The dual Syk/JAK inhibitor cerdulatinib antagonises B-cell receptor and microenvironmental signaling in chronic lymphocytic leukemia

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Abstract

Purpose—B-cell receptor (BCR)-associated kinase inhibitors such as ibrutinib have revolutionised the treatment of chronic lymphocytic leukemia (CLL). However, these agents are not curative and resistance is already emerging in a proportion of patients. Interleukin-4 (IL-4), expressed in CLL lymph nodes, can augment BCR-signalling and reduce the effectiveness of BCR-kinase inhibitors. Therefore simultaneous targeting of the IL-4- and BCR-signalling pathways by cerdulatinib, a novel dual Syk/JAK inhibitor currently in clinical trials (NCT01994382), may improve treatment responses in patients.

Experimental Design—PBMCs from CLL patients were treated with cerdulatinib alone or in combination with venetoclax. Cell death, chemokine and cell signalling assay were performed and analysed by flow cytometry, immunoblotting, Q-PCR and ELISA as indicated.

Results—At concentrations achievable in patients, cerdulatinib inhibited BCR- and IL-4-induced downstream signalling in CLL cells using multiple read-outs and prevented anti-IgM- and nurse-like cell (NLC)-mediated CCL3/CCL4 production. Cerdulatinib induced apoptosis of CLL cells, in a time- and concentration-dependent manner, and particularly in IGHV unmutated samples with

greater BCR-signalling capacity and response to IL-4, or samples expressing higher levels of sIgM, CD49d+ or ZAP70+. Cerdulatinib overcame anti-IgM, IL-4/CD40L or NLC-mediated protection by preventing upregulation of MCL-1- and BCL-X_L, however BCL-2 expression was unaffected. Furthermore in samples treated with IL-4/CD40L, cerdulatinib synergised with venetoclax *in vitro* to induce greater apoptosis than either drug alone.

Conclusion—Cerdulatinib is a promising therapeutic for the treatment of CLL either alone or in combination with venetoclax, with the potential to target critical survival pathways in this currently incurable disease.

Keywords

CLL; JAK3; STAT6; Cerdulatinib; Interleukin 4; B-cell receptor

Introduction

The importance of B-cell receptor (BCR) mediated signalling in the pathogenesis of chronic lymphocytic leukemia (CLL) has become even more apparent in recent years(1), and drugs which target kinases associated with this pathway are revolutionising the treatment of this disease(2–4). Recently approved agents for relapsed/refractory CLL include ibrutinib (BTK inhibitor) and idelalisib (PI3Kδ inhibitor)(5, 6). To date these compounds have not proved curative, which may in part be due to protection of the tumour by the microenvironment(7). Importantly, a proportion of patients are developing resistance to these new agents. Mechanisms include mutations in BTK or PLCγ for ibrutinib(8) or through as yet unknown factors(9). Spleen tyrosine kinase (Syk) belongs to the Syk/ZAP70 family of non-receptor kinases and plays a central role in the transmission of activating signals downstream of the BCR, and chemokine and integrin receptors within B cells(10). Indeed Syk inhibition has been used in treatment strategies for B-cell malignancies and autoimmune disease(10). Fostamatinib (R788), one of the first oral inhibitors of Syk, reduced CLL cell migration, chemokine secretion and BCR signalling in vitro(11), and induced a number of partial responses in patients with relapsed disease(12). More recently inhibition of Syk with entospletinib (GS-9973) was shown to overcome resistance to ibrutinib in vitro(13), indicating that inhibition of Syk maybe a promising therapeutic strategy for the treatment of patients with CLL, particularly following emergence of resistance to ibrutinib. Entospletinib alone has demonstrated an acceptable safety profile and clinical activity in patients with CLL(14) and a phase II clinical trial of this drug in CLL is currently ongoing (NCT01799889).

CLL cells are dependent upon various signals from the lymph node microenvironment for their survival(10). Using gene set enrichment analysis we recently identified an IL-4 gene signature which was enriched in lymph node tissue compared with matched blood and bone marrow(7). IL-4 signals in lymphocytes predominantly through the type 1 IL-4 receptor (IL-4R) via Janus protein tyrosine kinases JAK1 and JAK3 resulting in phosphorylation of STAT6 (pSTAT6)(15). IL-4 signaling is known to promote tumor survival and protect against therapy-induced cell death(7, 16), and is produced by T cells from CLL patients(17). Patients with progressive CLL have been reported to have greater numbers of T cells which spontaneously produce IL-4(18) and their tumor cells express significantly higher IL-4R

levels compared to normal B cells, which correlates with increased signaling to IL-4(19, 20). We recently showed that IL-4 increased surface IgM (sIgM) expression on CLL cells in vitro and potentiated BCR-mediated signaling(7). Furthermore, the ability of idelalisib or ibrutinib to inhibit BCR-mediated signalling was significantly impaired following IL-4 treatment(7).

The pharmacological targeting of JAK/STAT signalling has proved therapeutically useful in patients with autoimmune disorders, exemplified by approval of the JAK inhibitor tofacitinib for treatment of rheumatoid arthritis(21). Moreover, results from a phase II clinical trial using the JAK1/2 inhibitor ruxolitinib in 13 CLL patients showed rapid but transient decreases in lymphadenopathy and increased lymphocytosis(22). Together these data highlight a potential strategy for simultaneous inhibition of Syk and JAK in CLL.

Cerdulatinib (also termed PRT062070) is a novel dual Syk/JAK inhibitor which is currently in phase 1/2A clinical trials for CLL as well as other B-cell Non Hodgkin lymphomas (NCT01994382), with first in man clinical trial data and therapeutic responses in patients resistant to ibrutinib already reported(23). It is a novel reversible ATP-competitive dual Syk/JAK inhibitor that has been shown to inhibit anti-Ig and IL-4 signalling in vitro in normal human lymphocytes and to inhibit BCR-induced B-cell activation and splenomegaly in vivo in mice(24). Cerdulatinib also induced apoptosis in primary diffuse large B-cell lymphoma (DLBCL) cell lines in vitro and inhibited BCR-mediated signalling(25). Cerdulatinib treatment with once daily administration at 45mg dosing, achieved a Cmax of ~2μM (23). Here we show in vitro that primary CLL samples are sensitive to cerdulatinib, at concentrations achievable in patients. Cerdulatinib inhibited IL-4 and BCR-mediated signalling which resulted in apoptosis, particularly in samples with markers of progressive disease, and synergised with venetoclax (ABT-199) in vitro. These data suggest that cerdulatinib is a promising therapeutic strategy for the treatment of CLL, with the potential to target critical pro-survival signalling pathways and supports ongoing clinical trials.

Methods

CLL Patient samples acquisition

Peripheral blood samples were obtained from patients diagnosed with CLL according to the IWCLL-NCI 2008 criteria(26) in the Hematology department at the University of Southampton (n=53) and Leukemia Department at MD Anderson Cancer Center (n=24). Patient consent was obtained in accordance with the Declaration of Helsinki on protocols that were reviewed and approved by institutional review boards at both centers. Peripheral blood mononuclear cells were isolated via density gradient centrifugation over Ficoll-Paque (GE Healthcare, Waukesha, WI, USA). Samples were used fresh or were placed into fetal bovine serum (FBS) (BD Biosciences, San Diego, CA, USA) plus 10% dimethylsulfoxide (Sigma- Aldrich, St Louis, MO, USA) for viable frozen storage in liquid nitrogen as previously described(27).

Reagents

Tissue culture materials were from ThermoFisherScientific (Paisley, UK). Idelalisib, ABT-199, ibrutinib and SYK inhibitors fostamatinib and P505-15 were from Selleckchem (Stratech Scientific Ltd, UK). IL-4 and CD40L were from R&D Systems (Abingdon, UK). Cerdulatinib was provided under MTA by Portola Pharmaceuticals, USA.

