Clinical Cancer Research

The Dual Syk/JAK Inhibitor Cerdulatinib Antagonizes 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 revolutionized the treatment of chronic lymphocytic leukemia (CLL). However, these agents are not curative, and resistance is already emerging in a proportion of patients. IL4, expressed in CLL lymph nodes, can augment BCR signaling and reduce the effectiveness of BCR kinase inhibitors. Therefore, simultaneous targeting of the IL4- and BCR signaling pathways by cerdulatinib, a novel dual Syk/JAK inhibitor currently in clinical trials (NCT01994382), may improve treatment responses in patients.

Experimental Design: PBMCs from patients with CLL were treated *in vitro* with cerdulatinib alone or in combination with venetoclax. Cell death, chemokine, and cell signaling assay were performed and analyzed by flow cytometry, immunoblotting, q-PCR, and ELISA as indicated.

Results: At concentrations achievable in patients, cerdulatinib inhibited BCR- and IL4-induced downstream signaling in CLL cells using multiple readouts 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 signaling capacity and response to IL4, or samples expressing higher levels of sIgM, $CD49d^+$, or $ZAP70^+$. Cerdulatinib overcame anti-IgM, IL4/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 IL4/CD40L, cerdulatinib synergized with venetoclax *in vitro* to induce greater apoptosis than either drug alone.

Conclusions: 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. *Clin Cancer Res; 23(9); 2313–24. ©2016 AACR.*

Introduction

The importance of B-cell receptor (BCR)–mediated signaling in the pathogenesis of chronic lymphocytic leukemia (CLL) has become even more apparent in recent years (1), and drugs that target kinases associated with this pathway are revolutionizing the treatment of this disease (2–4). Recently approved agents for relapsed/refractory CLL include ibrutinib [Bruton tyrosine kinase (BTK) inhibitor] and idelalisib (PI3Kδ inhibitor; refs. 5, 6). To date, these compounds have not proved curative, which may in part be due to protection of the tumor by the microenvironment (7). Importantly, a proportion of patients is 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 nonreceptor 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 signaling *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

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Translational Relevance

B-cell receptor (BCR) kinase inhibitors are revolutionizing 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 IL4, which can prevent inhibition of the BCR signaling pathway by ibrutinib in CLL cells. Importantly, treatment with a JAK3 inhibitor tofacitinib can reverse IL4-induced effects. Herein, we demonstrated that cerdulatinib, the first dual Syk/JAK inhibitor, induced apoptosis of CLL cells following inhibition of the BCR/IL4 signaling pathways and overcame nurse-like cell or anti-IgM/CD40L + IL4-mediated protection at concentrations achievable in patients. Finally, combination studies with cerdulatinib and the Bcl-2 inhibitor venetoclax synergized 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.

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 *IL4* gene signature, which was enriched in lymph node tissue compared with matched blood and bone marrow (7). IL4 signals in lymphocytes predominantly through the type I IL4 receptor (IL4R) via Janus protein tyrosine kinases JAK1 and JAK3, resulting in phosphorylation of STAT6 (pSTAT6; ref. 15). IL4 signaling is known to promote tumor survival and protect against therapy-induced cell death (7, 16) and is produced by T cells from patients with CLL (17). Patients with progressive CLL have been reported to have greater numbers of T cells that spontaneously produce IL4 (18), and their tumor cells express significantly higher IL4R levels compared with normal B cells, which correlates with increased signaling to IL4 (19, 20). We recently showed that IL4 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 signaling was significantly impaired following IL4 treatment (7).

The pharmacologic targeting of JAK/STAT signaling 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 patients with CLL 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 I/IIA 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 IL4 signaling *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 cell lines *in vitro* and inhibited BCR-mediated signaling (25). Cerdulatinib treatment with once-daily administration at 45 mg dosing achieved a C_{max} of approximately 2 μmol/L (23). Here, we show *in vitro* that primary CLL samples are sensitive to cerdulatinib, at concentrations achievable in patients. Cerdulatinib inhibited IL4 and BCR-mediated signaling, which resulted in apoptosis, particularly in samples with markers of progressive disease, and synergized 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 prosurvival signaling pathways and supports ongoing clinical trials.

Materials and 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 (Southampton, United Knigdom; $n = 53$) and Leukemia Department at MD Anderson Cancer Center (Houston, TX; $n = 24$). 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). Samples were used fresh or were placed into FBS (BD Biosciences) plus 10% DMSO (Sigma- Aldrich) for viable frozen storage in liquid nitrogen as described previously (27).

Reagents

Tissue culture materials were from Thermo Fisher Scientific. Idelalisib, ABT-199, ibrutinib, and SYK inhibitors fostamatinib and P505-15 were from Selleckchem (Stratech Scientific Ltd.). IL4 and CD40L were from R&D Systems. Cerdulatinib was provided under MTA by Portola Pharmaceuticals.

Phosflow

Antibodies obtained from BD Biosciences include mouse anti-human CD19 peridinin–chlorophyll protein, CD5 Alexa Fluor 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. Goat anti-human IgD (Bethyl Laboratories), donkey anti-human IgM (Jackson ImmunoResearch), and recombinant human IL4 (R&D Systems) were used for whole-blood stimulations. PBS, BSA, and methanol were obtained from Sigma-Aldrich. 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 described previously (27). Soluble anti-IgM or IgD $F(ab')_2$ was used at the indicated concentrations, and bead-bound immobilized anti-IgM $F(ab')_2$ was used at a 2:1 ratio, bead:CLL cell as described previously (27).

