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The dual inhibitor of the phosphoinositol-3 and PIM kinases, IBL-202, is effective against chronic lymphocytic leukaemia cells under conditions that mimic the hypoxic tumour microenvironment

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Summary

Despite significant advances in treatment, chronic lymphocytic leukaemia (CLL) remains an incurable disease. Ibrutinib and idelalisib, which inhibit Bruton Tyrosine kinase (BTK) and phosphoinositol-3 (PI3) kinase-δ respectively, have become important treatment options for the disease and demonstrate the potential of targeting components of the B-cell receptor-signalling pathway. IBL-202 is a dual inhibitor of the PIM and PI3 kinases. Synergy between the pan-PIM inhibitor, pPIMi, and idelalisib against a range of haematological cell lines and primary CLL cells supports the rationale for preclinical studies of IBL-202 in CLL. Importantly, IBL-202, but not idelalisib, was cytotoxic against CLL cells under *in vitro* conditions that mimic the hypoxic tumour microenvironment. The significant effects of IBL-202 on CD49d and CXCR4 expression and migration, cycle and proliferation of CLL cells suggest the drug may also interfere with the migratory and proliferative capacity of the leukaemic cells. Collectively, these data demonstrate that dual inhibition of the PIM and PI3 kinases by IBL-202 may be an effective strategy for targeting CLL cells, particularly within the environmental niches known to confer drug-resistance.

Keywords: chronic lymphocytic leukaemia, microenvironment, phosphoinositol-3 kinase, proviral integration of Moloney murine leukaemia virus.

The treatment of chronic lymphocytic leukaemia (CLL) has been revolutionised in recent years by the advent of chemo-immunotherapy (Tam *et al*, 2008; Fischer *et al*, 2016) and more recently by development of inhibitors targeting components of the B-cell receptor (BCR) signalling pathway. In particular, ibrutinib and idelalisib, which target the Bruton tyrosine kinase (BTK) and phosphoinositol-3 (PI3) kinase respectively, have shown extremely promising results in clinical trials (Brown *et al*, 2014; Burger *et al*, 2015). Despite these significant advances, it remains unclear whether any treatment option is curative, with disease relapse still common. Therefore, it is imperative that development of novel treatment strategies remain a focus of research for this disease.

A better understanding of the signalling pathways that promote the survival and proliferation of CLL cells has been the driving force behind the development of the novel agents. The clinical efficacy of ibrutinib and idelalisib highlights the importance of the BCR pathway in CLL-cell biology. However, treatment resistance and toxicity represent significant challenges in the clinical management of patients on these new agents. Acquired mutations in several genes, including *BTK* and *PLCG2*, are now known to confer resistance to ibrutinib, while toxicity has been a limiting factor in further clinical trials of idelalisib (Woyach *et al*, 2014; Lampson *et al*, 2016; Ahn *et al*, 2017; Romero, 2017).

Ibrutinib and idelalisib inhibit the interaction of CLL cells with the tumour microenvironment (Fiorcari *et al*, 2013; Niemann *et al*, 2016; Ten Hacken & Burger, 2016), resulting in the lymphocytosis commonly observed with both drugs (Herman *et al*, 2014; Rossi & Gaidano, 2014). It is now generally believed that this lymphocytosis results from CLL-cell liberation from the lymph nodes and bone marrow; death of the leukaemic cells then occurs as both drugs prevent CLL

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cells from migrating to, and accumulating in, the lymph nodes and bone marrow (Cheng *et al*, 2014).

The role of the tumour microenvironment should be an important consideration in any pre-clinical assessment of novel agents for CLL because successful treatment of the disease relies on targeting the leukaemic cells within the bone marrow and lymph nodes. The interaction of CLL cells with stromal cells within these tissues confers resistance to a variety of drugs, including fludarabine (Kurtova *et al*, 2009) and ABT-737 (Vogler *et al*, 2009). It is becoming increasingly apparent that hypoxia may also play a significant role in the CLL tumour microenvironment (Huelsemann *et al*, 2015; Koczula *et al*, 2016; Valsecchi *et al*, 2016). A recent study showed that areas within the bone marrow are profoundly hypoxic (Spencer *et al*, 2014), suggesting that in order for CLL cells to populate these tissue regions they must have the capacity to adapt to and proliferate under low oxygen tensions. Evidence that hypoxia is a significant factor in the drug resistance of other cancer cell types (Muz *et al*, 2015), suggests that modelling this environmental factor *in vitro* may aid in the pre-clinical assessment of novel agents for CLL.

Multiple pathways, including those regulated by the hypoxia-inducible factor-1 alpha (Hif-1α, HIF1A) and NF-κB (Muz *et al*, 2015) transcription factors facilitate the adaption of cancer cells to hypoxia. In multiple cell types hypoxia results in changes in expression of a range of pro-survival, anti-apoptotic proteins including components of the PI3 kinase pathway (Courtnay *et al*, 2015), several members of the BCL2 family (Sermeus *et al*, 2012) and the proviral integration of Moloney murine leukaemia virus (PIM) kinases (Chen *et al*, 2009a).

The PIM kinases are a family of serine/threonine kinases with documented roles in drug-resistance (Chen *et al*, 2009a), apoptosis (Braso-Maristany *et al*, 2016), cell cycle (Chen *et al*, 2016) and DNA damage repair (Chen *et al*, 2016). In pancreatic ductal carcinoma (PDA) an increase in PIM1 expression induced during hypoxic stress was concomitant with resistance to oxaliplatin (O'Hayer *et al*, 2016), while induction of PIM2 expression in an *in vitro* stromal co-culture model of multiple myeloma was associated with resistance to the proteasome inhibitor bortezomib (Reidy *et al*, 2014). Pre-clinical studies suggest that further investigation of PIM kinase inhibitors may also be warranted in CLL (Chen *et al*, 2009c; Decker *et al*, 2014; Garcia *et al*, 2014), although no studies to date have examined the effects of inhibitors of either the PIM or PI3 kinases on CLL cells under reduced oxygen tensions.

Given the multitude of interactions between CLL cells and the microenvironment it is conceivable that simultaneous inhibition of multiple signalling pathways with novel drugs or logical drug combinations may be one approach towards eliminating CLL cells from within microenvironmental niches, thereby limiting the potential for clonal evolution and disease progression. Several recent studies illustrate this notion by describing potential new strategies for targeting CLL cells under *in vitro* conditions that confer resistance to more conventional treatments. Many of these, such as combinations of duvelisib or acalabrutinib with venetoclax (Deng *et al*, 2017; Patel *et al*, 2017), build upon recent clinical data by incorporating drugs targeting components of the BCR signalling pathway.

Studies in multiple myeloma (Reidy *et al*, 2014) and PDA (O'Hayer *et al*, 2016) demonstrate the potential of IBL-202, an inhibitor of both the PIM and PI3 kinases, for the treatment of a variety of tumour types under conditions that mimic the tumour microenvironment and confer resistance to more conventional therapies. In the current study, we explored the effects of IBL-202 under *in vitro* conditions that mimic the hypoxic CLL tumour microenvironment (Herishanu *et al*, 2011).

Materials and methods

Patient samples and cell lines

All patient samples were collected following informed consent under approval from the Northern Sydney Local Health District human research ethics committee and according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) from CLL patients were isolated by centrifugation through a Ficoll-density gradient and cryopreserved in 90% fetal calf serum (FCS)/10% dimethylsulphoxide (DMSO) until required. The proportion of CD5/CD19$^+$ cells in all PBMC fractions, as determined by flow cytometry, was >85% (data not shown). Assessment of ZAP70 and CD38 expression was carried out as previously described (Orchard *et al*, 2004). Samples were assigned to one of three *ATM*/*TP53* functional categories using a previously published methodology (Best *et al*, 2008; Tracy *et al*, 2017).

The OSU-CLL (CLL) cell line was obtained under a material transfer agreement with Ohio State University's human genetics sample bank. The OSU-CLL, MEC-1 (CLL/prolymphocytic), Raji (Burkitt lymphoma), Ramos (Burkitt lymphoma) and the CD40L (also termed CD40LG)-expressing fibroblast cell lines were maintained in RPMI-1640 media containing 10% FCS, 2 mmol/l L-glutamine and 1% penicillin/streptomycin.

