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SHORT REPORT

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# ERK1/2 pro-survival signalling is suppressed by pirtobrutinib in ibrutinib-resistant *MYD88*-mutated lymphoma cells

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**Summary**
Covalent Bruton's tyrosine kinase-inhibitors (cBTK-i) are highly active in *MYD88*-mutated (*MYD88*$^{Mut}$) Waldenstrom's macroglobulinaemia and suppress nuclear factor kappa-light-chain-enhancer of activated B cells and extracellular signal-regulated kinases-1/2 (ERK1/2)-related signalling. BTK$^{Cys481}$ mutations are associated with cBTK-i acquired resistance and are accompanied by reactivation of ERK1/2 that promotes inflammatory cytokine secretion and paracrine-mediated resistance of BTK wild-type (BTK$^{WT}$) tumour cells. Pirtobrutinib is a non-covalent BTK-inhibitor that binds at non-BTK$^{Cys481}$ sites. We show that pirtobrutinib blocked p-ERK1/2, ERK1/2-driven inflammatory cytokines, and overcame paracrine-mediated resistance in *MYD88*$^{Mut}$ lymphoma cells expressing mutated BTK$^{Cys481}$. Our results provide important mechanistic insights for the activity of pirtobrutinib in *MYD88*$^{Mut}$ lymphomas carrying BTK$^{Cys481}$ mutations.

**KEYWORDS**
ABC DLBCL, BTK, ERK1/2, ibrutinib, *MYD88*, pirtobrutinib, Waldenstrom's macroglobulinaemia

## INTRODUCTION

*MYD88* mutations (*MYD88*$^{Mut}$) are common in B-cell malignancies including Waldenstrom's macroglobulinaemia (WM) and ABC diffuse large B-cell lymphoma (ABC DLBCL).[1,2] *MYD88*$^{Mut}$ transcriptionally upregulates haematopoietic cell kinase (HCK) that triggers Bruton's tyrosine kinase (BTK)-dependent downstream pro-survival signalling including nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) and extracellular signal-regulated kinases-1/2 (ERK1/2).[3–5] ERK1/2 triggers interleukin (IL)-6 and IL-10 that support autocrine and/or paracrine-mediated survival signalling.[4,5] Approved covalent BTK-inhibitors (cBTK-i) including ibrutinib, zanubrutinib, acalabrutinib and tirabrutinib bind to BTK$^{Cys481}$ and are highly active in WM.[6] Progression due to acquired BTK$^{Cys481}$ mutations is

common in WM patients treated with cBTK-i and is accompanied by reactivation of ERK1/2-related survival signalling.[5,7] Pirtobrutinib is a non-covalent BTK-inhibitor that binds to BTK at non-Cys481 sites and has shown high levels of activity in both cBTK-i naïve and previously exposed WM patients.[8–10] Mechanistic details on the activity of pirtobrutinib in *MYD88*$^{Mut}$ lymphomas carrying mutated BTK are limited. We therefore examined the activity of pirtobrutinib on *MYD88*$^{Mut}$ lymphoma cells, including cells engineered to express mutated BTK$^{Cys481}$. We also focused these studies on the impact of pirtobrutinib on ERK1/2 enabled signalling given the importance of this pathway in autocrine- and paracrine-related survival signalling.

# MATERIALS AND METHODS

## Cell lines, patient cells and reagents

*MYD88*$^{L265P}$ expressing WM (BCWM.1, MWCL-1) and ABC DLBCL (TMD-8, HBL-1), and *MYD88*$^{WT}$ (OCI-Ly7, OCI-LY19, Ramos, RPMI-8226) cells were cultured as previously described.[3–5] Mononuclear cells from bone marrow aspirates of WM patients were treated overnight with ibrutinib or pirtobrutinib and apoptosis analyses performed on CD19$^{+}$ cells as described.[3,5] *MYD88* genotyping of primary patient cells was performed using allele-specific polymerase chain reaction assay. Ibrutinib and venetoclax were purchased from Selleck Chemicals (Houston, TX). Pirtobrutinib was provided by Eli Lilly/LOXO Pharmaceuticals.

