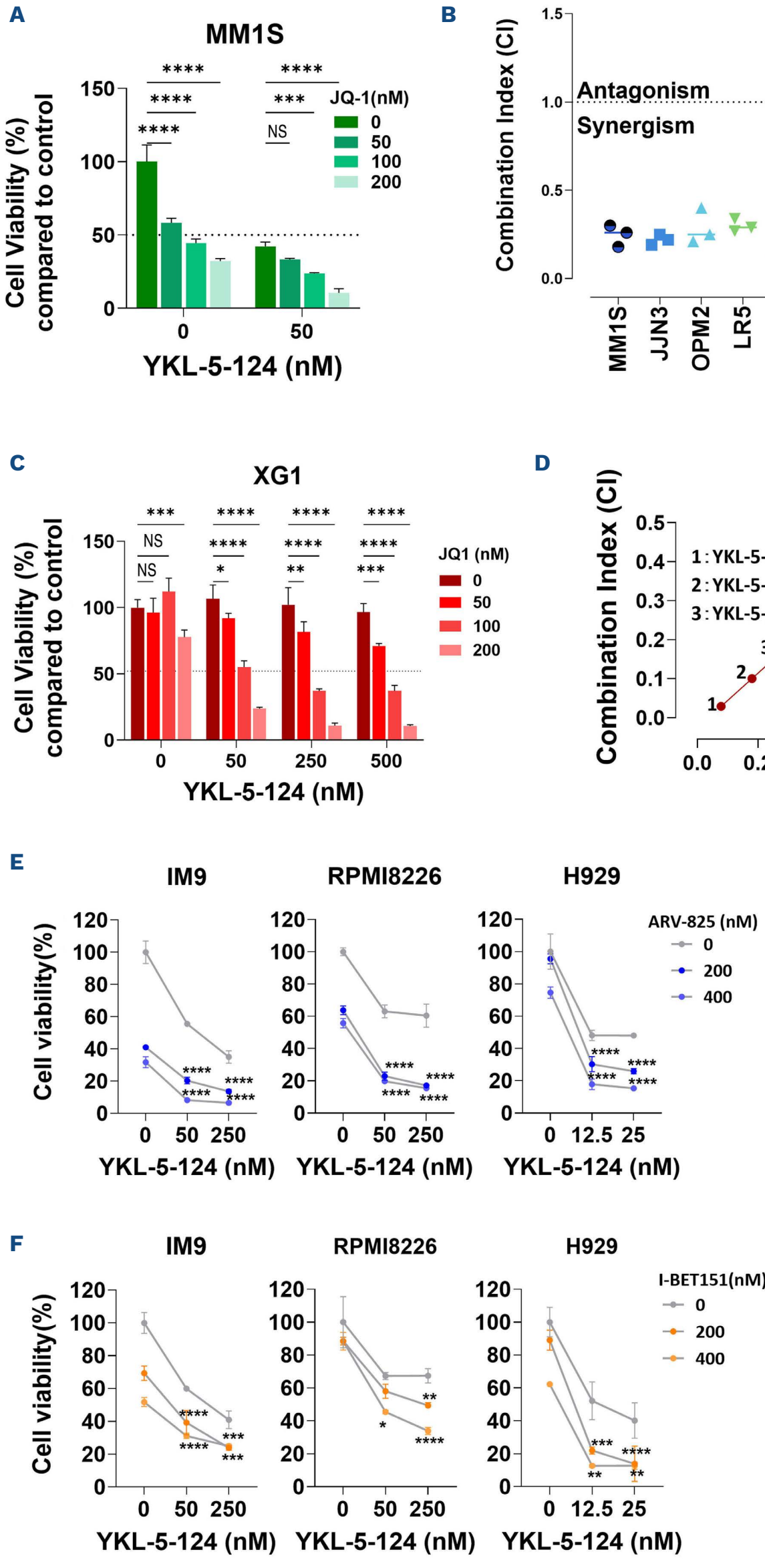


Figure 1. Enhanced suppression of multiple myeloma cell proliferation through dual inhibition strategy. (A, B) A panel of 11 multiple myeloma cell lines was treated with YKL-5-124 or JQ1 alone or in combination, and cell survival was assessed by a CellTiter-Glo uptake assay. For a representative cell line (MM1S), the percentage of cell viability compared to untreated cells is shown (mean ± standard deviation [SD]; N=3) (A). Synergism analysis was performed with Calcusyn software. A Combination Index (CI)=1 means an additive effect, a CI <1 means a synergistic effect, and a CI >1 means an antagonistic effect (B). ***P<0.001, ****P<0.0001. (C, D) XG1 cells were cultured in the presence of different concentrations of YKL-5-124 with or without JQ1 for 72 hours, and cell survival was assessed by a CellTiter-Glo uptake assay. Data are presented as the percentage of cell viability compared to untreated cells (mean ± SD; N=3) (C). Synergism analysis was performed with Calcusyn software (D). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (E, F) IM9, RPMI8226 and H929 cells were cultured in the presence of the BRD4 inhibitor ARV-825 (E) or the BRD4 degrader I-BET151 (F) with or without YKL-5-124. Cell viability was assessed by a CCK8 assay (mean ± SD; N=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

noted in vivo when JQ1 was combined with palbociclib, although the difference did not achieve statistical significance, likely due to the limited sample size (*Online Supplementary Figure S4D*).

Discussion

Alterations (loss- or gain-of-function) in factors and enzymes that control transcription and genome stability are frequently associated with transformation, contributing to the "transcriptional addiction" observed in cancer. Myeloma cells are characterized by enhancer alterations, such as Ig enhancer translocations to key drivers including CCND2, MAF, MYC, and MMSET; and chromatin modifiers have been successfully exploited as novel targets against myeloma cells.[1,25-29] Indeed, both genetic and pharmacological interventions aimed at BRD4 yield anti-myeloma effects in