Flow cytometry and calcium flux analysis

Cells were labeled with antibodies conjugated to various fluorochromes. All mean fluorescence intensities (MFIs) were measured relative to an isotype control. Detection of intracellular calcium was assessed as described previously (7). Ionomycin (1 μmol/L; 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 (Thermo Fisher Scientific), transferred to nitrocellulose membranes (GE Healthcare), and probed with anti-HSC70 (Santa Cruz Biotechnology) as loading control. All antibodies were from Cell Signaling Technology. Proteins were detected following incubation with HRP-linked secondary antibodies (Dako), enhanced chemiluminescence reagents (Thermo Scientific), and visualized using the ChemiDoc-It imaging system (UVP). Band intensities were quantified using ImageJ and normalized 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 ELISA

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-immobilized (BI) anti-IgM or by NLC coculture for 24 hours, followed by the quantification of the respective chemokines in the supernatant via ELISA in accordance with the supplier's instructions (R&D Systems). NLC cocultures were performed as described previously (28).

RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Total RNA was reverse-transcribed with M-MLV enzyme (Promega). Real-time PCR was performed on an ABI-7500 (Applied Biosystems) using Mcl-1 and Bcl-X_L TaqMan probes (Thermo Fisher). Each sample was analyzed in duplicate with *B2M* as a housekeeping control. The relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. Each sample was normalized to its nontreated 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 Student *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 described previously (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 signaling

Here, we demonstrated by immunoblotting that CLL cells treated with soluble or BI anti-IgM (Fig. 1A and B) or anti-IgD (Fig. 1C and 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 to 1 μmol/L, with small but variable sensitivities to the drug between patient samples (Supplementary Figs. S1–S4). These results are consistent and comparable with idelalisib and ibrutinib used here as controls to inhibit BCR signaling (Fig. 1A–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 signaling of $pSYK^{Y525/526}$, $pERK^{Y204}$, and $pAKT^{S473}$ by flow cytometry at drug concentrations equivalent to that shown by immunoblotting (Fig. 1E and Supplementary Fig. S5A) and strongly inhibited BCR-induced calcium flux at 1 μmol/L (Fig. 1F and G and Supplementary Fig. S5B and S5C). Together, these data confirm inhibition of BCR signaling *in vitro* by cerdulatinib at concentrations achievable in patients.

Cerdulatinib inhibits chemokine secretion in response to BCR ligation and NLC coculture

CLL cells are known to secrete chemokines, such as CCL3 and CCL4, to recruit T cells and monocytes into the lymph node (31). CLL cells treated with soluble anti-IgM (Fig. 2A), BI anti-IgM (Fig. 2B and C), or in coculture with NLC (Fig. 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 cocultured with NLC increased secretion of the chemokine CXCL13, but this was significantly inhibited by cerdulatinib compared with the vehicle control (Fig. 2D). However, compared with basal expression of CXCL13 by CLL cells cultured alone, chemokine levels remained elevated even in the presence of cerdulatinib and were in contrast to NLC-induced CCL3 and CCL4 production, where their expression was reduced below basal levels following drug treatment.

IL4-mediated signaling and increased IgM expression are inhibited by cerdulatinib

Next, we demonstrated that ibrutinib, idelalisib (Fig. 3A and B), and fostamatinib and P505-15 (PRT062607; Fig. 3C) were unable to inhibit phosphorylation of STAT6 (pSTAT6) following treatment with IL4 (10 ng/mL). In contrast, treatment with cerdulatinib abrogated IL4-induced pSTAT6 expression in a concentration-dependent manner, equivalent to that demonstrated with the specific JAK3 inhibitor tofacitinib, with approximately 40% and 90% of the IL4 signal inhibited by 0.3 and 1 μmol/L of the drug, respectively, by immunoblotting, shown in a representative sample (Fig. 3A) and summarized ($n = 8$; Fig. 3B). Cerdulatinib