All hypoxic culture experiments were conducted in a purpose-built X-Vivo culture cabinet (Biospherix Ltd., Parish, NY, USA). The oxygen tensions within the workspace and culture cabinets were calibrated relative to medical gas, according to the manufacturer's specifications. CO_2 was kept at a constant5% with changes in O_2 balanced with nitrogen.

Drug cytotoxicity and synergy

The cytotoxic effects of idelalisib (Selleck Chemicals, Houston, TX, USA), pPIMi and IBL-202 (Inflection Biosciences Ltd, Dublin, Ireland) were assessed using the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, as previously described (Crassini *et al*, 2015). Briefly, for MTT assays, CLL cells were seeded at 3×10^5 cells per well in a 96-well plate and treated with drugs at the concentrations indicated. Prior to the end of the treatment period, 20 μl of a 5 mg/ml MTT solution was added to each well and incubated at 37°C for 4 h. The cells were spun and the supernatant removed by aspiration. The blue crystals of formazan salt produced by the viable cells present were then solubilised by addition of DMSO. Readings were taken on a Powerwave HT microplate reader (Biotek, Winooski, VT, USA) at 570 nm, with a background subtraction at 690 nm.

To assess synergy between idelalisib and pPIMi, the two drugs were combined in a fixed ratio determined by the 50% inhibitory concentration (IC_{50}) of the two drugs as single agents. All IC_{50} values were calculated using the Biodatafit software (http://www.changbioscience.com/stat/ec50.html). The MTT assay was used to measure the proportion of viable cells remaining relative to untreated controls. A combination index was calculated for each fractional effect using the method of Chou and Talalay (1984); according to this calculation a fractional effect of 0.5, for example, indicates a 50% cell kill and combination indices of <1, =1 and >1 are indicative of synergy, additivity and antagonism, respectively.

The proportion of apoptotic CLL cells following culture or drug treatment was assessed using the mitochondrial membrane potential dye 1,1′,3,3,3′,3′-hexamethylindodicarbo cyanine iodide ($DiIC_1(5)$; Mitoprobe, Thermo Fisher Scientific, Waltham, MA, USA) and propidium iodide (Sigma-Aldrich, St Louis, MI, USA). At the end of the indicated treatment period the CLL cells were harvested and labelled with 50 nmol/l $DiIC_1(5)$ and 10 μg/ml propidium iodide for 10 min at 37°C. Data acquisition and analysis were performed on a FACS Fortessa flow cytometer running Diva 8 software (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell cycle and proliferation

The cell cycle distribution and proliferation rate of OSU-CLL cells were assessed by flow cytometry using propidium iodide and carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich) respectively. For cell cycle assessment cells were seeded at 1.5×10^5 cells per well of a 96-well plate and were either left untreated in media or treated with the doses of IBL-202 and for the times indicated under either normoxic or hypoxic conditions. The distribution of OSU-CLL cells within the cell cycle was assessed following culture in media alone (control) or treatment with the concentrations of IBL-202 indicated. The cells were harvested, washed, resuspended in 70% (v/v) ethanol and stored at −20°C. After a minimum of 24 h at −20°C, the cells were pelleted by centrifugation and stained with 40 μg/ml propidium iodide, 20 μg/ml RNAse, 0.1% Triton-X100 in phosphate-buffered saline (PBS) for 30 min at 37°C. Data was acquired on a FACS Fortessa flow cytometer and analysed using ModFit software (Verity Software House, Topsham, ME, USA).

Cell proliferation was assessed by staining the cells with the cell permeable, amine dye carboxyfluorescein succinimidyl ester (CFSE). Briefly, OSU-CLL cells were labelled with 2 μmol/l CFSE for 20 min at 37°C, washed in PBS and seeded at 1.5×10^5 cells per well of a 96-well plate. The cells were then either left untreated or were treated with IBL-202 at the doses and for the times indicated under either normoxic or hypoxic conditions. Apoptotic and dead cells were excluded from the analysis based on their forward/side scatter properties.

The effects of IBL-202 on the proliferation and cell cycling of primary CLL cells was assessed following stimulation with the CpG-oligonucleotide, Dsp30, in combination with interleukin 2 (IL2), according to the method described by Decker *et al* (2000). Briefly, 4×10^5 cells per well of a 96-well plate were seeded and cultured in medium alone or with medium containing 1 μmol/l Dsp30 and 200 units/ml IL2. Cultures of Dsp30/IL2-stimulated cells from each patient sample were also treated with 0.1, 0.5 or 1.0 μmol/l IBL-202. For cell proliferation, following 48-h culture the cells were pulsed with 5-bromo-2′-deoxyuridine (BrdU) and cultured for a further 24 h. The cells were then fixed and denatured in the plate and probed for BrdU incorporation using a BrdU cell proliferation kit (Merck, Burlington, MA, USA). Readings were taken at 570 nm on a Powerwave HT microplate reader (Biotek, Winooski, VT, USA). Data are expressed as a fold change in BrdU incorporation relative to the levels in cells cultured in medium alone. Cell cycle analysis of Dsp30/IL2-stimulated CLL cells from the same patients was performed as outlined above for the OSU-CLL cell line.

Immunophenotypic changes and cell migration

Expression of the integrin CD49d and chemokine receptor CXCR4 was assessed using specific phycoerythrin (PE)-labelled antibodies (Biolegend, San Diego, CA, USA) and flow cytometry. Primary CLL cells were treated with 1 μmol/l IBL-202 for 6 h, washed in fresh media followed by a 24 h culture on a confluent layer of CD40L-expressing fibroblasts. The percentage change in expression following treatment was calculated from the mean fluorescence intensity (MFI) of IBL-202-treated cells relative to cells cultured in media alone.

The effects of IBL-202 on the migratory capacity of CLL cells were determined by examining the ability of CLL cells to migrate towards stroma-derived factor (SDF-1α, also termed CXCL12), the ligand for CXCR4. CLL cells (2.5×10^6 per condition) from 6 patients were cultured either in medium alone or with 1 μmol/l IBL-202 for 18 h before being placed in the upper chamber of 5 μm pore cell culture inserts (Merck Millipore, Burlington, MA, USA). Either medium alone or medium containing 200 ng recombinant human SDF-1α (Peprotech, Rocky Hill, NJ, USA) was added to the well below each support. Cells were cultured

for a further 3 h after which the medium below the supports was collected and spun at 300 ***g*** to pellet the migrated cells. The pellet was then stained with antibodies against CD5 and CD19, conjugated to fluorescein isothiocyanate and PE fluorochromes respectively (Biolegend), and with the mitochondrial membrane potential dye $DiIC_1(5)$ to exclude any apoptotic cells. Following a wash in PBS, the number of viable CLL cells ($CD5^+/CD19^+/DiIC_1(5)^+$) in each culture fraction was assessed by flow cytometry by fixing the time of data acquisition to 120 s. Data are expressed as a fold change in the number of CLL cells enumerated in each fraction relative to untreated control cultures for each patient sample.

Assessment of mitochondrial ROS levels

Levels of mitochondria-specific reactive oxygen species (ROS) were determined using the MitoSox Red dye (Thermo Fisher Scientific, Waltham, MA, USA) and flow cytometry according to the manufacturer's specifications. Briefly, 3×10^5 primary CLL cells were cultured in media alone or with 1 μmol/l IBL-202 for 6 h, washed in fresh media and cultured for a further 24 h with CD40L-expressing fibroblasts. Cells were then harvested and stained with 5 μmol/l MitoSox Red for 10 min at 37°C. Following a wash in PBS (137 mmol/l NaCl, 10 mmol/l Phosphate, 2.7 mmol/l KCl, pH 7.4), viable cells were labelled with 50 nmol/l $DiIC_1(5)$ for a further 10 min at room temperature. An acquisition gate on the $DiIC_1(5)$ positive (viable) cell fraction was used to acquire a minimum of 10 000 events. The data shown is expressed as a fold change in the MitoSox Red mean fluorescent intensity (MFI) within the viable cell population relative to levels in control (untreated) cells.

