Inhibition of NF-κB-mediated signaling by the cyclin-dependent kinase inhibitor CR8 overcomes pro-survival stimuli to induce apoptosis in chronic lymphocytic leukemia cells

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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/year. Patients frequently become refractory to current immuno-chemotherapeutic regimes, highlighting the unmet medical need for additional therapeutic options. The tumour microenvironment within lymphoid organs of CLL patients 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 towards CLL cells and inhibits two key signals linked with progressive disease: Mcl-1 expression and NF-κB signaling at the transcriptional and post-translational level. Our studies demonstrate the promise of novel selective CDK inhibitors as therapies for CLL.

Abstract

Background: 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* pro-survival and pro-proliferative conditions to mimic microenvironmental signals in the lymphoid organs, to elucidate the mechanism of action of CR8 in quiescent and proliferating CLL cells utilising 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-co-culture 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 anti-apoptotic 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 IKK complex, resulting in stabilisation of IκBα expression.

Conclusions: CR8 is a potent CDK inhibitor that subverts pivotal pro-survival and pro-proliferative 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 regimes. First-line immuno-chemotherapy 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 anti-apoptotic 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 (CD154), and interleukin 4 (IL4) (5). CD40 stimulation *in vitro* promotes activation of nuclear factor-κB (NF-κB) signaling and upregulation of Bcl-x_L and Mcl-1, and survivin, mimicking the expression profile of CLL cells within lymph nodes (LNs) (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 pro-survival and pro-proliferative signals represent promising 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 fifteen 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 *in vivo* led to the development of more potent analogs (19). Here, we demonstrate 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 CD154/IL4 stimulation, inducing cell cycle arrest and apoptosis by inhibiting canonical NF-κB 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 (20, 21) and provided by ManRos Therapeutics (Roscoff, France). Synthesis of (R)-DH22 [**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**], (R)-Ness2 [**13**] is provided in (22). Flavopiridol [**14**] was obtained from Sigma-Aldrich Co. Ltd. (Gillingham, UK), SNS-032 [**15**] was synthesized in-house. Western blotting antibodies were sourced from Cell Signaling Technology (Danvers, MA), except anti-Bcl-2 (Millipore, Billerica, MA), anti-Bcl-x (BD Biosciences, Oxford, UK), anti-cFLIP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-RNA polymerase II (Ser2 and Ser5; Covance Research Products Emeryville, CA). Flow cytometry antibodies were purchased from BD Biosciences. Carboxyfluorescein succinimidyl ester (CFSE), colcemid and CountBright™ absolute counting beads were obtained from Invitrogen Ltd. (Paisley, UK).

Patient Samples and CLL cell isolation

Peripheral blood (PB) samples were obtained after informed consent, from patients with B-CLL that were treatment naïve (untreated) or had received treatment but not in the preceding three 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 (UK)). Linked clinical data of CLL patients were stored (Table 1). CLL lymphocytes were isolated and used freshly or cryopreserved as previously described (23). After separation, CLL cell purity was > 95% in all cases, determined by flow cytometry. Normal PB mononuclear cells (PBMCs) 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 PB of healthy donors.

Cell lines and cell culture conditions

CLL cells were cultured at $1x10^6$/ml in RPMI-1640 containing 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM 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, UK), were used in co-culture experiments to support CLL cell survival/proliferation. NT-L/CD154L cells were irradiated with 30 Gy and then co-cultured with CLL cells at 25:1 CLL:NT-L cells, for a minimum of 18 hr prior to treatment with CR8. Complete medium was supplemented with 10 ng/ml IL4 for CLL-CD154L proliferation experiments (Peprotech EC Ltd., London, UK) (23). Functional authentication of pro-survival and pro-proliferative 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-APC/7-AAD and flow cytometry data was acquired using a FACSCantoII flow cytometer (BD Biosciences) (23). Annexin V^- $7\text{-}AAD^-$ cells were considered viable.