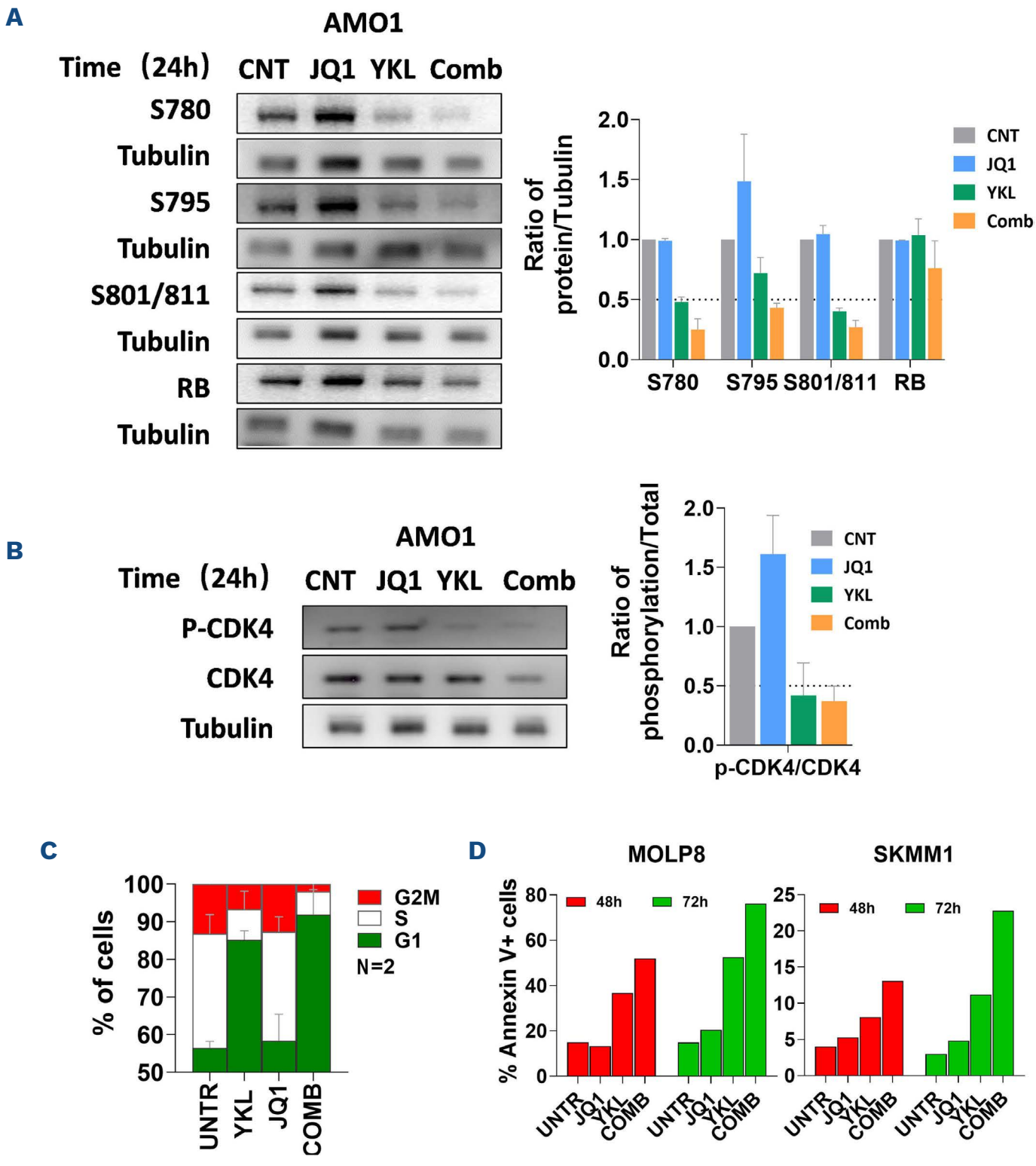
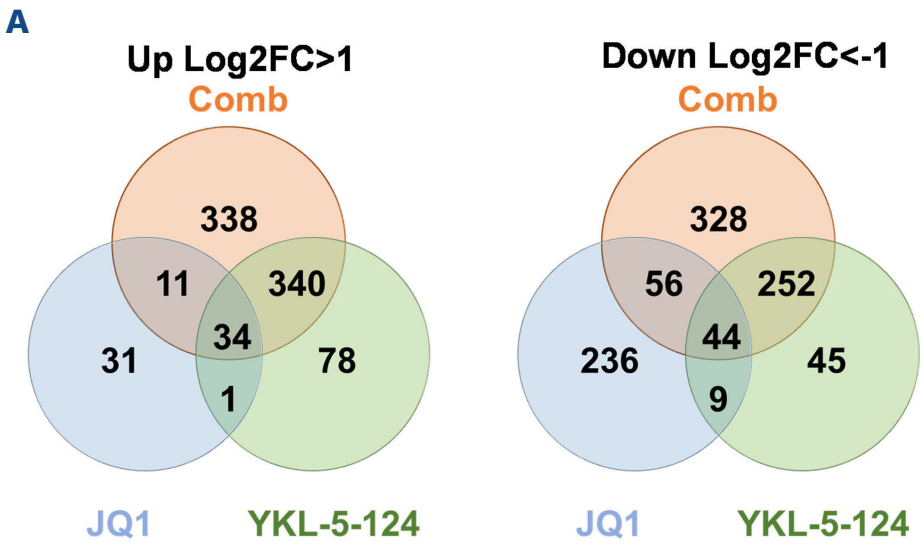
