INTRODUCTION

Bruton's tyrosine kinase (BTK) inhibitors (BTKi) have been approved by the FDA for the treatment of relapsed/refractory (R/R) MCL; however, intrinsic and acquired BTKi resistance remains a clinically unmet challenge. Therefore, overcoming BTKi resistance is critical to improving clinical patient outcome. Targeting compensatory pathways that drive BTKi resistance by single agents or in combination could potentially overcome BTKi resistance. Duvelisib is a dual PI3Kδ/γ inhibitor that has shown modest preclinical activity in both ibrutinib-sensitive and ibrutinib-resistant (IR) MCL. Venetoclax is a specific BCL-2 inhibitor that has been approved to treat relapsed chronic lymphocytic leukaemia (CLL) and shown clinical efficacy in MCL patients, especially when combined with ibrutinib. In IR MCL cells, sustained PI3K-mTOR activation results in high expression of BCL-2 and MCL-1. Duvelisib treatment decreased BCL-2 expression and sensitized venetoclax treatment in CLL. Therefore, we hypothesized that targeting PI3K by duvelisib and BCL-2 by venetoclax...
would result in a synergistic anti-MCL activity and overcome BTKi resistance. In this study, we investigated the in vitro and in vivo efficacy of duvelisib and venetoclax combination in IR MCL cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Mantle cell lymphoma cell lines JeKo-1, Maver-1, Z138, JeKo-R, JeKo BTK KD cells and JeKo-Luc cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. HS-5 cells were maintained in DMEM medium with 10% FBS and 1% penicillin/streptomycin.

2.2 | Primary patient samples

Patient samples were obtained through a protocol approved by the Institutional Review Board at MD Anderson Cancer Center.

2.3 | Reagents

Ibrutinib (S2680), venetoclax (S8048) and duvelisib (S7028) were purchased from Selleckchem.

2.4 | Cell viability, cell apoptosis assay and Western blotting

These assays were performed as described previously.

2.5 | Reverse phase protein array analysis

JeKo BTK KD cells were treated with duvelisib and/or venetoclax for 24 h and subjected to reverse phase protein array (RPPA) analysis as described previously. Proteins with >twofold change between the combination and vehicle were selected to generate the heatmap.

2.6 | Stroma-mediated cell migration and viability assay

The bottom chamber of the Transwell system was pre-seeded with $1 \times 10^5$ HS-5 and left to attach overnight. $1 \times 10^5$ MCL cells were labelled with CMFDA, pre-treated with duvelisib and/or venetoclax for 30 min and washed with PBS before adding to the upper chamber. Four hours later, CMFDA-positive cells (bottom chamber migrated MCL cells) were expressed as a percentile of cells/well. JeKo-1 cell viability in response to ibrutinib was tested in the presence and absence of HS-5 cells using a similar procedure but without the Transwell system.

2.7 | In vivo drug efficacy in mouse xenograft models

$5 \times 10^6$ JeKo-Luc cells were injected subcutaneously into each 6–8-week-old NOD SCID IL2R null (NSG) mice. Treatment was started 3 days following inoculation: vehicle, ibrutinib (50 mg/kg, oral, daily), venetoclax (50 mg/kg, oral, daily) and duvelisib (50 mg/kg, oral, daily) alone or in combination for 3 weeks. Tumours were measured weekly by IVIS Spectrum In Vivo Imaging System (Perkin Elmer).

2.8 | Animal study approval

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

2.9 | Statistics

Student’s t-test was used to calculate statistical significance.

3 | RESULTS AND DISCUSSION

3.1 | Duvelisib and venetoclax in combination synergistically inhibited the cell growth of ibrutinib-resistant MCL models

All ibrutinib-resistant cell lines and patient samples (except Pt4) were only slightly responsive to duvelisib as a single agent, whereas two cell lines (Z138 and Maver-1) and two patient samples (Pt1 and Pt3) were sensitive to venetoclax as a single agent (Figure 1A,B). A striking synergy was observed in 3/4 cell lines and 3/4 patient samples, while a weaker combination effect was observed in Maver and Pt4 (Figure 1A-C). This synergistic effect was also confirmed by isobologram analysis (Figure S1A-B). Consistently, cell apoptosis (30.3%–97.0%) was induced by the combination therapy (combination index 0.36–0.77) in all tested cell lines (Figure 1C). The patient characteristics of the four patients used is summarized in Table S1.