Immunoblotting

Changes in the protein expression of Akt, BAD, PIM 1,2 and 3, NF-κB, Mcl-1 [MCL1], NOXA [PMAIP1] and BCL2 were assessed by immunoblotting using specific antibodies directed against the total or phosphorylated forms of each protein. All primary antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). CLL cells (3×10^6 per condition) were lysed in radioimmunoprecipitate assay buffer (5 mmol/l Tris-HCl pH 7–8, 150 mmol/l NaCl, 0.1% sodium dodecylsulphate, 0.5% sodium deoxycholate and 1% Triton X-100) containing a cocktail of protease/phosphatase inhibitors (MS-SAFE, Sigma Aldrich, St Louis, MI, USA) for 30 min on ice with intermittent vortexing. The cell lysates were then heated at 70°C for 10 min in sample buffer and resolved through 4–12% Bolt pre-cast gels (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were blotted onto polyvinylidene difluoride membranes using the iBlot system (Thermo Fisher Scientific, Waltham, MA, USA), blocked in 0.5% dry milk powder/Tris-buffered saline with tween-20 (TBST; 137 mmol/l NaCl, 19 mmol/l Tris-HCl, 0.1% Tween-20) before being stained with primary antibody overnight at 4°C. Following extensive washing in TBST, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature, washed and incubated with enhanced chemiluminescent substrate (ECL; Advansta, Menlo Park, CA, USA). Images were acquired using the ChemiDoc XRS+ system (Biorad, Hercules, CA, USA). Densitometric calculation of changes in protein expression were performed using the ImageJ software (https://imagej.net/).

Statistical analyses

All statistical analyses were performed using the students *T*-test with P values <0.05 considered significant.

Results

Synergy between inhibition of PIM and PI3 kinases

We explored the rationale for dual targeting of the PI3 and PIM kinases by assessing synergy between the PI3 kinase-δ inhibitor idelalisib and the pan-PIM inhibitor, pPIMi (Inflection Biosciences, Dublin, Ireland). Dose response analyses were conducted against a panel of CLL patient samples ($n = 6$) and 4 haematological cell lines (MEC1, OSU-CLL, RAJI and RAMOS) using the MTT assay. The patient samples (CLL 4, 5, 8, 9, 11 and 18 – Table I) included 3 with evidence of TP53 dysfunction. Synergy was confirmed according to the method described by Chou and Talalay (1984).

We observed a greater decrease in viable cell number in response to the combination of the drugs compared to either drug as a single agent, indicated by the right-shift in the dose response curves for both CLL patient samples (Fig 1A, left) and cell lines (Fig 1A, right). The combination indices calculated for both primary CLL cells and cell lines is indicative of strong synergy between the drugs at all fractional effect levels (Fig 1B, left). The synergy observed was consistent with a significant decrease in the IC_{50} values for both pPIMi (data not shown) and idelalisib (Fig 1B, right) in the CLL patient samples and cell lines.

The interaction between CLL cells and the stroma that constitute the tumour microenvironment has a significant impact upon the sensitivity of the leukaemic cells to a variety of drugs, including fludarabine (Kurtova *et al*, 2009) and ABT-737 (Vogler *et al*, 2009). By employing the CD40L-expressing fibroblast model (Herishanu *et al*, 2011) we investigated the efficacy of combining pPIMi and idelalisib against primary CLL cells cultured under *in vitro* conditions that mimic the lymph node tumour microenvironment. CLL cells in co-culture with fibroblasts were treated with a range of doses of pPIMi and idelalisib alone or in combination. The proportion of viable cells remaining following a 6 h treatment followed by 24 h CD40L-fibroblast co-culture was assessed using the mitochondrial membrane potential dye

Table I. CLL patient sample details.

CLL patient	ZAP70 (%)	CD38 (%)	ATM/TP53 Function	17p (%)	11q (%)	Treatment History
1	0.5	0.9	N	+/+	+/+	FCR
2	ND	5.38	1	ND	ND	NT
3	66.82	84.24	N	+/− (12)	+/+	NT
4	77.8	13.8	N	+/+	+/+	FCR
5	3.52	0.83	N	+/+	+/+	FCR
6	74.73	16.98	1	+/− (23)	+/+	FCR
7	23.3	10.1	3	+/+	+/+	FCR
8	61.34	40.21	1	+/− (65)	+/+	FCR
9	37.33	89.6	1	+/− (88)	+/+	MULTIPLE
10	73.4	9.05	N	+/+	+/− (83)	FCR
11	72.2	83.1	N	+/+	+/+	FCR
12	60.3	45.5	1	+/−	+/+	FCR
13	94.06	99.86	3	+/− (13)	+/+	MULTIPLE
14	81.78	0.8	3	+/−	+/+	FCR
15	3.3	17.9	3	ND	ND	NT
16	1.38	4.46	N	+/+	+/+	NT
17	12.06	2.43	N	+/+	+/+	NT
18	4.52	1.02	1	+/− (85)	+/+	CLB

Cut-off values for ZAP70 and CD38 positivity were 10% and 7% respectively. ATM/TP53 functional categorisation was as follows: N, normal; 1, TP53 dysfunction; 2, ATM dysfunction; and 3, evidence of emerging TP53 dysfunction. Deletions of loci within chromosomes 17p and 11q, which encompass the *TP53* and *ATM* genes respectively, were assessed by fluorescent *in-situ* hybridisation. Where available, the clone size of deletions is shown in parentheses. Treatment history: FCR, fludarabine, cyclophosphamide, rituximab; CLB, chlorambucil; MULTIPLE, 3 or more lines of prior treatment; NT, no prior treatment; ND, no data available.

$DiIC_1(5)$, propidium iodide and flow cytometry. Representative images from one patient sample are shown (Fig 1C, left). We observed a greater-than-additive effect of the combination of the two drugs at 20 and 50 μmol/l which is suggestive of synergy under these conditions. The IC_{50} for idelalisib was not reached within the dose range analysed. For pPIMi as a single agent the IC_{50} was 94.63 ± 3.49 μmol/l. The IC_{50} value for both drugs in combination at a 1:1 ratio was significantly ($P = 0.03$) lower at 38.11 ± 2.27 μmol/l.

The dual PIM/PI3 kinase inhibitor, IBL-202, is effective under conditions that model the hypoxic tumour microenvironment

Having demonstrated the potential of simultaneously targeting both the PIM and PI3 kinases we next investigated the efficacy of IBL-202, a drug that inhibits isoforms in both kinase families, under conditions that mimic the lymph node microenvironment and the low oxygen tensions experienced by CLL cells *in vivo*. Modelling of this microenvironment *in vitro* was achieved by co-culturing CLL cells with CD40L-expressing fibroblasts at 1% O_2 in a purpose-built culture cabinet.

Initially, we were able to demonstrate a significant effect of hypoxia on CLL-cell survival. Primary CLL cells from 3 patients (CLL 1, 8, 16) including one with evidence of TP53 dysfunction, were cultured in media alone under normoxic (21% O_2) or hypoxic (1% O_2) conditions for up to 96 h. The proportion of viable cells remaining at each time point was assessed using $DiIC_1(5)$, propidium iodide and flow cytometry. The number of intact, viable cells remaining at the end of the 96-h culture was assessed using a haemocytometer and trypan blue exclusion. Culture under hypoxic conditions did not prevent the spontaneous apoptosis of CLL cells but significantly ($P < 0.05$) decreased the proportion of apoptotic cells at each time point, suggesting a decrease in the rate of cell death (Fig 2A, left). This was confirmed by the significantly ($P = 0.04$) higher number of viable cells present at the end of the 96 h period in cultures maintained at 1% O_2 (Fig 2A, right). In contrast, we observed no significant difference in the survival of primary CLL cells co-cultured with CD40L-expressing fibroblasts under normoxic or hypoxic conditions, with the proportion of viable cells exceeding 95% under both oxygen tensions (data not shown).

