Inhibition of NF-κB–Mediated Signaling by the Cyclin-Dependent Kinase Inhibitor CR8 Overcomes Prosurvival Stimuli to Induce Apoptosis in Chronic Lymphocytic Leukemia Cells


Abstract

Purpose: Chronic lymphocytic leukemia (CLL) is currently incurable with standard chemotherapeutic agents, highlighting the need for novel therapies. Overcoming proliferative and cytoprotective signals generated within the microenvironment of lymphoid organs is essential for limiting CLL progression and ultimately developing a cure.

Experimental Design: We assessed the potency of cyclin-dependent kinase (CDK) inhibitor CR8, a roscovitine analog, to induce apoptosis in primary CLL from distinct prognostic subsets using flow cytometry–based assays. CLL cells were cultured in in vitro prosurvival and proproliferative conditions to mimic microenvironmental signals in the lymphoid organs, to elucidate the mechanism of action of CR8 in quiescent and proliferating CLL cells using flow cytometry, Western blotting, and quantitative real-time PCR.

Results: CR8 was 100-fold more potent at inducing apoptosis in primary CLL cells than roscovitine, both in isolated culture and stromal-coculture conditions. Importantly, CR8 induced apoptosis in CD40-ligated CLL cells and preferentially targeted actively proliferating cells within these cultures. CR8 treatment induced downregulation of the antiapoptotic proteins Mcl-1 and XIAP, through inhibition of RNA polymerase II, and inhibition of NF-κB signaling at the transcriptional level and through inhibition of the inhibitor of IκB complex, resulting in stabilization of IκBa expression.

Conclusions: CR8 is a potent CDK inhibitor that subverts pivotal prosurvival and proproliferative signals present in the tumor microenvironment of CLL patient lymphoid organs. Our data support the clinical development of selective CDK inhibitors as novel therapies for CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is incurable with conventional chemotherapeutic regimens. First-line immunotherapy fludarabine, cyclophosphamide, and rituximab elicits a varying quality of remission (1), however, patients relapse due to re-emergence of minimal residual disease (MRD).

CLL progression involves cellular accumulation due to deregulated expression of antiapoptotic Bcl-2 protein family members leading to acquired resistance to apoptosis (2, 3) and enhanced proliferation within lymphoid organs through stromal niche interactions (4). Ki67+ CLL cells attract activated CD4+ T lymphocytes expressing CD40 ligand (CD40), and interleukin-4 (IL-4; ref. 5). CD40 stimulation in vitro promotes activation of NF-κB signaling and upregulation of Bcl-xL, Mcl-1, and survivin, mimicking the expression profile of CLL cells within lymph nodes (6–10). Clonal expansion in this environment enhances the likelihood of cytogenetic abnormalities, including 17p deletion (targeting p53) resulting in CLL that is largely resistant to standard chemotherapeutic agents (11, 12). Therefore, drugs that overcome prosurvival and proproliferative signals represent promising therapies for CLL.
Translational Relevance

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world, with an incidence rate of 3 in 100,000 people per year. Patients frequently become refractory to current immunotherapeutic regimens, highlighting the unmet medical need for additional therapeutic options. The tumor microenvironment within lymphoid organs of patients with CLL plays a pivotal role in promoting survival, proliferation, and chemoresistance of the leukemic clone; inhibiting the signals that orchestrate these events is key to disrupting disease progression. We establish that CR8, a second-generation roscovitine analog, exhibits significantly enhanced and selective cytotoxicity toward CLL cells and inhibits two key signals linked with progressive disease: Mcl-1 expression and NF-kB signaling at the transcriptional and posttranslational level. Our studies show the promise of novel selective cyclin-dependent kinase (CDK) inhibitors as therapies for CLL.

