

## **BTK-independent regulation of calcium signalling downstream of the B-cell receptor in malignant B-cells**

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## Abstract

BTK inhibitors (BTKi) have dramatically improved outcomes for patients with chronic lymphocytic leukaemia (CLL) and some forms of B-cell lymphoma. However, new strategies are needed to enhance responses. Here we have performed a detailed analysis of the effects of BTKi on B-cell receptor (BCR)-induced signalling using primary malignant cells from CLL patients and B-lymphoma cell lines. Although BTK is considered as a key activator of PLC $\gamma$ 2, BTKi (ibrutinib and acalabrutinib) failed to fully inhibit calcium responses in CLL samples with strong BCR signalling capacity. This BTKi-resistant calcium signalling was sufficient to engage downstream calcium-dependent transcription and suppress CLL cell apoptosis and was entirely independent of BTK and not just its kinase activity as similar results were obtained using a BTK-degrading PROTAC. BTK-independent calcium signalling was also observed in two B-lymphoma cell lines where BTKi had little effect on the initial phase of the calcium response but did accelerate the subsequent decline in intracellular calcium. In contrast to BTKi, calcium responses were completely blocked by inhibition of SYK in CLL and lymphoma cells. Engagement of BTK-independent calcium responses was associated with BTK-independent phosphorylation of PLC $\gamma$ 2 on Y<sup>753</sup> and Y<sup>759</sup> in both CLL and lymphoma cells. Moreover, in CLL samples, inhibition of RAC, which can mediate BTK-independent activation of PLC $\gamma$ 2, cooperated with ibrutinib to suppress calcium responses. BTK-independent calcium signalling may limit the effectiveness of BTKi to suppress BCR signalling responses and our results suggest inhibition of SYK or dual inhibition of BTK and RAC as alternative strategies to strengthen pathway blockade.

## 1. Introduction

Bruton's tyrosine kinase (BTK) is essential for normal B-cell responses and is a target for drug therapy of B-cell neoplasms [1, 2]. BTK participates in signalling downstream of various cell surface receptors [3-6], including the B-cell receptor (BCR) where BTK activation is initiated by SYK-dependent phosphorylation on Y<sup>551</sup> followed by auto-phosphorylation on Y<sup>223</sup> (Supplementary Fig. 1) [2, 7]. PI3K can also increase BTK activity as BTK is recruited to the plasma membrane via interaction of its pleckstrin homology (PH) domain with phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P<sub>3</sub>) generated by phosphatidylinositol 3-kinase (PI3K)-dependent phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) [8].

A key target for BTK-dependent phosphorylation is PLC $\gamma$ 2 which catalyses the breakdown of PI(4,5)P<sub>2</sub> to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) [7]. IP<sub>3</sub> induces release of calcium from the endoplasmic reticulum resulting in influx of extracellular calcium via store-operated calcium entry [9]. Increased intracellular calcium, together with DAG, stimulates PKC isoforms leading to activation of MAPKs and NF- $\kappa$ B [10]. Increased calcium also leads to nuclear translocation of NFAT, and NF- $\kappa$ B and NFAT in turn induce expression of target genes which promote B-cell survival (e.g., *BCL2A1*), growth (e.g., *MYC*) and cell cycle progression (e.g., *CCND2*) [11-14].

Although the precise mechanism by which BTK activates PLC $\gamma$ 2 remains incompletely understood, BTK can phosphorylate tyrosine residues within both the central (Y<sup>753</sup> and Y<sup>759</sup>) and C-terminal (Y<sup>1197</sup> and Y<sup>1217</sup>) domains of PLC $\gamma$ 2 and phosphorylation at these sites is associated with PLC $\gamma$ 2 activation [15-20]. However, the degree to which calcium release is dependent on BTK varies between cell types. For example, in DT40 chicken B cells, deletion of BTK completely ablates anti-IgM-induced calcium responses whereas Btk deletion in mouse B cells has only partial effects [21, 22]. In some settings BTK functions predominantly to maintain intracellular calcium levels rather than mediate the initial response following surface IgM (sIgM) stimulation [23, 24]. Similar to BTK, PLC $\gamma$ 2

can be recruited to the plasma membrane via binding of its PH-domain to PI(3,4,5)P<sub>3</sub> which enhances availability for BTK-dependent activation [8]. Phosphorylation-independent pathways of PLC $\gamma$ 2 activation have also been described. For example, RAC GTPases can activate PLC $\gamma$ 2 via a mechanism that involves protein-protein interactions but is independent of PLC $\gamma$ 2 phosphorylation [25-27].

BCR signalling plays a major role in the pathogenesis of various B-cell malignancies, including chronic lymphocytic leukaemia (CLL) [28, 29]. CLL comprises two major subsets with differing clinical outcomes; U-CLL is derived from pre-germinal centre B cells and has a poorer outcome compared to M-CLL of post-germinal centre origin [30]. Importantly, U-CLL samples tend to have stronger capacity to signal via sIgM and this signalling leads to activation of pro-proliferation and survival pathways [31-33]. Consistent with this, BTK inhibitors (BTKi), including ibrutinib and the second-generation inhibitor acalabrutinib, have been approved for treatment of CLL [34]. BCR signalling also contributes to the pathogenesis of B-cell lymphomas [29] and BTKi have been approved for treatment of mantle cell, marginal zone, primary central nervous system and lymphoblastic lymphomas and Waldenstrom's macroglobulinaemia, and have anti-lymphoma activity in some preclinical models of diffuse large B-cell lymphoma (DLBCL) [34-36].

Despite the clinical benefits of BTKi in CLL and some forms of B-cell lymphoma, significant challenges limit the effectiveness of BTK inhibition as a treatment strategy. For example, a substantial proportion of patients do not respond or develop resistance to BTKi [34]. It is possible that new strategies can be developed to disrupt more effectively the pivotal BTK/PLC $\gamma$ 2/calcium signalling cascade, but this will require detailed insight into the operation of this complex pathway in malignant B cells. In our previous study [37], we demonstrated that the ability of ibrutinib to inhibit anti-IgM-induced calcium responses varied between CLL samples revealing the presence of BTK-independent signalling to calcium responses. However, the features that influence the differential effects of ibrutinib between samples and the molecular pathways that mediate these BTK-independent calcium responses were not determined.

Here, we have performed a detailed analysis of the effects of various BTKi, including a BTK-targeted proteolysis-targeting chimera (PROTAC), on anti-IgM-induced calcium responses using malignant B cells from CLL patients and B-cell lymphoma cell lines. We show that the contribution of BTK to calcium responses varies substantially and identify signalling pathways which mediate BTK-independent calcium responses. Finally, we identify novel combination strategies that can be used to augment the ability of ibrutinib to suppress BCR-induced calcium responses in CLL cells.

## **2. Materials and methods**

### *2.1 Compounds and ligands*

Ibrutinib, idelalisib, acalabrutinib, EHT-1874 and tamatinib were from MedChemExpress (US). MT-802 and SJF-6625 [38] were a kind gift from Professor Craig Crews (Yale University, New Haven, US) or were from MedChemExpress (MT-802 only). All compounds were dissolved in dimethylsulfoxide (DMSO). sIgM stimulation was performed using either soluble or bead-bound goat F(ab')<sub>2</sub> anti-human IgM (or control F(ab')<sub>2</sub>) (both Southern Biotech) as described [39]. IL-4 was from R&D Systems and used at 10 ng/ml.