## Lentiviral transduction experiments

BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing or control lentiviral vectors were prepared and transduced into *MYD88*$^{Mut}$ WM and ABC DLBCL cells as before.[5] Following lentiviral transduction, stable cell lines were selected by culture with 0.5–1.0 μg/mL puromycin. Protein expression levels for BTK$^{WT}$ or BTK$^{Cys481Ser}$ transduced cells were confirmed by immunoblotting for BTK.

## Signalling studies

Immunoblotting for BTK and its downstream signalling proteins was performed using antibodies for BTK-pY223 (Abcam, Cambridge, MA); BTK (Santa Cruz Biotechnology, Dallas, TX); PLCγ2-pY759, PLCγ2, ERK1/2-pT202/pY204, ERK1/2 (Cell Signaling Technologies, Danvers, MA). GAPDH antibody (Santa Cruz Biotechnology) was used for loading control. BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing WM and ABC DLBCL cells were treated for 2 h with dimethylsulfoxide, ibrutinib or pirtobrutinib (0.1–0.5 μM) prior to immunoblotting.

## Multiplex cytokine and chemokine assay

To assess cytokine production after drug exposure, BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing BCWM.1 WM or TMD-8 ABC DLBCL cells were treated with vehicle control, ibrutinib or pirtobrutinib (0.25 μM) for 36 h at 37°C before supernatants were harvested, aliquoted and stored at −80°C. Sample aliquots were thawed on ice before addition to a customized multiplex plate (Invitrogen). The multiplex plate was read on a Bio-Rad Bio-Plex 3D system, and samples analysed using xPONENT software.

## Cytotoxicity and cell survival studies

The CellTiter-Glo® Luminescent cell viability assay (Promega, Madison, WI) was used to assess the dose–response of inhibitors alone or in combination as before.[3,5] Drug interactions were assessed by CalcuSyn 2.0 software (Biosoft, Cambridge, UK) based on Chou–Talalay method. The resulting combination index (CI) offers quantitative definition for additive effect (CI = 1.0), synergism (CI < 1.0) and antagonism (CI > 1.0) for evaluated drug combinations.[11] Cell survival was assessed using Annexin V-FITC and propidium iodide staining with the Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA).

## Co-culture experiments

For Transwell™ co-culture experiments, BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing TMD8 cells were pretreated with serially diluted ibrutinib in the upper chambers of 0.4 μm filtered 96-well plates for 6 h as before.[5] Non-transduced BTK$^{WT}$ TMD-8 cells were then seeded into the lower chambers. Fresh ibrutinib or pirtobrutinib was added to maintain the same concentration as the upper chamber, and cells incubated for 72 h. Non-transduced cells in lower compartment were then assessed for viability using the CellTiter-Glo® assay.

## Statistical analysis

The statistical significance of differences was analysed using one-way analysis of variance with Tukey's multiple comparisons test by Prism software. Differences were considered significant when $p \leq 0.05$.

# RESULTS

## Anti-proliferative and apoptotic activity for pirtobrutinib in *MYD88*$^{Mut}$ lymphoma cells

Akin to ibrutinib, pirtobrutinib showed selective anti-proliferative activity against *MYD88*$^{Mut}$ WM and ABC DLBCL cell lines (Figure 1A), as well as marked apoptotic

activity at pharmacologically achievable levels against primary treatment-naive *MYD88*$^{Mut}$ WM lymphoplasmacytic cells (Figure 1B).[9] Importantly, pirtobrutinib overcame ibrutinib resistance in *MYD88*$^{Mut}$ BCWM.1 WM and TMD-8 ABC DLBCL cells expressing mutated BTK (BTK$^{Cys481Ser}$) (Figure 1C). Furthermore, the combination of venetoclax and pirtobrutinib showed at least additive tumour cell killing at most combination doses in both BTK$^{WT}$ and BTK$^{Cys481Ser}$ expressing *MYD88*$^{Mut}$ WM and ABC DLBCL cells (Figure 1D).

