

Zanubrutinib and pirtobrutinib (ZAP) but not ibrutinib and pirtobrutinib (IAP) show synergistic tumor cell killing and suppression of BTK and downstream signaling in MYD88 mutated lymphoma cells.

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Introduction: Bruton's tyrosine kinase (BTK) plays a central role in oncogenic signaling in MYD88 mutated (MYD88^{Mut}) B-cell malignancies including Waldenström's Macroglobulinemia (WM) and ABC Subtype of Diffuse Large B-Cell Lymphoma (ABC DLBCL). The covalent BTK inhibitors (cBTK-i) zanubrutinib and ibrutinib are highly active and approved for the treatment of symptomatic WM. However, prolonged use of these cBTK-inhibitors is limited by the emergence of acquired resistance mutations, particularly those at BTK^{Cys481}. In WM patients, multiple BTK^{Cys481} acquired mutations can emerge within individual patients (Xu et al, Blood 2017). While combining non-covalent BTK-i (nc-BTK-i) which bind BTK at alternate sites to those of cBTK-i may be a strategy to prevent emergence of resistant disease, no published data exists on the potential synergistic impact for such combinations. We therefore evaluated the therapeutic potential of combining covalent (ibrutinib or zanubrutinib) and noncovalent (pirtobrutinib) BTKis to enhance anti-tumor efficacy and overcome resistance in MYD88^{Mut} B-cell malignancies.

Methods: We evaluated MYD88^{Mut}BCWM.1 and MWCL-1 WM, and HBL-1 and TMD8 ABC DLBCL cells in these experiments. BTK-inhibitors were obtained from MedChem Express. Cell proliferation and drug-drug interactions was evaluated over 72 hours using Cell Titer-Glo Viability Assay. Dose-response curves were calculated using GraphPad Prism. Synergy was assessed using Calcsyn 2.0. Cells were treated with 0.5 uM of compounds for 2 hours, followed by immunoblotting for key signaling proteins including phospho (Y223, Y551) and

total BTK; phospho- and total PLC γ 2; phospho- and total NF κ B-p65; phospho- and total ERK1/2; and GAPDH as a loading control.

Results: Drug synergy analysis revealed that the combination of zanubrutinib and pirtobrutinib (ZAP) exhibited strong synergistic effects across a broad concentration range in all MYD88^{Mut} WM and ABC-DLBCL cell lines evaluated. In contrast, the combination of ibrutinib and pirtobrutinib (IAP) showed predominantly non-additive or antagonistic interactions. In TMD8, ZAP had significantly lower combination indices (median 0.30) versus IAP (median 1.08; $p=0.001$), with similar findings in BCWM.1, MWCL-1 WM, and HBL-1 cells. Based on these findings, we focused on ZAP for further mechanistic evaluation in BCWM.1 and TMD8 lymphoma cells. By western blot analysis, ZAP showed robust inhibition of phosphorylation versus zanubrutinib or pirtobrutinib alone for BTK at both Y223 and Y551; PLC γ 2; NF κ B-p65; and ERK 1/2 in BCWM.1 and TMD8 cells. Moreover, ZAP showed more potent inhibition of BTK activity at both Y223 and Y551, as well as NF κ B-p65 versus IAP which was maintained outwards to 16 hours following treatment. Importantly, in TMD8 cells engineered to express the BTK Cys481Ser mutation, ZAP maintained strong inhibitory effects on BTK activation and its downstream signaling components, demonstrating efficacy in the context of acquired resistance. Moreover, synergistic interactions occurred in TMD8 cells engineered to express either wild-type (median 0.52) or Cys481Ser mutated (median 0.47) BTK following treatment with ZAP. Conversely, non-additive or antagonistic interactions occurred in TMD8 cells engineered to express either wild-type (median 1.23) or Cys481Ser mutated (median 0.71) BTK following treatment with IAP; $p<0.0001$ for wild-type BTK and $p=0.0115$ for Cys481Ser mutated comparisons. In vivo studies in TMD8 xenografted mice to evaluate the relative impact of ZAP vs. zanubrutinib or pirtobrutinib monotherapy on tumor response, overall survival and acquisition of tumor somatic variants associated with BTK-inhibitor resistance have been initiated and will be updated at the meeting.

Conclusion: The ZAP combination exhibited more potent synergistic inhibition of BTK signaling and downstream survival pathways versus zanubrutinib or pirtobrutinib alone or IAP in MYD88 mutated lymphomas. ZAP was also active in MYD88 mutated lymphoma cells expressing mutated BTK Cys481. The results support the investigation of ZAP as a novel therapeutic approach to overcome resistance and improve outcomes in patients with MYD88 mutated B-cell malignancies.

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