Protein kinase assays

Kinase activities were assayed in triplicate using Buffer D (10 mM $MgCl_2$, 1 mM EGTA (ethyleneglycoltetraacetic acid), 1 mM DTT (dithiothreitol), 25 mM Tris/HCl, 50 µg/ml Heparin) (19). Dose-response curves enabled IC_{50} calculation. *CDK2/cyclin A* and *CDK9/cyclin T* (human, recombinant, expressed in insect cells) were prepared as described (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* (YSPTSPSYSPTSPSYSPTSPSKKKK;

CDK9; Millegen, Labege, France), with 15 μM [γ-^{33}P] 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 mM DTT, 2 mM EDTA, 20 mM Tris pH 7.5 containing Complete protease inhibitor and PhosStop (Roche, Herts., UK)). Western blotting was carried out as described previously (23).

NF-κB activity assay

Cytoplasmic and nuclear protein lysates were isolated from 1x10^7 CLL cells/condition using the Nuclear Extract Kit and an ELISA-based method, TransAM (Active Motif, La Hulpe, Belgium), 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 real-time quantitative PCR

Total RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia, CA). 10-20 ng was used for cDNA synthesis using first-strand cDNA synthesis kit (Roche). Real-time PCR was performed using the TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA) (25). Relative gene expression was analyzed by the ΔΔCt method using GAPDH as reference control and an assigned calibrator (26). Inventoried primers and probes and PCR buffers were purchased from Applied Biosystems.

CLL cell proliferation assay

CFSE-labelled CLL cells were plated on irradiated CD154L/IL4 (27). CLL cells were treated with CR8 at day 3 to assess the effect on proliferation initiation, and harvested. For longer-term experiments, medium and IL4 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^- $7\text{-}AAD^-$) within proliferating co-cultures, CountBright™ absolute counting beads were added to CLL cells prior to 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 ($5x10^5$ cells).

Data and statistical analysis

Average responses from at least 3 individual donors are shown (mean ± SEM). Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software Inc., CA), 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., Ashland, OR).

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 IC_{50} 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 two 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; IC_{50} = 118.2 nM vs. 14.9 μM (n=8)). CLL cells were over two-fold more sensitive to CR8 treatment than freshly isolated or cryopreserved PBMCs from healthy individuals, and normal B cells were more sensitive compared with T lymphocytes (Fig. 1B; B - IC_{50} = 188 nM; T - IC_{50} = 632 nM and Supplementary Fig. S2). CR8 induced apoptosis in all CLL patient subsets at 300 nM, while displaying a varied response to 100 nM (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 nM CR8 (Fig. 1D).

Interestingly, CR8 was slower to induce CLL apoptosis, with ~25% cells undergoing apoptosis compared to ~ 70% at 8 hr 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 (IC_{50} FBS vs. NHS: CR8 – 62.99 vs. 321.4 nM; flavopiridol - 59.03 vs. 184.3 nM; roscovitine – 10.97 vs. 14.27 μM at 48 hr), however CR8 maintained the ability to induce apoptosis in the nanomolar range (Fig. 1F). Collectively, these results demonstrate that CR8 is a potent inducer of apoptosis in CLL cells.

CR8 induces apoptosis in pro-survival and pro-proliferative environments in vitro

To mimic signals generated within the tumour microenvironment, CLL cells were co-cultured with NT-L cells or CD154L/IL4 (6, 23)). While viability of untreated CLL cells was elevated upon co-culture 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 pro-survival-mediated signals delivered by the microenvironment. Normal B cells were further protected upon co-culture NT-L cells (Supplementary Fig. S4; B - IC_{50} = 245 nM). While the ability of CR8 to induce apoptosis in CLL cells co-cultured on CD154L/IL4 was diminished, a significant reduction in viable cells was evident (Fig. 2A), demonstrating that CR8 inhibits CD154L/IL4-mediated pro-survival signals.