The cyclin-dependent kinase (CDK) family regulates cell division, cell-cycle progression, and transcription and is often deregulated in cancerous cells (13). At least 15 CDK inhibitors have been in clinical trials for leukemias and solid tumors (13). Flavopiridol (Alvocidib), roscovitine (CYC202/Seliciclib), and SNS-032 induce apoptosis in CLL cells irrespective of ZAP-70 status and p53 function, indicating that CDK inhibitors should be effective at treating poor prognostic CLL subsets (14–17). Flavopiridol and SNS-032 are currently in clinical trials for CLL (18). Although roscovitine progressed through phase II clinical trials for non–small cell lung and nasopharyngeal cancers due to its strong selectivity for CDKs and its relative lack of toxicity (13), its weak potency and short half-life led to the development of more potent analogs (19). Here, we show that CR8, a novel roscovitine analog, possesses enhanced potency over roscovitine for inducing apoptosis in CLL cells, which is not reduced by mouse fibroblast L cell (NT-L)–mediated cytoprotection. CR8 treatment overcomes key survival and proliferative signals that accompany CDK inhibition and apoptosis by inhibiting canonical NF-kB signaling and Mcl-1 and XIAP expression via an inhibition of RNA polymerase II.

Materials and Methods

Reagents and antibodies

Roscovitine-purine analog structures are shown in Supplementary Fig. S1. (R)-roscovitine [1], (R)-CR8 [8], and (S)-CR8 [9] were synthesized as described previously (20, 21) and provided by ManRos Therapeutics. Synthesis of (R)-DH122 [2], (R)-ML20 [3], (R)-ML76 [4], (R)-CR3 [5], (R)-ML78 [6], (R)-CR4 [7], (R)-CR1 [10], (R)-CR2 [11], (R)-CR11 [12], and (R)-Ness2 [13] is provided in ref. (22).

Flavopiridol [14] was obtained from Sigma-Aldrich Co. Ltd., SNS-032 [15] was synthesized in-house. Western blotting antibodies were sourced from Cell Signaling Technology, except anti-Bcl-2 (Millipore), anti-Bcl-x (BD Biosciences), anti-cFLIP (Santa Cruz Biotechnology), and anti-phospho-RNA polymerase II (Ser2 and Ser5; Covance Research Products). Flow cytometry antibodies were purchased from BD Biosciences. Carboxyfluorescein succinimidyl ester (CFSE), colemid, and CountBright absolute counting beads were obtained from Invitrogen Ltd.

Patient samples and CLL cell isolation

Peripheral blood samples were obtained after informed consent, from patients with B-cell CLL (B-CLL) that were treatment naïve (untreated) or had received treatment but not in the preceding 3 months. The studies were approved by Local Ethics Committees [Comité de Protection des Personnes de Brest (France) and West of Scotland Research Ethics Service, NHS Greater Glasgow and Clyde (United Kingdom)]. Linked clinical data of patients with CLL were stored (Table 1). CLL lymphocytes were isolated and used freshly or cryopreserved as previously described (23). After separation, CLL cell purity was more than 95% in all cases, determined by flow cytometry. Normal peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density gradient centrifugation using Histopaque (Sigma-Aldrich) and used freshly or cryopreserved. Normal human serum (NHS) was isolated from peripheral blood of healthy donors.

Cell lines and cell culture conditions

CLL cells were cultured at 1 × 10⁶/mL in RPMI-1640 containing 10% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mmol/L L-glutamine (complete medium; Invitrogen Ltd.). Mouse fibroblast L cells (NT-L) and NT-L cells stably expressing CD154 (CD154L; confirmed by flow cytometry), a gift from Prof. J. Gordon (University of Birmingham, Birmingham, United Kingdom), were used in coculture experiments to support CLL cell survival/proliferation. NT-L/CD154L cells were irradiated with 30 Gy and then cocultured with CLL cells at 25:1 CLL:NT-L cells, for a minimum of 18 hours before treatment with CR8. Complete medium was supplemented with 10 mg/mL IL-4 for CLL/CD154L proliferation experiments (Peprotech EC Ltd.; ref. 23). Functional authentication of prosurvival and proproliferative effects of NTL/CD154L cell lines on CLL cells was carried out.

Assessment of apoptosis

Following treatment, CLL cells were harvested, stained with Annexin V–allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD), and flow cytometry data were acquired using a FACSCantoII flow cytometer (BD Biosciences; ref. 23). Annexin V–7-AAD–cells were considered viable.