Chronic lymphocytic leukaemia cells from 6 patients (CLL 2, 3, 6, 7, 12 and 17) cultured in media under normoxic or hypoxic conditions, were sensitive to IBL-202 in a dose dependent manner (Fig 2B, left), albeit with a significant difference in sensitivity under the two conditions; the IC_{50} for IBL-202 under normoxic and hypoxic conditions were 0.15 ± 0.07 and 0.39 ± 0.15 μmol/l respectively. All the CLL patient samples were significantly more sensitive to the

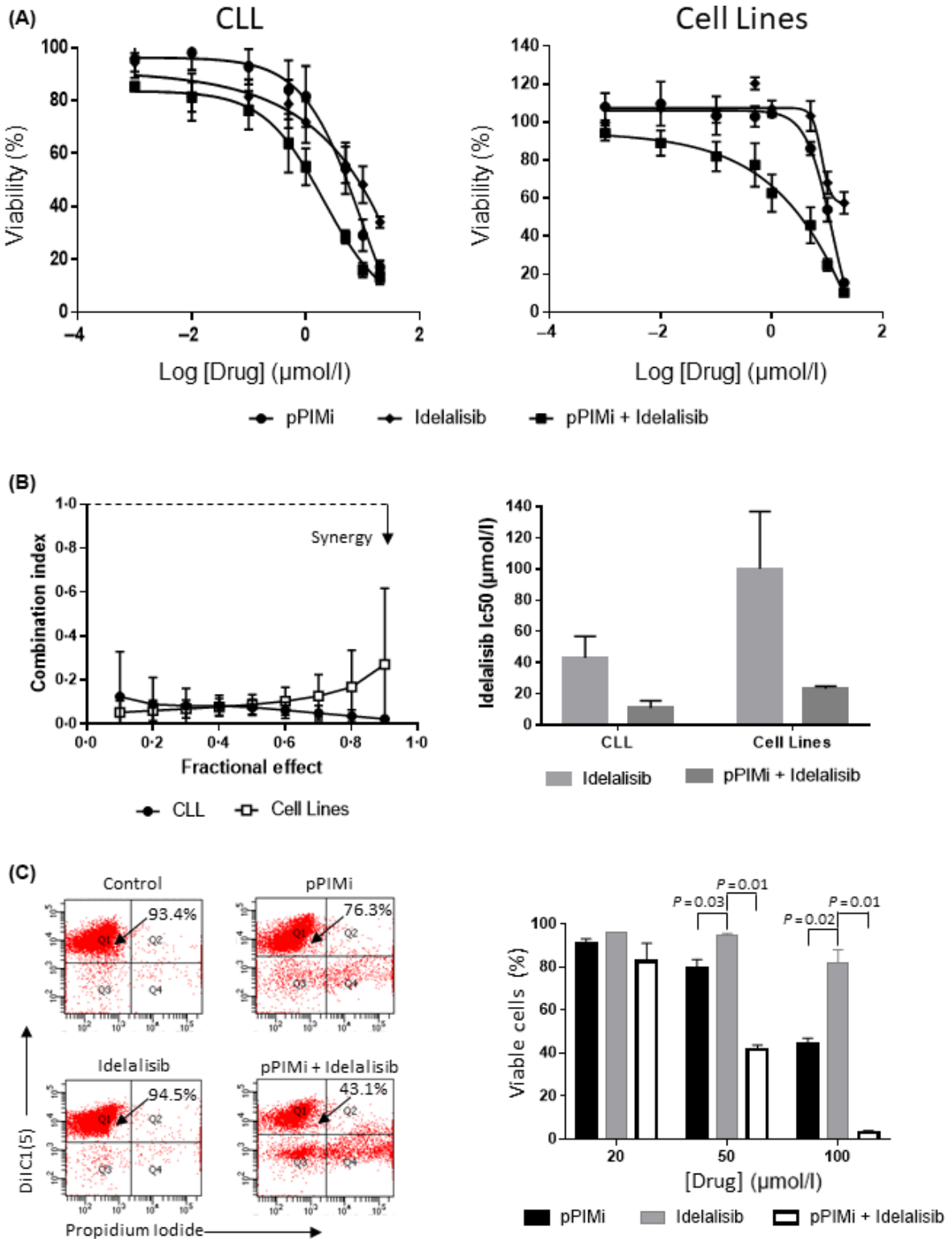


Fig 1. Synergy between pPIMi and idelalisib provides the rationale for dual targeting of the PIM and PI3 kinases in CLL. (A) Dose response analyses for pan-PIM inhibitor (pPIMi), idelalisib or the two drugs in combination at a 1:4 ratio were generated for primary chronic lymphocytic leukaemia (CLL) cells (n = 3) and cell lines (n = 4) by MTT assay (B) Synergy between pPIMi and idelalisib was confirmed in both primary CLL cell cultures and cell lines, as indicated by combination indices of <1, by MTT assay (left). The 50% inhibitory concentration for idelalisib as a single agent and in combination with pPIMi was assessed in CLL samples and cell lines. *$P < 0.01$. (C) CLL patient samples (n = 3) co-cultured with CD40L-expressing fibroblasts were treated with pPIMi or idelalisib or the drugs in combination at a 1:1 ratio. Cell viability was assessed by flow cytometry. Representative analysis plots for one patient sample treated with 20 μmol/l of each drug are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