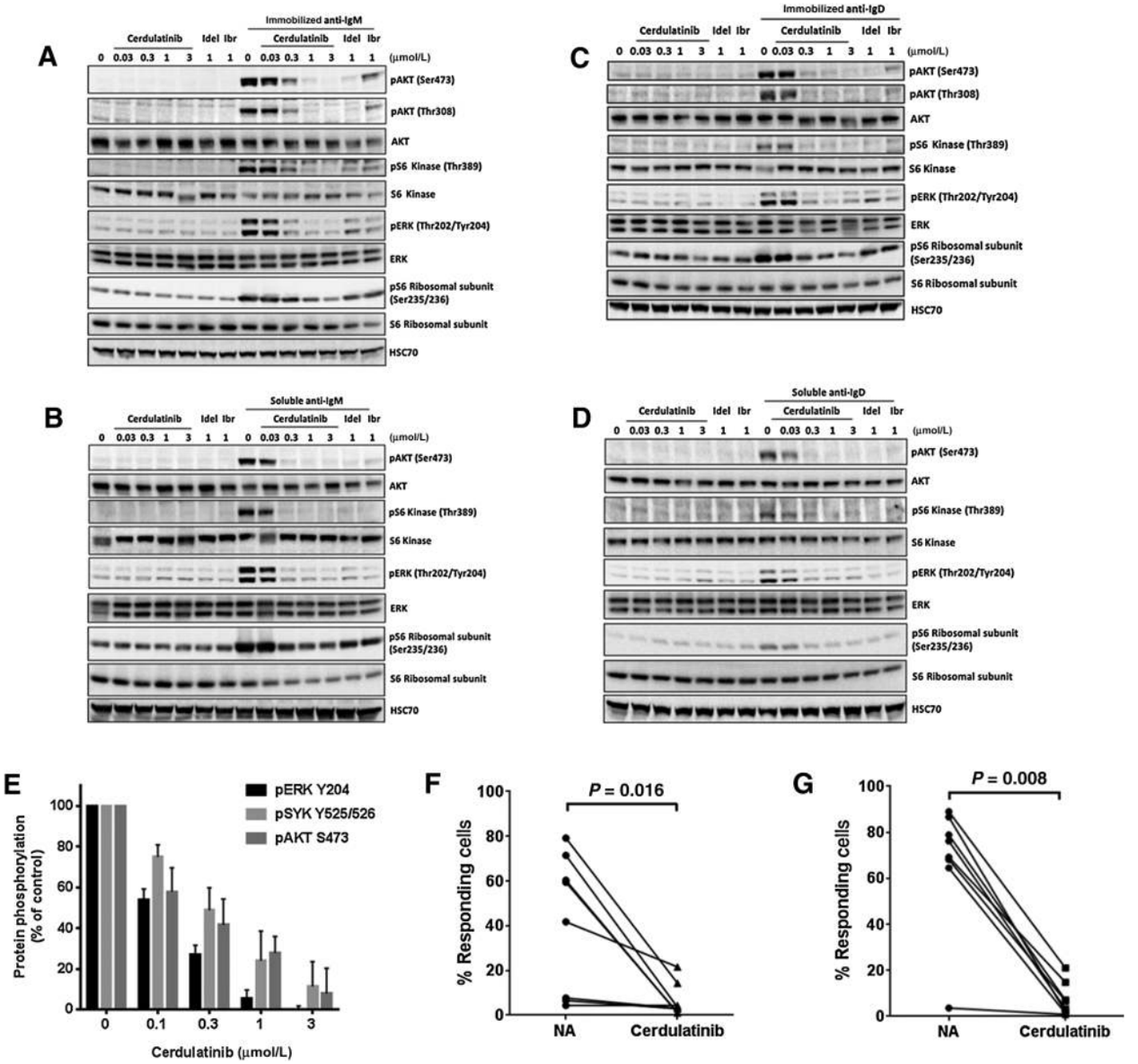

Figure 1.

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 1 hour and stimulated with BI anti-IgM (**A**) or anti-IgD (**C**) for 1.5 hours or soluble anti-IgM (**B**) or anti-IgD (**D**) for 15 or 5 minutes, 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** and **G,** $CD19^+$ B cells from a CLL patient were treated with cerdulatinib for 60 minutes and stimulated with soluble anti-IgM (**F**) or soluble anti-IgD (**G**), and calcium flux was assessed using flow cytometry as described previously. Bar graphs, means ± SEM.

inhibited equivalent pSTAT6 signaling in CLL B cells, T cells, and monocytes (Supplementary Fig. S6A–S6C) in response to IL4 in whole-blood assays by flow cytometry, with >58% and >80% pSTAT6 inhibition with 1 and 3 μmol/L of the drug, respectively. Next, we examined the effect of cerdulatinib upon IL4-induced expression of surface markers CD69 and CD25. Compared with the vehicle control, cerdulatinib significantly inhibited IL4-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 1 and 3 μmol/L, respectively (Supplementary Fig. S6B and S6D). Notably *in vitro*, higher concentrations of cerdulatinib were required to show full target inhibition in whole-blood assays compared with experiments performed in 10% FCS, due to plasma protein binding of the drug.

As we previously demonstrated that IL4 augments sIgM and inhibits CXCR4 expression in CLL cells (7), and that treatment

