Pirtobrutinib and venetoclax combination overcomes resistance to targeted and chimeric antigen receptor T-cell therapy in aggressive mantle cell lymphoma

Despite the remarkable success of targeted therapies for mantle cell lymphoma (MCL), including inhibitors of Bruton tyrosine kinase (BTK) and CD19-directed chimeric antigen receptor (CAR) T-cell therapy, resistance and disease relapse persist, so there is an urgent need to develop novel agents and combinatorial strategies against this deadly disease.[1,2] BTK is a key component of the B-cell receptor pathway, which regulates B-cell survival and proliferation. Ibrutinib, the first Food and Drug Administration-approved covalent BTK inhibitor, achieved overall response rates of 70-77% in patients with relapsed/refractory MCL,[3] which represented a major milestone in targeted MCL therapies.[4] As a key regulator of apoptosis, BCL-2 is aberrantly expressed in MCL, and its inhibition with venetoclax (ABT-199) induces massive apoptosis in MCL cells.[5] Notably, combinatorial ibrutinib and venetoclax yielded favorable complete response rates in MCL patients in a phase II study (71%)[6] and in the phase III SYMPATICO study (62%),[3] indicating that novel combinatorial approaches can be useful to overcome therapeutic resistance and increase durability of effective treatments for MCL.

Pirtobrutinib (LOXO-305) is a next-generation, highly selective, non-covalent BTK inhibitor.[7] Compared to traditional covalent BTK inhibitors, pirtobrutinib achieves remarkable target coverage regardless of the intrinsically high rate of BTK turnover, and lacks the off-target inhibition of other kinases.[8] In the phase I/II BRUIN study pirtobrutinib exhibited promising efficacy in heavily pretreated MCL patients irrespective of prior exposure to covalent BTK inhibitors.[9] Given the clinical success of combinatorial ibrutinib and venetoclax in MCL patients, we investigated and here report the antitumor effects of pirtobrutinib in combination with venetoclax in various MCL models *in vitro* and *in vivo* to provide proof of concept for further exploration in the clinic. The patients' apheresis samples used in this study were collected after obtaining informed consent and approval from the Institutional Review Board at The University of Texas MD Anderson Cancer Center and all experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

First, we performed *in vitro* cell viability assays to test the efficacy of the combination of pirtobrutinib and venetoclax in a panel of MCL cell lines and primary MCL patients' samples (Figure 1A, B). Compared to the effects of single agents, the cytotoxicity of the combination was enhanced in all the cells tested irrespective of their response to ibrutinib, venetoclax, and even anti-CD19 CAR T-cell therapy, indicating that this novel combination has promising potential for overcoming multiple types of therapeutic resistance in MCL. A dose-response viability assay for BTK knockdown JeKo-1 cells (JeKo BTK KD_2) (*Online Supplementary Figure S1A*)[10] and ibrutinib-resistant JeKo-1 cells (JeKo-ibrutinib-R)[11] showed a clear synergistic effect in reducing cell viability with combination indexes[12] <1 (0.54 and 0.47, respectively) (*Online Supplementary Figure S1B*). As a functional outcome, the annexin V/propidium iodide apoptosis assay revealed enhanced cytotoxicity of the combination compared to single agents in ibrutinib-resistant cell lines (JeKo BTK KD cells and JeKo-ibrutinib-R) and a venetoclax-resistant cell line (Mino-venetoclax-R)[13] (*Online Supplementary Figure S1C*). Consistent with this, reverse-phase protein array analysis on JeKo-ibrutinib-R cells confirmed enhanced apoptosis for the combination, as demonstrated by increases in the stress response proteins p-NDRG1 and p-JNK, the DNA damage marker γH2AX, and cleaved apoptotic caspases (*Online Supplementary Figure S1D*). In further accordance with these results, western blotting assay verified that the late-stage cell death markers cleaved caspase 3 and cleaved PARP were markedly increased by the combinatorial treatment (*Online Supplementary Figure S1E*).