Protein kinase assays

Kinase activities were assayed in triplicate using Buffer D [10 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L...
dithiothreitol (DTT), 25 mmol/L Tris–HCl, and 50 μg/mL heparin; ref. 19]. Dose–response curves enabled IC₅₀ calculation. CDK2/cyclin A and CDK9/cyclin T (human, recombinant, expressed in insect cells) were prepared as described previously (19, 22). Kinase activity was assayed with either 1 mg/mL histone H1 type III-S (Sigma-Aldrich; CDK2) or 8.07 μg/assay CDK7/9 tide (YSPTSPSYSPYSPTSPSKKKK; CDK9; Millegen), with 15 μmol/L [γ-³²P] ATP (3,000 Ci/mmol; 10 mCi/mL) in a 30 μL final volume.

Western blotting
Protein lysates were prepared in lysis buffer [1% Triton, 1 mmol/L DTT, 2 mmol/L EDTA, 20 mmol/L Tris pH 7.5 containing complete protease inhibitor, and PhosStop (Roche)]. Western blotting was carried out as described previously (23).

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**NOTE:** —, samples that do not carry a 17p/11q cytogenetic abnormality.

ZAP-70 analysis was conducted by immunohistochemistry in our regional hematology laboratory. CLL cells were treated with 100 nmol/L CR8 for 24 hours and the percentage of viable cells was determined by carrying out Annexin V/7-AAD flow-cytometric analysis. Annexin V−7-AAD− cells were considered viable. Results are shown as percentage viability relative to untreated control (100%). ND, not determined.

NF-κB activity assay
Cytoplasmic and nuclear protein lysates were isolated from 1 × 10⁷ CLL cells/condition using the Nuclear Extract Kit and an ELISA-based method, TransAM (Active Motif), was used to quantify RelA DNA-binding activity according to the manufacturer’s protocol.

Cell-cycle analysis
After treatment cells were harvested, washed in PBS, and stained with propidium iodide (PI; Sigma-Aldrich), before acquiring data on the flow cytometer (24).
RNA isolation and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Ten to 20 ng was used for cDNA synthesis using first-strand cDNA synthesis kit (Roche). Real-time PCR (RT-PCR) was conducted using the TaqMan PCR Master Mix (Applied Biosystems; ref. 25). Relative gene expression was analyzed by the ΔΔCt method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference control and an assigned calibrator (26). Invented primers and probes and PCR buffers were purchased from Applied Biosystems.

CLL cell proliferation assay

CFSE-labeled CLL cells were plated on irradiated CD154L/IL-4 (27). CLL cells were treated with CR8 at day 3 to assess the effect on proliferation initiation and harvested. For longer experiments, medium and IL-4 were replenished every 3 days and fresh CD154L cells were added at day 6. Cells treated with CR8 at day 9, were stained with anti-CD19-APC-Cy7, Annexin V–APC and 7-AAD. Unstained and CFSE-positive CLL cells treated with colcemid (undivided) were included. To calculate absolute numbers of live CLL cells (CD19+/Annexin V–/7-AAD–) within proliferating cocultures, CountBright absolute counting beads were added to CLL cells before fluorescence-activated cell sorting (FACS). Percentage recovery of input CLL cells was calculated by dividing the absolute number of viable cells of all divisions, corrected for cell division, by the total number of input cells (5 x 10^6 cells).

Data and statistical analysis

Average responses from at least 3 individual donors are shown (mean ± SEM). Statistical analysis was conducted using GraphPad Prism 4 (GraphPad Software Inc.), using Students paired or unpaired t test (*, P < 0.05; **, P < 0.005; ***, P < 0.001). Flow cytometry data were analyzed using FlowJo (Tree Star Inc.).