### *2.2 Cells*

Analysis of CLL samples (Supplementary Table 1) was performed after receiving ethical approval (South Central - Hampshire B Research Ethics Committee) and in accordance with the Declaration of Helsinki of 1975. All patients provided written informed consent. Heparinized peripheral blood mononuclear cells were obtained from patients attending clinic at the Southampton General Hospital. *IGHV* mutation status, expression of cell surface IgM, CD5 and CD19 were determined as described [40]. Cryopreserved samples were recovered and rested for one hour in complete RPMI-1640 (RPMI-1640 medium (Sigma) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin) at 37 °C prior to use. Cell viability was determined by trypan blue exclusion and was ≥90% in all

cases.

TMD8 and OCI-LY7 cells (kind gifts of Professors Louis Staudt (National Cancer Institute, US) and Jude Fitzgibbon (Bart's Cancer Centre, UK), respectively) were cultured in complete RPMI-1640 supplemented with 10% FBS or IMDM supplemented with 20% FBS, respectively. Cell line identity was routinely confirmed using short tandem repeat analysis (Powerplex 16 System, Promega) and absence of mycoplasma was confirmed using Mycoplasma PCR detection kit (Applied Biological Materials). Cell lines were typically cultured for a maximum of 6-8 weeks.

### *2.3 Calcium response assays*

Intracellular calcium responses were determined following stimulation with soluble goat F(ab')<sub>2</sub> anti-IgM (Southern Biotech), as previously described [40]. The calcium ionophore ionomycin was used as a positive control to confirm that cells retained the capacity to release calcium. Calcium responses were quantified in CLL samples by calculating the maximum percentage of cells with increased calcium above a basal threshold (peak response), as previously described [40], whereas, for B-cell lines, the area-under-the curve of the response (AUC) was calculated from the point of addition of anti-IgM to addition of ionomycin.

### *2.4 Quantitative polymerase chain reaction (Q-PCR)*

Total mRNA was isolated using the Qiagen RNA extraction kit and cDNA synthesis was performed using MMLV reverse transcriptase and oligo-dT primers (both Promega, Southampton, UK). *MYC*, *CCND2*, *BCL2A1* and *B2M* mRNA expression was quantified by Q-PCR using probes Hs00153408\_m1, Hs00277041\_m1, Hs00187845\_m1 and Hs00984230\_m1, respectively (Life Technologies). RNA abundance was determined for each RNA against a standard curve, providing cDNA values and relative RNA expression was calculated by normalizing the obtained values against *B2M* expression.

### *2.5 Immunoblotting*

SDS-PAGE was performed using equal protein loading following quantitation of protein content using the BioRad Protein Assay and with the following antibodies; anti-SYK, anti-phosphorylated-SYK (Y<sup>525/526</sup>), anti-BLNK, anti-PLC $\gamma$ 2, anti-BTK, anti-phosphorylated BTK (Y<sup>223</sup>) (all from Cell Signaling Technology), anti-phosphorylated-BLNK (Y<sup>84</sup>), anti-phosphorylated BTK (Y<sup>551</sup>), anti-phosphorylated-PLC $\gamma$ 2 (Y<sup>753</sup>, Y<sup>759</sup> or Y<sup>1217</sup>) (all from Abcam), anti-PARP (BD Biosciences), anti-MCL1 (Santa Cruz Biotechnologies) and anti-GAPDH (Invitrogen). Secondary antibodies were horseradish peroxidase-conjugated antibodies (GE Healthcare). Images were captured using the ChemiDoc-It Imaging System with a BioChem HR camera (UVP) and quantified using ImageJ (<http://imagej.nih.gov/ij/>). Expression of phosphorylated proteins was normalized to the equivalent total protein. PARP cleavage was quantified by calculating the proportion of cleaved PARP as a percentage of total PARP expression (i.e., cleaved plus uncleaved). Heatmaps were prepared by setting values for control antibody-treated cells to 0 and values for anti-IgM/DMSO-treated cells to 1.0.

## 2.6 Statistics

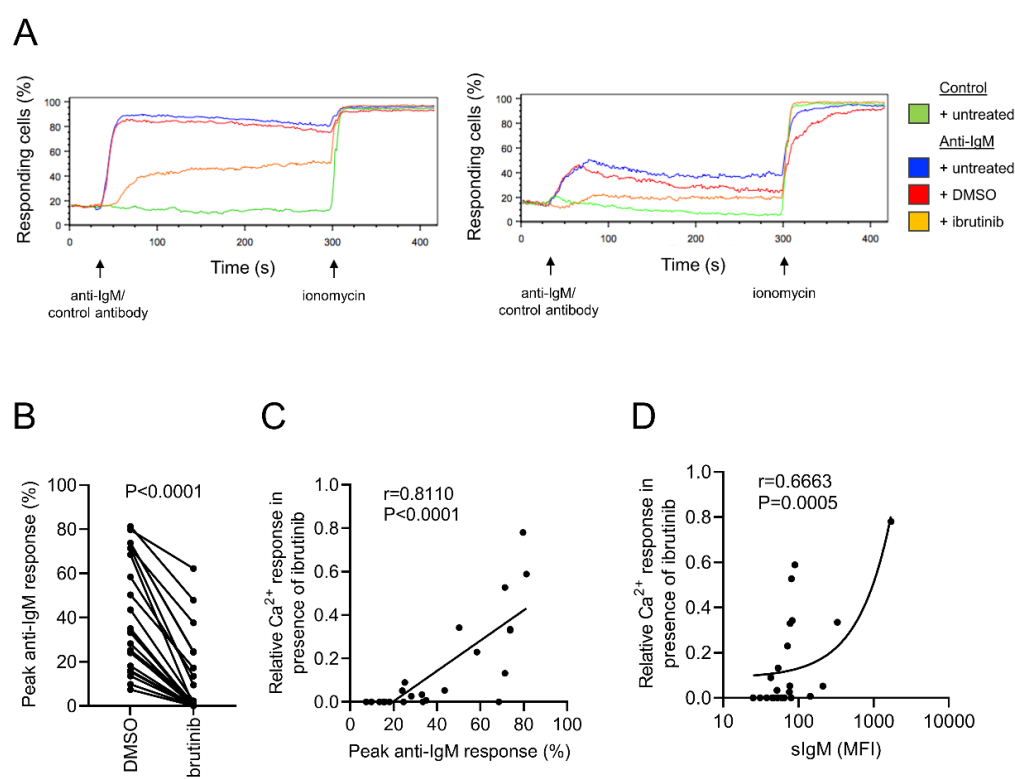
Statistical comparisons were performed in Prism9 (GraphPad Software, La Jolla, CA, USA) using Student's t-tests and one-sample t-tests, or Spearman's correlations, Wilcoxon signed ranked tests and Wilcoxon matched-pairs signed rank tests, depending on whether data were normally distributed or not, respectively, according to Shapiro-Wilk's tests.