CR8 induced apoptosis via a caspase-dependent mechanism, with almost complete inhibition of apoptosis upon pre-treatment 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 and (19)). CDK9-targeted phosphorylation site (Ser2) of RNA polymerase II was inhibited upon CR8 treatment for 8

hr, while the CDK7 targeted phosphorylation site (Ser5) was unaffected (Fig. 2B). Moreover, *MCL1* and *XIAP* transcripts were significantly decreased at 18 hr post-CR8 treatment in all culture conditions (Fig. 2C). Co-culture of CLL cells on CD154L/IL4 induces a marked upregulation of anti-apoptotic proteins Bcl-x_L and Mcl-1 (Fig. 2D and (23)). Incubation of CLL cells with CR8 for 8 hr, resulted in a significant downregulation of Mcl-1 protein expression on CD154L/IL4, which was sustained after 18 hr of treatment. XIAP levels were also reduced when CLL cells were treated either on plastic or in NT-L at 8 hr (Fig. 2D). Bcl2 levels were unaffected by co-culture or subsequent treatment with CR8 (Fig. 2D). These findings demonstrate that CR8 treatment reduces Mcl-1 and XIAP expression to assist in the induction of CLL apoptosis upon co-culture with CD154L/IL4.

CR8 inhibits the NF-κB signaling pathway in CLL cells

NF-κB-mediated signaling represents a pro-survival pathway in CLL cells, upregulated in CLL patient LNs (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 co-culture on CD154L/IL4 induced an upregulation of IκBα$^{S32/36}$ 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 IκBα kinase (IKK) 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 co-cultured on CD154L/IL4 resulted in downregulation of phospho-IκBα, stabilising 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 stabilisation and elevation of IκBα after 24 hr CR8 treatment and a decrease in $RelA^{S536}$ phosphorylation and RelA DNA binding activity (Figs. 3C & 3D). 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/IL4-mediated proliferation in CLL cells

To determine whether CR8 could inhibit CLL proliferation, CLL cells were labeled with CFSE to track cell division, and co-cultured with CD154L/IL4, treating cells with CR8 from day 3. While 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 anti-proliferative effect on CLL cells, demonstrated by the significant elevation in CFSE MFI at days 9 and 12 of CLL-CD154L/IL4 co-culture (Fig. 4A). To further investigate the anti-proliferative role of CR8, CLL cells treated at day 3 were co-cultured for a further 3 days, then cell cycle phases were analyzed by PI analysis. As expected, a significant proportion of cells apoptosed upon CR8 treatment (Fig. 4B-left), supporting data in Fig. 2A. Within the live cells, there was a significant elevation of cells in the G_1 phase of the cell cycle upon CR8 treatment, with a corresponding decrease in $S/G_2/M$ compared with untreated cells, indicative of CR8 inducing a G_1 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 co-cultured with CD154L/IL4 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 co-cultured with CD154L/IL4 for 9 days prior to treatment with CR8. A significant increase in the percentage of early apoptotic cells (Annexin V^+ 7-AAD^-) was observed, with the $CFSE^{lo}$ population being preferentially targeted, demonstrating that CR8 induced apoptosis within actively proliferating CLL cells (Fig. 5B). This was supported by an enrichment of $CFSE^{hi}$ CLL cells (non-dividing/slow-dividing cells) after treatment with CR8 for 72 hr (Fig. 5C). Moreover, absolute cell numbers decreased in all dividing CLL populations upon treatment with ≥ 300 nM 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 demonstrate 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 ~IC_{50} concentrations of CR8 (100 nM), indicating that higher concentrations of CDK inhibitors may be required *in vivo* to gain clinical benefit in pre-treated 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 CLL patients, possibly because the trial consisted of a heavily pre-treated patient cohort (29).

CR8 exhibited only a modest improvement as a CDK inhibitor compared with roscovitine, despite an enhanced ability to induce CLL apoptosis (19, 30), while 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 while purine CDK inhibitors have little effect (IC_{50} values flavopiridol, SNS-032, roscovitine, CR8: 0.45, 0.02, 130, >30 μM, respectively (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 (IC_{50} = 246.9 vs. 146.7 nM 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 tumour 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 CLL patients exhibiting progressive disease, whilst 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/IL4, indicating that CDK9 targeting may be sufficient to induce CLL cell apoptosis in the LN microenvironment (37). As shown previously, Mcl-1 expression may also be regulated at the post-transcriptional level, due to the abrogation of Mcl-1 protein expression (16, 30, 37). Mcl-1 proteasomal degradation, coupled with maintenance or upregulation of pro-apoptotic, Mcl-1 binding partner Noxa upon treatment of cells with CR8, roscovitine or PHA767491 can drive cells towards 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 demonstrate 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/IL4-stimulated IKK phosphorylation of RelA and IκBα, leading to inhibition of RelA activity. Repression of NF-κB signaling by CDK inhibitors has not previously been demonstrated in CLL cells, however studies demonstrate flavopiridol and roscovitine can inhibit NF-κB signaling in other cell types through inhibition of IKK activity

(40, 41). Interestingly, the mechanism utilised 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 anti-proliferative 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 CLL patients exhibiting progressive disease. Recent reports from Phase II clinical trials using flavopiridol demonstrate 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 to induce cell cycle arrest and cell death, thus possessing the potential to impede progressive CLL, and suggesting that significant reduction in tumour load to below the level of MRD detection may be achievable.