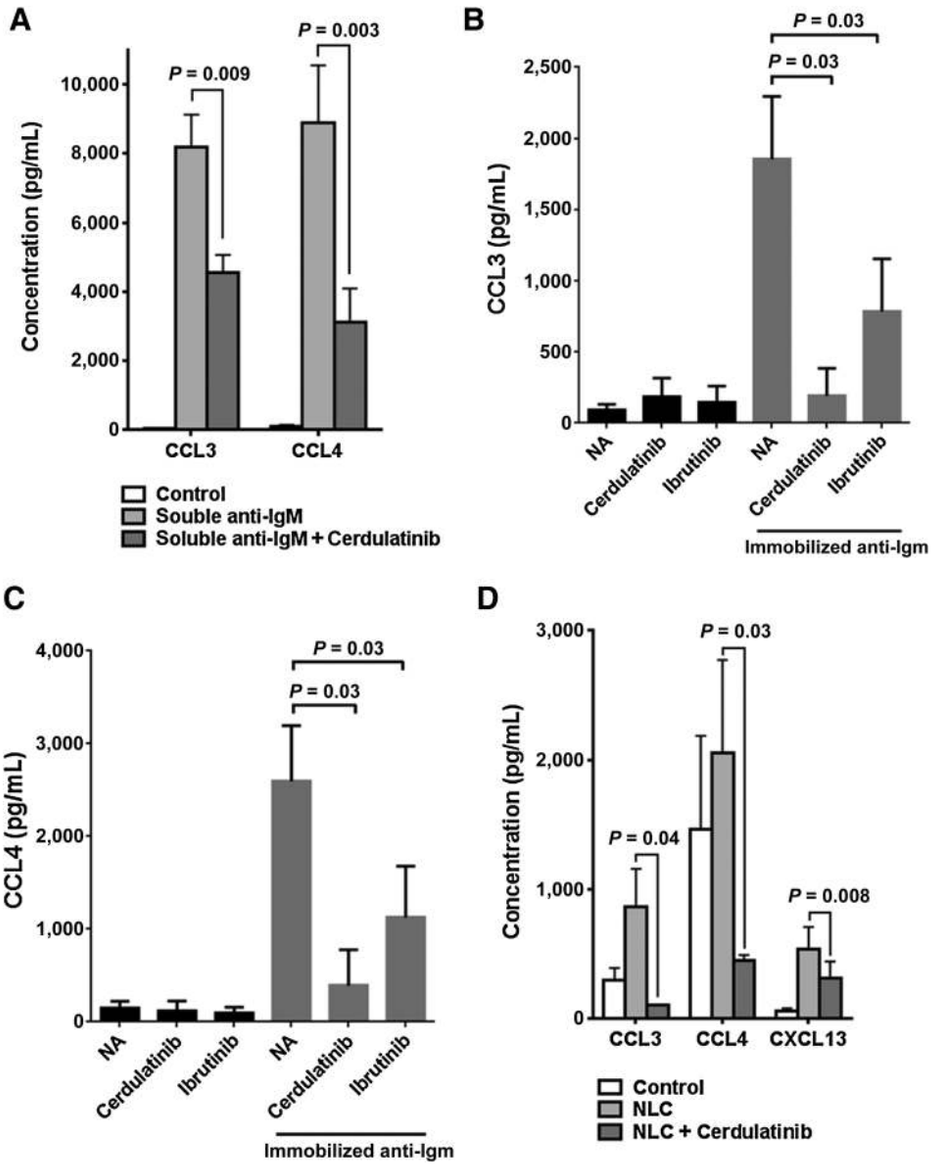

Figure 2.
Regulation of chemokine expression by cerdulatinib. CLL cells were stimulated with soluble (**A**; $n = 5$) or immobilized (**B** and **C**; $n = 8$) anti-IgM in the presence or absence of cerdulatinib (**A,** 2 μmol/L; B, 1 μmol/L) or ibrutinib (1 μmol/L) for 24 hours. CCL3 and CCL4 levels were quantified in the supernatant by ELISA. **D,** CLL cells in coculture with NLC were treated with cerdulatinib (2 μmol/L) for 24 hours, followed by the quantification of CCL3/4 and CXCL13 in the culture supernatants ($n = 5$). Bar graphs, means ± SEM.

with the JAK1/3 inhibitor tofacitinib prevented these cytokine-mediated effects (7), we investigated whether cerdulatinib modulated expression of these receptors. Similarly, cerdulatinib prevented IL4-mediated increases in sIgM (Fig. 3D) and decreases in CXCR4 on CLL cells (Fig. 3E), to the same extent as tofacitinib (CP-690550) alone. In contrast, idelalisib and ibrutinib had no effect on IL4-induced IgM expression (data not shown).

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

Pharmacologic 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 (0.003–3 μmol/L) 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% to 50%, in a concentration- and time-dependent manner (Fig. 4A), increased expression of the catalytically active cleaved caspase-3 subunit, and induced PARP cleavage (Fig. 4B). Drug-induced cell death appeared to be caspase dependent as coincubation with the caspase inhibitor ZVAD significantly reduced CLL cell apoptosis (Fig. 4B and C). As CLL cells have a highly heterogeneous clinical course, we assessed the ability of cerdulatinib to induce apoptosis between samples with different prognostic markers at 48 hours (in line with other BCR kinase inhibitors *in vitro*). Indeed, IGHV-unmutated CLL samples with a greater BCR signaling capacity and response to IL4, or samples expressing higher levels of $CD49d^+$ or $ZAP70^+$, were more sensitive to drug-induced killing (Fig. 4D–F), compared with 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; Fig. 4G). Therefore, cerdulatinib may achieve superior responses in samples with a greater signaling

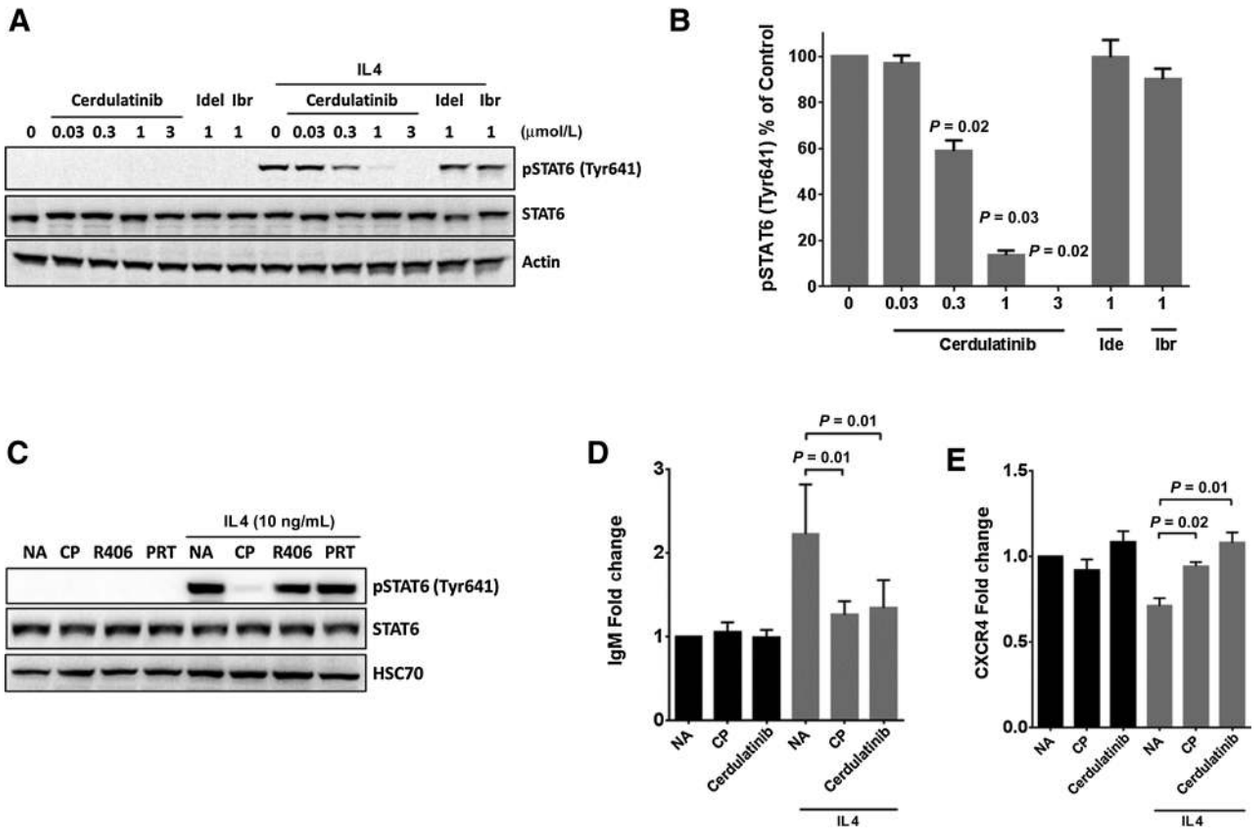

Figure 3.