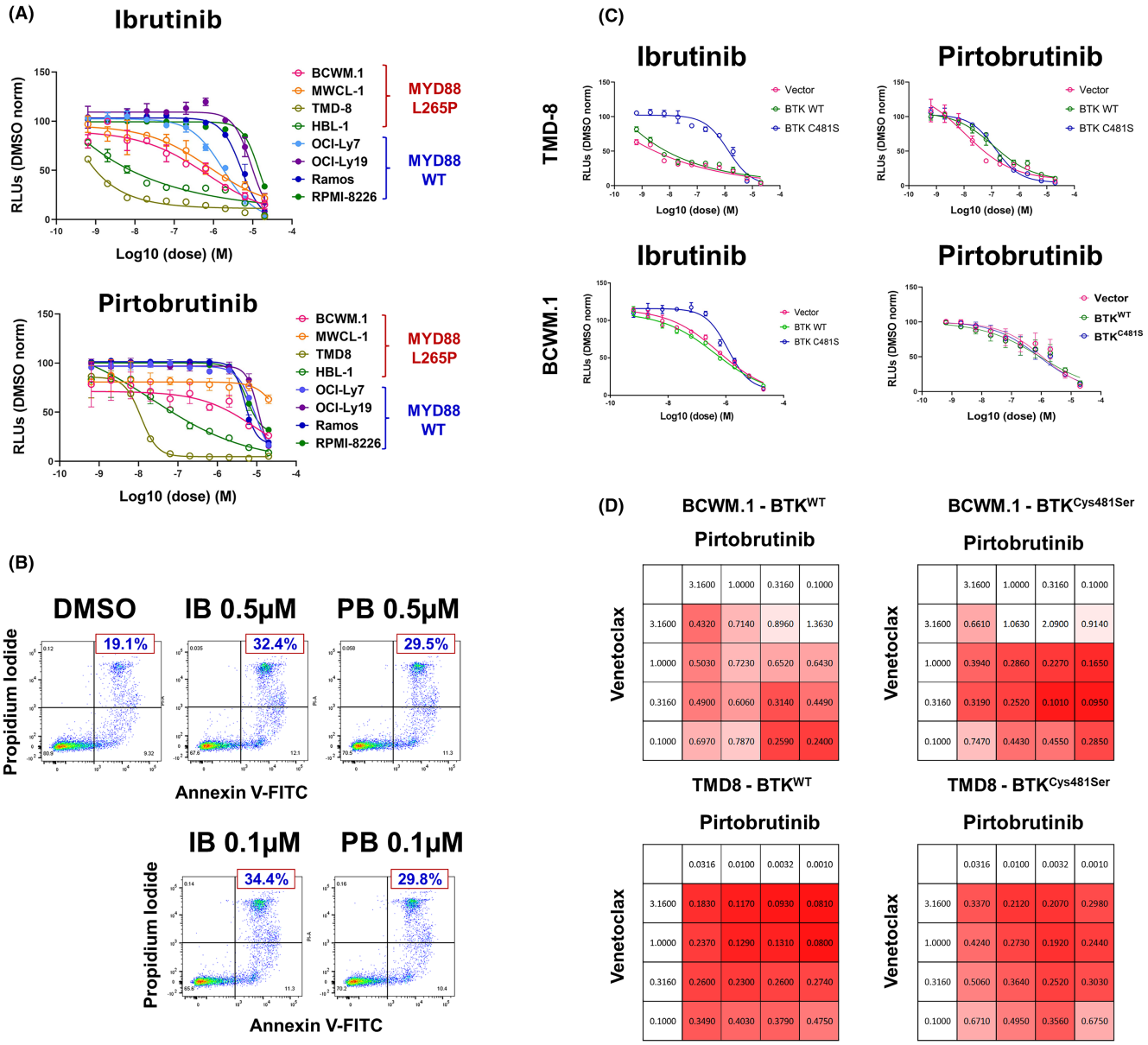


**FIGURE 1** Activity of pirtobrutinib in *MYD88*-mutated WM and ABC DLBCL cells. (A) Dose–response curves for *MYD88*$^{WT}$ (OCI-Ly7, OCI-Ly19, Ramos, RPMI-8226) and *MYD88*$^{L265P}$ (BCWM.1, MWCL-1, TMD-8, HBL-1) cell lines following treatment with ibrutinib or pirtobrutinib for 72 h at indicated concentrations. (B) Apoptosis studies assessed by propidium iodide (PI-A) and Annexin V (FITC-A) staining for CD19-gated *MYD88*$^{L265P}$-mutated primary cells from the bone marrow of a symptomatic, untreated WM patient. (C) *MYD88*-mutated WM (BCWM.1) and ABC DLBCL (TMD8) cells were transduced with vector alone, or vectors expressing either wild type (BTK$^{WT}$) or mutant (BTK$^{C481S}$). Dose-dependent survival determined by CellTiter-Glo Luminescent cell viability assay following treatment with ibrutinib or pirtobrutinib at the indicated doses for 72 h. (D) In vitro studies for synergistic interactions of pirtobrutinib and venetoclax in BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing *MYD88*-mutated BCWM.1 WM and TMD-8 ABC DLBCL cells. The combination index (CI) and normalized isobologram analyses are depicted. CI < 1 (indicated in shades of red) or the dots under the oblique line in the isobologram plots indicate a synergistic effect for the combination.