Phosflow

Antibodies obtained from BD Biosciences (San Jose, CA) include mouse anti-human CD19 peridinin-chlorophyll protein, CD5 alexafluor 700, CD20 phycoerythrin cyanine 7, CD14 allophycocyanin, CD3 phycoerythrin cyanine fluorescent 594, STAT6 tyrosine 641 phycoerythrin, and pAKT^{S473} phycoerythrin cyanine fluorescent 594. SYK$^{Y525/526}$ phycoerythrin and ERK^{Y204} allophycocyanin were obtained from Cell Signaling Technologies (Danvers, MA). Goat anti-human IgD (Bethyl Laboratories, Montgomery, TX), donkey anti-human IgM (Jackson Immunoresearch, (West Grove, PA) and recombinant human IL-4 (R&D Systems, Minneapolis, MN) were used for whole blood stimulations. Phosphate buffered saline (PBS), bovine serum albumin (BSA), and methanol were obtained from Sigma-Aldrich (St. Louis, MO). FACS/Lyse solution was obtained from BD Biosciences.

CLL cell culture, anti-IgM/D stimulation and protein extraction

Primary human CLL cells were cultured and protein extracted as previously described(27). Soluble anti-IgM or IgD F(ab')$_2$ was used at the indicated concentrations and bead bound immobilised anti-IgM F(ab')$_2$ was used at a 2:1 ratio, bead:CLL cell as previously described(27).

Flow Cytometry and calcium flux analysis

Cells were labelled with antibodies conjugated to various fluorochromes. All mean fluorescence intensity (MFI) were measured relative to an isotype control. Detection of intracellular calcium was assessed as previously described(7). 1μM Ionomycin (Sigma) was added as a positive control and all analysis was performed using Flowjo v10 software.

Immunoblotting

Proteins were separated on 10% or 12% polyacrylamide gels (ThermoFisherScientific, Paisley, Scotland), transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) and probed with anti-HSC70 (Santa Cruz, CA) as loading control. All antibodies were from Cell Signaling Technology (Hitchin, UK). Proteins were detected following incubation with HRP-linked secondary antibodies (Dako, UK), enhanced chemiluminescence reagents (Thermo Scientific, Rockford, IL) and visualised using the ChemiDoc-It imaging system (UVP). Band intensities were quantified using ImageJ and normalised to HSC70.

Viability assay

CLL cells were treated as indicated for 24, 48 or 72 hours and viability assessed by annexin V-FITC or annexin V-APC and propidium iodide (PI) negativity or PI/DiOC$_6$ positivity using flow cytometry.

CCL3, CCL4 and CXCL13 enzyme-linked immunosorbent assays

To study the effects of cerdulatinib on BCR- or Nurse like cell (NLC)-dependent secretion of CCL3, CCL4 and CXCL13, CLL cells were treated with 10 μg/ml anti-IgM, bead immobilised anti-IgM or by NLC-co-culture for 24h. Followed by the quantification of the respective chemokines in the supernatant via enzyme-linked immunosorbent assays in accordance with the supplier's instructions (R&D, Abingdon UK and Minneapolis, MN). NLC co-culture were performed as previously described[28].

RT-PCR

Total RNA was isolated with the RNeasy mini kit (Qiagen, UK) as described by the manufacturer. Total RNA was reverse-transcribed with M-MLV enzyme (Promega, UK). Real time PCR was performed on an ABI-7500 (Applied Biosystems, UK) using Mcl-1 and Bcl-X$_L$ taqman probes (ThermoFisher, UK). Each sample was analysed in duplicate with *B2M* as a house-keeping control. The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. Each sample was normalized to its non-treated matched sample.

Statistical analysis

The normal distribution of the samples was tested by D'Agostino-Pearson test. Statistical differences between groups were evaluated by paired or unpaired students T test when samples were normally distributed or by the Mann-Whitney U test when samples were not. Statistical analysis was performed using GraphPad Prism v6 (GraphPad Software Inc). Additive and synergistic drug interactions were assessed as previously described[7, 29, 30]. Basically observed survival was plotted against expected survival ((cerdulatinib×ABT-199)/ 100). XY line indicates observed survival equals expected survival. Samples beneath the line indicate synergistic interactions whereby observed survival is less than expected survival. Samples above the line indicate additive interactions whereby observed survival is less than expected survival but greater than survival for the most active drug alone. Antagonistic interactions whereby observed survival is less than the most active single drug alone were not observed in this study for any patient.

Results

Cerdulatinib inhibits BCR-induced signalling

Here we demonstrated by immunoblotting that CLL cells treated with soluble or bead immobilised (BI) anti-IgM (Figure 1A-B) or anti-IgD (Figure 1C-D) induced phosphorylation (p) of pAKT^{S473}, pS6K^{T389}, pS6 ribosomal subunit$^{S235/236}$, pERK$^{T202/Y204}$ and pAKT^{T308} (BI anti-Ig only). We demonstrate for the first time that these BCR-induced signals were inhibited by cerdulatinib in a dose dependent manner and most strongly between 0.3-1μM, with small but variable sensitivities to the drug between patient samples

(Supplementary Figures 1-4). These results are consistent and comparable to idelalisib and ibrutinib used here as controls to inhibit BCR signalling (Figure 1A-B, 1C-D). To confirm our findings were specific for B cells from CLL PBMCs, we performed flow cytometry for pSYK$^{Y525/526}$, pERK^{Y204} and pAKT^{S473} and calcium flux analysis in CD19+ samples. Cerdulatinib inhibited anti-IgM or anti-IgD-induced signalling of pSYK$^{Y525/526}$, pERK^{Y204} and pAKT^{S473} by flow cytometry at drug concentrations equivalent to that shown by immunoblotting (Figure 1E, Supplementary Figure 5A) and strongly inhibited BCR-induced calcium flux at 1μM (Figure 1F-G, Supplementary Figure 5B-C). Together these data confirm inhibition of BCR signalling in vitro by cerdulatinib at concentrations achievable in patients.

Cerdulatinib inhibits chemokine secretion in response to BCR ligation and NLC co-culture

CLL cells are known to secrete chemokines such as CCL3 and CCL4 in order to recruit T cells and monocytes into the lymph node(31). CLL cells treated with soluble anti-IgM (Figure 2A), BI anti-IgM (Figure 2B-C) or in co-culture with NLC (Figure 2D) secreted CCL3 and CCL4 at similar concentrations to that shown previously(11, 28). However at concentrations achievable in patients, cerdulatinib markedly reduced chemokine production by CLL cells with all stimuli, indicating that cerdulatinib may affect T-cell/monocyte recruitment by CLL cells into the lymph node. In addition we demonstrated that CLL cells co-cultured with NLC increased secretion of the chemokine CXCL13, but this was significantly inhibited by cerdulatinib compared to the vehicle control (Figure 2D). However, compared to basal expression of CXCL13 by CLL cells cultured alone, chemokine levels remained elevated even in the presence of cerdulatinib and was in contrast to NLC-induced CCL3 and CCL4 production, where their expression was reduced below basal levels following drug treatment.

