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

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Running Title: Combined pirtobrutinib and venetoclax in MCL

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Disclosures

M.W. is 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, Oncertal, Pharmacyclics, VelosBio, Vincerx. M.W. also received a speaker honorarium 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 no competing financial interests.
Authors’ contributions

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

Ethics approval and consent to participate

The patient apheresis samples were collected after obtaining informed consent and approval from the Institutional Review Board at The University of Texas MD Anderson Cancer Center. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

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Data sharing statement

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

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Despite the remarkable success of targeted therapies for mantle cell lymphoma (MCL), including Bruton's tyrosine kinase (BTK) inhibitors and CD19-directed chimeric antigen receptor (CAR) T-cell therapy, resistance and disease relapse persist, presenting an urgent need to develop novel agents and combinatorial strategies against this deadly disease. BTK is a key component of the B-cell receptor pathway, which regulates B-cell survival and proliferation. Ibrutinib, the first FDA-approved covalent BTK inhibitor, achieved overall response rates of 70-77% in patients with relapsed/refractory MCL, which represented a major milestone in targeted MCL therapies. 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. Notably, combinatorial ibrutinib and venetoclax yielded favorable complete response rates in MCL patients in a phase II study (71%) and in the phase III SYMPATICO study (62%), 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. 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. The phase I/II BRUIN study demonstrated that pirtobrutinib exhibited promising efficacy in heavily pretreated MCL patients irrespective of prior exposure to covalent BTK inhibitors. 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.

First, we performed in vitro cell viability assays to test the efficacy of this combination strategy in a panel of MCL cell lines and primary MCL patient samples (Figure 1A and B). Compared to 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 the BTK knockdown JeKo-1 (JeKo BTK KD_2, Supplementary Figure S1A) and
ibrutinib-resistant JeKo-1 (JeKo-ibrutinib-R)\textsuperscript{11} cells showed a clear synergistic effect in reducing cell viability with combination indexes\textsuperscript{12} < 1 (i.e., 0.54 and 0.47, respectively) (Supplementary Figure S1B). As a functional outcome, the annexin V/PI 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)\textsuperscript{13} (Supplementary Figure S1C). Consistent with this, reverse-phase protein array (RPPA) analysis on JeKo-ibrutinib-R cells confirmed enhanced apoptosis for the combination as demonstrated by increases in stress response proteins p-NDRG1 and p-JNK, the DNA damage marker γH2AX, and cleaved apoptotic caspases (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 (Supplementary Figure S1E).

To determine the \textit{in vivo} anti-MCL efficacy, we tested the pirtobrutinib and venetoclax combination in an aggressive patient-derived xenograft (PDX) mouse model generated from a dual ibrutinib- and CD19-targeted CAR T-cell-resistant patient (PT15 in Figure 1B). Compared to 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 (β2M) in mouse serum (Supplementary Figure S2A). Strikingly, the combination therapy completely suppressed tumor development long after the other three groups reached the humane endpoint and caused no noticeable adverse effects in the mice (e.g., loss of body weight or hair) during the entirety of the treatment (Supplementary Figure S2B). The combination treatment was discontinued at day 85 to track the effect on tumor progression. These mice remained tumor-free for more than two months. Kaplan-Meier survival analysis demonstrated that the combination therapy dramatically prolonged mouse survival with a median survival greater than 150 d, compared to 81 d for pirtobrutinib and 80 d for 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 \textit{in vivo} finding, we generated a xenograft model by inoculating Mino-venetoclax-R cells subcutaneously into NSG mice. The tumor-bearing mice were administered pirtobrutinib and venetocla
alone or in combination for 25 d and then euthanized. The monotherapy moderately reduced the rate of tumor growth, while the combinatorial treatment almost completely prevented tumor growth as reflected in tumor volume and mass (Figure 2A and 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 (RNA-seq) 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 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 terms of the genes that are differentially expressed in combination therapy but not in 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 (GSEA) was performed to determine the associated cancer hallmark gene sets. Compared to single treatments alone and vehicle, combination therapy exhibited suppression in mTORC1 signaling, MYC targets, E2F targets, oxidative phosphorylation (OXPHOS), fatty acid metabolism, and adipogenesis pathways (false discovery rate < 0.1) (Figure 2E and Supplementary Figure S3). It is noteworthy that upregulation of these oncogenic and metabolic pathways has been previously reported to drive ibrutinib resistance in MCL.\textsuperscript{14,15} Further, 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.
References