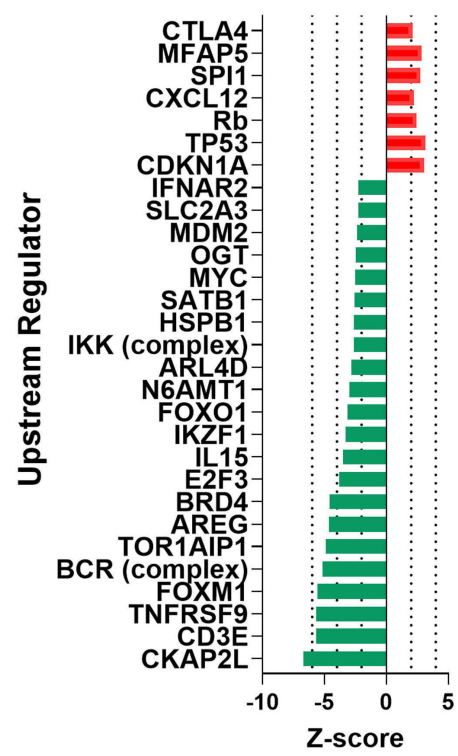
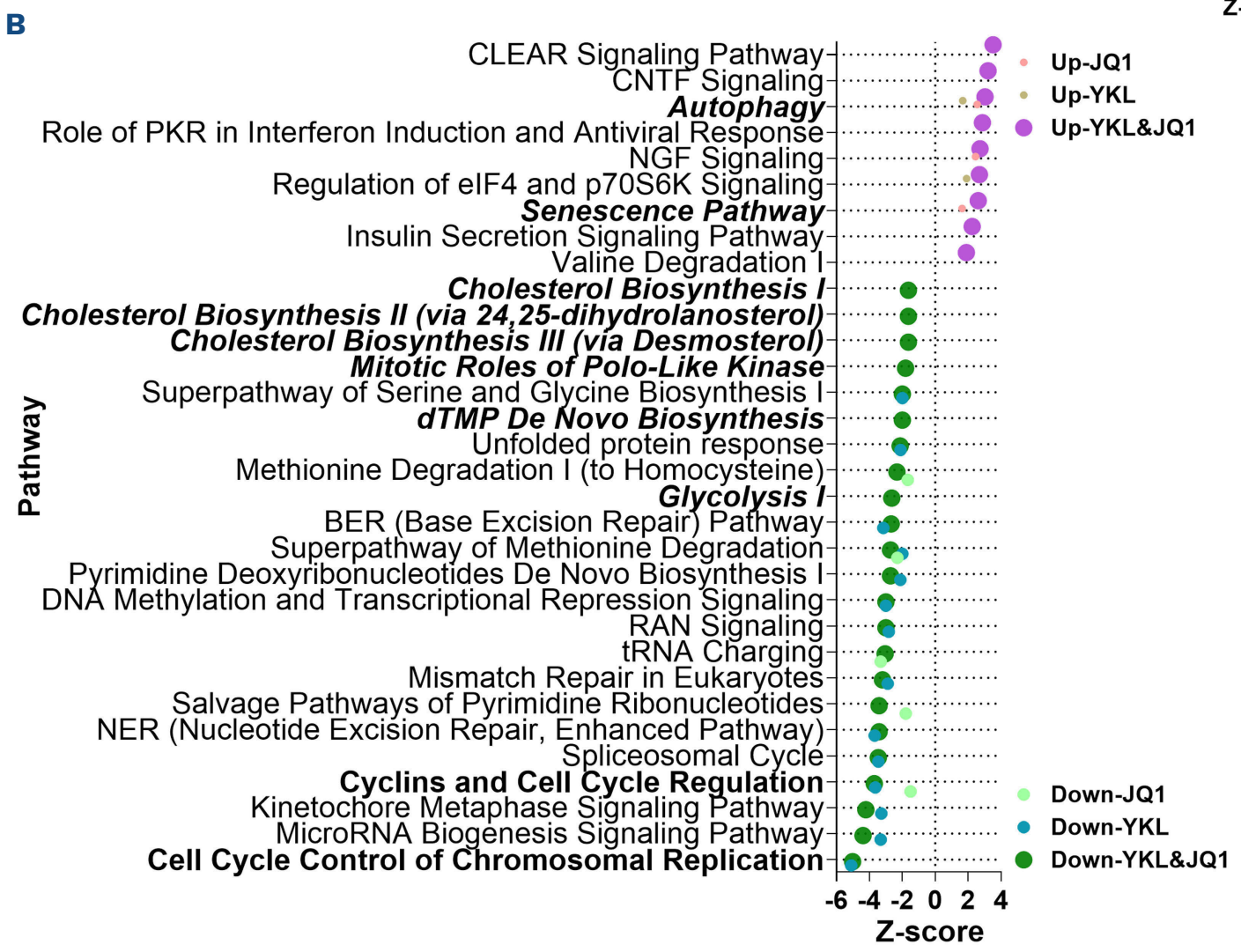
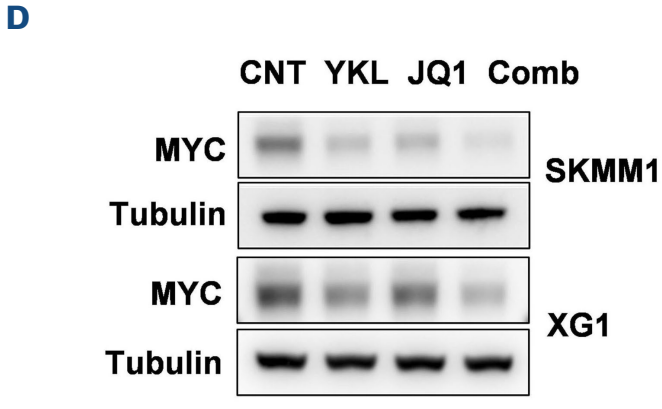