## 3. Results

### *3.1 The ability of ibrutinib to inhibit anti-IgM-induced calcium responses varies between CLL samples*

We first investigated the effect of ibrutinib on calcium responses in a cohort of 23 CLL samples using anti-IgM to mimic engagement of the BCR by antigen. The sample cohort was selected to comprise signal responsive samples (i.e., >5% cells

with increased intracellular calcium following stimulation with anti-IgM) from both the U-CLL and M-CLL subsets [40]. Soluble anti-IgM was used to stimulate the BCR in calcium assays, as described [40], and ibrutinib was used at 1  $\mu$ M which is sufficient to fully inhibit BTK in CLL cells [41]. Consistent with our previous study [37], ibrutinib significantly reduced anti-IgM-induced calcium responses (Fig. 1A,B). However, there was clear inter-sample variation in the extent of inhibition which ranged from 100% to ~20%. Titration experiments demonstrated that the extent of inhibition was very similar for cells pre-treated with 1  $\mu$ M or 100 nM ibrutinib confirming that the partial inhibitory effects observed for ibrutinib at 1  $\mu$ M were not a consequence of inadequate drug exposure (Supplementary Fig. 2). Ibrutinib was still relatively effective at 10 nM but inhibitory activity was substantially reduced at 1 nM.



**Fig. 1. Inhibition of anti-IgM-induced calcium response in CLL cells.** (A-D) CLL samples ( $n=23$ ) were pre-treated with ibrutinib (1  $\mu$ M), DMSO, or left untreated for 1 hour before analysis of anti-IgM-induced calcium responses. (A) Representative results for two samples showing relatively strong and weak inhibitory effects of ibrutinib, respectively. Arrows indicate point of addition of anti-IgM or control antibody and then ionomycin. (B) Results for all samples showing peak percentage of responding cells in DMSO or ibrutinib pre-treated cells (Wilcoxon matched-pairs signed rank test). (C,D) Correlations between the effect of ibrutinib (peak percentage of responding cells in presence of ibrutinib/peak



percentage of responding cells in presence of DMSO) and (C) peak percentage of responding cells in the absence of ibrutinib and (D) sIgM expression (with results of linear regression and Spearman's correlation).

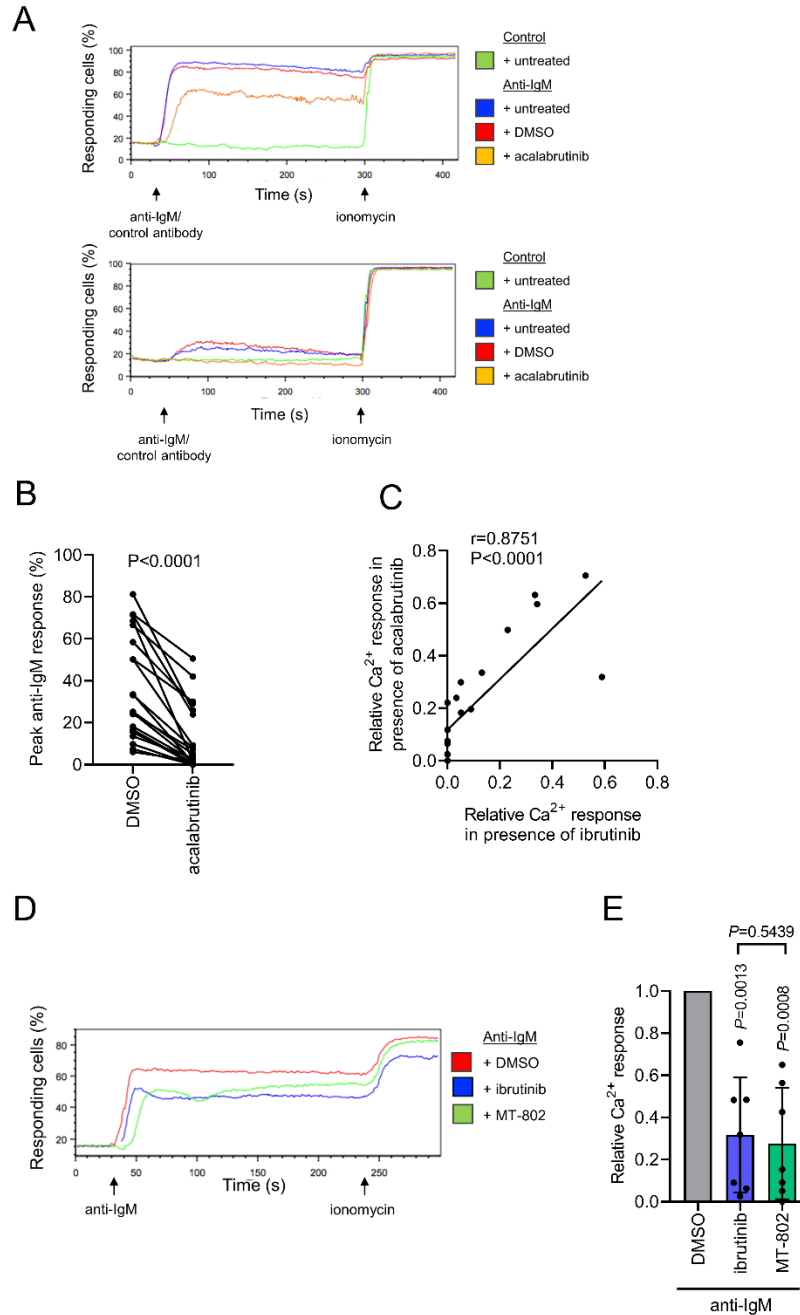
We previously showed that pre-treatment of CLL cells with IL-4 reduced the ability of ibrutinib to inhibit calcium responses [37]. Since IL-4 increases sIgM expression and signal capacity of CLL cells, this suggested that variable responses to ibrutinib of CLL samples without IL-4 might also be linked to variation in sIgM expression/signal strength. To probe this, we correlated the extent of inhibition of calcium release by ibrutinib with sIgM expression and signal strength (i.e., anti-IgM-induced calcium release in the absence of ibrutinib). This revealed strong, positive correlations between the relative ineffectiveness of ibrutinib-mediated inhibition with sIgM signalling capacity and sIgM expression (Fig. 1C,D). Thus, ibrutinib was least able to inhibit calcium responses in samples with the strongest sIgM signalling. We did not attempt to correlate the variability in ibrutinib effectiveness with clinical features due to the selected nature of the cohort which comprised only sIgM signal responsive samples.

We compared the effect of BTK inhibition using ibrutinib on calcium responses with that of inhibition of SYK, a proximal kinase in the BCR signalling cascade (Supplementary Fig. 1). For SYK inhibition, we used tamatinib which is the active form of fostamatinib, the first SYK inhibitor shown to have clinical activity in CLL [42]. Tamatinib is typically used at concentrations up to 5  $\mu$ M in CLL cells [43-45] but we used a higher concentration of 10  $\mu$ M to ensure full inhibition of SYK. At this concentration tamatinib very effectively suppressed anti-IgM-induced calcium responses in all samples (Supplementary Fig. 3). It is possible that some effects of tamatinib in these experiments may be mediated by off-target inhibition, but these results clearly demonstrate that anti-IgM-induced calcium responses can be fully blocked by pharmacological inhibition in all samples.