To understand the underlying mechanism of action, we performed an unbiased proteomic analysis by reverse-phase protein array (RPPA) analysis with 425 antibodies encompassing multiple signalling pathways (Figure 1D). PI3K/AKT/mTOR, PKC and PLK1 signalling pathways were predominantly downregulated in the combination-treated cells, validating the intended targets. PI3K/AKT/mTOR plays a crucial role in IR, and blocking this pathway is a rational strategy for overcoming IR. PKC activation correlates with ibrutinib relapse in CLL. BTK activates and upregulates the expression of BCL-2 family. PLK1, a central cell cycle regulator at G2/M phase, is associated with IR as previously shown. Moreover, the most upregulated proteins in the combination group were apoptosis-related proteins such as caspases and Histone-H3 (Figure 1D). These
results were further validated by Western blotting (Figure 1E). Together, these data demonstrate that the combination of duvelisib and venetoclax inhibits Ibrutinib resistance. 13 PI3K-Akt-mTOR pathway and activation of pro-apoptotic signalling.

3.2 | Duvelisib and venetoclax in combination inhibit significantly TME-mediated cell migration

The tumour microenvironment (TME) plays a critical role in drug resistance in many cancer types. 13 PI3K-AKT signalling plays a crucial role in regulating the tumour-TME interplay, and targeting this pathway via duvelisib has shown to disrupt the malignant B-cell homing process. 14-16 To investigate whether duvelisib and venetoclax in combination synergistically block directional cell migration, we performed a Transwell migration assay using IR JeKo BTK KD and human stroma cell line HS-5 cell monolayer as the TME attractant (Figure S2A). A 30-min pre-treatment with the venetoclax and duvelisib combination significantly reduced cell migration towards the HS-5 monolayer compared with controls (p = 0.027) and with venetoclax alone (p = 0.036 and p = 0.056, respectively; Figure 1F and Figure S2B). These data suggest that this combination has the potential to interfere with the chemotactic effect and, therefore, impacting directional migration.

3.3 | The combination of duvelisib and venetoclax overcomes TME-mediated ibrutinib-resistance

To investigate the role of TME in tumour phenotypic changes, we cocultured ibrutinib-sensitive JeKo-1 cells with HS-5 cells. JeKo-1 cells

![Image](image-url)
became IR when co-cultured with HS-5 cells in vitro ($p < 0.0001$; Figure 2A). The effect of TME shifting the JeKo-1 phenotype from sensitive to IR was also confirmed in vivo as mice bearing JeKo-Luc-derived xenografts showed no response to ibrutinib treatment ($p = 0.0003$) to the combination (Figure 2G,H). Altogether, these data demonstrate that this combination may potentially subvert primary, acquired and TME-associated MCL IR in both, in vitro and in vivo.

Here, we demonstrated that duvelisib and venetoclax in combination overcomes IR-MCL synergistically in vitro and in vivo. We investigated different types of IR including intrinsic, acquired and TME-associated resistance mechanisms. The combination reduced tumour growth and migration in all IR cell types, indicating that this is a robust and sustained therapeutic strategy for overcoming...
IR. While the exact mechanism is still under investigation, we report here that PI3K-AKT/mTOR and Bcl-2 signalling are the main IR phenotype drivers. There are four PI3K inhibitors: idelalisib,17 duvelisib,18 alpelisib19 and copanlisib20 that have been approved by FDA. We tested three of the four PI3K inhibitors in MCL cells, excluding alpelisib (targeting PI3Kδ) since PI3Kδ is highly expressed in haematologic malignancies,21 including MCL (Figure S3). Both duvelisib (targeting PI3Kδ/PI3Kγ) and copanlisib (pan-class I PI3K inhibitor), but not idelalisib (targeting PI3Kδ), showed potent anti-MCL activity (data not shown). MCL cells express all four isoforms of PI3K (Figure S3), so it is likely that targeting PI3Kδ only by idelalisib is not sufficient for inducing cytotoxicity in MCL. Expression of other isoforms in addition to PI3Kδ may compensate for the PI3K-mediated tumour cell survival and growth. Our data on duvelisib and venetoclax in combination provides evidence to support the dual targeting of PI3K and BCL-2 as a promising therapeutic strategy to overcome ibrutinib resistance in R/R MCL. It is likely copanlisib and venetoclax in combination will also show anti-MCL synergy.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Haige Ye: Data curation (equal); Formal analysis (equal); Investigation (lead); Methodology (equal); Writing – review & editing (supporting).

Shengjian Huang: Data curation (equal); Formal analysis (equal); Investigation (supporting); Methodology (equal); Writing – original draft (lead); Writing – review & editing (supporting).

Yang Liu: Formal analysis (supporting); Investigation (supporting).

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Michael Wang: Conceptualization (lead); Funding acquisition (lead); Resources (lead); Supervision (supporting); Writing – review & editing (supporting).

Vivian Changying Jiang: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Project administration (lead); Supervision (lead); Writing – original draft (supporting); Writing – review & editing (lead).

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