Results

Roscovitine analogs trigger CLL cell death in vitro

CLL cells were treated with roscovitine analogs to determine the percentage of cell death by flow cytometry and calculate IC50 values (Table 2). The kinase inhibitory activity of these compounds was determined on purified, recombinant CDK2/cyclin A and CDK9/cyclin T. Addition of a 2-pyridyl substituent at position 4 of the phenyl ring [8, 9] leads to a major improvement in potency of the compounds ability to induce CLL apoptosis, with the S-CR8 isomer [9] more potent than the R isomer [8]. Addition of a pyridyl substituent at position 3 of the phenyl ring did not enhance the activity as efficiently [10–13], confirming results obtained with neuroblastoma cells SH-SY5Y (19). A good correlation was observed between CDK9 inhibition and induction of CLL cell death (Table 2). (S)-CR8 [9] induced CLL cell apoptosis at a similar nanomolar concentration as 2 reference CDK inhibitors, flavopiridol [14] and SNS-032 [15], therefore (S)-CR8 was selected for more detailed studies.

CR8 induces apoptosis in all CLL patient subgroups

Comparison of (S)-CR8 (now CR8) and roscovitine confirmed that CR8 induced apoptosis in CLL cells with 100-fold greater potency than roscovitine [Fig. 1A; IC50 = 118.2 nmol/L vs. 14.9 μmol/L (n = 8)]. CLL cells were over 2-fold more sensitive to CR8 treatment than freshly isolated or cryopreserved PBMCs from healthy individuals, and

| Table 2. Effects of CDK inhibitors on CLL survival and the catalytic activity of CDK2 and CDK9 |
|-------------------------------|------------|------------|------------|
| #                | Kinase inhibitors | Cell death induction IC50 | CDK2/ cyclin A | CDK9/ cyclin T |
| 1                | Roscovitine      | 8.96        | 0.23        | 0.7          |
| 2                | DH22             | >10         | 0.66        | 1.5          |
| 3                | ML20             | 6.1         | 1.7         | 2            |
| 4                | ML76             | >10         | 0.25        | 3            |
| 5                | CR3              | 1.25        | 0.6         | 1.2          |
| 6                | CR4              | 1.15        | 0.38        | 0.54         |
| 7                | CR8 (R)          | 0.18        | 0.16        | 0.35         |
| 8                | CR8 (S)          | 0.09        | 0.14        | 0.21         |
| 9                | CR1              | 2.13        | 0.42        | 1.7          |
| 10               | CR2              | 5.5         | 0.15        | 0.81         |
| 11               | CR11             | 0.73        | 0.09        | 0.42         |
| 12               | Ness2            | 1.26        | 0.28        | 1.4          |
| 13               | Flavopiridol     | 0.140       | 0.042       | 0.003        |
| 14               | SNS-032          | 0.064       | 0.022       | 0.0065       |

NOTE: The ability of selected kinase inhibitors to induce cell death was tested on freshly isolated CLL cells obtained from previously untreated patients (n = 5). Cell viability was measured using flow cytometry to exclude Annexin V and PI-positive cells following 24-hour inhibitor treatment. The same compounds were tested on 2 purified CDKs. IC50 values, calculated from the dose–response curves, are reported in μmol/L.
normal B cells were more sensitive compared with T lymphocytes (Fig. 1B; IC₅₀ = 188 nmol/L; T; IC₅₀ = 632 nmol/L; Supplementary Fig. S2). CR8 induced apoptosis in all CLL patient subsets at 300 nmol/L, while displaying a varied response to 100 nmol/L (Supplementary Fig. S3 and Fig. 1C). Indeed, patients carrying poor prognostic markers (ZAP-70⁺, 11q/17p cytogenetic markers) or more advanced CLL (Binet stage C and/or previously treated) were significantly less responsive to 100 nmol/L CR8 (Fig. 1D).

Interestingly, CR8 was slower to induce CLL apoptosis, with approximately 25% cells undergoing apoptosis compared with approximately 70% at 8-hour treatment with flavopiridol (Fig. 1E). To determine the impact of human plasma protein binding, CLL cells were treated with CR8, flavopiridol, or roscovitine with either FBS or NHS. The potency of these drugs to induce apoptosis was reduced in the presence of NHS (IC₅₀ FBS vs. NHS: CR8, 62.99 vs. 321.4 nmol/L; flavopiridol, 59.03 vs. 184.3 nmol/L; roscovitine, 10.97 vs. 14.27 μmol/L at 48 hours), however, CR8 maintained the ability to induce apoptosis in the nanoparticles range (Fig. 1F). Collectively, these results show that CR8 is a potent inducer of apoptosis in CLL cells.