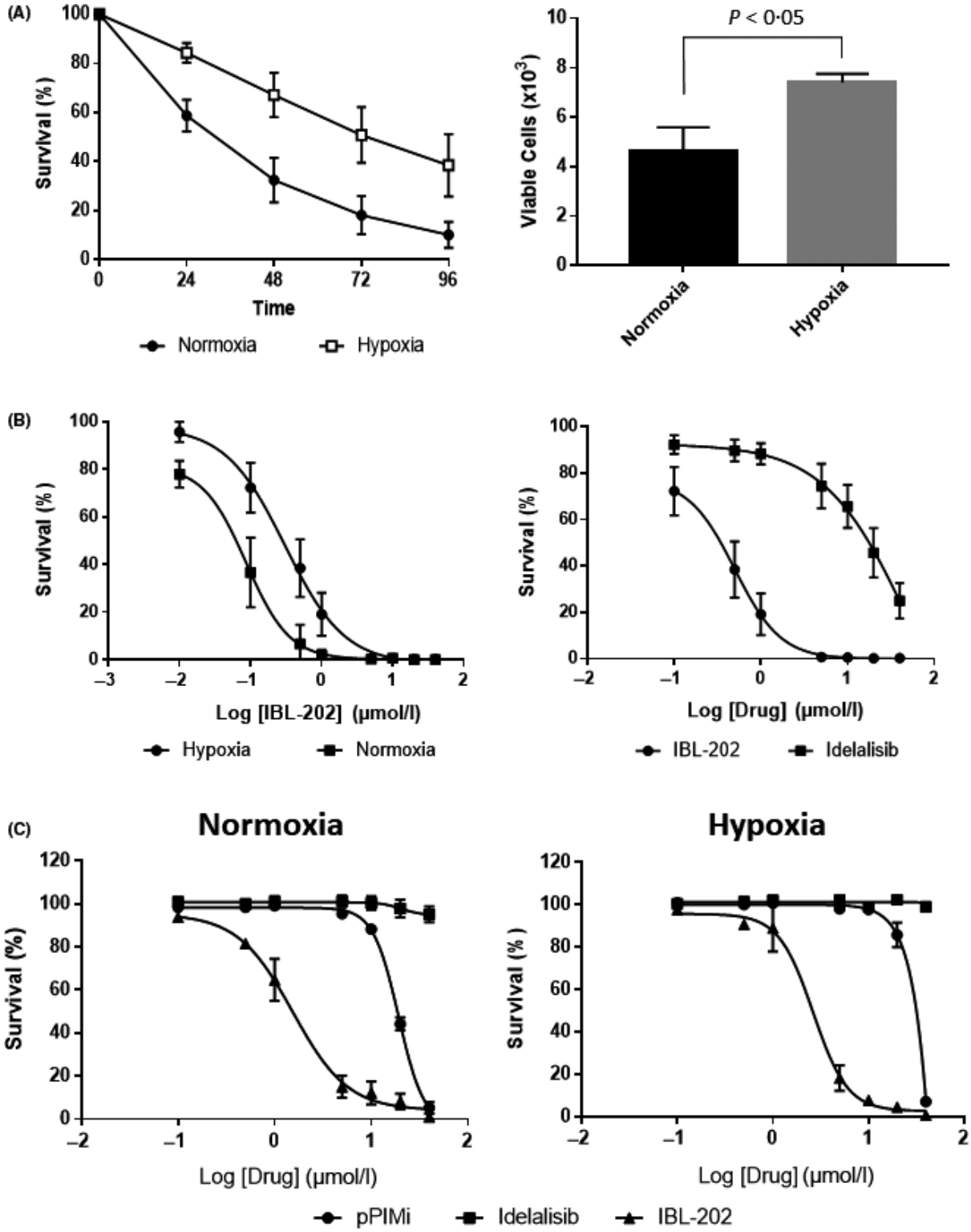


Fig 2. CLL cells cultured under hypoxic conditions show a reduced rate of apoptosis and are significantly more sensitive to IBL-202 than to PIMi or idelalisib. (A) Primary chronic lymphocytic leukaemia (CLL)cells from 3 patients were cultured in media alone under normoxic or hypoxic conditions for up to 96 h. The proportion of viable cells at each time point and at the end of the 96-h culture period were assessed by flow cytometry and trypan blue exclusion respectively. (B) Dose response analyses for IBL-202 against primary CLL patient samples ($n = 6$) cultured in media alone under either normoxic or hypoxic conditions and in comparison to idelalisib under normoxic conditions. (C) Dose response analyses for pan-PIM inhibitor (pPIMi), idelalisib and IBL-202 against CLL patient samples co-cultured with CD40L-expressing fibroblasts under normoxic (left) or hypoxic (right) conditions.

cytotoxic effects of IBL-202 than to idelalisib at 1% O_2 (Fig 2B, right).

Chronic lymphocytic leukaemia cells co-cultured with CD40L-expressing fibroblasts were insensitive to any cytotoxic effect of idelalisib within the dose range studied, irrespective of oxygen tension but responded to pPIMi and IBL-202 in a dose-dependent manner (Fig 2C). All 6 of the CLL patient samples studied were significantly more sensitive to IBL-202 than to either pPIMi or idelalisib under either normoxic or hypoxic conditions.

IBL202 reduces the cell cycle progression and proliferation rate of CLL cells

Next, we investigated the impact of IBL-202 on the cell-cycle distribution and proliferation rate of OSU-CLL cells using propidium iodide or CFSE staining and flow cytometry. Hypoxic culture reduced the proportion of OSU-CLL cells in S-phase, with a concomitant increase in the G2/M proportion of cells. IBL-202, under both normoxic and hypoxic conditions, significantly increased the proportion of cells in the S and G2/M phases with a decrease in the proportion in G0/G1, relative to vehicle-treated control cells (Fig 3A). To confirm whether IBL-202 was inducing a G2/M phase arrest, OSU-CLL cells were cultured under hypoxic conditions and either left untreated or were treated with 1 μmol/l IBL-202 for 24–96 h. The proportion of cells in the G2/M phases increased over the time course with a concomitant decrease in the proportion of cells in the S and G0/G1 phases. (Fig 3A, lower right).

Consistent with the cell-cycle effects observed, hypoxia and IBL-202 also had marked effects on the proliferation rate of OSU-CLL cells. The representative histograms (Fig 3B, upper) show the decay in CFSE fluorescence with time under normoxic (left) and hypoxic (right) conditions from which the growth curves were calculated. Cells maintained at 1% O_2 proliferated at a significantly slower rate compared to those in 21% O_2 (Fig 3B, lower). IBL-202 significantly slowed the rate of OSU-CLL cell proliferation under normoxic and hypoxic conditions; under normoxic conditions IBL-202 treatment resulted in a significant decrease in proliferation rate at 0.1 μmol/l and an almost complete arrest of proliferation at 0.5 and 1 μmol/l (Fig 3B, lower left). IBL-202 also had a significant effect on the proliferation rate under hypoxic conditions at doses of 0.5 and 1.0 μmol/l (Fig 3B, right). There was no significant difference in the proliferation rate of cells during treatment with 0.5 or 1.0 μmol/l IBL-202 under normoxic compared with hypoxic conditions.

Culture of primary CLL cells from 4 patients with the CpG-oligonucleotide Dsp30 in combination with IL2 induced a significant increase in the number of proliferating cells compared to unstimulated controls, as determined by the relative fold-change in BrdU incorporation (Fig 3C, left). IBL-202 at 0.1, 0.5 and 1.0 μmol/l significantly ($P < 0.02$) reduced the fold change in cell proliferation, as shown by the reduction in BrdU incorporation. At 0.5 and 1.0 μmol/l IBL-202 treatment showed no significant difference ($P = 0.2$ and 0.6 respectively) in BrdU incorporation compared with cells cultured in media alone, suggesting that at these concentrations IBL-202 abrogated the effects of Dsp30/IL2 on cell proliferation. Consistent with its effects on the proliferation of primary CLL cells, IBL-202 also significantly reduced the proportion of cells in the S and G2/M cell cycle phases (Fig 3C, right).

IBL202 down-regulates the expression of CD49d and CXCR4

The capacity of CLL cells to migrate to and populate the tumour microenvironment is conferred by expression of chemokine receptors and adhesion molecules respectively. Among the most well documented of these molecules are the receptor for SDF-1α, CXCR4 (CD184) (Mohle *et al*, 1999), and the adhesion molecule CD49d (Bulian *et al*, 2014).

We examined whether IBL-202 treatment alters surface expression of CXCR4 and CD49d on CLL cells and determined the impact of CXCR4 down-regulation on the ability of CLL cells to migrate towards the ligand for this receptor, SDF-1α. Patient samples ($n = 3$, CLL 4, 6 and 13) were cultured in media in the presence or absence of 1 μmol/l IBL-202 for 24 h under normoxic conditions. The cells were then harvested and stained with antibodies against CXCR4 or CD49d and co-stained with $DilC_1(5)$ to enable exclusion of apoptotic cells from the analysis. IBL-202 significantly down-regulated expression of both CXCR4 ($P = 0.04$) and CD49d ($P = 0.01$) on CLL cells by an average of 40.29% and 61.22% respectively compared to untreated controls (Fig 3D, left and centre).

Stroma-derived factor-1α significantly ($P = 0.001$) increased the number of CLL cells that migrated across the permeable supports in all 4 of the patient samples assessed, from a mean of 189.3 ± 111.4 to 1632 ± 736.4 cells (Fig 3D, right). Consistent with the effects of the drug on CXCR4 expression, pre-treatment with IBL-202 significantly ($P = 0.01$) decreased the number of CLL cells that migrated in the presence of SDF-1α relative to controls to an average of 435 ± 243.4 cells. No significant difference was observed in the number of CLL cells that migrated following culture in medium alone or following pre-treatment with IBL-202 ($P = 0.1$), suggesting IBL-202 treatment completely abrogated SDF-1α-induced CLL cell migration.