Figure Legends

Figure 1. The pirtobrutinib-venetoclax combination exhibits enhanced anti-MCL efficacy in vitro and in the dual ibrutinib/CAR T-cell resistant PDX mouse model. (A) The pirtobrutinib and venetoclax combination was tested for effects using CellTiter-Glo luminescence assay (Promega) after 72 h treatment of a panel of 10 MCL cell lines. JeKo-ibrutinib (IBN)-R, Mino-venetoclax (VEN)-R, Rec-VEN-R and Granta-519-VEN-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. PBN = 7.5 μM & VEN = 25 nM for JeKo-1, Rec-1, SP-49, and Z-138 cell lines; PBN = 15 μM & VEN = 50 nM for the other cell lines. (B) Apheresis samples from 15 patients (PT1-15) were collected and purified for cell viability assay post 24 h treatment. Patient treatment status is indicated under the x-axis. (C) Isolated MCL cells from an established dual ibrutinib/CAR T-cell resistant PDX 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 for the vehicle, pirtobrutinib, and venetoclax groups are reported as the mean ± SEM. (D) Kaplan-Meier survival curves of tumor-bearing mice treated with pirtobrutinib and venetoclax were used to estimate the survival rate. Humane endpoint was reached if a tumor diameter exceeded 15 mm. The p value for survival was determined by log-rank (Mantel-Cox) test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. PBN = pirtobrutinib; VEN = venetoclax; Combo = combination.

Figure 2. Pirtobrutinib and venetoclax combination synergistically prevented tumor growth in the Mino-venetoclax-R xenograft mouse model. (A&B) 6- to 8-week-old NSG mice 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 tumor became palpable and continued for 25 d. 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 ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001). (C) Heatmap shows...
the expression of differentially expressed genes detected by RNA-seq of tumors derived from mice treated with vehicle and the pirtobrutinib-venetoclax combination. (D) Venn diagram displays the number of overlapped and unique differentially expressed genes for the treatment groups versus vehicle. (E) Dot plot shows significantly enriched cancer hallmark pathways (y-axis) in each treatment group compared to control (false discovery rate < 0.1). Dot is scaled by enrichment ratio and colored by significance. Shape represents regulation direction (circle: downregulation, triangle: upregulation). Veh = vehicle; PBN = pirtobrutinib; VEN = venetoclax; Combo = combination.
Supplementary Data

A

B

JeKo-IBN-R

JeKo BTK KD_2

CI = 0.47
CI = 0.54

C

D

E

Fibronectin
Aurora-B
ER-α_pS118
H2AX_pS139
IGFBP2
JNK_pT183_Y185
Caspase-7-cleaved
Caspase-8-cleaved
CREB_pS133
Histone-H3
Aurora-ABC_pT288
NDRG1_pT346
p90RSK_pT573
Bim
PLK1
ACC_pS79
S6_pS240_S244
Lck
SIRP-alpha
CDK1_pT14
Rb_pS807_S811
Lyn

**

DMSO  PBN  VEN  Combo

Apoptotic cells (%)
Supplementary Figure S1. **In vitro** anti-MCL activity of the pirtobrutinib-venetoclax combination in resistant MCL cell lines. (A) BTK expression levels for parental JeKo-1 cells and two BTK knockdown JeKo-1 clones. (B) Dose-response cell viability assay was performed for JeKo BTK KD _2 and JeKo-ibrutinib-R cells after a 72 h treatment using the CellTiter-Glo luminescence assay (Promega). The combination Index (CI) (where synergistic, additive, and antagonistic effects are defined by CI < 1, CI = 1, and CI > 1, respectively) is indicated in the individual panels. (C) Annexin V/PI apoptosis assay was performed for the indicated resistant MCL cell lines after 24 h treatment with DMSO, pirtobrutinib, venetoclax or the pirtobrutinib-venetoclax combination. **p < 0.01. (D) Heatmap showed RPPA-based protein expression profiles of JeKo-IBN-R cells in response to 12 h treatment of pirtobrutinib, venetoclax, and their combination. Names of proteins of interest are written in red. (E) JeKo-BTK KD _2 and JeKo-IBN-R cells were treated with pirtobrutinib or/and venetoclax for 24 h. Apoptosis induction is indicated by cleaved caspase 3/7 and PARP. GAPDH was used as a loading control.
Supplementary Figure S2. The pirtobrutinib-venetoclax combination showed enhanced anti-MCL efficacy in Mino-venetoclax-R xenograft model with a favorable safety profile. (A) Mouse tail vein blood was collected
periodically and β2M levels in the serum were measured with a β2M ELISA kit to assess the tumor burden. (B) Mouse body weight was monitored throughout the treatment period as indicated. (C) GSEA enrichment plots show downregulation of mTORC1, MYC targets, OXPHOS, and glycolysis pathways in the combination group compared to vehicle. (D) Box plots display the expression of representative differentially expressed genes in the combination group versus both single treatments and vehicle group. n = 3 per group. *p < 0.05. **p < 0.01. ***p < 0.001.

Supplementary Figure S3. Heatmap shows the expression of the most downregulated gene targets in oncogenic and metabolism pathways by combination treatment in Mino-venetoclax-R xenograft model. Veh = vehicle; PBN = pirtobrutinib; VEN = venetoclax; Combo = combination.