Figure 2. YKL-5-124 synergizes with JQ1 to induce cell cycle arrest and apoptosis in multiple myeloma cells. (A, B) AMO1 cells were treated with 100 nM YKL-5-124 or 100 nM JQ1 alone or in combination for 24 hours, and western blot analysis was performed using indicated antibodies against p-RB (s780, s795 or s801/811), RB, p-CDK4, and CDK4, with tubulin as a loading control (left panel). The ratios of phosphorylation of RB/tubulin (A) and p-CDK4/CDK4 (B) were analyzed by Image J software (right panel). (C) Multiple myeloma cells were treated with 50 nM YKL-5-124 or 50 nM JQ1 alone or in combination for 24 hours. Cell cycle was evaluated by flow cytometric analysis following propidium iodide (PI) staining and analyzed with ModFit LT 5.0 software. Data represent the mean of values obtained from two cell lines (MOLP8 and SKMM1). (D) MOLP8 and SKMM1 cells were treated with 50 nM YKL-5-124 or 50 nM JQ1 alone or in combination for 48 or 72 hours. Apoptotic cell death was assessed by flow cytometric analysis following annexin V^+/PI staining. CNT: control; Comb: combination.









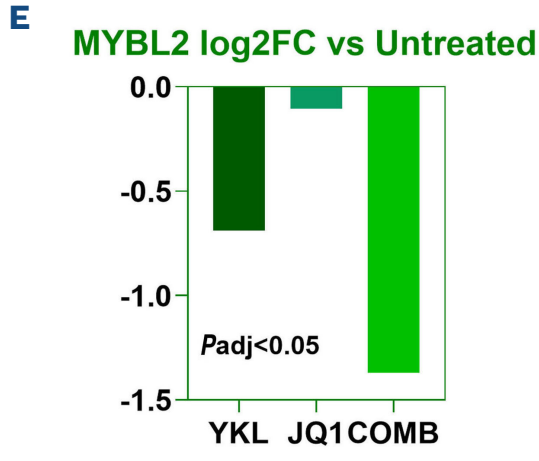


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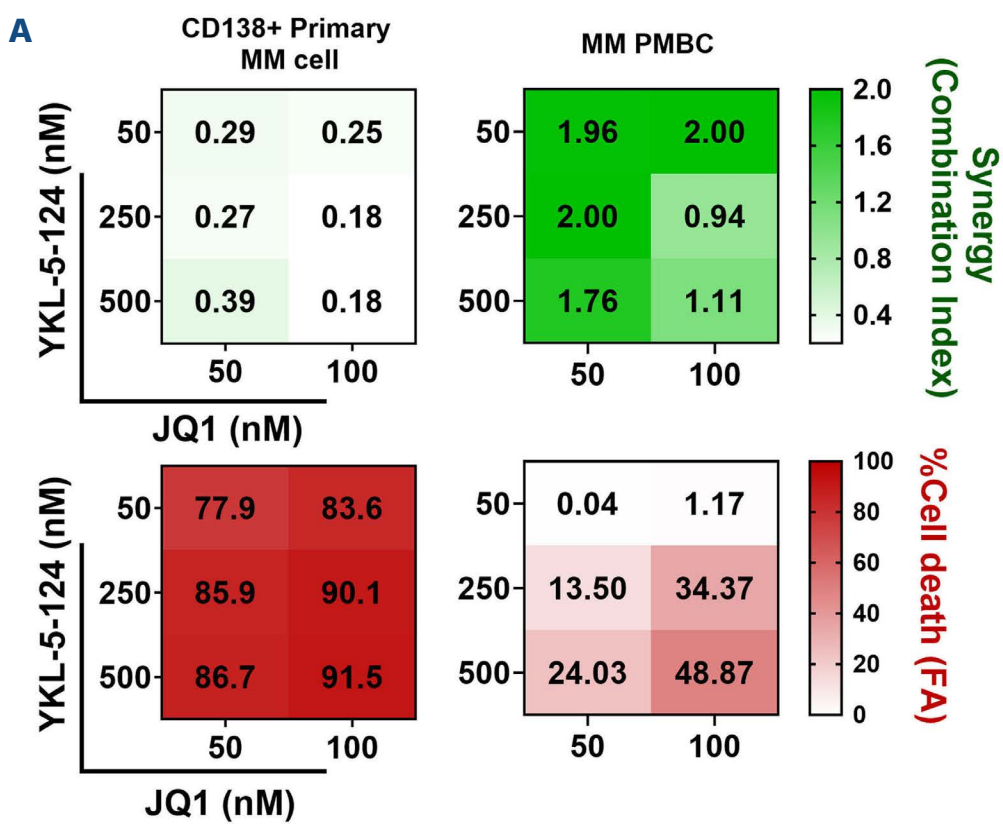
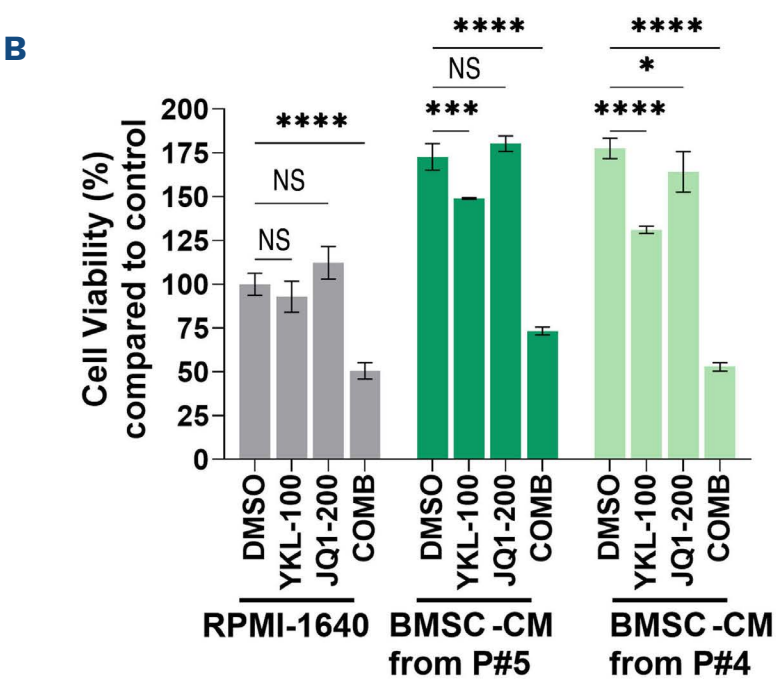
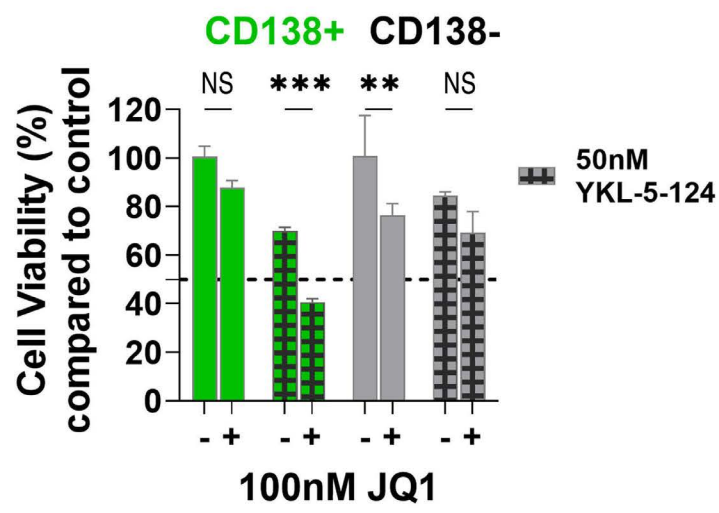
Figure 3. JQ1 and YKL-5-124 combination treatment induces transcriptomic alterations in multiple myeloma cells. (A) AMO1 cells were treated with 200 nM JQ1 and 50 nM YKL-5-124 for 24 hours individually and in combination and then subjected to RNA-sequencing