[11] Standard error bars of triplicate samples are shown. Representative experiments that were repeated at least twice are depicted. ABC DLBCL, ABC diffuse large B-cell lymphoma; BTK, Bruton's tyrosine kinase; DMSO, dimethylsulfoxide; WM, Waldenstrom's macroglobulinaemia.

## Impact of pirtobrutinib on BTK and ERK1/2 signalling in *MYD88*$^{Mut}$ lymphoma cells bearing the BTK$^{Cys481Ser}$ mutation

In previous studies, we showed that the activation of ERK1/2 occurred in response to expression of BTK$^{Cys481Ser}$ and promoted resistance to ibrutinib in *MYD88*$^{Mut}$ WM and ABC DLBCL cells. Moreover, ERK1/2 activation in response to BTK$^{Cys481Ser}$ expression triggered IL-6 and IL-10 release in the presence of ibrutinib that supported paracrine-mediated resistance of co-cultured BTK$^{WT}$ *MYD88*$^{Mut}$ cells.[5] We show in these studies that while ibrutinib or pirtobrutinib abrogated p-BTK and p-ERK1/2 in BTK$^{WT}$ expressing *MYD88*$^{Mut}$ WM and ABC DLBCL cells, only pirtobrutinib blocked p-BTK and p-ERK1/2 in cells expressing BTK$^{Cys481Ser}$ (Figure 2A).