Regulation of STAT6 phosphorylation and sIgM and CXCR4 expression by IL4 and cerdulatinib. CLL cells were treated with cerdulatinib, idelalisib (Idel), or ibrutinib (Ibr) at the stated concentrations for 60 minutes, then stimulated with IL4 (10 ng/mL) for a further 60 minutes. **A** and **B,** Phosphorylated STAT6 (pSTAT6), STAT6, and actin were assessed using immunoblotting in a representative sample (**A**) and summarized (**B**; $n = 8$). **C,** CLL cells were treated with tofacitinib (JAK3 inhibitor; CP) and SYK inhibitors fostamatinib (R406) and P505-15 (PRT) prior to IL4 treatment and immunoblotting performed for pSTAT6, STAT6, and HSC70 in a representative sample. **D** and **E,** CLL cells were incubated with cerdulatinib (1 μmol/L) or the JAK1/3 inhibitor tofacitinib (CP; 1 μmol/L) for 60 minutes then with IL4 (10 ng/mL) for a further 23 hours. **D** and **E,** Surface IgM (**D**) and surface CXCR4 (**E**) were assessed using flow cytometry. Bar graphs, means ± SEM.

capacity, as 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 signaling following anti-IgM engagement (36, 37), treatment with CD40L (31) and IL4 (16, 31), or coculture with NLC (31) are known to protect CLL cells from basal and therapy-induced killing and mimic prosurvival signals within the lymph node. Therefore, we evaluated CLL samples treated with soluble or BI anti-IgM (Fig. 5A and B and Supplementary Fig. S7A), cocultured with NLC (Fig. 5C) or with a combination of CD40L (300 ng/mL) and IL4 (10 ng/mL; Supplementary Fig. S7B and S7C) prior to treatment with cerdulatinib. Using 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 48 hours (Fig. 5A and B and Supplementary Fig. S7A). However, treatment with cerdulatinib significantly impaired this BCR-induced survival and promoted further reductions in CLL viability compared with unstimulated CLL cells. In comparison with the untreated controls, treatment with cerdulatinib also overcame NLC-mediated protection and induced greater levels of CLL cell apoptosis in a time-dependent manner (24–72 hours; Fig. 5C). Subsequently, we treated CLL samples with IL4/CD40L to study the effect of cerdulatinib in a more controlled setting and to replicate signals produced by T cells. We identified that IL4/CD40L promoted CLL survival in line with previous observations (38); in contrast, treatment with cerdulatinib (1–3 μmol/L) reversed the protection conferred by these growth factors (Supplementary Fig. S7B and S7C). Overall, these data suggest cerdulatinib can induce CLL cell death *in vitro* irrespective of key prosurvival signals.

In line with previously published data (32, 36, 38), BI anti-IgM and IL4/CD40L treatment induced expression of antiapoptotic proteins MCL-1 and BCL-X_L, shown by immunoblotting in a representative sample (Fig. 5D and E) and summarized ($n = 8$; Supplementary Fig. S8A–S8G). Simultaneous inhibition of Syk and JAK by cerdulatinib (1 and 3 μmol/L) significantly decreased BI anti-IgM and IL4/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 IL4/CD40L treatment, although to a lesser extent than that at the protein level, similar to previous published findings with Mcl-1 (Supplementary Fig. S9A–S9D; ref. 36). Treatment with cerdulatinib showed