CR8 induces apoptosis in prosurvival and proproliferative environments in vitro

To mimic signals generated within the tumor microenvironment, CLL cells were cocultured with NT-L cells or CD154L/IL-4 (6, 23). Although viability of untreated CLL cells was elevated upon coculture with NT-L cells (Fig. 2A), no significant difference was noted between CLL cells treated with CR8 on plastic or NT-L cells, indicating that CR8 overcomes prosurvival-mediated signals delivered by the microenvironment. Normal B cells were further protected upon coculture with NT-L cells (Supplementary Fig. S4; B, IC₅₀ = 245 nmol/L). Although the ability of CR8 to induce apoptosis in CLL cells cocultured on CD154L/IL-4 was diminished, a significant reduction in viable cells was evident (Fig. 2A), showing that CR8 inhibits CD154L/IL-4–mediated prosurvival signals.

CR8 induced apoptosis via a caspase-dependent mechanism, with almost complete inhibition of apoptosis upon pretreatment of CLL cells with pan-caspase inhibitor ZVAD-fmk (Supplementary Fig. S5A). In addition, CR8 treatment lead to a loss of mitochondrial membrane potential, increased levels of activated caspase-3 and PARP cleavage (Supplementary Fig. S5B and Fig. 2B).

CR8 selectively inhibits CDK1, 2, 3, 5, and 9 (Table 2; ref. 19). CDK9-targeted phosphorylation site (Ser2) of RNA polymerase II was inhibited upon CR8 treatment for 8 hours, whereas the CDK7-targeted phosphorylation site (Ser5) was unaffected (Fig. 2B). Moreover, MCL1 and XIAP transcripts were significantly decreased at 18 hours post-CR8 treatment in all culture conditions (Fig. 2C). Coculture of CLL cells on CD154/IL-4 induces a marked upregulation of antiapoptotic proteins Bcl-xl and Mcl-1 (Fig. 2D; ref. 23). Incubation of CLL cells with CR8 for 8 hours, resulted in a significant downregulation of Mcl-1 protein expression on CD154L/IL-4, which was sustained after 18 hours of treatment. XIAP levels were also reduced when CLL cells were treated either on plastic or in NT-L at 8 hours (Fig. 2D). Bcl2 levels were unaffected by coculture or subsequent treatment with CR8 (Fig. 2D). These findings show that CR8 treatment reduces Mcl-1 and XIAP expression to assist in the induction of CLL apoptosis upon coculture with CD154L/IL-4.

CR8 inhibits the NF-κB signaling pathway in CLL cells

NF-κB–mediated signaling represents a prosurvival pathway in CLL cells, upregulated in CLL patient lymph nodes (5). CR8 treatment reduced NF-κB transcription factor gene expression in all culture conditions, negatively impacting on their availability for NF-κB–mediated gene transcription in CLL cells (Fig. 3A). CLL coculture on CD154L/IL-4 induced an upregulation of IκBα⁰⁰⁰³⁄³³ phosphorylation and elevation in RelA activity, indicative of an activation of canonical NF-κB signaling (Fig. 3B and Supplementary Fig. S6). This is supported by previous findings that CD40 ligation on CLL cells leads to the activation of inhibitor of IκB kinase (IκK) resulting in phosphorylation, ubiquitination, and degradation of IκBα, releasing RelA and p50 to translocate to the nucleus and initiate transcription (28). Moreover, the NF-κB–regulated protein cellular FLICE inhibitory protein (cFLIP) was upregulated (Fig. 3B). CR8 treatment of CLL cells cocultured on CD154L/IL-4 resulted in downregulation of phosphorylated IκBα, stabilizing IκBα expression, and inhibiting NF-κB signaling. This was supported by downregulation in cFLIP protein expression and inhibition of CFLAR transcription (gene-encoding cFLIP) upon CR8 treatment (Fig. 3B). Further analysis revealed stabilization and elevation of IκBα after 24 hours CR8 treatment and a decrease in RelA⁰⁰⁰³⁄³³ phosphorylation and RelA DNA-binding activity (Fig. 3C and D). As RelA and IκBα are downstream substrates of the IKK complex, these studies indicate that CR8 inhibits canonical NF-κB activity through IKK complex inhibition.