IL-4 mediated signalling and increased IgM expression are inhibited by cerdulatinib

Next we demonstrated that ibrutinib, idelalisib (Figure 3A-B), and fostamatinib and P505-15 (PRT062607) (Figure 3C) were unable to inhibit phosphorylation of STAT6 (pSTAT6) following treatment with IL-4 (10ng/ml). In contrast, treatment with cerdulatinib abrogated IL-4-induced pSTAT6 expression in a concentration dependent manner, equivalent to that demonstrated with the specific JAK3 inhibitor tofacitinib, with ~40% and ~90% of the IL-4 signal inhibited by 0.3μM and 1μM of the drug respectively by immunoblotting, shown in a representative sample (Figure 3A) and summarised (n=8) (Figure 3B). Cerdulatinib inhibited equivalent pSTAT6 signalling in CLL B cells, T cells and monocytes (Supplementary Figure 6A-C) in response to IL-4 in whole blood assays by flow cytometry, with >58% and >80% pSTAT6 inhibition with 1μM and 3μM of the drug respectively. Next, we examined the effect of cerdulatinib upon IL-4-induced expression of surface markers CD69 and CD25. Compared to the vehicle control, cerdulatinib significantly inhibited IL-4-induced expression of CD69 and CD25 in whole blood assays in a dose dependent manner, with >50% and >80% inhibition in primary CLL samples at 1μM and 3μM respectively (Supplementary Figure 6B & 6D). Notably in vitro, higher concentrations of cerdulatinib were required to show full target inhibition in whole blood assays compared to experiments performed in 10% FCS, due to plasma protein binding of the drug.

Since we previously demonstrated that IL-4 augments sIgM and inhibits CXCR4 expression in CLL cells(7), and that treatment with the JAK1/3 inhibitor tofacitinib prevented these cytokine-mediated effects(7), we investigated whether cerdulatinib modulated expression of these receptors. Similarly, cerdulatinib prevented IL-4-mediated increases in sIgM (Figure 3D) and decreases in CXCR4 on CLL cells (Figure 3E), to the same extent as tofacitinib (CP) alone. In contrast, idelalisib and ibrutinib had no effect on IL-4 induced IgM expression (data not shown).

Cerdulatinib reduces CLL cell viability in a concentration, time and caspase dependent manner

Pharmacological inhibition of Syk by fostamatinib, P505-15 and entospletinib in CLL cells has previously been shown to be cytotoxic in vitro(32–34). Therefore we examined the effect of cerdulatinib (0.003-3μM) on 24 different CLL cases (12 U-CLL and 12 M-CLL) at 24, 48 and 72 hours using an annexin V/PI cell viability assay by flow cytometry. Cerdulatinib reduced CLL cell viability (cells negative for PI and Annexin V) by 30-50%, in a concentration and time dependent manner (Figure 4A), increased expression of the catalytically active cleaved caspase 3 subunit and induced PARP cleavage (Figure 4B). Drug-induced cell death appeared to be caspase dependent since co-incubation with the caspase inhibitor ZVAD significantly reduced CLL cell apoptosis (Figure 4B-C). Since CLL cells have a highly heterogeneous clinical course we assessed the ability of cerdulatinib to induce apoptosis between samples with different prognostic markers at 48h (in line with other BCR-kinase inhibitors in vitro). Indeed, IGHV unmutated CLL samples with a greater BCR-signalling capacity and response to IL-4, or samples expressing higher levels of CD49d+ or ZAP70+, were more sensitive to drug-induced killing (Figure 4D-F), compared to samples with mutated-IGHV genes, or which were CD49d or ZAP-70 negative. Interestingly, cerdulatinib also induced greater levels of cell death in samples expressing higher levels of sIgM (MFI >50) (Figure 4G). Therefore cerdulatinib may achieve superior responses in samples with a greater signalling capacity, since sIgM expression correlates with the ability of the CLL cell to flux calcium(35).

Cerdulatinib reduces cell viability in the presence of microenvironmental support

BCR signalling following anti-IgM engagement(36, 37), treatment with CD40L(31) and IL-4(16, 31) or co-culture with NLC(31) are known to protect CLL cells from basal and therapy induced killing and mimic pro-survival signals within the lymph node. Therefore, we evaluated CLL samples treated with soluble or BI anti-IgM (Figure 5A-B, Supplementary Figures 7A), co-cultured with NLC (Figure 5C) or with a combination of CD40L (300ng/ml) and IL-4 (10ng/ml) (Supplementary Figure 7B-C) prior to treatment with cerdulatinib. Using propidium iodide (PI)/DiOC$_6$ (soluble anti-IgM) or annexin V/PI and PARP cleavage (BI anti-IgM) viability assays, we demonstrated that treatment with both soluble and BI anti-IgM increased CLL viability at 24 and 48h (Figure 5A-B, Supplementary Figure 7A). However treatment with cerdulatinib significantly impaired this BCR-induced survival and promoted further reductions in CLL viability compared to unstimulated CLL cells. In comparison to the untreated controls, treatment with cerdulatinib also overcame NLC-mediated protection and induced greater levels of CLL cell apoptosis in a time dependent manner (24-72h) (Figure 5C). Subsequently we treated CLL samples with

IL-4/CD40L to study the effect of cerdulatinib in a more controlled setting and to replicate signals produced by T-cells. We identified that IL-4/CD40L promoted CLL survival in line with previous observations(38), in contrast treatment with cerdulatinib (1-3μM) reversed the protection conferred by these growth factors (Supplementary Figure 7B-C). Overall these data suggest cerdulatinib can induce CLL cell death in vitro irrespective of key pro-survival signals.

In line with previously published data(32, 36, 38), BI anti-IgM and IL-4/CD40L treatment induced expression of anti-apoptotic proteins MCL-1 and BCL-X_L, shown by immunoblotting in a representative sample (Figure 5D-E) and summarised (n=8) (Supplementary Figure 8A-G).

Simultaneous inhibition of Syk and JAK by cerdulatinib (1 and 3μM) significantly decreased BI anti-IgM and IL-4/CD40L-induced MCL-1 and BCL-X_L protein expression, but had no discernible effect on Bcl-2. At the RNA level, only BCL-X_L expression was induced following IL-4/CD40L treatment, although to a lesser extent than that at the protein level, similar to previous published findings with Mcl-1 (36) (Supplementary Figure 9A-D). Treatment with cerdulatinib showed no reproducible inhibition of basal MCL-1 or BCL-X_L protein or RNA expression (Supplementary Figure 8 & 9), however in 2/7 samples where basal MCL-1 and BCL-X_L protein expression was higher (Patient 276D and 674C), cerdulatinib treatment reduced their expression (Figure 5D).

Cerdulatinib and venetoclax synergise to induces substantial apoptosis in the presence of IL-4/CD40L

It has recently been proposed that the BCR-kinase inhibitor ibrutinib may be beneficially combined with the BCL-2 inhibitor venetoclax (ABT-199) in CLL(39), and resistance to venetoclax in non-Hodgkin lymphoma cell lines can be overcome by simultaneous treatment with idelalisib in vitro(40). Since we have shown that cerdulatinib could inhibit MCL-1 and BCL-X_L expression induced by IL-4/CD40L and anti-IgM ligation, but not BCL-2, we investigated whether cerdulatinib would synergise with venetoclax in vitro to augment CLL cell killing. CLL cells were treated with IL-4/CD40L for 6hr and then incubated with either venetoclax or cerdulatinib alone or in combination for a further 24h. Venetoclax significantly reduced CLL cell viability in the absence of IL-4/CD40L compared with the vehicle control. However treatment with IL-4/CD40L substantially protected CLL cells against venetoclax induced apoptosis (Figure 6A). In contrast, CLL cells treated with IL-4/CD40L and a combination of venetoclax and cerdulatinib, reduced CLL cell viability to a greater extent than with either drug alone (Figure 6A-B). Since cerdulatinib (1μM) alone did not reproducibly induce cell death >50%, we were unable to use conventional combination indices (CI) analysis to evaluate synergy between the drugs. Consequently we used the fractional 2-drug analysis method previously described for CLL(29, 30, 38) to evaluate the synergistic interaction between cerdulatinib and venetoclax (Figure 6C). Values above the diagonal line represent additive interaction whilst those under the line are synergistic. In the presence of IL-4/CD40L, a synergistic relationship was observed with cerdulatinib (1μM) in combination with either 10nM or 100nM venetoclax in the majority of samples (n=8/9)

(Figure 6C), indicating that cerdulatinib in combination with BCL-2 inhibitors may be a useful strategy for targeting cells within the protective lymph node niche.