To determine the *in vivo* anti-MCL efficacy of pirtobrutinib and venetoclax, we tested the combination in an aggressive patient-derived xenograft mouse model generated from a dual ibrutinib- and CD19-targeted CAR T-cell-resistant patient (PT15 in Figure 1B). Compared to a vehicle, each of the single-drug treatments decreased tumor growth in mice, as confirmed by measurement of both tumor volume (Figure 1C) and levels of the tumor marker β_2-microglobulin in mouse serum (*Online Supplementary Figure S2A*). Strikingly, the combination therapy completely suppressed tumor development long after the mice in the other three groups had reached the humane endpoint, and caused no noticeable adverse effects in the mice (e.g., loss of body weight or hair) during the entire treatment (*Online Supplementary Figure S2B*). The combination treatment was discontinued on day 85 to track the effect on tumor progression. The mice remained tumor-free for more than 2 months. Kaplan-Meier survival analysis demonstrated that the combination therapy dramatically prolonged

mouse survival with a median survival longer than 150 days, compared to 81 days for the mice treated with pirtobrutinib and 80 days for those given venetoclax, indicating that this novel combination regimen may be a promising strategy to overcome ibrutinib- and CAR T-cell therapy dual-resistant MCL (Figure 1D).

To validate this *in vivo* finding, we generated a xenograft model by inoculating Mino-venetoclax-R cells subcu-

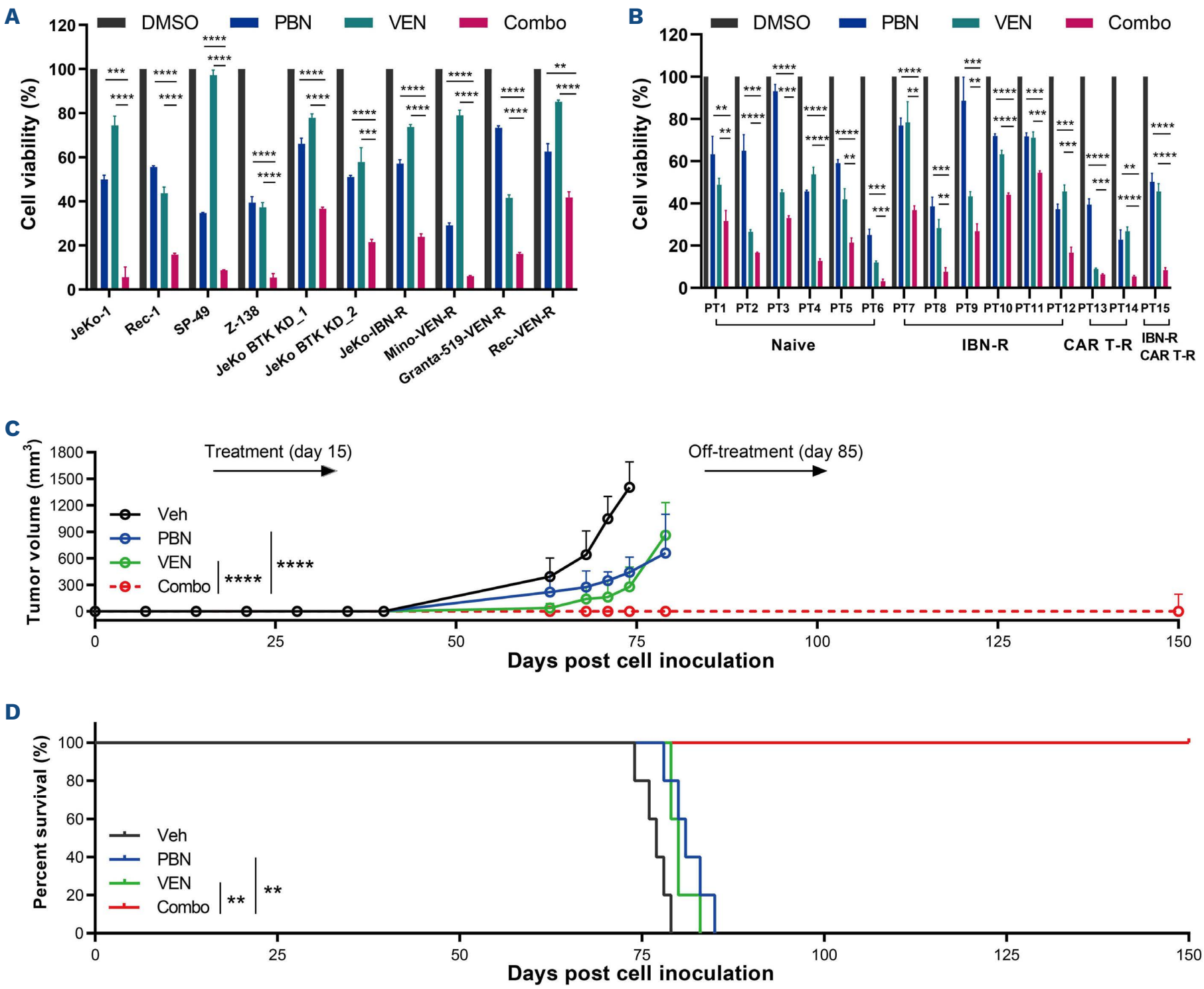
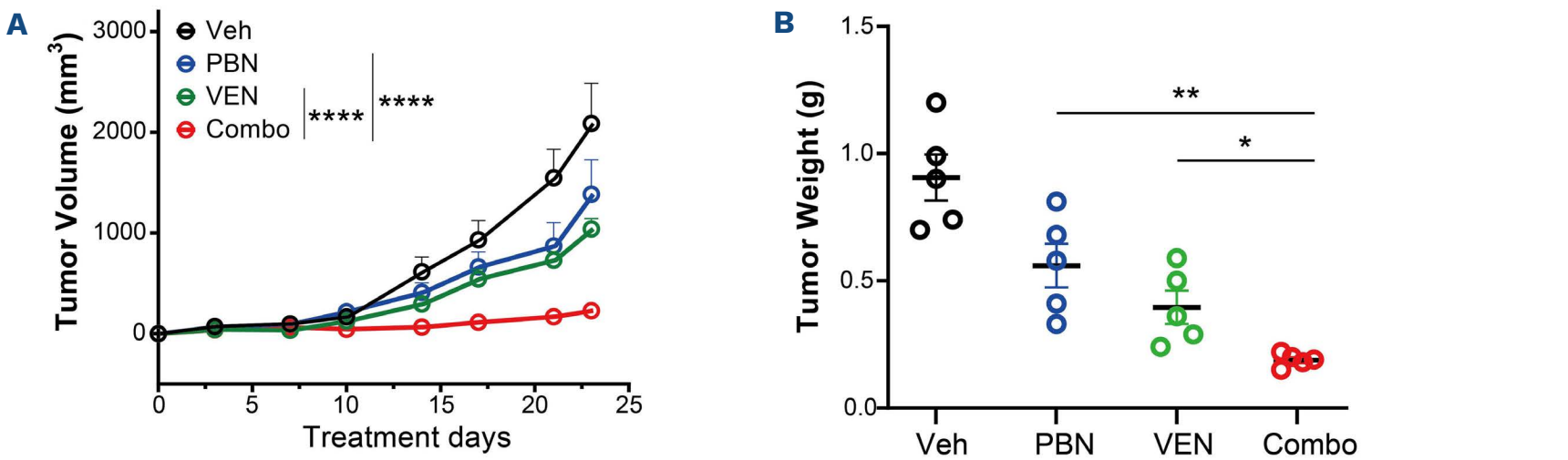