analysis. The Venn diagram shows overlap of genes in these three groups (log_2 fold change >1, adjusted P<0.05 or log_2 fold change <-1, adjusted P<0.05). (B) Canonical pathway analysis was done using ingenuity pathway analysis (IPA). Top predicted negative canonical pathways are shown in the bubble plot. (C) Biological upstream regulators associated with CDK7 and BET inhibition were identified using IPA. Top predicted upstream regulators for RNA-sequencing data in the group treated with the YKL-5-124 and JQ1 combination are shown in the table (red = activated, green = inhibited). (D) SKMM1 and XG1 cells were treated with 100 nM JQ1 and 100 nM YKL-5-124 for 24 hours individually and in combination, and western blot analysis was performed using antibodies against MYC and tubulin. (E) MYBL2 log_2 fold change following treatment with single agents or the combination regimen compared to the dimethyl sulfoxide control. Log2FC: log_2 fold change, COMB: combination.

preclinical models.[11,13] However, despite being a promising target in preclinical models with known c-MYC dependency, monotherapy trials assessing the efficacy and safety profile of BET inhibitors in MM fell short of expectations. In the OTX015 clinical trial, activity was not noted in any of the 12 MM patients, with only two (17%) experiencing stable disease.[13] Moreover, BET inhibitors demonstrated a relatively high incidence of treatment-emergent adverse events, with thrombocytopenia, nausea, diarrhea, and fatigue ranking as the top four adverse events of all grades.[14]