Discussion

BCR-mediated signalling is crucial for the pathogenesis of CLL, promoting survival and reducing the effectiveness of therapy(1, 32, 36, 37). BCR-kinase inhibitors ibrutinib and idelalisib have transformed the treatment landscape for CLL patients, with profound clinical responses(3, 9, 41), however there is limited clinical follow-up (idelalisib ~45 months(9) and ibrutinib ~42 months(3)). Therefore, whether patients will still tolerate these BCR-kinase inhibitors when they have been continually treated for longer periods of time, or whether we will see greater incidence of Richters transformation, or a larger proportion of patients developing drug resistance, remains to be seen. Indeed, patients who discontinued ibrutinib in one study had an extremely poor median survival of only 3.1 months(42), although these patients were heavily pre-treated and may not be representative of outcomes in patients after ibrutinib as an initial therapy. Novel therapies or treatment strategies are therefore required once resistance develops. Importantly, the Syk inhibitor entospletinib was shown in vitro to inhibit BCR-signalling and induce apoptosis irrespective of the samples sensitivity to ibrutinib(13). Syk is the apical kinase downstream of the BCR and its inhibition prevents anti-IgM downstream signalling pathways, as indicated by the Syk inhibitors fostamatinib(11, 32) and P505-15(33). To examine the extremes of the BCR-signalling response, we treated CLL cells with soluble and bead immobilised (BI) anti-IgM or –IgD. Soluble anti-IgM signalling is curtailed within 0.5-1 hour, following endocytosis of its receptor, in contrast BI anti-IgM was sustained up to 8h, possibly due to its inability to be endocytosed(43). Consequently, BI anti-IgM induces a stronger and prolonged signal compared to soluble antibody, theoretically replicating more closely antigen presentation by a cell(43). The Syk/JAK inhibitor cerdulatinib was able to overcome both soluble and immobilised anti-IgM and for the first time anti-IgD-induced signalling.

Recent investigation by our group demonstrated that IL-4 can protect against ibrutinib and idelalisib induced apoptosis(7), in line with previous publications(44, 45), and reduce the effectiveness of idelalisib and ibrutinib to inhibit BCR-signalling(7). Importantly, these effects could be reversed following JAK1/3 inhibition by tofacitinib(7). Intriguingly the JAK inhibitor ruxolitinib (n=13), decreased lymphadenopathy and increased lymphocytosis in CLL patients(22), suggesting a role for JAK-inhibition in regulating egress or preventing influx of tumor cell into the lymph node. These effects appeared transient and for that reason the authors hypothesized that a combination of ruxolitinib with BCR-kinase inhibitors may achieve superior results(22). Therefore preventing cytokine- and BCR-mediated signalling by JAK and Syk inhibition respectively may produce therapeutically greater responses in patients compared to suppression of either pathway alone. Indeed, combining established BCR-kinase inhibitors with JAK inhibitors or utilising antibodies which block the cytokine receptor may be a useful strategy to achieve this goal. Moreover, these therapeutic strategies are not limited to CLL and could be transferable to follicular lymphoma (FL) where there is greater evidence for IL-4 signalling in its biology. This may be particularly useful in cases of FL, where the recently identified STAT6 mutation at amino acid residue 419 conferred heightened IL-4-induced activation of target genes(46).

The phase I dose escalation study with cerdulatinib in patients with relapsed/refractory B cell malignancies has completed enrolment, with no dose limiting toxicities observed to date(23). A phase II dose (35mg twice daily) has been established which achieved a steady-state Cmin of 1μM, and is demonstrating effective anti-tumor activity. Lymphocytosis was also evident, complementing data shown with other BCR-kinase inhibitors. In this study cerdulatinib was not studied in mouse models due to substantial differences in the pharmacokinetics between mice and humans, where the half-life of the drug was 30min and 14h respectively, making comparisons between mice and humans treated with cerdulatinib difficult. Here we demonstrated that in contrast to ibrutinib, idelalisib, fostamatinib or P505-15, cerdulatinib inhibited IL-4-induced signalling in CLL samples using multiple readouts. This is in contrast to previous reports which identified ibrutinib as an inhibitor of JAK3 in a kinase assay(47) and therefore would be predicted to inhibit IL-4-induced pSTAT6 expression. Idelalisib and ibrutinib could not prevent IL-4 induced IgM expression which has recently been described to enhance BCR signaling in CLL (7). In contrast, treatment with cerdulatinib prevented IL-4-induced sIgM expression and IL-4-suppression of CXCR4, which was consistent with the JAK1/3 inhibitor tofacitinib(7). This highlights a role for cerdulatinib in the suppression of anti-IgM and CXCL12 pro-survival signalling within the CLL lymph node and in overcoming IL-4-mediated resistance to ibrutinib and idelalisib(7).

In whole blood assays we observed a reduced inhibition of anti-IgM and IL-4 signalling, this is because plasma protein binding for cerdulatinib is ˜78%, so higher amounts of drug are required to show full target inhibition in whole blood assays compared to experiments performed in 10% FCS. Moreover, the inhibition of BCR and IL-4 mediated signalling post oral cerdulatinib administration in whole blood from patient samples has been published in poster format (Flinn et al, ASCO, 2015) (23). Whereby 40mg cerdulatinib inhibited BCR-induced phosphorylated of pERK and pSYK and IL-4-induced pSTAT6 between 80-90% in circulating lymphocytes as a consequence of repeated dosing of the patient.

Cerdulatinib induced CLL cell apoptosis in a time, concentration and caspase dependent manner, consistent with previously published data using SYK(32–34), BTK and PI3Kδ(44, 45) inhibitors. Inhibition of JAK1/3 by tofacitinib alone did not induce apoptosis (data not shown), in agreement with previous studies(16), and may suggest that CLL cell apoptosis by cerdulatinib is largely dependent on its inhibition of Syk. Importantly, Coffey et al demonstrated cerdulatinib did not induce apoptosis of normal B cells(24) and patients tolerate the drug extremely well(23).

High plasma concentrations of CCL3 and CCL4 are associated with an inferior clinical outcome(48) and their expression is induced following treatment with anti-IgM or in co-culture with NLC(28). Cerdulatinib inhibited anti-IgM and NLC-induced CCL3 and CCL4 secretion by CLL cells, in agreement with that shown for Syk inhibitors PRT318, P505-15(33) and R406(11). Consequently cerdulatinib may reduce T cell and monocyte recruitment by these chemokines into the lymph node where these cells are known to play a role in promoting tumor survival, and warrants further investigation in patients.

Cerdulatinib overcame protection conferred by anti-IgM signalling, co-culture with NLC or treatment with IL-4 and CD40L. Treatment with anti-IgM or IL-4 and CD40L induced expression of MCL-1 and BCL-X_L. Longo et al suggested that MCL-1 expression was pivotal for CLL cell survival since siRNA knockdown of the gene resulted in rapid tumor apoptosis, in contrast BCL-X_L siRNA knockdown had no effect on apoptosis(36). However, our previous studies demonstrated simultaneous inhibition of MCL-1 and BCL-X_L was required in vitro, following IL-4/CD40L treatment, to obtained optimal apoptosis(38). Here we demonstrated cerdulatinib significantly inhibits BI anti-IgM- and IL-4/CD40L-induced MCL-1 and BCL-X_L protein expression. Although MCL-1 protein levels were reduced to a lesser extent following IL-4/CD40L activation compared to anti-IgM treatment, this may simply reflect a reduced ability by cerdulatinib to completely inhibit CD40L-induced signalling.