Figure 1. The pirtobrutinib-venetoclax combination exhibits enhanced efficacy against mantle cell lymphoma *in vitro* and in a dual ibrutinib/chimeric antigen receptor T-cell resistant patient-derived xenograft mouse model. (A) The pirtobrutinib and venetoclax combination was tested for effects using a CellTiter-Glo luminescence assay (Promega) after 72 h treatment of a panel of ten mantle cell lymphoma (MCL) cell lines. JeKo-ibrutinib (IBN)-R, Mino-venetoclax-R, Rec-venetoclax-R and Granta-519-venetoclax-R were generated by culturing parental cells with progressively increasing concentrations of ibrutinib or venetoclax. Two BTK knockdown JeKo-1 clones (JeKo BTK KD_1 and JeKo BTK KD_2) were generated using CRISPR/Cas9-mediated editing. Pirtobrutinib 7.5 μM and Venetoclax 25 nM were used for the JeKo-1, Rec-1, SP-49, and Z-138 cell lines; pirtobrutinib 15 μM and venetoclax 50 nM were used for the other cell lines. (B) Apheresis samples from 15 patients (PT1-15) were collected and purified for cell viability assay after 24 h of treatment. The patients' treatment status is indicated under the x-axis. (C) Isolated MCL cells from an established dual ibrutinib/chimeric antigen receptor T-cell-resistant patient-derived xenograft model were engrafted subcutaneously into 6- to 8-week-old NSG mice. Pirtobrutinib (50 mg/kg, oral gavage, BID) and venetoclax (10 mg/kg, oral gavage, QD) were administered as single agents or in combination when tumors became palpable. Tumor volume was calculated using the formula V = (L x W x W)/2 to assess tumor burden. Tumor volumes of animals in the groups treated with the vehicle, pirtobrutinib, venetoclax and the combination of pirtobrutinib and venetoclax are reported as the mean ± standard error of mean. (D) Kaplan-Meier survival curves of tumor-bearing mice treated with pirtobrutinib and venetoclax were used to estimate the survival rate. The humane endpoint was reached if a tumor diameter exceeded 15 mm. The *P* value for survival was determined by a log-rank (Mantel-Cox) test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. DMSO: dimethylsulfoxide; PBN: pirtobrutinib; VEN: venetoclax; Combo: combination; IBN: ibrutinib; R: resistant; CAR T: chimeric antigen receptor T-cells.