IBL-202 blocks activity of PI3 kinase/AKT pathway and increases levels of mitochondrial ROS under hypoxic conditions

Next, we investigated the mechanisms of action of IBL-202 under hypoxic co-culture conditions by examining changes in protein expression and levels of mitochondria-specific ROS. Co-culture of CLL cells with CD40L-expressing

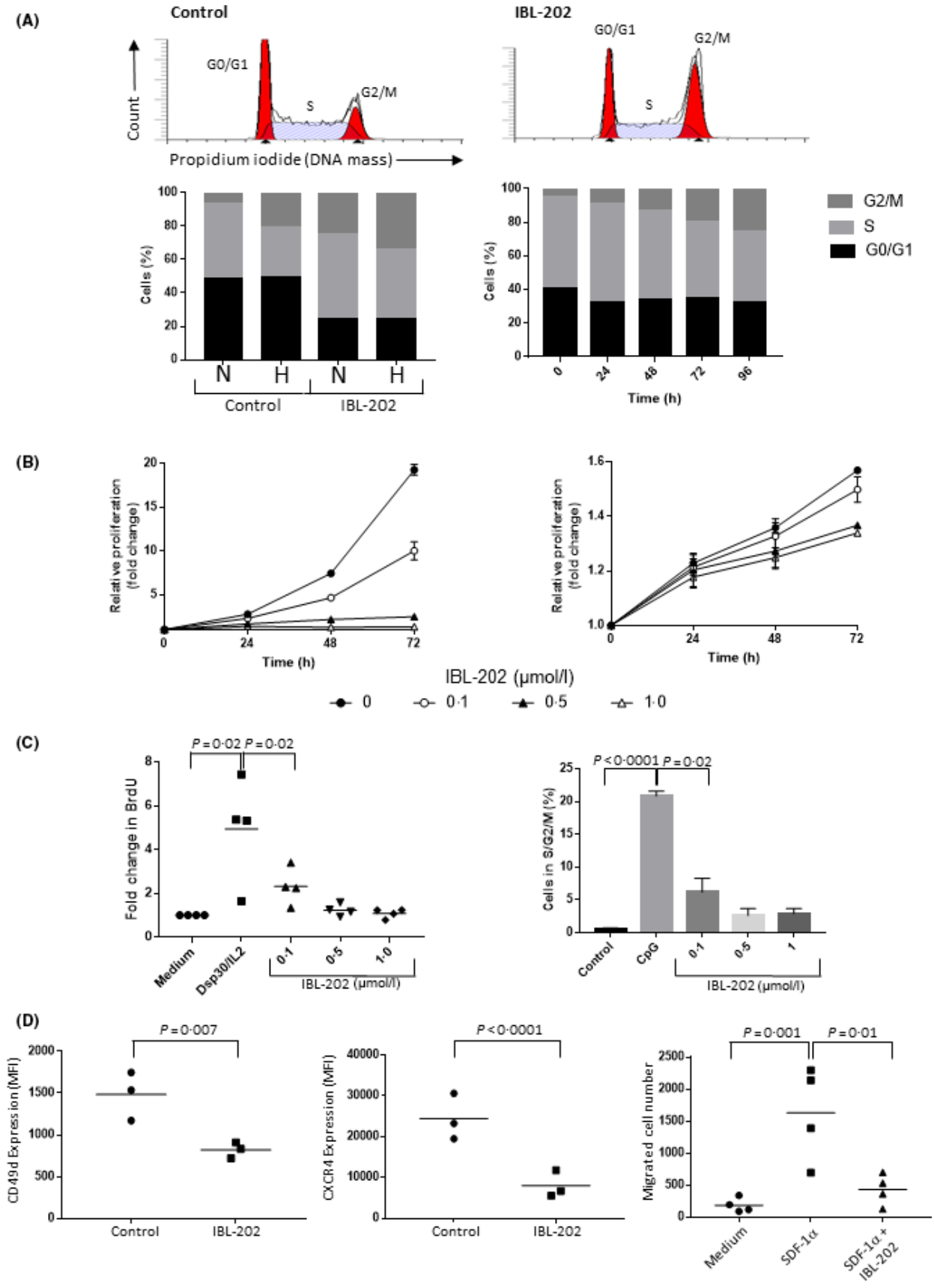


Fig 3. IBL-202 has significant cytostatic effects against the OSU-CLL cell line and decreases expression of CD49d and CXCR4 on primary CLL cells. (A) The effects of IBL-202 on cell cycle distribution of OSU-CLL cells under normoxic or hypoxic conditions were assessed by flow cytometry. (B) OSU-CLL cell proliferation under normoxic or hypoxic conditions and following treatment with IBL-202 was assessed by CFSE staining and flow cytometry. The upper histograms are representative images from one patient sample cultured under normoxic (left) or hypoxic (right) conditions for up to 72 h, as indicated. (C) Changes in expression of CD49d and CXCR4 and on migration of primary CLL cells down an SDF-1α gradient ($n = 3$) were determined by flow cytometry following treatment with IBL-202. BrdU, 5-bromo-2′-deoxyuridine; H, hypoxic; MFI, mean fluorescence intensity; N, normoxic. [Colour figure can be viewed at wileyonlinelibrary.com]

fibroblasts under hypoxic conditions up-regulated the expression and phosphorylation of AKT and BAD (Fig 4A), known substrates for the PI3 and PIM kinases respectively. As expected, IBL-202 almost completely abrogated phosphorylation of AKT but, interestingly, pPIMi, albeit to a lesser extent, also decreased phosphorylation of AKT in all 3 samples. In 2 of the 3 samples we also observed a decrease in the phosphorylation of BAD relative to total BAD expression following treatment with either pPIMi or IBL-202.

Expression of PIM1, 2 and 3 was evident in patient samples cultured in media under hypoxic conditions. Expression of PIM1 and 3 but not PIM 2 was significantly increased by co-culture with CD40L-fibroblasts. Neither pPIMi nor IBL-202 had any effect on the expression of any of the three PIM isoforms.

Given the well-documented roles of ROS in mediating cellular responses to changes in oxygen tension (Waypa *et al*, 2016) we explored the effects of hypoxia and CD40L-fibroblast co-culture on intracellular levels of mitochondrial ROS and investigated the possibility that these may be involved in the sensitivity of CLL cells to pPIMi and IBL-202. CLL cells from 3 patients (CLL 5, 14 and 18) were cultured in medium alone or with CD40L-fibroblasts for 24 h under normoxic or hypoxic conditions. Levels of mitochondrial ROS were measured using MitoSox red and flow cytometry. Consistent with the supportive effects of the stromal cells, we observed a significant ($P = 0.002$) decrease in mitochondrial ROS levels in CLL cells co-cultured with CD40L-fibroblasts relative to cells cultured in media (Fig 4B, upper panel). A similar effect was observed in CLL cells cultured in media or with CD40L-fibroblasts under hypoxic conditions relative to normoxic conditions ($P = 0.02$ and $P = 0.006$ respectively); the decrease in mitochondrial ROS levels in CLL cells cultured in media under hypoxic conditions is consistent with the effects of hypoxia we observed on CLL-cell survival (Fig 2A). Treatment with IBL-202, but not pPIMi, resulted in a significant ($P = 0.04$) increase in mitochondrial ROS levels in CLL cells co-cultured with fibroblasts under hypoxic conditions (Fig 4B, lower panel).

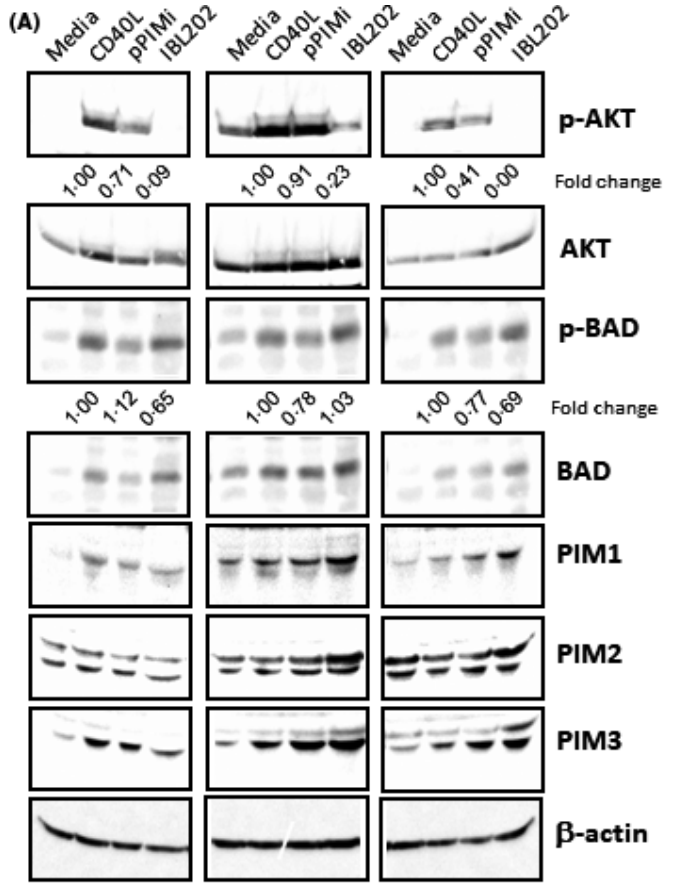
IBL-202 inhibits phosphorylation of NF-κB and increases the NOXA/Mcl-1 ratio under hypoxic co-culture conditions