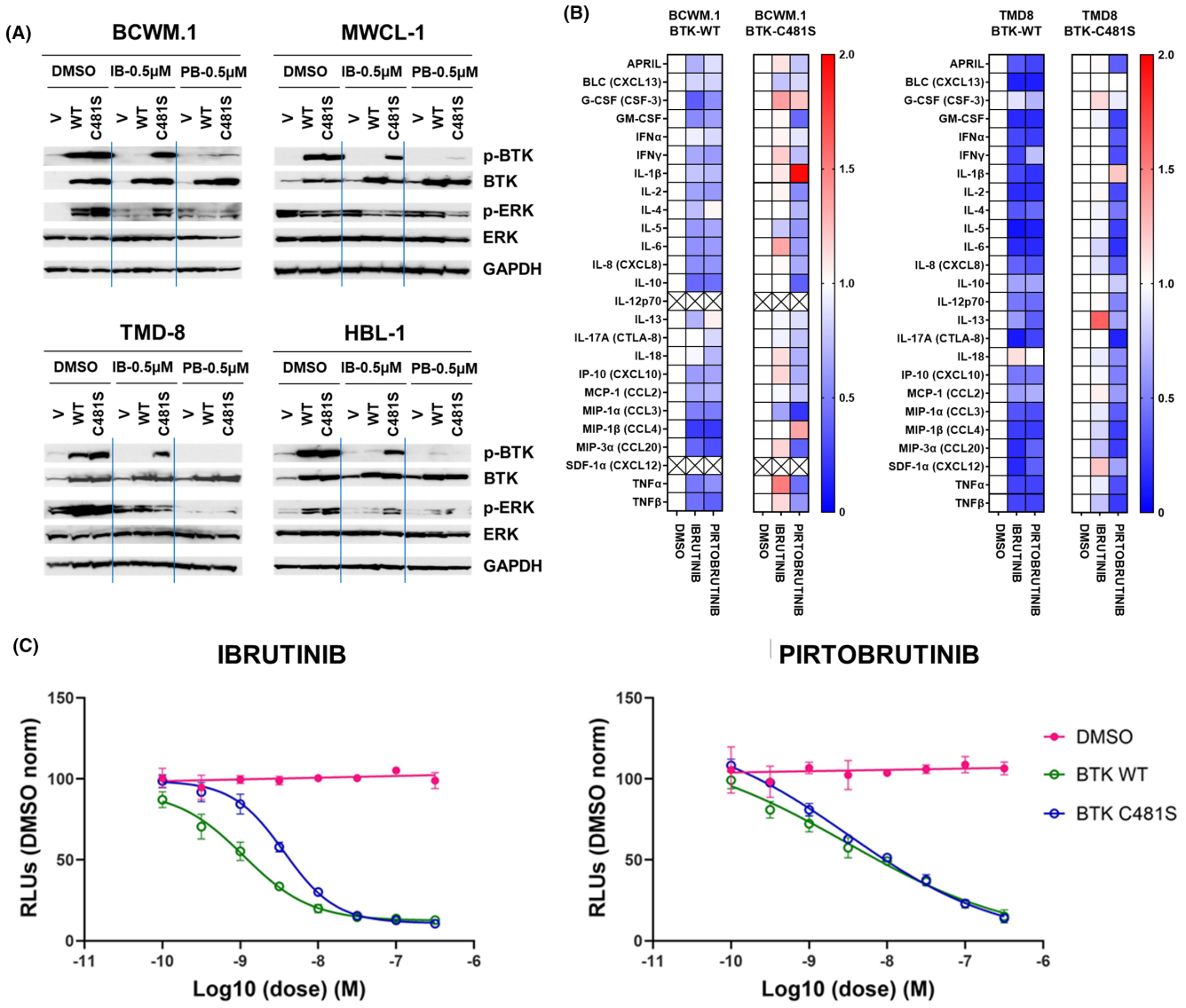


**FIGURE 2** Impact of pirtobrutinib on pro-survival signalling in wild-type and mutant BTK$^{Cys481}$ expressing *MYD88*-mutated WM and ABC DLBCL cells. (A) p-BTK$^{Y223}$ and p-ERK1/2$^{T202/Y204}$ signalling in vector only, BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing *MYD88*-mutated BCWM.1 and MWCL-1 WM and TMD-8 and HBL-1 ABC DLBCL cells following incubation for 2 h with vehicle control (DMSO), pirtobrutinib (0.5 μM) or ibrutinib (0.5 μM). Total BTK and ERK1/2 levels are also shown, along GAPDH as loading control. (B) Cytokine and chemokine release by multiplex assay in BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing BCWM.1 or TMD-8 cells following treatment with vehicle control (DMSO), ibrutinib (0.25 μM) or pirtobrutinib (0.25 μM) for 36 h at 37°C. (C) For Transwell™ co-culture experiments, BTK$^{WT}$ or BTK$^{Cys481Ser}$ expressing TMD-8 ABC DLBCL cells were pretreated with either a vehicle control (DMSO), ibrutinib or pirtobrutinib at indicated concentrations in the upper chambers of 0.4 μm filtered plates for 6 h. Non-transduced counterparts were then cultured for 72 h with added ibrutinib or pirtobrutinib at the indicated concentrations in the lower compartments, and then assessed for viability using the CellTiter-Glo® assay. The single (DMSO) line shows viability of non-transduced cells with drug vehicle control alone. *X*-axis shows ibrutinib log concentration. Standard error bars of triplicate samples are shown. Representative experiments that were repeated at least twice are depicted. ABC DLBCL, ABC diffuse large B-cell lymphoma; BTK, Bruton's tyrosine kinase; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinases; RLU, relative light units; WM, Waldenstrom's macroglobulinaemia.