Since cerdulatinib reduced MCL-1 and BCL-X_L expression but had no effect on BCL-2 expression, we hypothesized that cerdulatinib in combination with the BCL-2 inhibitor venetoclax may achieve greater cell death. Indeed, cerdulatinib in combination with venetoclax synergised to produce greater levels of CLL apoptosis than either drug alone following treatment with IL-4/CD40L. This is important because venetoclax has recently been approved for the treatment of CLL patients with 17p del(49) and rapidly clears CLL cells from the blood(50). However signals within the tumor microenvironment (IL-4/CD40L and anti-IgM) are known to protect CLL cells from venetoclax-induced killing by augmenting MCL-1 expression(37, 38, 51). These data indicate that cerdulatinib in combination with venetoclax may overcome these microenvironmental signals, providing a promising clinical strategy. In conclusion, this study demonstrates the advantages for dual Syk/JAK inhibition in CLL and supports ongoing clinical trials using cerdulatinib for haematological malignancies alone and in combination with venetoclax.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Statement of translational relevance

B cell receptor (BCR) kinase inhibitors are revolutionising the treatment of patients with chronic lymphocytic leukemia (CLL). However resistance to the BCR-kinase inhibitor ibrutinib, is already emerging through mutations in BTK and PLCγ2 or by other as yet unknown reasons. We recently demonstrated this resistance may be mediated by interleukin-4 (IL4), which can prevent inhibition of the BCR-signalling pathway by ibrutinib in CLL cells. Importantly treatment with a JAK3-inhibitor tofacitinib can reverse IL-4-induced effects. Herein we demonstrated that cerdulatinib, the first dual Syk/JAK inhibitor, induced apoptosis of CLL cells following inhibition of the BCR/IL-4 signalling pathways and overcame nurse-like cell or anti-IgM/CD40L+IL-4-mediated protection at concentrations achievable in patients. Finally combination studies with cerdulatinib and the Bcl-2-inhibitor venetoclax synergised to augment apoptosis. These results provide compelling evidence for the use of cerdulatinib as a single agent or in combination with Bcl-2 inhibitors to more effectively treat patients with CLL.
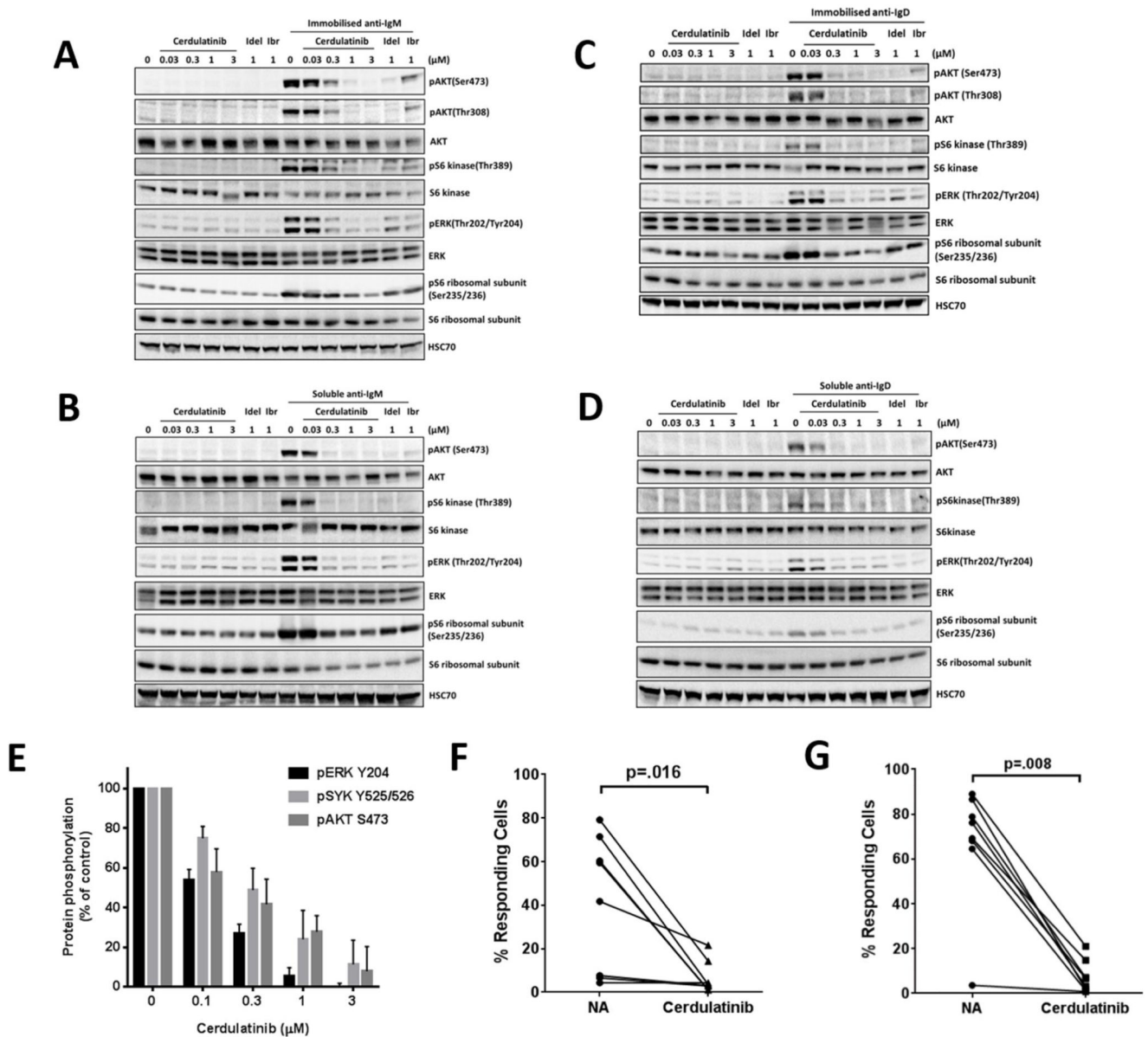
Figure 1. Regulation of Anti-IgM and Anti-IgD induced signaling by cerdulatinib.
CLL cells were treated with cerdulatinib, idelalisib (Idel) or ibrutinib (Ibr) at the stated
concentrations for 1h and stimulated with; **(A-D)** bead immobilised (BI) **(A)** anti-IgM or **(C)**
anti-IgD for 1.5hr or **(B)** soluble anti-IgM or **(D)** anti-IgD for 15min or 5min respectively.
Levels of phosphorylated AKT (pAKT Ser473), ERK (pERK Thr202/Tyr204), S6kinase
(pS6K Thr389) and S6 ribosomal subunit (pS6 Ser235/236) were assessed by
immunoblotting. **(E)** CLL whole blood was treated in the presence or absence of increasing
concentrations of cerdulatinib prior to activation with soluble anti-IgM and anti-IgD.
Phosphorylated (p)ERK Y204, pSYK Y525/526 and pAKT S473 were assessed in CD19+
cells via phospho-specific flow cytometry. **(F-G)** CD19+ B cells from a CLL patient were
treated with cerdulatinib for 60 minutes and stimulated with **(F)** soluble anti-IgM or **(G)**