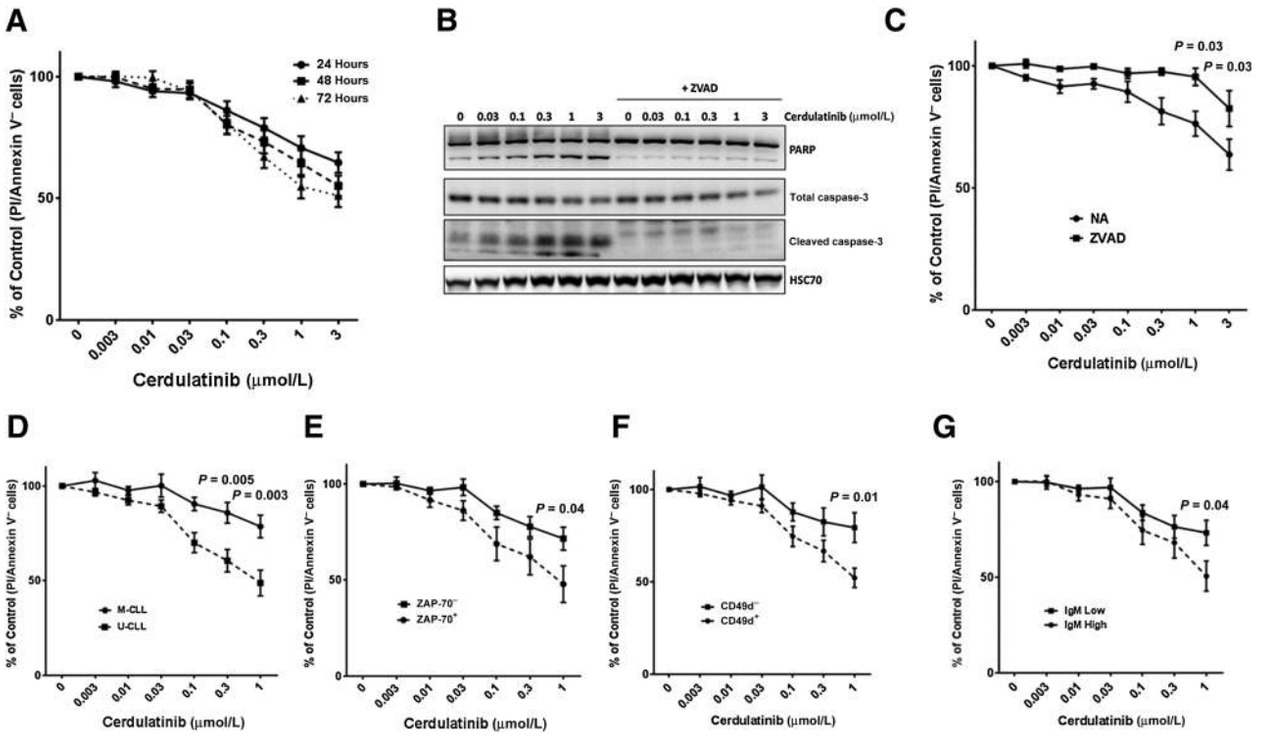

Figure 4.

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 72 hours and apoptosis assessed using PI/Annexin V by flow cytometry. Data is percentage of control (PI/Annexin V–negative cells). **B** and **C,** CLL cells were treated with cerdulatinib at the indicated concentrations in the presence or absence of 100 μmol/L pan-caspase inhibitor ZVAD for 24 hours and assessed for PARP and caspase-3 cleavage by immunoblotting (B) or Annexin V/PI analysis (**C**) by flow cytometry. **D–G,** Samples were characterized from analysis in A by IGHV mutational status (**D**), ZAP-70 (**E**), CD49d (**F**), and IgM expression (**G**). Bar graphs, means ± SEM.

no reproducible inhibition of basal MCL-1 or BCL-X_L protein or RNA expression (Supplementary Figs. S8 and S9); however in two of seven samples where basal MCL-1 and BCL-X_L protein expression was higher (patient 276D and 674C), cerdulatinib treatment reduced their expression (Fig. 5D).

Cerdulatinib and venetoclax synergize to induce substantial apoptosis in the presence of IL4/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). As we have shown that cerdulatinib could inhibit MCL-1 and BCL-X_L expression induced by IL4/CD40L and anti-IgM ligation, but not BCL-2, we investigated whether cerdulatinib would synergize with venetoclax *in vitro* to augment CLL cell killing. CLL cells were treated with IL4/CD40L for 6 hours and then incubated with either venetoclax or cerdulatinib alone or in combination for a further 24 hours. Venetoclax significantly reduced CLL cell viability in the absence of IL4/CD40L compared with the vehicle control. However, treatment with IL4/CD40L substantially protected CLL cells against venetoclax-induced apoptosis (Fig. 6A). In contrast, CLL cells treated with IL4/CD40L and a combination of venetoclax and cerdulatinib reduced CLL cell viability to a greater extent than with either drug alone (Fig. 6A and B). As cerdulatinib (1 μmol/L) alone did not reproducibly induce cell death >50%, we were unable to use conventional combination indices analysis to evaluate synergy between the drugs. Consequently, we used the fractional 2-drug analysis method described previously for CLL (29, 30, 38) to evaluate the synergistic interaction between cerdulatinib and venetoclax (Fig. 6C). Values above the diagonal line represent additive interaction, whereas those under the line are synergistic. In the presence of IL4/CD40L, a synergistic relationship was observed with cerdulatinib (1 μmol/L) in combination with either 10 or 100 nmol/L venetoclax in the majority of samples ($n = 8/9$; Fig. 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 signaling 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 patients with CLL, 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 Richter transformation, or a larger proportion of patients

