

Dual BTK and BCL-2 inhibition disrupts the molecular basis of CXCR4 mutation-driven resistance in Waldenström macroglobulinemia

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Background: Somatic *CXCR4* mutations (*CXCR4*^{MUT}) occur in 20–40% of patients with Waldenström macroglobulinemia (WM) and are associated with enhanced survival signaling, retention of malignant cells within the bone marrow microenvironment, and reduced sensitivity to Bruton's tyrosine kinase inhibitors (BTKi), particularly the 1st generation BTKi, ibrutinib (IBR).

Aims: To elucidate the mechanisms of CXCR4-mediated BTKi resistance, the most prevalent *CXCR4*^{MUT} were introduced into experimental models *in vitro* and *in vivo*. We then evaluated the efficacy of IBR or the 2nd generation BTKi zanubrutinib (ZAN) in combination with the BCL-2 inhibitor venetoclax (VEN), to delineate the molecular, functional, and microenvironmental mechanisms underlying their synergistic activity.

Methods: MWCL-1, BCWM-1 and OCI-LY3 cells were transfected with *CXCR4*^{S338X}, *CXCR4*^{R334X}, *CXCR4*^{G336X}, *CXCR4*^{E343K} or *CXCR4*^{E343X} plasmids using Lipofectamine™ 3000. CXCR4 expression was determined using qPCR and flow cytometry (FC). Transfected cells were cultured in the presence of IBR (5μM), ZAN (5μM), VEN (1μM) or BTKi/VEN combination for 48 h. Proliferation was estimated using CCK-8. Cell cycle and apoptosis were evaluated by propidium iodide and Annexin V/7-AAD staining (FC), respectively. RNA-Seq analysis was performed in MWCL-1 and BCWM-1 cells transfected with *CXCR4*^{MUT} or wild-type plasmids with or without BTKi, VEN or their combination. Cytokine and growth-factor dynamics were profiled using Milliplex assays. Therapeutic efficacy and tolerability of ZAN/VEN combination (25mg/kg each) were validated in a *CXCR4*^{S338X} xenograft NOD/SCID mouse model. Tumor burden, angiogenesis, circulating tumor cells, serum IgM levels, and systemic cytokine secretion were assessed using confocal microscopy, FC and ELISA, respectively. Patient serum cytokine profiles before and after BTKi therapy were also measured by Milliplex assay.

Results: *CXCR4^{MUT}* conferred marked resistance to single-agent BTKi and reduced sensitivity to VEN. In contrast, combined BTK and BCL-2 inhibition profoundly reduced proliferation, enforced G₁/G₀ arrest, and triggered robust apoptosis across all *CXCR4^{MUT}* cells. ZAN consistently outperformed IBR both alone and in combination with VEN. RNA-Seq revealed that *CXCR4^{MUT}*-mediated resistance was driven by sustained AKT, RAS, and NFκB signaling. Combination treatment suppressed BCR/NFκB signaling and activated apoptotic transcriptional networks in all WHIM-like *CXCR4^{MUT}* cells. BTK/BCL-2 inhibition induced substantially broader transcriptional alterations compared to BTKi monotherapy, suppressing genes implicated in B-cell activation, proliferation, and inflammatory signaling, including *CD79A*, *MAP3K20*, *RASD2*, *CADM1*, and *CXCR4*, whereas the limited differences versus VEN alone indicate that VEN predominantly shapes the combination transcriptome. Cytokine profiling showed that dual inhibition also reshaped the cytokine milieu, suppressing inflammatory and tumor-supportive microenvironmental factors. In the *CXCR4^{S338X}* mouse xenograft, ZAN/VEN administration was well tolerated and induced tumor regression up to 95.39 ± 0.64% (p<0.0001, n=4 vs. untreated), eliminated circulating tumor cells, reduced angiogenesis and IgM secretion, accompanied by favorable systemic cytokine modulation. Furthermore, analysis in serum samples from WM patients (n=6) undergoing therapy with the above agents corroborated the preclinical findings, demonstrating decreased levels of IL-6, IL-10, GM-CSF, and TNFα following ZAN-based therapy.

Conclusion: Dual BTK/BCL-2 targeting overcomes *CXCR4^{MUT}*-mediated resistance in WM by silencing survival signaling and inducing apoptosis. The ZAN/VEN combination delivers deep antitumor activity beyond monotherapy. These findings support combination strategies for WM patients with *CXCR4^{MUT}* genotype.

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