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Figure 1. CR8 exhibits 100-fold increased potency over roscovitine. Cells were incubated with drug or left untreated. Apoptosis was analyzed by flow cytometry using Annexin V/7-AAD. A. CLL cell viability at 24 hr incubation (n=8); B. T and B lymphocytes from freshly-isolated PBMCs of healthy donors (n=5) were identified as $CD3^+$ and $CD19^+$ respectively. Apoptosis was analyzed after 24 hr drug treatment; C. CLL cells (n=29) were treated with increasing concentrations of CR8 (left). Assessment of apoptosis in individual CLL samples treated with 100 nM CR8 (right). p value generated by a paired *t* test; D. CLL viability in patient samples from distinct prognostic CLL subgroups were incubated with 100 nM CR8 for 24 hr or left untreated. Results show percentage reduction in cell viability from untreated control and p values generated by an unpaired *t* test; E: CLL (n=3) viability was analyzed; F. CLL cells were treated with drug in the presence of FBS (solid lines) or NHS (dashed lines) for 48 hr. Apoptosis was analyzed. All results are percentage viability relative to control ± SEM unless otherwise stated.

Figure 2. CR8 overcomes pro-survival signals to induce apoptosis in CLL cells co-cultured on NT-L, targeting CDK substrates. CLL cells were cultured in medium alone (plastic), NT-L or CD154L/IL4 overnight, then treated with CR8 for 24 (left) or 48 hr (right), or left untreated. A. Percentage of viable cells was determined by excluding Annexin V^+/7-AAD^+ 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 blots shown from independent experiments carried on at least six individual patients. C. RNA/cDNA was prepared from cells treated with CR8 for 18 hr 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).

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/IL4 overnight, then treated with 300 nM CR8 or left untreated. A. RNA/cDNA was prepared from CLL cells treated with CR8 for 18 hr 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 hr 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 hr 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 CLL patients ± SEM. All p values generated by paired *t* test.

Figure 4. CR8 treatment inhibits the initiation of CLL cell proliferation upon co-culture with CD154L/IL4. A. CFSE-labeled CLL cells were co-cultured with CD154L/IL4 for 3 days before treatment with CR8. $CFSE^-$ unstained control (pale grey, unfilled), $CFSE^+$-colcemid non-proliferating control (pale grey, filled), $CFSE^+$-untreated (black), and $CFSE^+$-treated with 300 nM CR8 (dark grey, unfilled). At day 6, cells were analyzed by flow cytometry to determine CFSE MFI. Upper histogram shows an individual CLL sample. Lower graph shows the CFSE MFI of CLL samples at days 6, 9 and 12 of analysis after treatment with CR8 at day 3, as indicated. B-left - CLL cells were co-cultured with

CD154L/IL4 for 3 days and then treated with CR8. After 3 days (day 6), PI analysis was carried out to define the cell cycle phases. The data is an average of 6 individual CLL samples ± SEM. Right - The same PI analysis excluding the sub-G_1 apoptotic CLL cells. The data shown is an average of 6 individual CLL samples ± SEM and p values generated by a paired *t* test.

Figure 5. CR8 treatment preferentially targets proliferating CLL cells for apoptosis. CLL cells were co-cultured with CD154L/IL4 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 is an average percentage of the original input ($5x10^5$ 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 UT control ($CD19^+$Annexin V^+7-AAD^-) present in proliferating CLL cell populations (n=9 ± SEM); C. CFSE MFI of CLL post treatment (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).