CR8 inhibits the initiation of CD154L/IL-4–mediated proliferation in CLL cells

To determine whether CR8 could inhibit CLL proliferation, CLL cells were labeled with CFSE to track cell division, and cocultured with CD154L/IL-4, treating cells with CR8 from day 3. Although at least one cell division was evident in untreated cells at day 6 [indicated by a reduction in CFSE mean fluorescence intensity (MFI)], CR8-treated CLL cells did not divide and maintained a similar MFI as cells treated with colcemid (Fig. 4A). CR8 treatment maintained this antiproliferative effect on CLL cells, showed by the significant elevation in CFSE MFI at days 9 and 12 of CLL-CD154L/IL-4 coculture (Fig. 4A). To further investigate the antiproliferative role of CR8, CLL cells treated at day 3 were cocultured for a further 3 days, then cell-cycle phases were analyzed by PI analysis. As expected, a significant proportion of cells apoptosis upon CR8 treatment (Fig. 4B, left), supporting data in Fig. 2A. Within the live cells, there was a significant elevation of cells in the G₁ phase of the cell cycle.
Figure 2. CR8 overcomes prosurvival signals to induce apoptosis in CLL cells cocultured on NT-L, targeting CDK substrates. CLL cells were cultured in medium alone (plastic), NT-L, or CD154L/IL-4 overnight, then treated with CR8 for 24 (left) or 48 hours (right), or left untreated. A, percentage of viable cells was determined by excluding Annexin V<sup>+</sup>/7-AAD<sup>+</sup> cells ± SEM (n = 7) and P values generated by a paired t test. B, lysates were prepared for Western blotting to assess PARP cleavage, CDK substrate (phospho-RNA polymerase II-Ser2 or -Ser5 and total RNA polymerase II), and protein loading (GAPDH/β-tubulin). Representative Western blot analyses shown from independent experiments carried out on at least 6 individual patients. C, RNA/cDNA was prepared from cells treated with CR8 for 18 hours as indicated, and mRNA expression levels of MCL1 (left) and XIAP (right) were determined by TaqMan RT-PCR. Each gene is expressed relative to GAPDH reference gene and calibrated to untreated plastic sample. Data are mean ± SEM (n = 4), and P values generated by a paired two-tailed t test. D, lysates were prepared for Western blotting to assess the expression of proteins related to apoptosis and protein loading (actin). *, P < 0.05; **, P < 0.005; ***, P < 0.001.
Figure 3. CR8 treatment of CLL cells reduces canonical NF-κB–mediated signaling and activity. CLL cells were cultured on plastic, NT-L cells, or CD154L/IL-4 overnight, then treated with 300 nmol/L CR8 or left untreated. A, RNA/cDNA was prepared from CLL cells treated with CR8 for 18 hours and mRNA expression levels of NF-κB–transcription factors determined by TaqMan RT-PCR. All data are fold change in expression of the gene of interest, relative to GAPDH reference gene, calibrated to untreated CLL cells on plastic (n = 4 ± SEM). B, lysates were prepared from cells treated with CR8 for 8 hours for Western blotting to assess the activation status of proteins related to NF-κB signaling and protein loading (β-tubulin). RNA/cDNA was prepared from cells treated with CR8 for 18 hours and CFLAR expression level was determined by TaqMan RT-PCR. C, lysates were prepared from treated cells for Western blotting to assess the activation status of proteins related to NF-κB signaling and protein loading (β-tubulin). D, nuclear fractions were prepared from CLL cells. RelA activity was assessed and results are an average of 3 individual patients with CLL ± SEM. All P values generated by paired t test.

*, P < 0.05; **, P < 0.005; ***, P < 0.001.
cycle upon CR8 treatment, with a corresponding decrease in S/G2–M compared with untreated cells, indicative of CR8 inducing a G1 arrest and inhibiting initiation of CLL cell proliferation (Fig. 4B, right).