One of the main challenges of monotherapy is the potential for cancer cells to develop resistance over time. Cancer cells,







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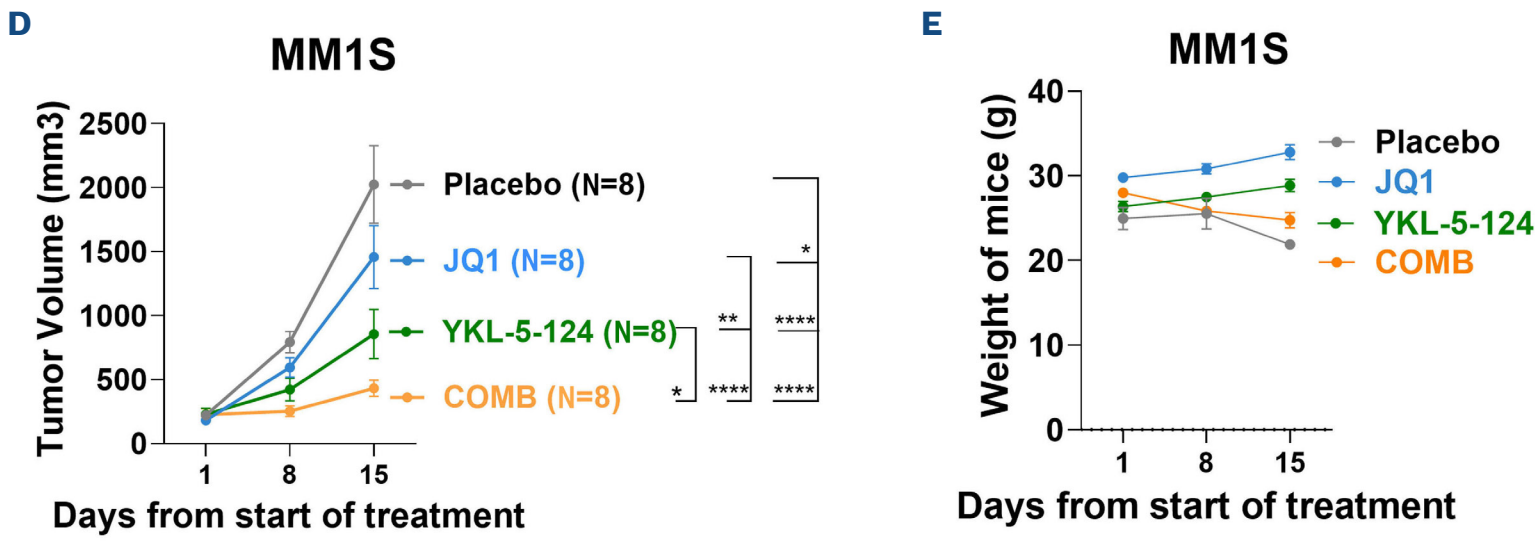

Figure 4. Therapeutic potential of combination therapy *ex vivo* in primary cells and *in vivo* in a humanized myeloma mouse model. (A) CD138$^+$ primary cells and peripheral blood mononuclear cells were cultured in the presence of different concentrations of YKL-5-124 with or without JQ1 for 24 hours and cell survival was assessed by a CellTiter-Glo uptake assay. Data are presented as synergy (combination index) and cell death (fraction affected). (B) Multiple myeloma cells were cultured with and without supernatant from primary bone marrow stromal cell cultures obtained from bone marrow mononuclear cells from two myeloma patients, in the presence of YKL-5-124 or JQ1 alone and in combination for 24 hours. Data represent the mean value ± standard deviation (SD) of one experiment performed in triplicate. *P<0.05, ***P<0.001, ****P<0.0001. (C) Bone marrow mononuclear cells from one myeloma patient were cultured in the absence or presence of 50 nM YKL-5-124 or 100 nM JQ1 alone and in combination for 24 hours. Cell viability of both CD138$^+$ and CD138$^-$ cells was assessed and expressed as percentage of live cells compared to untreated cells. **P<0.01, ***P<0.001. (D, E). Sub-lethally irradiated SCID mice were injected subcutaneously with MM1S cells. Mice were randomized to receive YKL-5-124 (2.5 mg/kg, i.p, 5 days/week), JQ1 (50 mg/kg, 2 days/week) or a combination for 2 weeks. Tumor volume was evaluated by caliper measurement. Fold change increase of tumor volume compared to start of treatment (day 1) (mean ± SD) (D) and body weight (E) are shown. *P* values indicate statistically significant differences between groups. *P<0.05, **P<0.01, ****P<0.0001. MM: multiple myeloma; PBMC: peripheral blood mononuclear cells; FA: fraction affected; NS: not significant; BMSC: bone marrow stromal cells; CM: conditioned medium; COMB: combination.