CLL Sample I.D.	Age	Sex	Binet Stage	Treated	ZAP-70 Status *	FISH 11q/17p	% viability of control at 100 nM CR8
7	73	M	C	Y	Pos	11q-	108.75
8	59	F	A	N	Neg	11q-	65.92
14	62	M	C	Y	Pos	17p-	96.87
21	65	F	C	Y	Pos	ND	102.15
28	69	F	A	N	Pos	17p-	91.30
31	76	M	B	N	Pos	-	ND
34	64	M	B	Y	Pos	11q-	89.86
35	68	M	B	N	Pos	11q-	45.79
41	59	M	A	N	Neg	-	42.64
42	77	F	B	Y	Pos	-	62.32
44	63	F	A	N	Neg	-	24.16
45	78	M	B	Y	Pos	-	43.40
47	58	M	B	N	Neg	-	60.29
50	61	M	C	N	Pos	-	59.53
51	76	M	B	N	Neg	-	38.27
52	78	F	B	Y	Neg	11q-	21.16
54	56	F	A	N	Neg	-	29.66
60	59	F	A	N	Pos	-	58.90
64	64	M	C	Y	Neg	-	104.72
68	57	F	A	Y	Pos	-	56.40
69	43	M	A	N	Pos	-	ND
70	74	F	C	N	Neg	-	34.46
75	76	M	C	N	ND	ND	74.77
80	58	M	C	N	Neg	17p-	101.4
87	57	M	C	N	Neg	-	55.60
91	62	M	C	Y	Pos	11q-	81.80
92	74	M	C	Y	Pos	11q-	98.75
93	65	M	C	Y	Pos	17p-	97.70
94	68	M	B	N	Pos	11q-	65.71
98	68	M	B	N	Neg	17p-	82.08
102	71	F	C	Y	Pos	11q-	57.56

Table 1: Summary of the clinical CLL samples. FISH – fluorescence *in situ* hybridisation. "-" denotes samples that do not carry a 17p/11q cytogenetic abnormality. * ZAP-70 analysis was performed by immuno-histochemistry in our regional haematology laboratory. CLL cells were treated with 100 nM CR8 for 24 hr and the percentage of viable cells was determined by carrying out Annexin V/7-AAD flow cytometric analysis. Annexin V^- $7\text{-}AAD^-$ cells were considered viable. Results are shown as percentage viability relative to untreated control (100%). ND = not determined.

#	Kinase inhibitors	Cell death induction (IC_{50})	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	ML78	1.35	1.4	1.4
7	CR4	1.15	0.38	0.54
8	CR8 (R)	0.18	0.16	0.35
9	**CR8 (S)**	**0.09**	**0.14**	**0.21**
10	CR1	2.13	0.42	1.7
11	CR2	5.5	0.15	0.81
12	CR11	0.73	0.09	0.42
13	Ness2	1.26	0.28	1.4
14	Flavopiridol	0.140	0.042	0.003
15	SNS-032	0.064	0.022	0.0065

Table 2. Effects of CDK inhibitors on CLL survival and the catalytic activity of CDK2 and CDK9. 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 hr inhibitor treatment. The same compounds were tested on two purified CDKs. IC_{50} values, calculated from the dose-response curves, are reported in μM.