CR8 induces apoptosis in proliferating CLL cells

To determine whether CR8 induces apoptosis in actively proliferating CLL cells, cells were cocultured with CD154L/IL-4 for 9 days to initiate proliferation and then treated with CR8. Absolute cell numbers decreased over time in the presence of CR8, suggesting that CR8 targets proliferating cells (Fig. 5A). To address this directly, CFSE-CLL cells were cocultured with CD154L/IL-4 for 9 days before treatment with CR8. A significant increase in the percentage of early apoptotic cells (Annexin V+/7-AAD−) was observed, with the CFSElo population being preferentially targeted, showing that CR8 induced apoptosis within actively proliferating CLL cells (Fig. 5B). This was supported by an enrichment of CFSEhi CLL cells (nondividing/slow-dividing cells) after treatment with CR8 for 72 hours (Fig. 5C). Moreover, absolute cell numbers decreased in all dividing CLL populations upon treatment with 300 nmol/L or more CR8 (Fig. 5D), confirming that CR8 induces apoptosis in proliferating CLL cells.

Discussion

CDKs represent a promising protein family for targeted inhibition due to their deregulation in many cancer types (13). We show that the roscovitine analog CR8 possesses a 100-fold enhanced potency for inducing cell death in CLL cells compared with roscovitine, with effectiveness in the nanomolar concentration range. We noted that poor prognostic patients and previously treated patients were less responsive to approximately IC50 concentrations of CR8 (100 nmol/L), indicating that higher concentrations of CDK inhibitors may be required in vivo to gain clinical benefit in pretreated patients. A recent report on the phase I clinical trial results for SNS-032, an inhibitor of CDK2, 7, and 9, indicated limited clinical activity in patients with CLL, possibly because the trial consisted of a heavily pretreated patient cohort (29).
Figure 5. CR8 treatment preferentially targets proliferating CLL cells for apoptosis. CLL cells were cocultured with CD154L/IL-4 for 9 days and then treated with increasing CR8 concentrations or left untreated for up to 3 days. P values generated by a paired t test. A, cell recovery was calculated on subsequent days using absolute cell counting beads. The data shown are an average percentage of the original input (5 × 10^5 cells) from 7 individual CLL samples ± SEM. B, the percentage of apoptotic CLL cells was determined by gating for CD19^− (to exclude NT-L cells) 7-AAD^− (excluding dead cells). The percentage of apoptotic cells (Annexin V^+) is shown in an individual patient (left) and a graph showing the average percentage of early apoptotic CLL cells relative to untreated (UT) control (CD19^− Annexin V^− 7-AAD^−) present in proliferating CLL cell populations (n = 9 ± SEM). C, CFSE MFI of CLL posttreatment (CLL samples ± SEM relative to UT control; n = 9). D, flow-cytometric analysis in the presence of absolute cell counting beads enabled calculation of the number of live CLL cells (CD19^− Annexin V^− 7-AAD^−). Graph showing the average relative number of live CLL cells in each division (n = 8). *P < 0.05; **P < 0.001.

CR8 exhibited only a modest improvement as a CDK inhibitor compared with roscovitine, despite an enhanced ability to induce CLL apoptosis (19, 30), whereas CR8 was less potent than flavopiridol at inhibiting CDK2 and CDK9 but induced CLL cell death in the same nanomolar range. These findings may reflect differences in the kinome each
drug interacts with. Indeed, flavopiridol is quite unselective, so side effects are more likely to occur than with CR8, which has a limited number of targets. Conversely, roscovitine is more selective than CR8, which may explain why CR8 has an enhanced ability to induce cell death compared with roscovitine. Therefore, combined inhibition of CDK1, 2, 3, 5, 9, CK1, and ERK1/2 by CR8 could result in enhanced cellular potency (19). Notably, flavopiridol and SNS-032 are potent inhibitors of GSK-3, whereas purine CDK inhibitors have little effect (IC50 values flavopiridol, SNS-032, roscovitine, CR8: 0.45, 0.02, 130, >30 μmol/L, respectively; ref. 19, 31). The kinase profile inhibited by these CDK inhibitors may also explain the increased selectivity that CR8 displays for CLL cells over PBMCs, compared with flavopiridol (IC50 = 246.9 vs. 146.7 nmol/L in PBMCs treated with CR8 vs. flavopiridol respectively), possibly relating to the ability of flavopiridol to generate DNA double-strand breaks (32). In addition, CR8 induces apoptosis in CLL cells with slower kinetics compared with flavopiridol, which may reduce the likelihood of tumor cell lysis observed in patients treated with flavopiridol and SNS-032 (29, 33). These studies highlight the selectivity and potency of CR8 for inhibiting CDKs and inducing CLL apoptosis.