soluble anti-IgD and calcium flux assessed using flow cytometry as previously described. Bar graphs depict means ± SEM
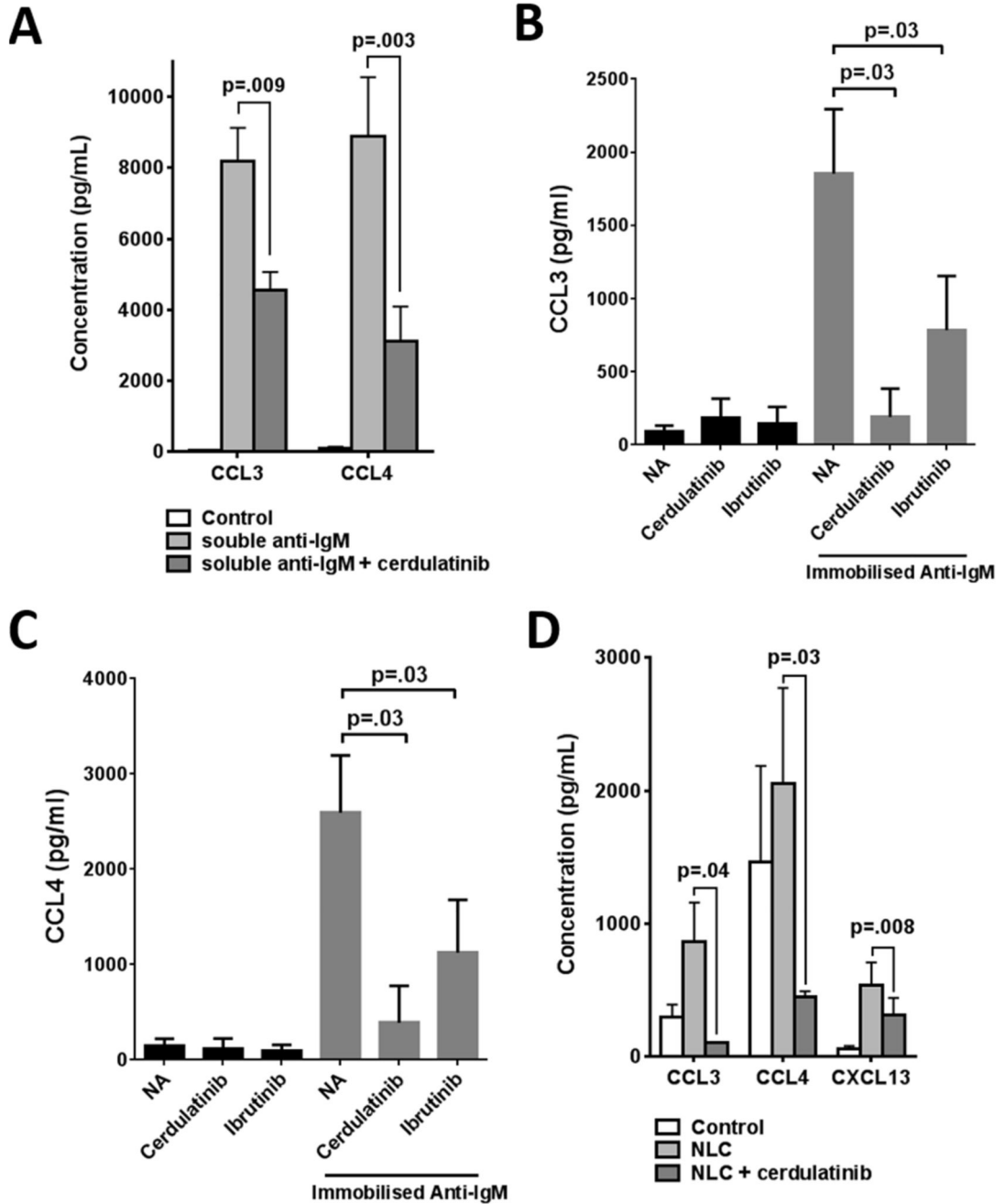
Figure 2. Regulation of chemokine expression by cerdulatinib
CLL cells were stimulated with **(A)** soluble (n=5) or **(B-C)** immobilised (n=8) anti-IgM in the presence or absence of cerdulatinib (**A,** 2μM, **B**, 1μM) or ibrutinib (1μM) for 24h. CCL3 and CCL4 levels were quantified in the supernatant by enzyme-linked immunosorbant assays (ELISA). **(D)** CLL cells in co-culture with NLC were treated with cerdulatinib (2μM) for 24h followed by the quantification of CCL3/4 and CXCL13 in the culture supernatants (n=5). Bar graphs depict means ± SEM.
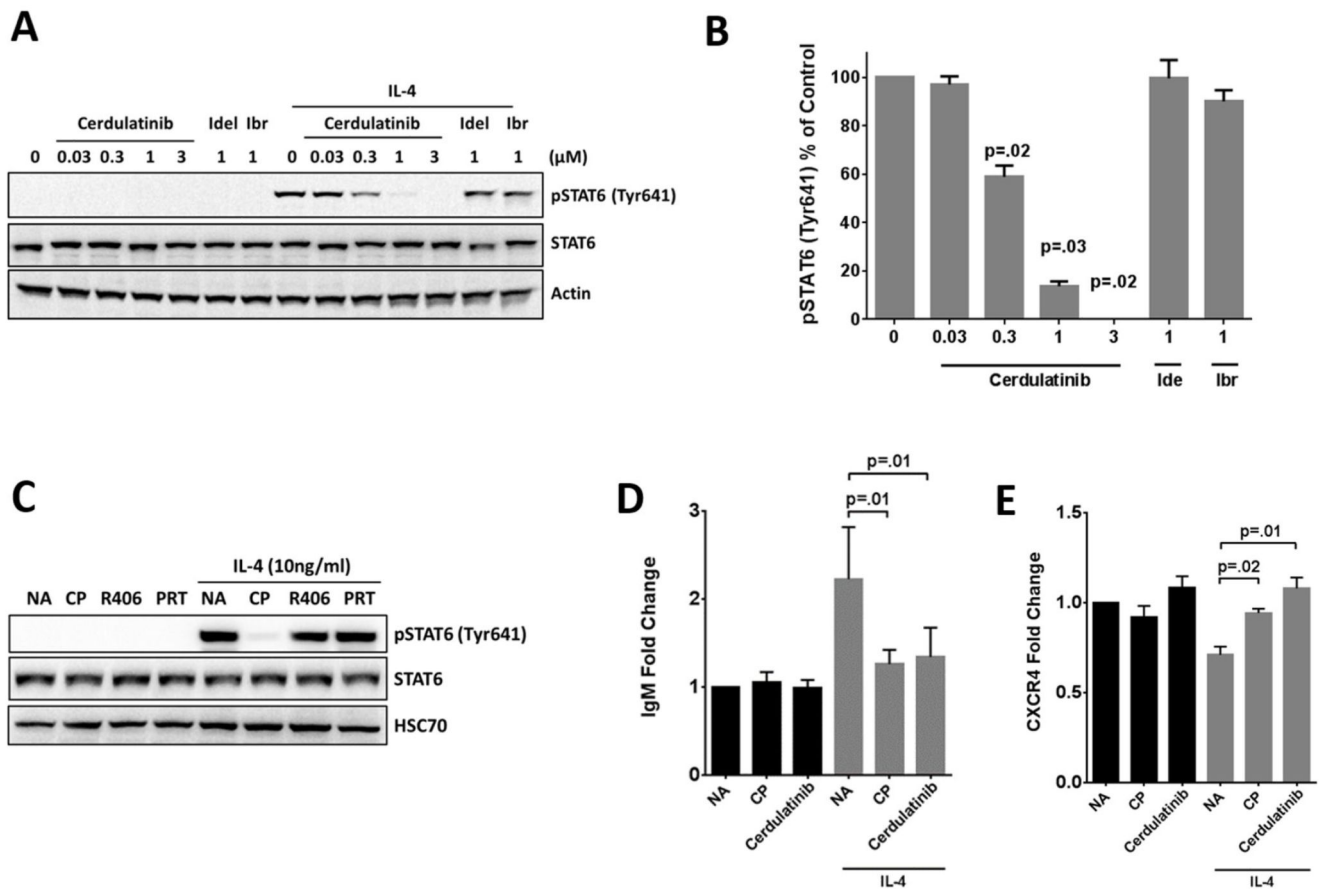
Figure 3. Regulation of STAT6 phosphorylation, and sIgM and CXCR4 expression by IL-4 and cerdulatinib