Figure 1 - Cosimo *et al.*,

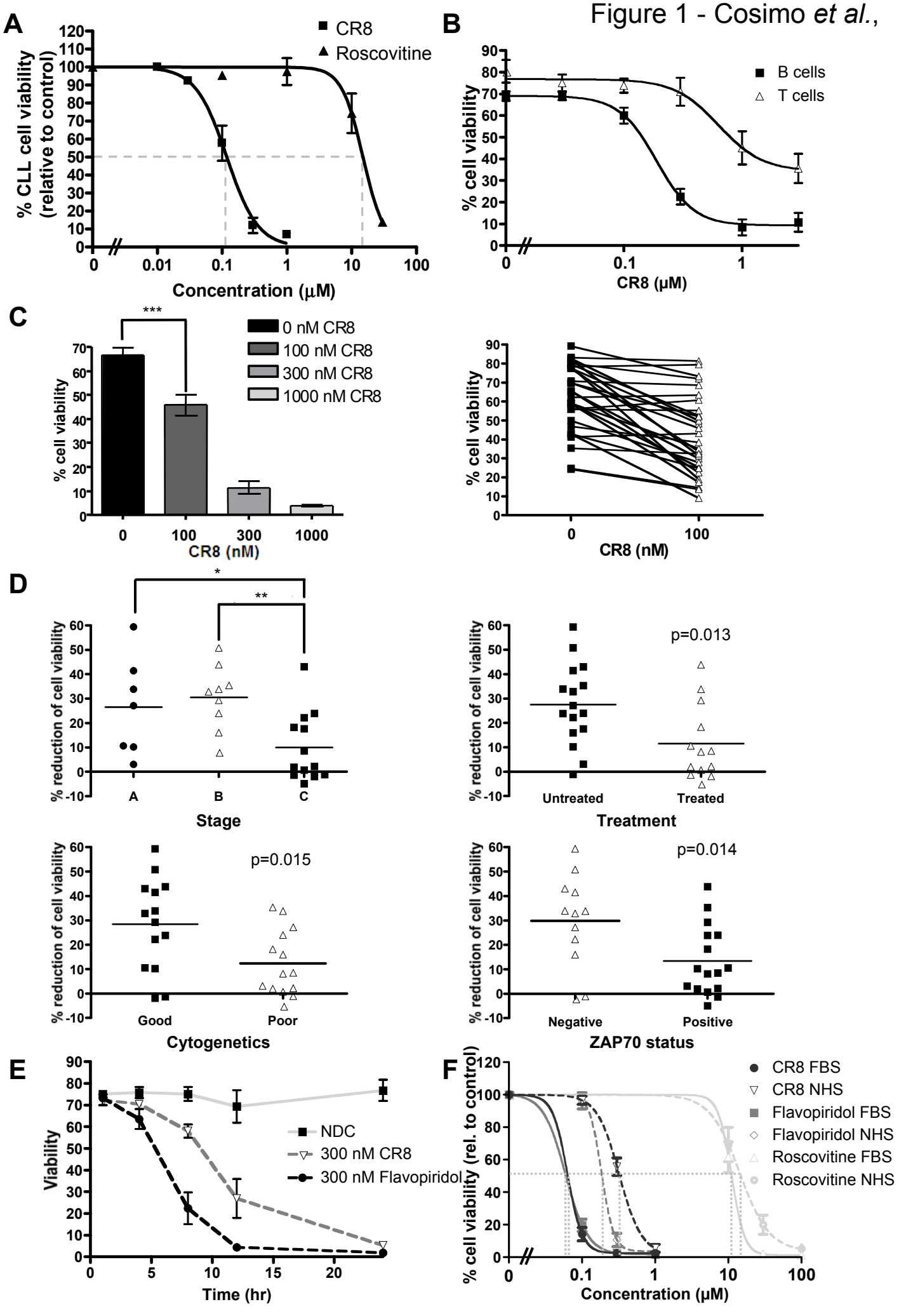
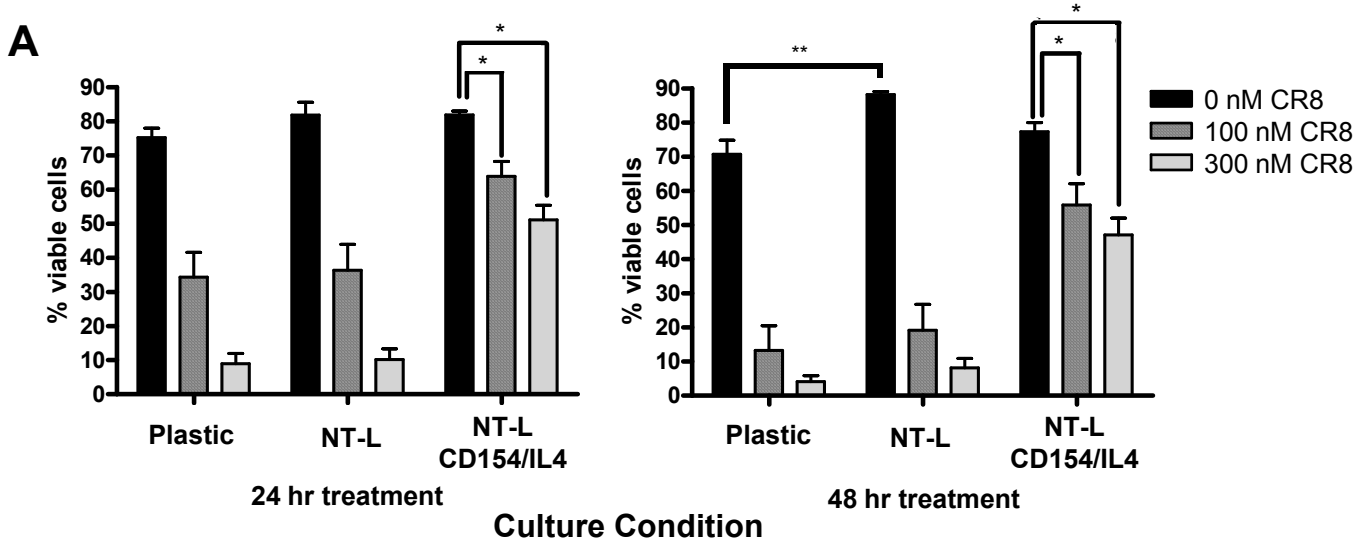