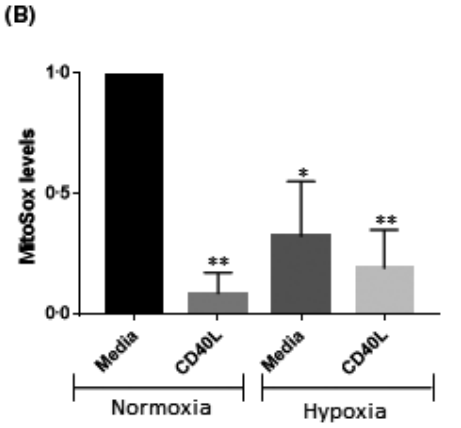
To further elucidate the mechanisms of action of IBL-202 under hypoxic fibroblast co-culture conditions we examined samples from 6 CLL patients (CLL 4, 5, 12, 14, 15, 18) for changes in the expression and phosphorylation of NF-κB and the BCL2 family proteins Mcl-1, NOXA and BCL2 following IBL-202 treatment. Representative immunoblots from 2 patient samples are shown (Fig 4C). Co-culture of CLL cells with CD40L-fibroblasts resulted in an increase in expression of phosphorylated NF-κB under both normoxic and hypoxic conditions relative to cells cultured in media alone. In 3/6 and 5/6 samples respectively, pPIMi and IBL-202 reduced the phosphorylation of pNF-κB relative to levels in cells co-cultured with CD40L fibroblasts under hypoxic conditions. Mcl-1 expression was increased in CLL cells following fibroblast co-culture under normoxic, but not hypoxic conditions. We also observed a significant increase in expression of the Mcl-1 antagonist NOXA following treatment with IBL-202. Densitometric calculation of the NOXA/Mcl-1 ratio showed a significant ($P = 0.006$) increase in this ratio in all 6 of the patient samples following IBL-202 treatment compared to levels in cells cultured in either media or with stromal cells under hypoxic conditions (Fig 4C). No significant effects of any of the culture conditions or drug treatments were observed on the expression of BCL2.

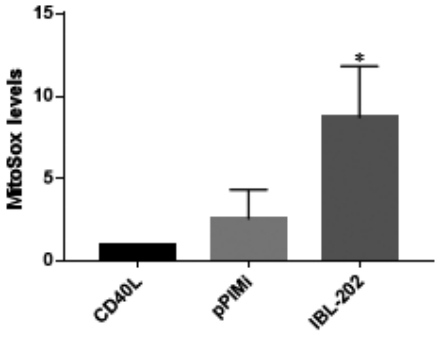
Discussion

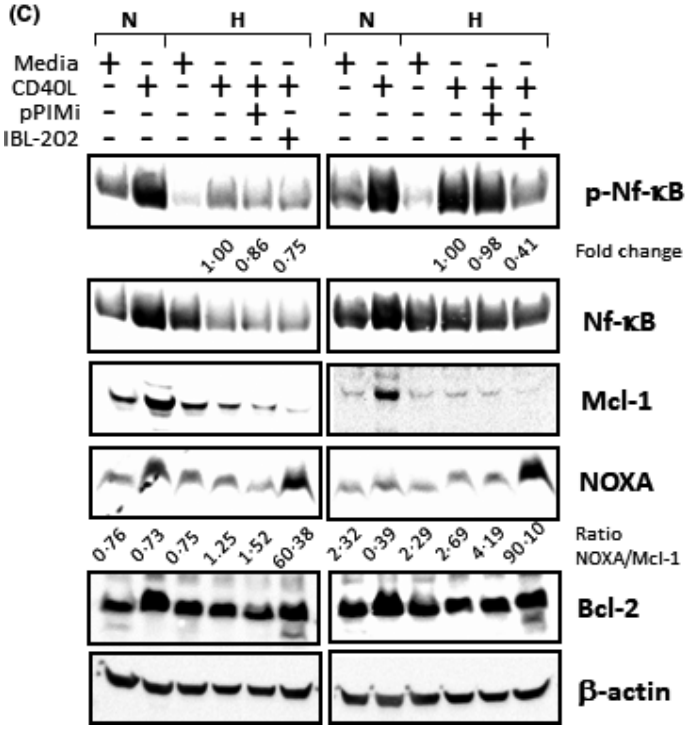
A rational approach in developing novel therapeutic regimens for CLL is to explore drug combinations that build upon the efficacy of BCR pathway-targeted therapies. This has been the focus of several recent studies, including trials of ibrutinib plus rituximab (Jain *et al*, 2017) and idelalisib plus ofatumumab (Jones *et al*, 2017). At the same time, a growing understanding of the biology of the CLL cell is providing an ever-expanding range of potential novel drug targets and combinations.

Pre-clinical studies in CLL (Chen *et al*, 2009c; Decker *et al*, 2014; Cervantes-Gomez *et al*, 2015), other haematological malignancies (Garcia *et al*, 2014; Keane *et al*, 2015; Brunen *et al*, 2016; Nair *et al*, 2017; Paino *et al*, 2017) and solid tumours (Chen *et al*, 2009b, 2016; Foulks *et al*, 2014; Braso-Maristany *et al*, 2016; O'Hayer *et al*, 2016; Warfel *et al*, 2016), suggest that, owing to their important roles in tumour cell biology, the PIM family of kinases may represent targets for novel, single agent or combination therapies. This concept is illustrated by a recent study in which the combination of a PIM and a PI3 kinase inhibitor, NMS-P645 and GDC-0941 respectively, had significantly more anti-proliferative activity than either inhibitor alone against pancreatic cancer cells (Mologni *et al*, 2017). Recent data also support a









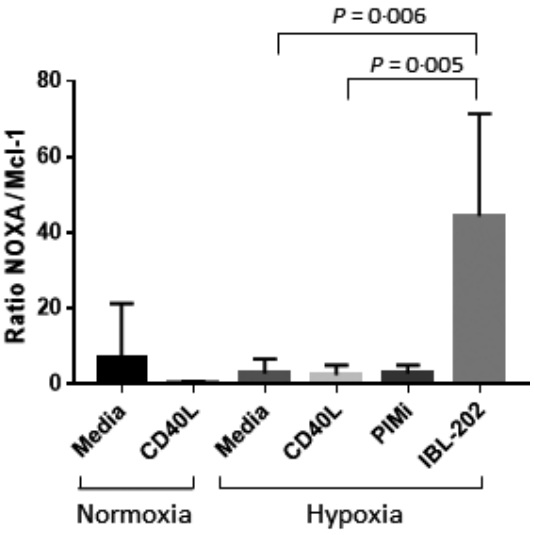


Fig 4. IBL-202 increases levels of mitochondrial reactive oxygen species and increases the NOXA/Mcl-1 ratio in CLL cells under hypoxic conditions. (A) Expression of total and phosphorylated (p-) forms of AKT and BAD was assessed by Western blotting. β-actin expression was determined as a loading control. The numbers under each lane indicate the fold change in the ratio of the phosphorylated form to total form of each protein, normalised to β-actin, relative to levels in CD40L-fibroblast co-cultured controls. (B) Levels of mitochondria-specific reactive oxygen species (ROS) were measured in 6 patient samples using the MitoSox Red dye and flow cytometry under the culture and treatment conditions indicated. Data is expressed relative to ROS levels in cells cultured in normoxic media (upper histogram) or hypoxic CD40L-fibroblast co-culture (lower histogram). $*P < 0.01$ relative to control (C) Expression of NF-κB, Mcl-1, NOXA and BCL2 was assessed by Western blotting in primary CLL cells from 6 patients cultured under normoxic or hypoxic conditions and in the presence or absence of CD40L-expressing fibroblasts. Representative data from 2 patient samples is shown. The values shown under each lane indicate the denistometric ratio of NOXA/Mcl-1 expression. β-Actin expression was determined as a loading control. The histogram is the cumulative data of the NOXA/Mcl-1 ratio from the 6 patient samples under the culture and treatment conditions indicated.

rationale for dual targeting of these pathways in acute myeloid leukaemia (AML), in which resistance to PIM kinase inhibition was shown to be regulated by feedback from the AKT/mTOR pathway (Brunen *et al*, 2016).