CLL cells were treated with cerdulatinib, idelalisib (Idel) or ibrutinib (Ibr) at the stated concentrations for 60mins then stimulated with IL-4 (10ng/ml) for a further 60mins. **(A)** Phosphorylated STAT6 (pSTAT6), STAT6 and actin were assessed using immunoblotting in a representative sample and **(B)** summarised (n=8). **(C)** CLL cells were treated with tofacitinib (JAK3 inhibitor) (CP), and SYK inhibitors fostamatinib (R406) and P505-15 (PRT) prior to IL-4 treatment and immunoblotting performed for pSTAT6, STAT6 and HSC70 in a representative sample. **(D,E)** CLL cells were incubated with cerdulatinib (1μM) or the JAK1/3 inhibitor tofacitinib (CP) (1μM) for 60mins then with IL-4 (10ng/ml) for a further 23hr. **(D)** Surface IgM and **(E)** surface CXCR4 were assessed using flow cytometry. Bar graphs depict means ± SEM
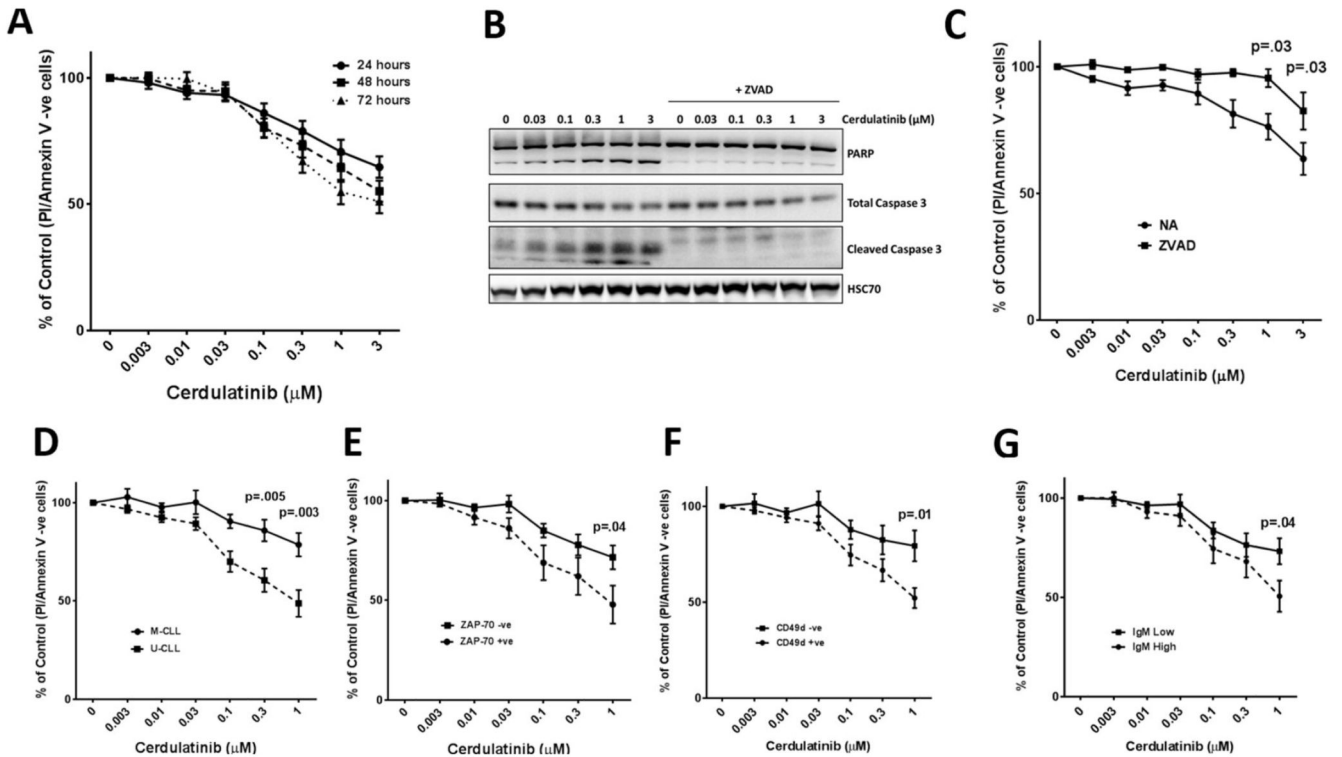
Figure 4. Cerdulatinib induces apoptosis in a concentration, time and caspase dependent manner
(A) CLL cells from 24 different patients were treated with cerdulatinib as indicated for 24, 48 and 72hrs and apoptosis assessed using PI/Annexin V by flow cytometry. Data is % of control (PI/Annexin V negative cells). **(B-C)** CLL cells were treated with cerdulatinib at the indicated concentrations in the presence or absence of 100μM pan caspase inhibitor ZVAD for 24hr and assessed for **(B)** PARP and caspase 3 cleavage by immunoblotting or **(C)** annexin V/PI analysis by flow cytometry. **(D-G)** Samples were characterised from analysis in **(A)** by; **(D)** IGHV mutational status, **(E)** ZAP-70, **(F)** CD49d and **(G)** IgM expression. Bar graphs depict means ± SEM
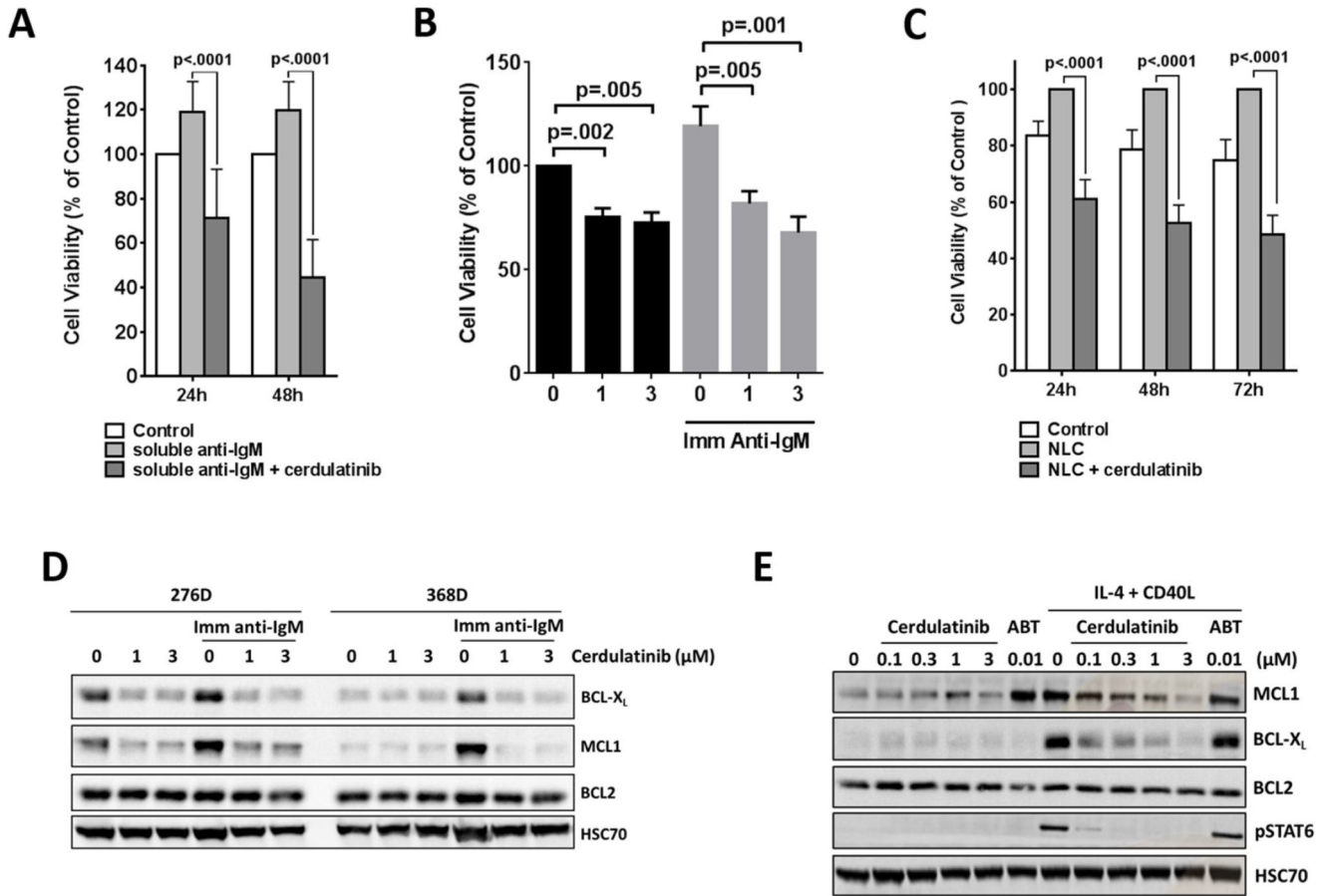
Figure 5. Cerdulatinib overcomes the protection of CLL cells by conditions which mimic the tumour microenvironment