### *3.2 Effect of acalabrutinib and a BTK-targeted PROTAC on anti-IgM-induced calcium responses in CLL cells*

Ibrutinib is a relatively poorly selective BTKi, and it was possible that its partial effects on calcium responses were a consequence of off-target effects (e.g. mediated by inhibition of inhibitory phosphorylation). We therefore performed similar experiments to investigate the effect of a second-generation BTK inhibitor, acalabrutinib, on anti-IgM-induced calcium responses. Acalabrutinib is a covalent BTK inhibitor with reduced off-target effects compared to ibrutinib [46]. Similar to ibrutinib, acalabrutinib significantly reduced anti-IgM responses but with inter-sample variation in the extent of inhibition (100 to ~30%) (Fig. 2A,B). Acalabrutinib showed a similar concentration dependency as ibrutinib (Supplementary Fig. 2) and relative lack of effectiveness of acalabrutinib-mediated inhibition was also correlated with sIgM signalling capacity and sIgM expression (Supplementary Fig. 4). Of the 21 samples analysed for response to acalabrutinib, 19 had been analysed for response to ibrutinib and in these shared samples there was a very strong positive correlation between the extent of inhibition by the two BTKi (Fig. 2C). Therefore, partial inhibition of calcium responses is a class effect shared amongst these BTKi.

Although previous analysis of BTK has focused on its kinase function, studies have revealed unexpected kinase-independent functions of BTK as re-expression of kinase inactive BTK mutants can at least partially reconstitute some BTK functions [22, 23, 47, 48]. We therefore compared calcium responses in cells treated with ibrutinib or the BTK-targeted PROTAC, MT-802 [38, 49]. MT-802 comprises an ibrutinib-based moiety which binds BTK, coupled via a linker to a pomalidomide-derived motif which recruits BTK to the cereblon E3 ubiquitin ligase complex thereby triggering BTK ubiquitination and degradation via the proteasome. Whereas inhibitory effects of ibrutinib would be restricted to those that are mediated via BTK's kinase activity, MT-802 inhibits all functions of BTK. Based on dose response and time course experiments (Supplementary Fig. 5), treatment of CLL cells with 500 nM MT-802 for 4 hours was selected for these experiments. However, there was no difference in the ability of ibrutinib or MT-802 to inhibit anti-IgM-induced calcium responses (Fig. 2D,E). Therefore, the inability of BTKi to fully block anti-IgM-induced calcium responses reveals the operation of a signalling pathway which is entirely independent of BTK and not just its kinase activity.



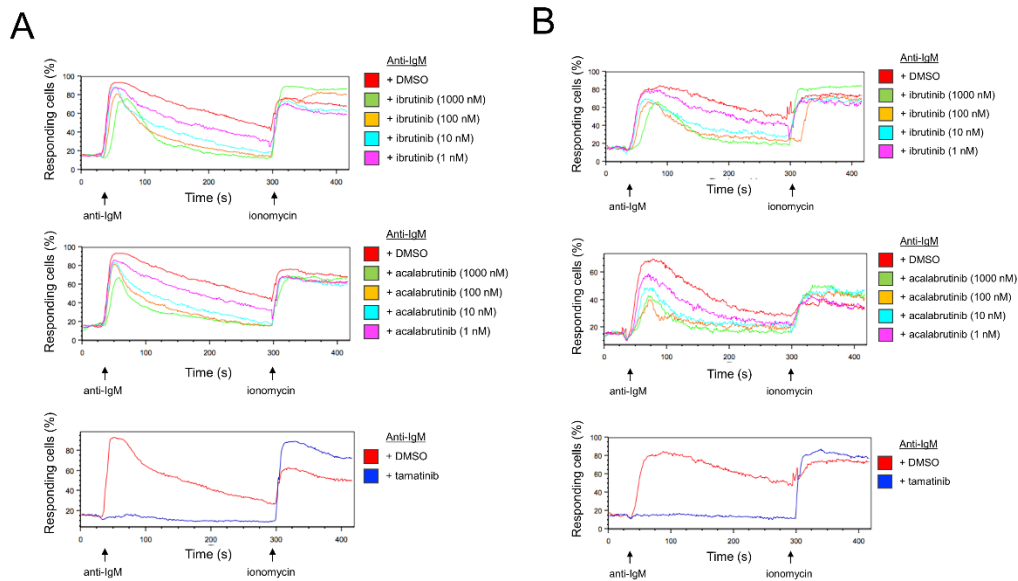
**Fig. 2. Effect of acalabrutinib and MT-802 on anti-IgM-induced calcium responses in CLL cells.** (A-C) CLL samples ( $n=21$ ) were pre-treated with acalabrutinib ( $1 \mu\text{M}$ ), DMSO, or left untreated for 1 hour before analysis of anti-IgM-induced calcium responses. (A) Representative results for two samples and (B) results for all samples showing peak percentage of responding cells in DMSO or acalabrutinib-treated cells (Wilcoxon matched-pairs signed rank test). (C) Correlation between extent of inhibition of anti-IgM-induced calcium response for ibrutinib or acalabrutinib-treated samples ( $n=19$ ) (with results of linear regression and Spearman's correlation). (D,E) CLL samples ( $n=8$ ) were pre-treated with MT-802 ( $500 \text{ nM}$ ) for 4 hours, or with ibrutinib ( $1 \mu\text{M}$ ) or DMSO for 1 hour, or left untreated, before analysis of anti-IgM-induced calcium responses. (D) Representative results and (E) results for all samples showing individual samples and means ( $\pm\text{SD}$ ), with values for DMSO/anti-IgM-treated cells set to 1.0. The statistical significance of the differences is shown (vertical P-values show one sample t-tests for comparisons to control and horizontal P-value is Student's t-tests for the comparison of ibrutinib and MT-802-treated cells).

### *3.3 Effect of BTKi on calcium responses in B-lymphoma cell lines*

Previous studies demonstrated that BTK's role in mediating calcium responses downstream of the BCR varies between models [21-24]. Since BTKi have also been approved for treatment of some forms of B-cell lymphoma and have activity in preclinical models of DLBCL [34-36] we extended our analysis to investigate the effects of BTKi in the B-lymphoma derived cell lines, OCI-LY7 and TMD8 which are derived from the germinal centre B (GCB) and activated B-cell (ABC) subsets of DLBCL, respectively. We selected these two lines from an initial panel of 6 (the other cell lines tested were HT, HBL1, OCI-LY18 and SU-DHL-6) as they gave robust and reproducible anti-IgM-induced calcium responses. Of note, TMD8 cells contain mutations of *MYD88* and *CD79B* which give rise to the My-T-BCR complex that may confer strong clinical responses to ibrutinib in treated DLBCL patients [50]. Although constitutive BCR signalling (i.e., in the absence of anti-IgM) has been described in DLBCL cell lines [35, 51, 52], this is likely to be low-level and cannot readily be distinguished from signalling induced by other cell surface receptors. We therefore continued to use anti-IgM to directly stimulate the BCR in these experiments.

Anti-IgM induced a strong response in OCI-LY7 and TMD8 cells with essentially all cells showing a calcium response (Fig. 3A,B). Interestingly, the predominant effect of ibrutinib or acalabrutinib in OCI-LY7 cells was to accelerate the decline in calcium response following its initial peak whereas the peak of the response was only modestly reduced in cells treated with the highest concentration of inhibitors. A similar acceleration of the decline in calcium response was observed in TMD8 cells, although the effect of BTKi on the peak response was perhaps more pronounced in these cells. Quantitation of these effects by analysis of AUC confirmed that BTKi significantly reduced anti-IgM-induced calcium responses in OCI-LY7 and TMD8 cells in a dose-dependent manner, whereas effects on the peak response were modest (Supplementary Fig. 6). Thus, in contrast to CLL cells where BTK (variably) mediates the initial phase of the calcium response, BTK appears to play a role predominantly in sustaining the response in these B-lymphoma cells. Similar to CLL cells, anti-IgM-induced calcium responses were

very effectively inhibited in cells treated with tamatinib (Fig. 3A; Supplementary Fig. 6).