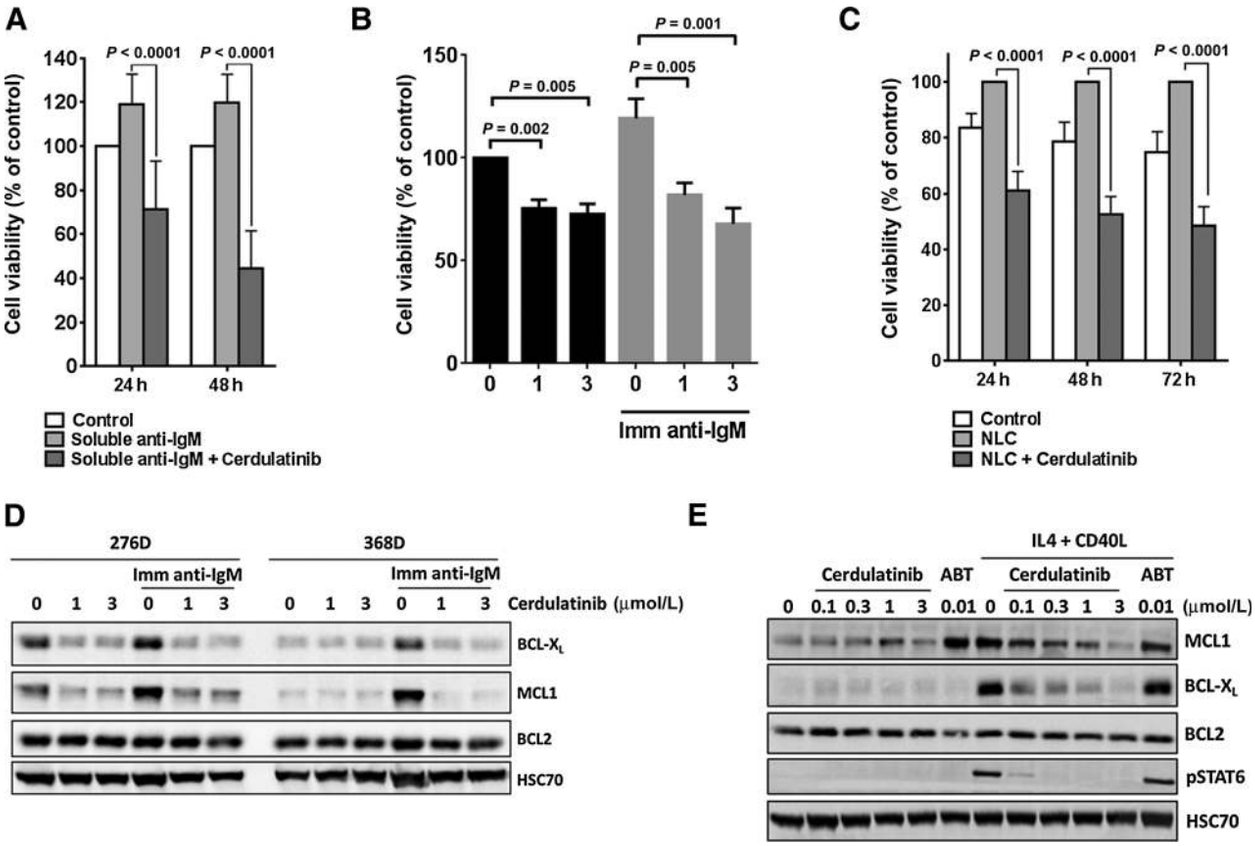

Figure 5.

Cerdulatinib overcomes the protection of CLL cells by conditions that mimic the tumor microenvironment. **A,** CLL cells were treated with cerdulatinib (2 μmol/L) for 24 to 48 hours in the presence or absence of soluble anti-IgM (10 μg/mL). Cell viability was determined by PI and DiOC6 double staining by flow cytometry ($n = 12$). **B,** CLL cells were treated with bead-immobilized (imm) anti-IgM in the presence or absence of 1 and 3 μmol/L 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 NLCs ($n = 6$). **D** and **E,** CLL cells were treated with cerdulatinib or venetoclax in the presence or absence of immobilized anti-IgM (**D**) or IL4 (10 ng/mL) and CD40L (300 ng/mL) for 24 hours (**E**) and protein expression of Bcl-X_L, Mcl-1, pSTAT6, HSC70, and Bcl-2 expression evaluated by immunoblotting. Bar graphs, means ± SEM.

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 pretreated 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 signaling and induced apoptosis irrespective of the sample sensitivity to ibrutinib (13). Syk is the apical kinase downstream of the BCR, and its inhibition prevents anti-IgM downstream signaling pathways, as indicated by the Syk inhibitors fostamatinib (11, 32) and P505-15 (33). To examine the extremes of the BCR signaling response, we treated CLL cells with soluble and BI anti-IgM or -IgD. Soluble anti-IgM signaling is curtailed within 0.5 to 1 hour, following endocytosis of its receptor; in contrast, BI anti-IgM was sustained up to 8 hours, possibly due to its inability to be endocytosed (43). Consequently, BI anti-IgM induces a stronger and prolonged signal compared with soluble antibody, theoretically replicating more closely antigen presentation by a cell (43). The Syk/JAK inhibitor cerdulatinib was able to overcome both soluble and immobilized anti-IgM and for the first time anti-IgD–induced signaling.

Recent investigation by our group demonstrated that IL4 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 signaling (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 patients with CLL (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 signaling by JAK and Syk inhibition, respectively, may produce therapeutically greater responses in patients compared with suppression of either pathway alone. Indeed, combining established BCR kinase inhibitors with JAK inhibitors or utilizing antibodies that block the cytokine receptor may be a useful strategy to achieve