(A) CLL cells were treated with cerdulatinib (2μM) for 24-48h in the presence or absence of soluble anti-IgM (10μg/ml). Cell viability was determined by propidium iodide and DiOC6 double staining by flow cytometry (n=12) (B) CLL cells were treated with bead immobilised (imm) anti-IgM in the presence or absence of 1 & 3μM cerdulatinib. Viability was assessed by PI/Annexin V staining using flow cytometry (n=8). (C) CLL cells were treated and assessed as indicated in (A) but in the presence or absence of nurse-like cells (NLC) (n=6). (D-E) CLL cells were treated with cerdulatinib or venetoclax in the presence or absence of (D) immobilised anti-IgM or (E) IL-4 (10ng/ml) and CD40L (300ng/ml) for 24h and protein expression of Bcl-X$_L$, Mcl-1, pSTAT6, HSC70 and Bcl-2 expression evaluated by immunoblotting. Bar graphs depict means ± SEM
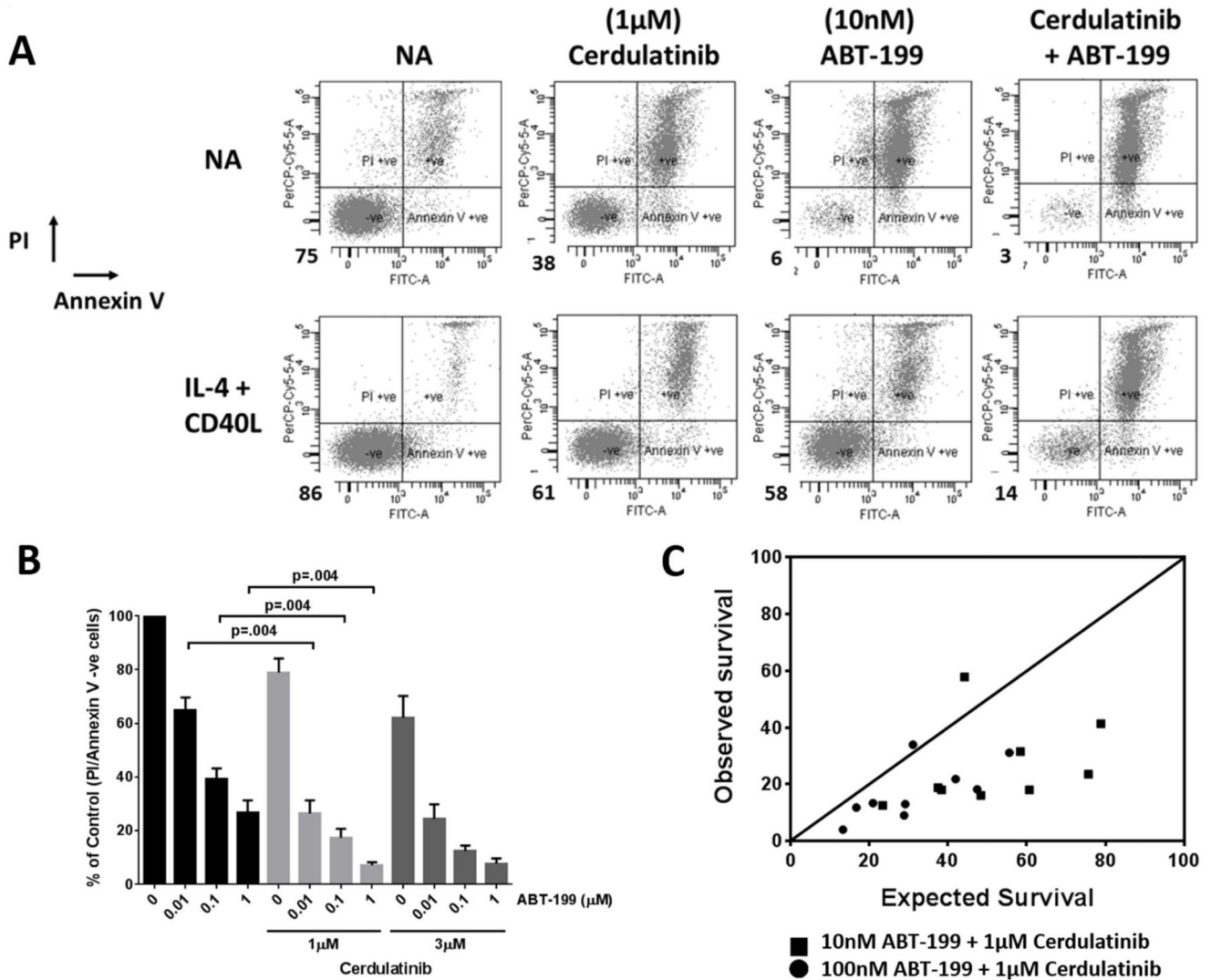
Figure 6. Cerdulatinib synergises with Venetoclax to kill CLL cells
CLL cells were incubated with IL-4 (10ng/ml) and CD40L (300ng/ml) for 6h then treated with cerdulatinib and/or venetoclax (ABT-199) as indicated for a further 24hr. Viability was assessed using **(A)** flow cytometry (PI/Annexin V negative cells) and a representative flow cytometry plot is shown and **(B)** summarised (n=9) showing % of control (PI/Annexin V negative cells). **(C)** Synergistic interactions between cerdulatinib and venetoclax were evaluated as indicated and described in the Methods. XY line indicates observed survival=expected survival. Points beneath the line indicate synergistic interactions and points above the line indicate additive interactions.