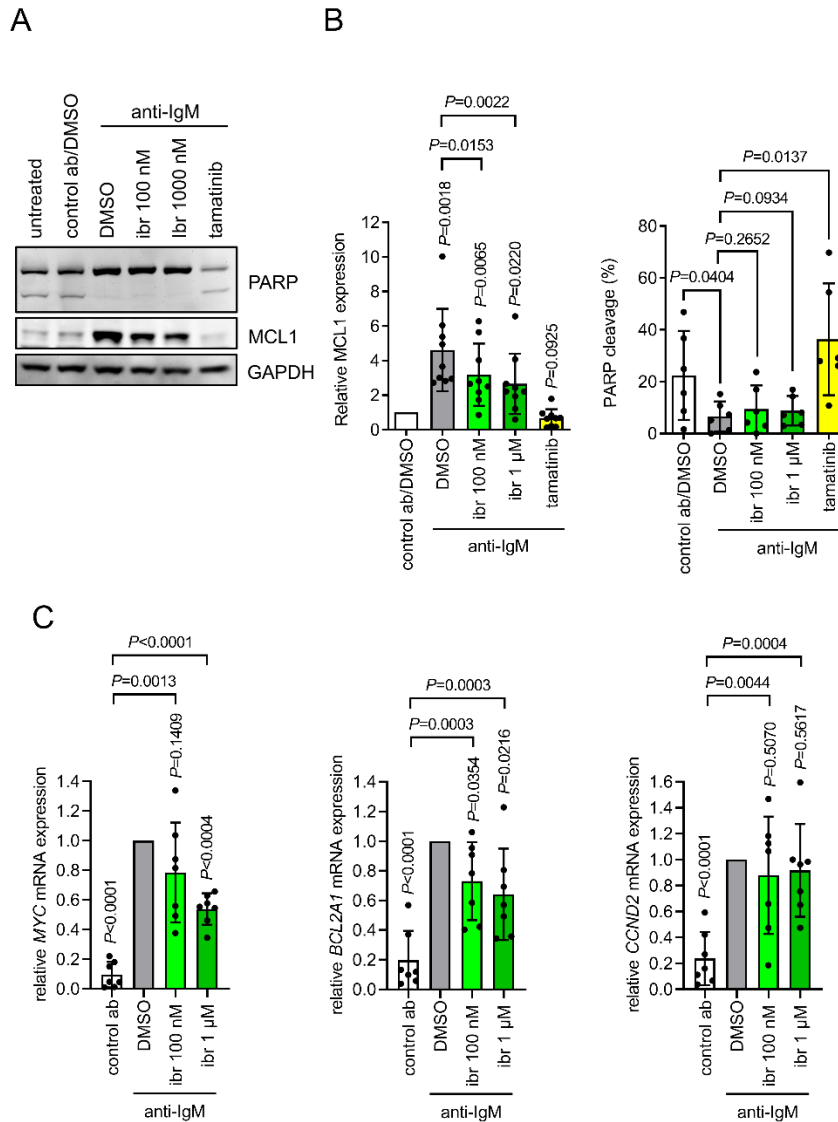


**Fig. 3. Effect of BTK inhibition on anti-IgM-induced calcium responses in B-lymphoma cells.** (A) OCI-LY7 and (B) TMD8 cells were pre-treated with the indicated concentrations of ibrutinib or acalabrutinib, tamatinib (10  $\mu$ M) or DMSO for 1 hour before analysis of anti-IgM-induced calcium responses. Results shown are representative of 3 individual experiments for both cell lines and the point of addition of anti-IgM and then ionomycin is indicated by arrows. Quantitation of multiple experiments is provided in Supplementary Fig. 6.

### 3.4 Effect of ibrutinib on anti-IgM-induced downstream responses in CLL cells

Having established that ibrutinib only partially inhibits anti-IgM calcium release, we next investigated the effects of this residual signalling on downstream responses in CLL cells, including suppression of cell death. Since soluble anti-IgM promotes CLL cell apoptosis, these experiments were performed using anti-IgM beads which suppress spontaneous apoptosis of CLL cells [31, 33]. It was possible that the effect of ibrutinib on calcium responses differed between cells treated with soluble anti-IgM or anti-IgM beads. However, it was not possible to perform direct analysis of calcium responses in these experiments as the high autofluorescence of anti-IgM beads interferes with analysis of calcium levels using flow cytometry [53]. Therefore, we also used Q-PCR to analyse expression of *MYC*, *CCND2* and

*BCL2A1* RNAs. These encode key regulators of B-cell survival, growth and proliferation but are also target genes for the calcium-dependent transcription factors NFAT and NF- $\kappa$ B and therefore provide downstream read-outs for calcium release.



**Fig. 4. Effect of ibrutinib on anti-IgM-induced transcriptional responses and survival in CLL cells.** (A,B) CLL samples were pre-treated with the indicated concentrations of ibrutinib or tamatinib (10  $\mu$ M) for 1 hour and then incubated with anti-IgM or control beads for 24 hours. Expression of PARP, MCL1 and GAPDH (loading control) was analysed by immunoblotting. (A) Representative results and (B) summary of relative MCL1 expression and percentage PARP cleavage and for all samples analysed ( $n=9$  for MCL1 and  $n=6$  for PARP). Graphs show results for individual samples (and means  $\pm$ SD) with results for MCL1 expression set to 1.0 for control antibody treated cells. The statistical significance of the differences is shown (vertical P-values show one sample t-tests for comparisons to control and horizontal P-values show Student's t-tests for comparisons between samples). (C) CLL samples ( $n=7$ ) were pre-treated with the indicated concentrations of ibrutinib (ibr) for 1 hour and then incubated with anti-IgM or control beads for 6 hours before Q-PCR