including myeloma cells, can adapt and evolve, often rendering single-agent treatments ineffective in the long term. Additionally, targeting a single pathway may not be sufficient to completely halt tumor growth, as cancer cells can utilize alternative pathways to bypass the effects of an inhibitor. The therapeutic application of cell cycle kinases in MM, for example, has generally encountered obstacles due to a lack of efficacy as single agents, suggesting that targeting cell cycle regulation is insufficient to elicit a sustained response in MM.[28] This aligns with our investigations into YKL-5-124, a specific CDK7 inhibitor,[19] in MM cells, in which we observed that selective perturbation of CDK7 activity alone was insufficient to consistently downregulate most super-enhancer-driven gene expression programs in MM cells.

We therefore tested whether combining inhibitors for CDK7 and BET bromodomain proteins would overcome each of their limitations as single agents. We found that low doses of YKL-5-124 and JQ1 proved highly synergistic and synthetically lethal in MM and WM cells, including in resistant settings. This drug combination robustly inhibited cell proliferation and triggered apoptosis. We also demonstrated that the synergistic cytotoxicity observed *in vitro* translates into antitumor effects *in vivo*, supporting the therapeutic potential of targeting CDK7 and BRD4 in combination in MM. In line with our findings, recent reports have documented synergistic effects between JQ1 and THZ1 (a CDK7 inhibitor with additional inhibitory activity toward CDK11 and CDK12) against glioma, MYCN-amplified neuroblastoma and medulloblastoma.[23] Notably, the combination of CDK7 inhibition with JQ1 exhibited more pronounced antiproliferative activity compared to JQ1 combined with CDK4/6 inhibition. By targeting multiple nodes within the cell cycle machinery, CDK7 inhibition likely exerts a more comprehensive blockade of cell cycle progression and may more effectively bypass the plasticity and compensatory/adaptation mechanisms observed with CDK4/6 and CDK2 inhibitors.[30]

Recent analysis of CRISPR screening data in breast cancer has shown that deletion of positive regulators of G1-S progression (e.g., *CCND1* and *CDK4*) sensitizes cells to JQ1, whereas deletion of negative regulators (e.g., *RB1* and *CDKN1A*) increases JQ1 resistance.[31,32] Given that YLK-5-124 indirectly inhibits a positive regulator (CDK4) and activates a negative regulator (RB), it effectively increases the sensitivity to JQ1 and may overcome acquired and intrinsic resistance, as shown in the XG1 MM cells. This delicate balance likely underpins the observed synergistic cell death elicited by the combination therapy.

In summary, our study elucidates several mechanisms contributing to the heightened efficacy of the combination therapy in our MM system. These mechanisms include CDK7-mediated G1 arrest and activation of RB, alongside the modulation of distinct and overlapping super-enhancer dependencies. Overall, our findings provide compelling preclinical evidence supporting the therapeutic strategy of

targeting CDK7 in combination with epigenetic therapies relying on BRD4 inhibition for the treatment of patients with MM and WM.

Disclosures

NCM is a consultant for BMS, Janssen, Oncopep, Amgen, Karyopharm, Legened, AbbVie, Takeda and GSK and serves on the board of directors of, and holds stocks options in, Oncopep. The other authors have no conflicts of interest.

Contributions

MF designed and conducted the study and wrote the manuscript. YY performed the in vitro and in vivo experiments and analyzed the data. SD, JFN, MY, CC and VJW helped with the in vitro and in vivo experiments. NM provided critical evaluation of experimental data and the manuscript.

Acknowledgments

We would like to thank Christina Usher for her comments on the manuscript.

Funding

This study was supported by National Institutes of Health (NIH)/National Cancer Institute (NCI) P01 grant (CA155258-10) to MF and National Natural Science Foundation of China (81870163) and Major Basic Research Project of the Natural Science Foundation of the Jiangsu Higher Education Institutions (19KJA560001) to YY.

Data-sharing statement

The data that support the findings of this study are available on request from the corresponding author.

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