## Impact of pirtobrutinib on inflammatory cytokine release by *MYD88*$^{Mut}$ lymphoma cells

We next assessed inflammatory cytokine and chemokine production following ibrutinib or pirtobrutinib treatment in *MYD88*$^{Mut}$ WM and ABC DLBCL cells expressing either BTK$^{WT}$ or BTK$^{Cys481Ser}$ protein. As shown in Figure 2B, treatment of BTK$^{WT}$ expressing *MYD88*$^{Mut}$ BCWM.1 or TMD-8 cells with ibrutinib or pirtobrutinib broadly blocked inflammatory cytokine and chemokine production, including IL-6 and IL-10. Conversely, in BTK$^{Cys481Ser}$ expressing BCWM.1 and TMD-8 cells, only pirtobrutinib blocked most inflammatory cytokines and chemokines that included APRIL, CXCL10, CXCL12, G-CSF, IL-6, IL-10, IL-13, IL-18, interferon-γ, tumour necrosis factor (TNF)-α, TNF-β, MIP1-α and/or MIP3-α. The inhibition of APRIL, IL-6, IL-10 and TNF-α production is likely related to ERK1/2 inhibition as informed by our prior study in BTK$^{Cys481}$ expressing BCWM.1 and TMD-8 cells. Since many of these cytokines have a role in promoting WM growth and/or survival (APRIL, IL-6, CXCL12); IgM secretion (IL-6); as well as autocrine- and paracrine-mediated drug resistance (CXCL12, IL-6, IL-10), their inhibition is clinically relevant.[5,12–14]

## Impact of pirtobrutinib on paracrine-mediated resistance to ibrutinib

The role of IL-6 and IL-10 in mediating paracrine-mediated resistance to ibrutinib was previously explored by us in co-culture experiments wherein BTK$^{WT}$ *MYD88*$^{Mut}$ lymphoma cells were co-cultured in Transwell plates with their BTK$^{Cys481}$ or BTK$^{WT}$ expressing counterparts.[5] BTK$^{WT}$ cells showed resistance to ibrutinib when co-cultured with BTK$^{Cys481}$ expressing cells. Herein, we show that pirtobrutinib overcame paracrine-mediated resistance in BTK$^{WT}$ TMD-8 cells induced by BTK$^{Cys481}$ expressing TMD-8 cells, whereas paracrine-mediated resistance persisted for ibrutinib-treated cells (Figure 2C).

# DISCUSSION

Our results provide important new insights into the activity of pirtobrutinib in *MYD88*$^{Mut}$ lymphomas. We show that pirtobrutinib exhibited selective activity against a broad panel of *MYD88*$^{Mut}$ lymphoma cells, that included WM and ABC DLBCL cell lines, as well as primary *MYD88*$^{Mut}$ WM cells. Pirtobrutinib also overcame resistance against ibrutinib-resistant WM (BCWM.1) and ABC DLBCL (TMD-8) cells engineered to express mutated BTK$^{Cys481}$ that is associated with acquired resistance to ibrutinib and other covalent BTK-inhibitors in WM patients.[7] Our findings affirm and expand on those previously reported by Gomez et al.[12] who showed activity for pirtobrutinib in *MYD88*$^{Mut}$ TMD-8 ABC DLBCL cells expressing either wild-type or mutated BTK$^{Cys481}$.