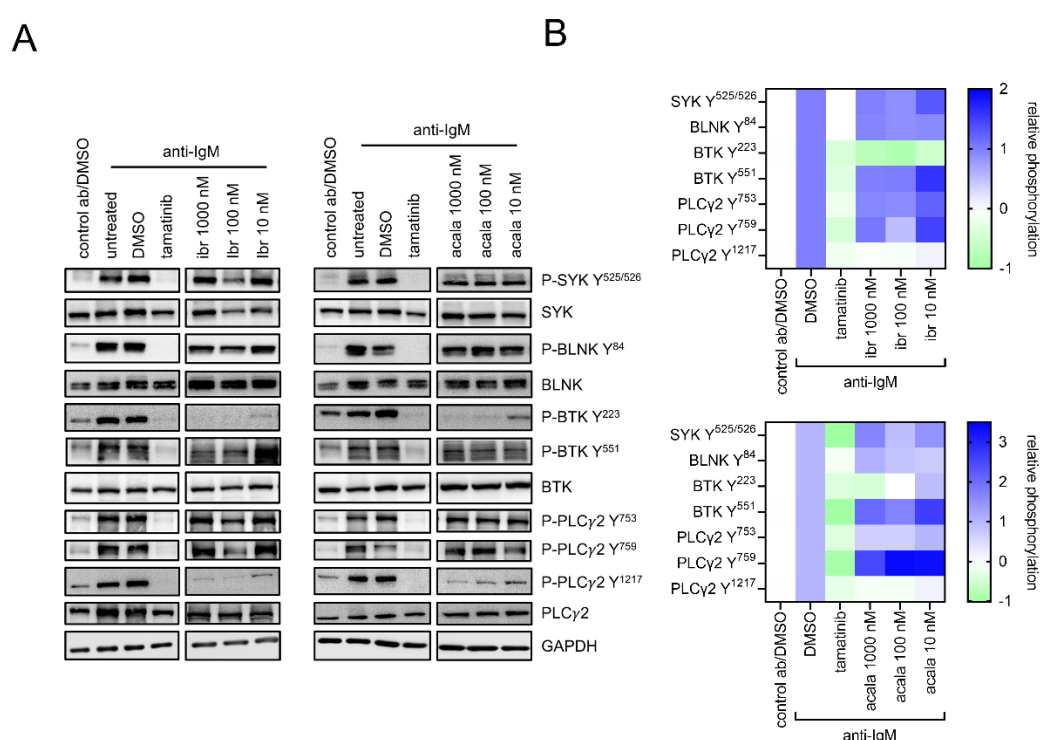
analysis of *MYC*, *BCL2A1* and *CCND2* RNA expression. Graphs show results for individual samples (and means  $\pm$ SD) with results for anti-IgM/DMSO-treated cells set to 1.0.

As previously described [33], anti-IgM beads effectively repressed spontaneous apoptosis of CLL cells measured by cleavage of PARP (Fig 4A,B). However, this was essentially unaffected by ibrutinib. We also analysed effects of ibrutinib on expression of the anti-apoptotic protein MCL1 which is an important mediator of the pro-survival effects of anti-IgM in CLL cells [33]. Induction of MCL1 by anti-IgM is predominantly mediated via translational regulation [54] and its expression was therefore quantified using immunoblotting. Consistent with the relative inability of ibrutinib to reverse anti-IgM-mediated survival effects, ibrutinib only partially reduced induction of MCL1 (Fig 4A,B). Parallel analysis of calcium-regulated gene expression confirmed that (similar to partial inhibition of anti-IgM-induced calcium responses) ibrutinib also only partly repressed calcium responses in these experiments using anti-IgM beads as induction of *BCL2A1* and *MYC* RNA expression was only partially reduced by ibrutinib, and ibrutinib had no effect on induction of *CCND2* RNA (Fig. 4C). In contrast to ibrutinib, SYK inhibition using tamatinib completely reversed anti-IgM-induced apoptosis suppression and induction of MCL1 protein (Fig. 4AB), and induction of *MYC*, *BCL2A1* and *CCND2* RNA expression (Supplementary Fig. 3).

### *3.5 Effect of BTKi on anti-IgM-induced PLC $\gamma$ 2 phosphorylation*

BCR-induced calcium responses are dependent on activation of PLC $\gamma$ 2 which catalyses the production of IP<sub>3</sub> to trigger calcium release from the endoplasmic reticulum. BTK can directly phosphorylate and activate PLC $\gamma$ 2, and we therefore analysed the effect of BTKi on anti-IgM-induced PLC $\gamma$ 2 phosphorylation. Experiments were first performed using B-lymphoma cell lines as these had the strongest signalling responses to anti-IgM. Interestingly, phosphorylation of the SH2-SH3-linker region sites (Y<sup>753</sup> and Y<sup>759</sup>) in OCI-LY7 cells was not inhibited by ibrutinib or acalabrutinib at concentrations up to 1  $\mu$ M (Fig. 5A,B). By contrast, phosphorylation of PLC $\gamma$ 2 at Y<sup>1217</sup> was strongly inhibited by ibrutinib or acalabrutinib at concentrations down to 10 nM. As expected, ibrutinib and acalabrutinib also inhibited autophosphorylation of BTK (on Y<sup>223</sup>) but had little effect

on upstream phosphorylation of SYK, or SYK-dependent phosphorylation of BTK (on Y<sup>551</sup>) or BLNK, an adaptor protein that facilitates binding of BTK to PLCγ2 [55]. However, all of these phosphorylations were strongly inhibited by tamatinib (Fig. 5A,B). Therefore, in OCI-LY7 cells, phosphorylation of PLCγ2 on a subset of activating sites (Y<sup>753</sup> and Y<sup>759</sup>) is induced following sIgM stimulation but occurs independently of BTK. Similar results were obtained for analysis of ibrutinib in TMD8 cells (Supplementary Fig. 7).



**Fig. 5. Effect of BTK inhibition on anti-IgM-induced PLCγ2 phosphorylation in OCI-LY7 cells.** (A,B) OCI-LY7 cells were pre-treated with the indicated concentrations of ibrutinib (ibr) or acalabrutinib (acala), or tamatinib (10 μM) for 1 hour and then treated with anti-IgM or control antibody for 30 seconds. Expression of the indicated proteins was analysed by immunoblotting. (A) Representative results and (B) quantitation derived from 3 independent experiments. Uncut immunoblot images are provided in Supplementary File 2.

We attempted to extend this analysis of the activation of the BCR signalling pathway to CLL samples. However, even when stimulated with anti-IgM beads the relatively low level of signalling in CLL cells made immunoblot analysis challenging, and we therefore treated CLL cells with IL-4 to further enhance responses. We confirmed that incubation with IL-4 increased sIgM expression and anti-IgM-induced calcium responses (Suppl. Fig. 8). Similar to results in OCI-LY7 cells,



ibrutinib did not inhibit phosphorylation of PLC $\gamma$ 2 on Y<sup>753</sup> and Y<sup>759</sup>, although this was effectively reduced by tamatinib (Suppl. Fig. 8). Phosphorylation of SYK and of BTK on Y<sup>551</sup> was also inhibited by tamatinib, but not ibrutinib. It was difficult to detect increased PLC $\gamma$ 2 Y<sup>1217</sup> phosphorylation and, as previously shown, CLL cells appeared to have high levels of BTK Y<sup>223</sup> which was only modestly increased following sIgM stimulation [56]. However, ibrutinib appeared to reduce both modifications.