Synergy between idelalisib and the pan-PIM inhibitor, pPIMi (Fig 1), supports the rationale for simultaneously inhibiting members of both kinase families in CLL. The high degree of synergy between pPIMi and idelalisib associated with the significant decrease in the Ic_{50} for idelalisib we observed (Fig 1) supports the notion that combinations of inhibitors targeting the PI3 and PIM kinases may be an effective strategy, which has the potential to reduce the toxicity associated with idelalisib (Lampson *et al*, 2016). Interestingly, in our study, combining idelalisib with pPIMi reduced the IC_{50} for idelalisib to within the reported steady-state plasma concentrations for the drug under current dosing schedules (Brown *et al*, 2014). A greater-than-additive effect of pPIMi in combination with idelalisib was also evident against CLL cells under *in vitro* conditions that mimic the tumour microenvironment and confer resistance to the cytotoxic effects of idelalisib (Fig 1C). The synergy between pPIMi and idelalisib we demonstrate in the current study and similar data from the other forms of cancer mentioned, provided a strong rationale for investigating the efficacy of the dual PIM/PI3 kinase inhibitor, IBL-202.

In the current study, we explored the effects of IBL-202 against CLL cells under conditions that mimic the tumour microenvironment. Given the growing body of evidence suggesting CLL cells may adapt to, survive and even proliferate under hypoxic conditions (Shachar *et al*, 2012; Koczula *et al*, 2016; Valsecchi *et al*, 2016) and that activity of the PIM kinases is oxygen-sensitive (Chen *et al*, 2009a; Warfel *et al*, 2016), we assessed the effects of IBL-202 under *in vitro* conditions which are believed to more accurately represent those experienced by CLL cells in the lymph node and marrow (Herishanu *et al*, 2011). The significant decrease in the spontaneous apoptosis rate of primary CLL cells (Fig 2A) and the continued proliferation (albeit at a slower rate) of the OSU-CLL cell line (Fig 3B) under hypoxic conditions are consistent with the notion that hypoxia may play a role in the survival of CLL cells *in vivo* and that CLL cells can adapt to and proliferate even under reduced oxygen tensions.

Chronic lymphocytic leukaemia cells cultured with or without stromal cells under normoxic or hypoxic conditions were sensitive to IBL-202 in a dose-dependent manner and significantly more-so than to either idelalisib or PIMi (Fig 2C). The decrease in sensitivity of CLL cells co-cultured with CD40L-expressing fibroblasts to pPIMi, idelalisib and IBL-202 was not surprising, given the increased phosphorylation of AKT and expression of PIM-1 and PIM-3 observed (Fig 4A) and the documented effects of stroma on the drug-sensitivity of CLL cells (Kurtova *et al*, 2009) (Vogler *et al*, 2009).

In addition to its cytotoxic effects IBL-202 also induced an accumulation and arrest of OSU-CLL cells in the G2/M phase under both normoxic and hypoxic conditions, an observation consistent with a previous study of PI3 kinase inhibition in Chinese hamster ovary (CHO) cells (Opstal & Boonstra, 2006). The cell-cycle effects of IBL-202 were concomitant with a slowing of the proliferation rate of OSU-CLL cells under normoxic and hypoxic conditions and of primary CLL cells stimulated with Dsp30/IL2 under normoxic conditions. The reduced proliferation of cancer cells under hypoxic conditions is believed to contribute to resistance against a range of conventional chemotherapeutic agents (Yokoi & Fidler, 2004; Song *et al*, 2006; Sullivan *et al*, 2008).

Our data, showing that the cytotoxic effects of IBL-202 under hypoxic conditions were concomitant with a significant increase in ROS levels, support previous observations that increased ROS levels in CLL cells may be associated with drug sensitivity and induction of apoptosis (Tonino *et al*, 2011). Both fibroblast co-culture and hypoxia reduced sensitivity to IBL-202, which may, in part, be related to both the increase in AKT activity and reduction in mitochondrial ROS levels. The decrease in mitochondrial ROS levels we observed under hypoxic conditions (Fig 4B) reinforces the notion that CLL cells have an innate capacity to adapt to hypoxia (Koczula *et al*, 2016) through mechanisms that appear to involve regulation of mitochondrial ROS levels. Our data are consistent with those of Tonino *et al* (2011), in suggesting that ROS are involved in the apoptotic processes in CLL cells, via a mechanism involving upregulation of the BH3 protein NOXA.

The current study also suggests that, similar to idelalisib (Fiorcari *et al*, 2013; Pepper *et al*, 2015), IBL-202 may

interfere with the mechanisms that enable CLL cells to populate the lymph nodes and bone marrow (Fig 3C). The significant reductions in expression of the integrin CD49d and the chemokine receptor CXCR4 and the impaired ability of CLL cells to migrate down an SDF-1α gradient (Fig 3B) suggest IBL-202 has the potential to reduce both the homing and retention of CLL cells in the microenvironment, in addition to the significant direct cytotoxic actions described.

The mechanisms of action of IBL-202 were further investigated by examining the expression of the BCL2 family member BAD, a well-documented substrate of both the PIM (Macdonald *et al*, 2006) and PI3 kinases (Song *et al*, 2005). The increase in phosphorylation of BAD we observed following co-culture with CD40L-fibroblasts is consistent with the increase in both AKT activity and increased expression of PIM1 and PIM3. Similarly, the decrease in AKT and BAD phosphorylation in 2 of the 3 samples following treatment with IBL-202 is consistent with the cytotoxic effects of this drug (Fig 4A). Interestingly, the effect of pPIMi on the phosphorylation of AKT we observed in all 3 samples (Fig 4A) supports the notion of cross-talk between the PIM and PI3 kinase pathways, as demonstrated in the aforementioned study on AML (Brunen *et al*, 2016).

Several of the proteins of the BCL2 family, including BAD, are regulated by activity of the NF-κB transcription factor (Herishanu *et al*, 2011). Consistent with its pro-survival effects, co-culture of CLL cells with CD40L fibroblasts under normoxic conditions significantly up-regulated NF-κB expression and phosphorylation, Mcl-1 expression and decreased the NOXA/Mcl-1 ratio in all 6 samples assessed (Fig 4C). The importance of the NOXA/Mcl-1 ratio is highlighted in studies of CLL and AML in which sensitivity to the BH3-mimetics ABT-737 and ABT-199 (venetoclax) under hypoxic conditions was shown to be dependent on Mcl-1 expression (Huelsemann *et al*, 2015) and on the NOXA/Mcl-1 balance (Konopleva *et al*, 2006; Tromp *et al*, 2012). Furthermore, our observations are consistent with those of Huelsemann *et al* (2015), in that they suggest Mcl-1 upregulation following CD40 engagement is dependent on local oxygen availability and NF-κB activity and that NF-κB activity may be involved in regulating NOXA and Mcl-1 expression in response to IBL-202.

In conclusion, we present data suggesting that dual inhibition of the PIM and PI3 kinasesby IBL-202 may represent an effective treatment strategy for targeting CLL cells in the tumour microenvironment, regardless of the oxygenation status of the tissue. IBL-202 had significant cytotoxic and cytostatic effects under these conditions and down-regulated expression of key proteins involved in the homing and migration of CLL cells to the tumour microenvironment.

Author contributions

OGB, SPM, RIC, MO'D and MO'N designed the study. MO'N contributed the inhibitors pPIMi and IBL-202. KC, YS and OGB performed experiments. All authors contributed towards preparation of the manuscript.

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