taneously into NSG mice. The tumor-bearing mice were administered pirtobrutinib and venetoclax alone or in combination for 25 days and then euthanized. The monotherapy moderately reduced the rate of tumor growth, while the combinatorial treatment almost completely prevented tumor growth, as reflected by tumor volume and mass (Figure 2A, B). These results indicate that the combination holds promise to overcome venetoclax resistance in MCL. To evaluate the transcriptome determinants associated with this combination, whole-transcriptome RNA sequencing studies were performed on tumor cells harvested from mice treated with vehicle, pirtobrutinib, venetoclax, and the combination of the latter two. A total of 967 genes were differentially expressed in the combined treatment group relative to the group given the vehicle control, of which 478 genes were significantly upregulated while 489 genes were downregulated (absolute [log fold change] >1 and adjusted *P* value <0.05) (Figure 2C). In



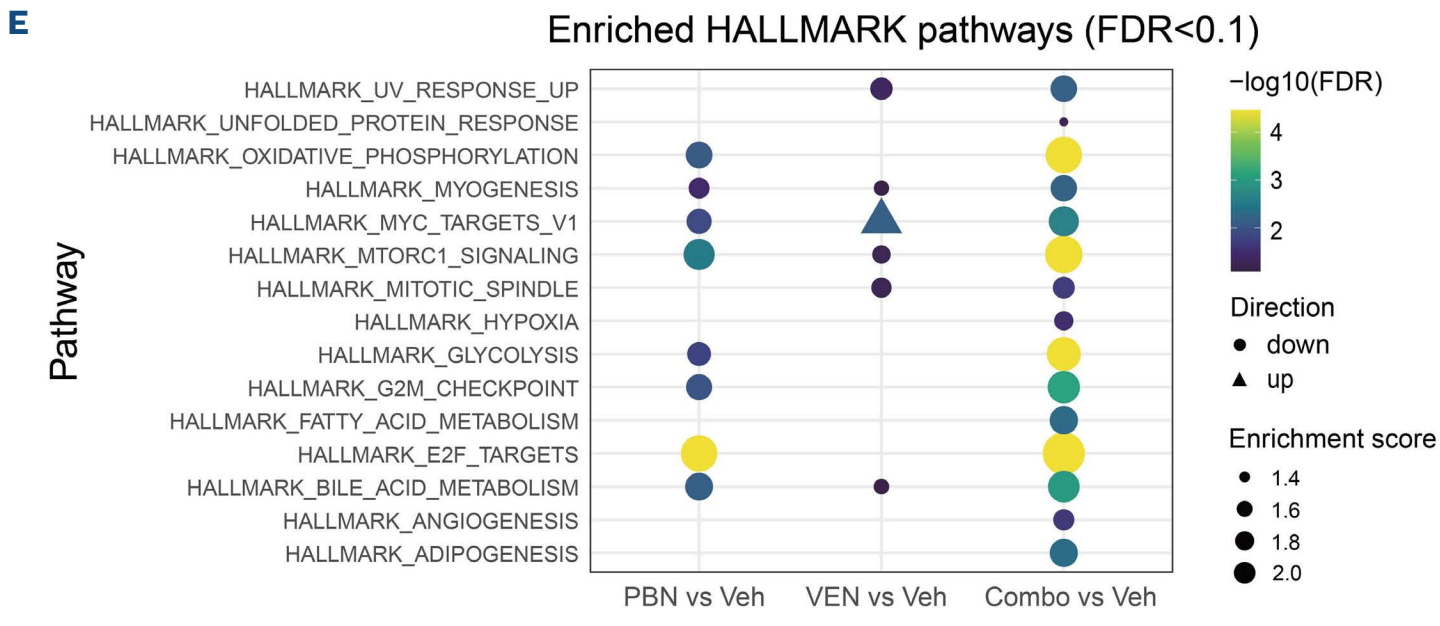


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Figure 2. The pirtobrutinib and venetoclax combination synergistically prevented tumor growth in the Mino-venetoclax-R xenograft mouse model. (A, B) NSG mice aged 6 to 8 weeks old were subcutaneously engrafted with Mino-venetoclax-R cells, then pirtobrutinib (50 mg/kg, oral, BID) and venetoclax (10 mg/kg, oral, QD) treatment was initiated when tumors became palpable and was continued for 25 days. Tumor volume was monitored and calculated using the formula V = (L x W x W)/2. Tumor weights were recorded at the end of the experiment. Data are represented as mean ± standard error of mean (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001). (C) Heatmap showing the expression of differentially expressed genes detected by RNA-sequencing of tumors derived from mice treated with vehicle and the pirtobrutinib-venetoclax combination. (D) Venn diagram displaying the number of overlapped and unique differentially expressed genes for the treatment groups *versus* vehicle. (E) Dot plot showing significantly enriched cancer hallmark pathways (y-axis) in each treatment group compared to control (false discovery rate <0.1). Dots are scaled by enrichment ratio and colored by significance. Shapes represent regulation direction (circle: downregulation, triangle: upregulation). Veh: vehicle; PBN: pirtobrutinib; VEN: venetoclax; Combo: combination; DEG: differentially expressed genes; FDR: false discovery rate.

terms of the genes that were differentially expressed in cells from animals exposed to the combination therapy but not to either monotherapy relative to vehicle, 224 genes (51.9%) were found to be upregulated and 251 genes (60.8%) were downregulated (adjusted *P* value <0.05) (Figure 2D). Gene set enrichment analysis was performed to determine the associated cancer hallmark gene sets. Compared to single treatments alone and vehicle, combination therapy suppressed mTORC1 signaling, MYC targets, E2F targets, oxidative phosphorylation (OXPHOS), fatty acid metabolism, and adipogenesis pathways (false discovery rate <0.1) (Figure 2E, *Online Supplementary Figure S3*). It is noteworthy that upregulation of these oncogenic and metabolic pathways has been previously reported to drive ibrutinib resistance in MCL.[14,15] Furthermore, not only OXPHOS but also glycolysis, the major energy production pathway, was downregulated in the combination group, indicating that mitochondrial energy production for cancer cells to grow and survive was efficiently blocked by this combination therapy.