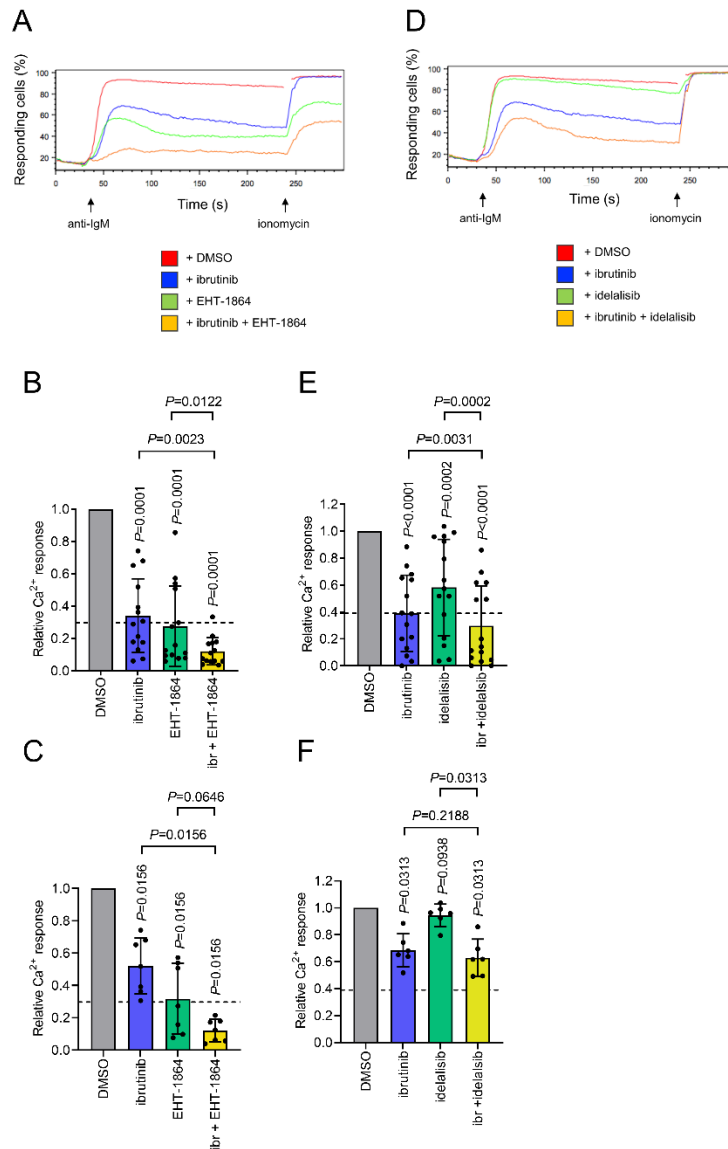
### *3.6 Effect of ibrutinib in combination with RAC or PI3K $\delta$ inhibition*

Analysis of PLC $\gamma$ 2 phosphorylation suggested that retained induction of phosphorylation of PLC $\gamma$ 2 on Y<sup>753</sup> and Y<sup>759</sup> contributed to activation of PLC $\gamma$ 2 despite BTK inhibition. However, other signalling pathways have been shown to contribute to PLC $\gamma$ 2 activation, including RAC GTPases and PI3K, and these may operate independently of BTK [8, 25-27].

EHT-1864 was used to investigate the role of RACs at 25  $\mu$ M, in line with previous studies [57-59]. EHT-1864 significantly reduced anti-IgM-induced calcium responses but, like BTKi, the extent of inhibition varied between samples (Fig. 6A,B). There was only a weak correlation between the extent of inhibition by EHT-1864 and ibrutinib (at 100 nM) for individual samples revealing little overlap between EHT-1864 and ibrutinib-sensitivity (Supplementary Fig. 9). The combination of EHT-1864 and ibrutinib increased inhibition compared to ibrutinib or EHT-1864 alone (Fig. 6B). Importantly, combining ibrutinib and EHT-1864 increased inhibition of calcium responses compared to ibrutinib alone in the subset of samples which were least sensitive to ibrutinib alone (Fig. 6C) but not samples which responded well to ibrutinib alone (Supplementary Fig. 9).

Parallel experiments were performed using idelalisib to investigate the role of PI3K $\delta$ . Idelalisib was used at 1  $\mu$ M in line with previous studies [60]. The combination of idelalisib and ibrutinib increased the extent of inhibition compared to ibrutinib or idelalisib alone in the full cohort (Figure 6D,E). However, compared to EHT-1864, there was a stronger correlation between the extent of inhibition by idelalisib and ibrutinib for individual samples indicating shared sensitivity to these

drugs (Supplementary Fig. 9). Moreover, in samples which were least sensitive to ibrutinib alone, the combination of idelalisib and ibrutinib did not substantially increase inhibition compared to ibrutinib alone (Fig. 6F) whereas there was a clear combinatorial effect in samples which responded well to ibrutinib alone (Supplementary Fig. 9). Taken together these results suggest that both RAC GTPases and PI3K $\delta$  contribute to PLC $\gamma$ 2 activation downstream of sIgM in CLL cells. However, whereas RACs appear to operate in a pathway parallel to BTK, PI3K $\delta$  appears to act in the same pathway.



**Fig. 6. Effect of combined inhibition of BTK and RAC or PI3K $\delta$  on anti-IgM-induced calcium responses in CLL cells.** CLL samples were pre-treated with (A-C) DMSO, ibrutinib (100 nM), EHT-1874 (25  $\mu$ M) or ibrutinib and EHT-1874 ( $n=14$ ), or (D-F) DMSO, ibrutinib (100 nM), idelalisib (1  $\mu$ M) or ibrutinib and idelalisib ( $n=15$ ) for 1 hour before analysis of anti-IgM-induced calcium responses. (A,D) Representative results. Graphs

show relative anti-IgM-induced calcium responses for individual samples (and means  $\pm$ SD) with results for DMSO/anti-IgM-treated cells set to 1.0 for (B,E) all samples and (C,F) samples with <median response to ibrutinib alone indicated by horizontal line). The statistical significance of the differences is shown (vertical P-values show Wilcoxon signed ranked test for comparisons to control and horizontal P-values are Wilcoxon matched-pairs signed rank tests for the indicated comparisons). Results for samples with >median response to ibrutinib alone are shown in Supplementary Fig. 9.

We investigated the effect of EHT-1864 or idelalisib, in combination with ibrutinib on calcium responses in OCI-LY7 and TMD8 cells. As shown above (Fig. 4), ibrutinib alone accelerated the decline in anti-IgM-induced calcium responses but did not substantially affect the peak response in OCI-LY7 cells (Supplementary Fig. 10). The response was only modestly reduced by EHT-1864 or idelalisib when tested alone (and like BTKi these inhibitors had no effect on the initial response) (Supplementary Fig. 10). Moreover, the combination of ibrutinib with either EHT-1864 or idelalisib had relatively little effect on calcium responses compared to ibrutinib alone. Very similar results were obtained using TMD8 cells (Supplementary Fig. 10). Therefore, in these lymphoma cells, neither RACs nor PI3K $\delta$  appear to make a major contribution to PLC $\gamma$ 2 activation downstream of sIgM.

#### **4. Discussion**

BTK is a central player in the signalling pathway that leads from the BCR to activation of PLC $\gamma$ 2 and subsequent engagement of downstream calcium-dependent responses [2]. Lack of active BTK in humans (in X-linked agammaglobulinaemia) is associated with severe immunodeficiency whereas BTK activity plays a major role in the pathogenesis of B-cell malignancies [28, 29]. BTKi have revolutionised the treatment of patients with CLL and some forms of lymphoma. However, resistance and toxicity remain significant challenges [34] and new approaches to inhibit the critical BTK/PLC $\gamma$ 2/calcium signalling cascade are required. In this work, we performed a detailed analysis of the role of BTK in triggering calcium responses in malignant B cells following BCR activation.