Importantly, we show that, in addition to abrogating BTK activity, pirtobrutinib blocked ERK1/2 activation and a multitude of ERK1/2-dependent inflammatory cytokines such as IL-6 and IL-10 in both wild-type and mutant BTK$^{Cys481}$ expressing *MYD88*$^{Mut}$ lymphoma cells.[5] IL-6 and IL-10 are particularly important given their role in both autocrine- and paracrine-mediated growth and survival of *MYD88*$^{Mut}$ lymphoma cells. IL-6 triggers the activation of HCK, which enables broad pro-survival signalling, while IL-6 or IL-10 mediate paracrine resistance to ibrutinib in *MYD88*$^{Mut}$ lymphoma cells that express wild-type BTK.[4,5] In co-culture experiments reported herein, we showed that pirtobrutinib overcame paracrine resistance set off by BTK$^{Cys481}$-mutated lymphoma cells (Figure S1).

Our studies focused on modelling acquired BTK$^{Cys481}$ mutations, which identify as the most common acquired somatic event associated with ibrutinib resistance in WM.[7] Other acquired genomic alterations associated with ibrutinib resistance have also been observed in WM patients, including truncation or loss of the ERK1/2 regulator DOK2.[15] Further work in clarifying the activity of pirtobrutinib in non-BTK$^{Cys481}$-mediated ibrutinib resistance are needed. The suppression of ERK1/2 signalling by pirtobrutinib has also been reported by Aslan et al.[16] in MEC-1 CLL cells engineered to express mutated BTK$^{Cys481}$, though the mechanistic consequences of ERK1/2 activation were not addressed. ERK1/2 reactivation in patients progressing on pirtobrutinib was also shown by Naeem et al.,[17] highlighting the importance of this pathway and potentially our findings in other B-cell malignancies that are driven by non-*MYD88* mutations. The study also provides further support for targeting ERK1/2 pathway as a strategy for overcoming BTK-inhibitor resistance. Several small molecules are currently in development.

Consistent with our findings, pirtobrutinib has shown remarkably high levels of clinical activity and durability in WM patients previously treated with a cBTK-i.[9,10] Our studies further suggest that the combination of pirtobrutinib and venetoclax may represent a particularly optimal combination for treating *MYD88*$^{Mut}$ WM since at least additive tumour cell killing was observed at most combination doses of pirtobrutinib and venetoclax in *MYD88*$^{Mut}$ lymphoma cell lines. A study examining the combination of pirtobrutinib and venetoclax has been initiated by us in previously treated WM patients (NCT05734495).

In summary, our findings provide mechanistic support for the development of pirtobrutinib alone and in combination with BCL2 inhibitors in *MYD88*$^{Mut}$-driven lymphomas, including those resistant to covalent BTK-inhibitors on the bases of BTK$^{Cys481}$ mutations.

## AUTHOR CONTRIBUTIONS

SPT, MM and GY conceived the experimental design and wrote the first draft. MM, XL, AK, NT, AC, JNG, SL, MLG, JMH and JC performed experiments and critical review of study results. SS, CAF, ARB, JJC and KM provided patient care and samples. ARB, GvK, SS, JJC and LMP provided

study input and clinical trial design. All authors reviewed the first draft and provided revisions. The final draft was approved by all authors.

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## CONFLICT OF INTEREST STATEMENT

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## DATA AVAILABILITY STATEMENT

Data in support of the findings for this study are available from the corresponding author (SPT) and are available upon reasonable request.

## ETHICS APPROVAL STATEMENT

Sample use was approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board.

## PATIENT CONSENT STATEMENT

Written patient consent was obtained for human samples used in these studies using an institutional review board approved consent form.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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