Mcl-1 expression is normally tightly regulated, however, it is upregulated in patients with CLL exhibiting progressive disease, whereas exhibiting low/negligible expression in normal cells, and enhanced Mcl-1 expression is a predictive factor for chemoresistance (2, 34–36). CR8 treatment reduces Mcl-1 transcripts, mainly by inhibiting CDK9-mediated phosphorylation of RNA polymerase II (19). PHA76749, a dual cdc7/CDK9 inhibitor, also induces a reduction in Mcl-1 transcript expression causing apoptosis in CLL cells stimulated with CD154/IL-4, indicating that CDK9 targeting may be sufficient to induce CLL cell apoptosis in the lymph node microenvironment (37). As shown previously, Mcl-1 expression may also be regulated at the posttranscriptional level, due to the abrogation of Mcl-1 protein expression (16, 30, 37). Mcl-1 proteasomal degradation, coupled with maintenance or upregulation of proapoptotic, Mcl-1–binding partner Noxa upon treatment of cells with CR8, roscovitine, or PHA767491 can drive cells toward apoptosis (30, 37).

NF-κB signaling is constitutively active in CLL cells, with higher RelA/p65 activity correlating with chemoresistance and poorer clinical outcome (9, 10, 38). We show that CR8 inhibits NF-κB–mediated signals at the transcriptional and protein level. Transcriptional downregulation by CDK inhibition is well established in a number of cell types including CLL cells (16, 39), and our data show that CR8 treatment inhibits expression of the NF-κB transcription factors in CLL cells, and NF-κB–regulated gene products associated with CLL cell survival, likely through inhibition of CDK9 activity. In addition, CR8 inhibited CD154/IL-4–stimulated IκK phosphorylation of RelA and IkBα, leading to inhibition of RelA activity. Repression of NF-κB signaling by CDK inhibitors has not been previously shown in CLL cells, however, studies show flavopiridol and roscovitine can inhibit NF-κB signaling in other cell types through inhibition of IKK activity (40, 41). Interestingly, the mechanism used by CDK inhibitors to abrogate NF-κB differs between cell types, as roscovitine was only capable of inhibiting NF-κB at the transcriptional level in neutrophils (42).

CLL clonal expansion is a direct indicator of disease progression, with an overall growth of 0.35%/day correlating with poorer disease outcome (4). The ability of CR8 to inhibit CLL growth has important implications, as it reduces the possibility of clonal evolution during proliferation. CR8 induced an antiproliferative effect on CLL cells during the initiation of proliferation and induced apoptosis in actively proliferating CLL cells, suggesting that this compound may have clinical benefit in patients with CLL exhibiting progressive disease. Recent reports from phase II clinical trials using flavopiridol show clinical responses in fludarabine-refractory patients, reducing bulky lymphadenopathy and enabling almost a year of progression-free survival in relapsed patients (33, 43). Collectively, our findings establish that CR8 inhibits both survival and proliferative signals enabling almost a year of progression-free survival in relapsed patients (33, 43). Collectively, our findings establish that CR8 inhibits both survival and proliferative signals to induce cell-cycle arrest and cell death, thus possessing the potential to impede progressive CLL, and suggesting that significant reduction in tumor load to below the level of MRD detection may be achievable.

Disclosure of Potential Conflicts of Interest
N. Oumata has ownership interest (including patents) as coinventor of the CR8 patent and is an employee of ManRos Therapeutics. H. Galons has ownership interest (including patents) in the CR8 patent and is a Cofounder and consultant/advisory board member of ManRos Therapeutics. L. Meijer is the President and CSO of ManRos Therapeutics and has ownership interest (including patents) in a patent on CR8. No potential conflicts of interest were disclosed by the other authors.

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