We first demonstrated that the ability of both ibrutinib and acalabrutinib to inhibit anti-IgM-induced calcium responses varied widely between CLL samples. This was tightly linked to sIgM signal strength as these BTKi were least effective in samples with the strongest sIgM signalling capacity suggesting that it is the acquisition of BTK-independent signalling to PLC $\gamma$ 2 alongside canonical, BTK-dependent responses that drives strong sIgM signalling. Partial inhibition of anti-IgM-induced calcium responses in samples with strong signalling is consistent with our previous observation that pre-treatment of CLL cells with IL-4 (which increases sIgM expression/signalling capacity) renders CLL cells relatively less susceptible to inhibition by ibrutinib [37]. However, the results presented here extend that finding by demonstrating that natural variation in sIgM expression/signal capacity between samples (which is closely linked to clinical outcome [30]) also appears to influence effectiveness of responses to BTKi. Importantly, inhibition of BTK using a BTK-targeted PROTAC was also only partly capable of inhibiting calcium responses in samples which were relatively less sensitive to ibrutinib. Thus, although BTK can function independently of its kinase activity [22-24, 48], BTKi-resistant signalling to calcium in CLL cells was completely independent of BTK and not just its kinase activity.

In addition to variation between CLL samples, our study revealed distinct roles for BTK in mediating calcium responses between CLL samples and B-lymphoma cells. Thus, in CLL cells, BTK contributed to the initial wave of calcium response, whereas in both OCI-LY7 and TMD8 cells, the predominant function of BTK was to sustain the response as the subsequent decline in calcium was accelerated in cells treated with BTKi. It is possible that this difference between CLL and lymphoma cells is a consequence of comparing primary cells and established cell lines, respectively. Moreover, it is not clear whether this phenomenon applies more widely to other lymphoma-derived cells or is a peculiarity of the particular lines studied here. However, this dichotomy in the role of BTK reflects variation in other studies where BTK appears to be essential or only partly required for the initial phase of the calcium response or contributes predominantly to maintaining calcium levels depending on the B-cell model analysed [21-24].

In both CLL and B-lymphoma cells we identified BTK-independent phosphorylation of PLC $\gamma$ 2 on Y<sup>753</sup> and Y<sup>759</sup> following sIgM stimulation. By contrast, phosphorylation of Y<sup>1217</sup> in the PLC $\gamma$ 2 C-terminal appeared to be more tightly dependent on BTK activity. These results are surprising as previous studies have shown that anti-IgM-induced phosphorylation of PLC $\gamma$ 2 on Y<sup>753</sup> and Y<sup>759</sup> is BTK-dependent, whereas the role of BTK in mediating Y<sup>1217</sup> phosphorylation differs between studies [15-18]. However, it suggests that an alternate kinase is responsible for the Y<sup>753</sup> and Y<sup>759</sup> phosphorylation observed in this study and since mutation of Y<sup>1197</sup> and Y<sup>1217</sup> does not entirely ablate PLC $\gamma$ 2 activity [16], this retained phosphorylation may mediate at least partial PLC $\gamma$ 2 activation despite BTK inhibition. The nature of the kinase responsible for BTK-independent Y<sup>753</sup>/Y<sup>759</sup> is unclear. Although SYK appears to contribute to PLC $\gamma$ 2 activation predominantly via phosphorylation of BTK and BLNK, SYK can phosphorylate PLC $\gamma$ 2 *in vitro* [15]. Therefore, it is possible that when activated at a high level, SYK directly phosphorylates and activates PLC $\gamma$ 2 in CLL and lymphoma cells. Consistent with this, SYK inhibition completely inhibited anti-IgM-induced calcium responses, calcium-dependent transcription and survival in CLL cells.

In addition to its phosphorylation, PLC $\gamma$ 2 can be regulated by RAC GTPases and PI3K. RACs are activated downstream of VAV following BCR activation and stimulate PLC $\gamma$ 2 activity by direct protein-protein interactions independently of phosphorylation [26, 61]. We demonstrated that RAC inhibition reduced calcium responses in CLL cells and that co-inhibition of RACs and BTK co-operated to inhibit calcium responses. Importantly, the effect of RAC inhibition was greatest in samples that were relatively less sensitive to BTKi, indicating that RAC-mediated PLC $\gamma$ 2 may represent a significant BTK-independent pathway of activation.

PI3K $\delta$  inhibition also co-operated with BTKi to enhance inhibition of calcium responses in CLL cells. However, in contrast to RACs, PI3K $\delta$  appeared to operate in a BTK-dependent manner as samples which were sensitive to BTKi were also relatively sensitive to idelalisib and the inhibitory effect of BTK and PI3K $\delta$  co-inhibition was strongest in samples that were relatively sensitive to BTKi alone. PI3K $\delta$  likely contributes to calcium responses downstream of sIgM in CLL cells via generation of PIP<sub>3</sub> which creates a docking site for BTK and PLC $\gamma$ 2 to potentiate

canonical, phosphorylation-dependent activation of PLC $\gamma$ 2 by BTK [8]. In contrast to CLL cells, neither RAC or PI3K $\delta$  inhibition (alone or with BTK inhibition) had a substantial effect on anti-IgM-induced calcium responses in B-lymphoma cells. Overall, only SYK inhibition was capable of effectively inhibiting calcium responses in these cell lines.

Although our study was restricted to samples from BTKi-naïve patients, it is interesting to consider these results in light of mechanisms of resistance to BTKi in patients. Ibrutinib resistance in CLL patients is associated with a range of acquired mutations, including of *BTK* and *PLCG2*. Whereas *BTK* mutations typically render BTK less sensitive to inhibition by ibrutinib [34], *PLCG2* mutations can sensitise PLC $\gamma$ 2 to RAC-mediated activation [62]. Thus, the RAC-mediated pathway that we have shown here to contribute to BTK-independence in treatment-naïve cases can be selected for by mutation of PLC $\gamma$ 2 in ibrutinib-resistance. In addition to *BTK* and *PLCG2* mutations, ibrutinib-resistance is associated with activating mutations in the NF- $\kappa$ B pathway, consistent with the idea that this is a key effector pathway operating downstream of PLC $\gamma$ 2 [63]. Finally, it is also interesting to note that, in an *in vitro* model, epigenetic upregulation of RAC2 was identified as a key pathway of ibrutinib resistance in ABC DLBCL cells [64].

Overall, our study sheds important new light on the nature of BTK-independent signalling pathways leading to calcium responses in malignant B cells and indicates alternate targeting strategies. SYK inhibition remains a potential therapeutic strategy for B-cell malignancies [65-67] and this may be most relevant for CLL cases with strong signalling or in lymphoma where it was the only approach tested here that achieved substantial inhibition of responses. Combinations of RAC inhibitors with BTKi may also be an effective approach, especially in CLL cases with strong signalling that responded modestly to BTKi alone. Although our study has focused on BCR signalling, it is important to note that BTK contributes to signalling downstream of various receptors in addition to the BCR [3-6] and it is possible that inhibition of these pathways is important for the clinical effectiveness of BTKi. It will be interesting to determine whether the extent to which BTK mediates such responses also differs between samples and, if so, what alternate signalling pathways mediate BTK by-pass.

### **Author contributions**

RA, FKS, FF, AJS, GP, BV-A conceived and designed the study; RA, AW, EAL, FKS, FF, AJL, AJS, GP and BV-A were involved in acquisition, analysis, or interpretation of data; RA, GP and BV-A drafted the work; all authors approved the submitted version of the manuscript.

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### **Conflict-of-interests**

The authors report no relevant conflicts-of-interest.

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