In summary, our findings demonstrated that the combination of pirtobrutinib and venetoclax had enhanced antitumor efficacy over both monotherapies in preclinical, resistant MCL models and support future investigation of this promising regimen in other B-cell malignancies. Transcriptome profiling revealed a significantly downregulated gene expression signature associated with oncogenic MYC targets, mTORC1 signaling, and metabolic pathways such as glycolysis and OXPHOS. Further mechanistic studies are warranted to elucidate the underpinnings of this combinatorial efficacy. A phase II clinical trial (NCT05529069) based on this study has been activated at MD Anderson Cancer Center.

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https://doi.org/10.3324/haematol.2022.282031

Received: September 1, 2022.
Accepted: November 28, 2022.
Early view: December 7, 2022.

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Disclosures

MW is a consultant to AbbVie, Acerta Pharma, AstraZeneca, BeiGene, BioInvent, Deciphera, InnoCare, Janssen, Kite Pharma, Leukemia & Lymphoma Society, Lilly, Merck, Milken Institute, Oncternal, Parexel, Pepromene Bio, Pharmacyclics, and VelosBio, and he has received research support from Acerta Pharma, AstraZeneca, BeiGene, BioInvent, Celgene, Genmab, Genentech, Innocare, Janssen, Juno Therapeutics, Kite Pharma, Lilly, Loxo Oncology, Molecular Templates, Oncternal, Pharmacyclics, VelosBio and Vincerx. MW has also received speaker's honoraria from AbbVie, Acerta Pharma, AstraZeneca, BeiGene, BioInvent, Dava Oncology, Eastern Virginia Medical School, IDEOlogy Health, Janssen, Kite Pharma, Leukemia & Lymphoma Society, LLC TS Oncology, Medscape, Meeting Minds Experts, MJH Life Sciences, Merck, Moffit Cancer Center, Oncology Specialty Group, OncLive, Pharmacyclics, Physicians Education Resources (PER), Practice Point Communications (PPC), and Studio ER Congressi. All other authors declare that they have no competing financial interests.

Contributions

MW and YL conceived and designed the study; YL, VCJ, AJ, JM, YL, YC, IH and WW performed the experiments; FY and YL analyzed the data; YL wrote the manuscript; MW, FY, HL, YY, LN and JJ edited the manuscript.

Acknowledgments

The authors thank the patients and their families for their contribution to this research study. Thanks to Paul C. Dolber and Numsen Hail for offering help in reviewing and revising the manuscript.

Funding

This work was supported by an SRA grant from LOXO Oncology at Lilly. LOXO Oncology at Lilly provided the pirtobrutinib.

Data-sharing statement

The original data and protocols can be obtained upon reasonable request.

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