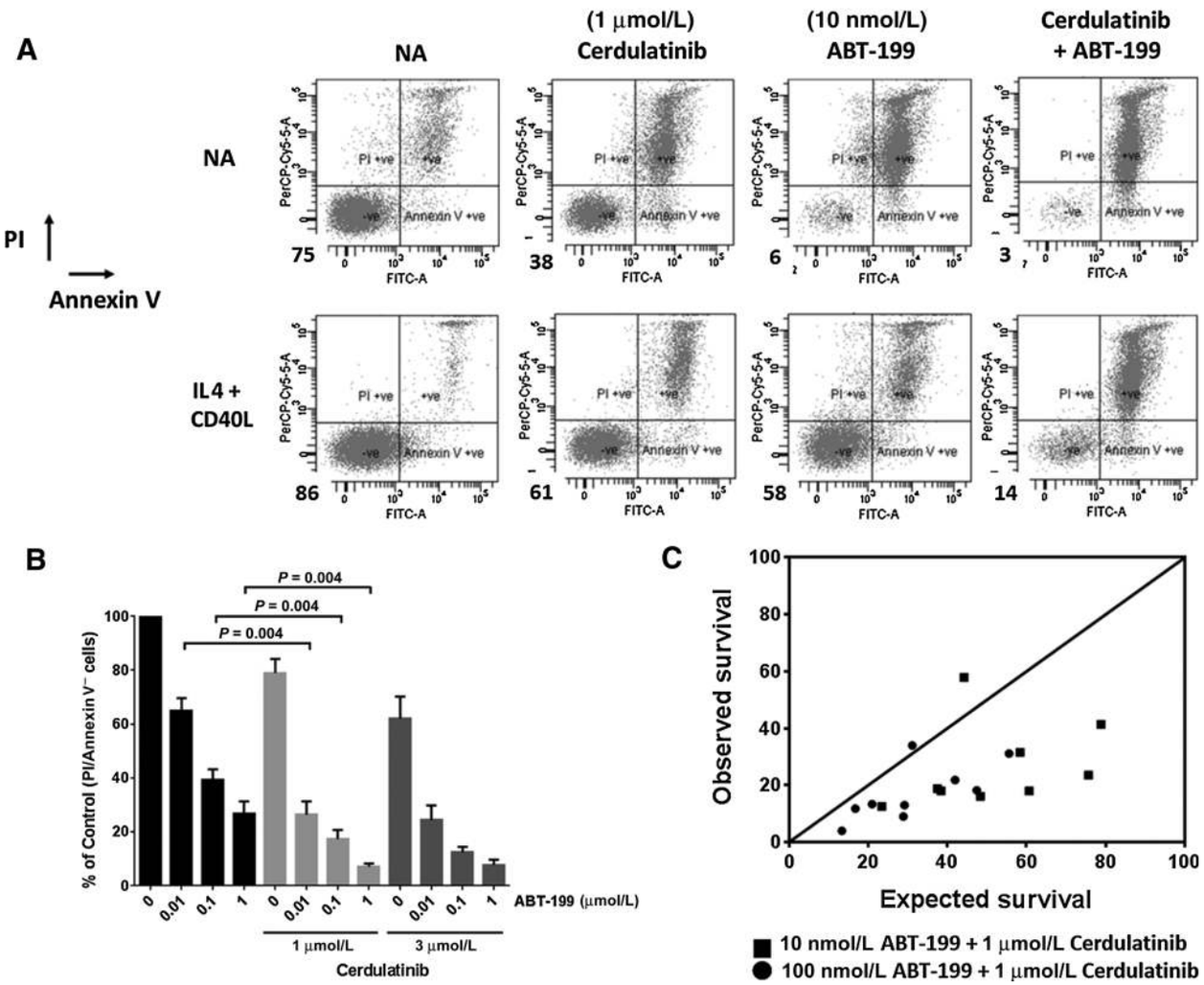

Figure 6.

Cerdulatinib synergizes with venetoclax to kill CLL cells. CLL cells were incubated with IL4 (10 ng/mL) and CD40L (300 ng/mL) for 6 hours and then treated with cerdulatinib and/or venetoclax (ABT-199) as indicated for a further 24 hours. Viability was assessed using flow cytometry (**A**; PI/Annexin V–negative cells) and a representative flow cytometry plot is shown and summarized (**B**; $n = 9$) showing percentage of control (PI/Annexin V–negative cells). **C,** Synergistic interactions between cerdulatinib and venetoclax were evaluated as indicated and described in the Materials and Methods section. XY line, observed survival = expected survival. Points beneath the line, synergistic interactions; points above the line, additive interactions.

this goal. Moreover, these therapeutic strategies are not limited to CLL and could be transferable to follicular lymphoma, where there is greater evidence for IL4 signaling in its biology. This may be particularly useful in cases of follicular lymphoma, where the recently identified STAT6 mutation at amino acid residue 419 conferred heightened IL4-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 (35 mg twice daily) has been established, which achieved a steady-state C_{min} of 1 μmol/L and is demonstrating effective antitumor 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 30 minutes and 14 hours, 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 IL4-induced signaling in CLL samples using multiple readouts. This is in contrast to previous reports that identified ibrutinib as an inhibitor of JAK3 in a kinase assay (47) and therefore would be predicted to inhibit IL4-induced pSTAT6 expression. Idelalisib and ibrutinib could not prevent IL4-induced IgM expression, which has recently been described to enhance BCR signaling in CLL (7). In contrast, treatment with cerdulatinib prevented IL4-induced sIgM expression and IL4 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 prosurvival signaling within the CLL lymph node and in overcoming IL4-mediated resistance to ibrutinib and idelalisib (7).

In whole-blood assays, we observed a reduced inhibition of anti-IgM and IL4 signaling; this is because plasma protein binding for cerdulatinib is approximately 78%, so higher amounts of drug are required to show full target inhibition in whole-blood assays compared with experiments performed in 10% FCS. Moreover, the inhibition of BCR and IL4-mediated signaling after oral cerdulatinib administration in whole blood from patient samples has been published in a poster format (Flinn and colleagues, ASCO, 2015; ref. 23), whereby 40 mg cerdulatinib inhibited BCR-induced phosphorylation of pERK and pSYK and IL4-induced pSTAT6 between 80% to 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 and colleagues 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 coculture 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 signaling, coculture with NLC, or treatment with IL4 and CD40L. Treatment with anti-IgM or IL4 and CD40L induced expression of MCL-1 and BCL-X_L. Longo and colleagues suggested that MCL-1 expression was pivotal for CLL cell survival, as 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 IL4/CD40L treatment, to obtain optimal apoptosis (38). Here, we demonstrated cerdulatinib significantly inhibits BI anti-IgM- and IL4/CD40L-induced MCL-1 and BCL-X_L protein expression. Although MCL-1 protein levels were reduced to a lesser extent following IL4/CD40L activation compared with anti-IgM treatment, this may simply reflect a reduced ability by cerdulatinib to completely inhibit CD40L-induced signaling.

As 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 synergized to produce greater levels of CLL apoptosis than either drug alone following treatment with IL4/CD40L. This is important because venetoclax has recently been approved for the treatment of patients with CLL with 17p del (49) and rapidly clears CLL cells from the blood (50). However, signals within the tumor microenvironment (IL4/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 hematologic malignancies alone and in combination with venetoclax.

Disclosure of Potential Conflicts of Interest

A. Davies reports receiving other commercial research support from Aceta, Celgene, Gilead, GlaxoSmithKline, Janssen, Pfizer, Roche, and Takeda and is a consultant/advisory board member for Celgene, Karyopharma, Roche, and Takeda. P.B. Conley holds ownership interest (including patents) in Portola Pharmaceuticals. A.J. Steele reports receiving speakers bureau honoraria from Portola Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: M.D. Blunt, S. Koehrer, A. Davies, P.W.M. Johnson, P.B. Conley, A. Pandey, J.C. Strefford, F.K. Stevenson, G. Packham, F. Forconi, G.P. Coffey, J.A. Burger, A.J. Steele
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.D. Smith, F. Forconi, A.J. Steele
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