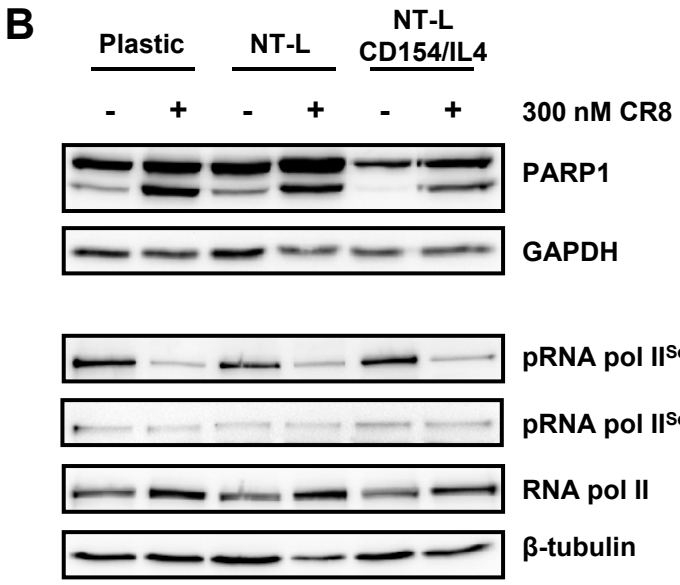
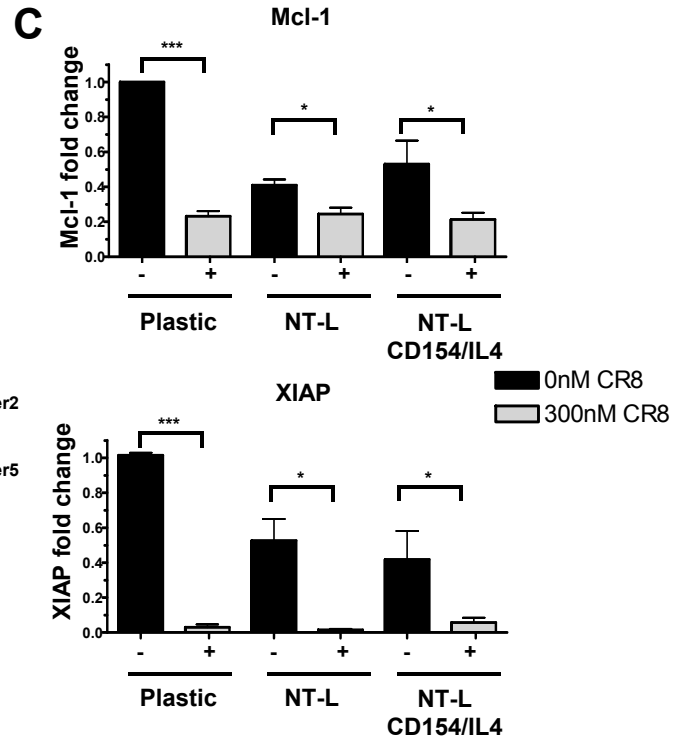


Figure 2 - Cosimo *et al.*,







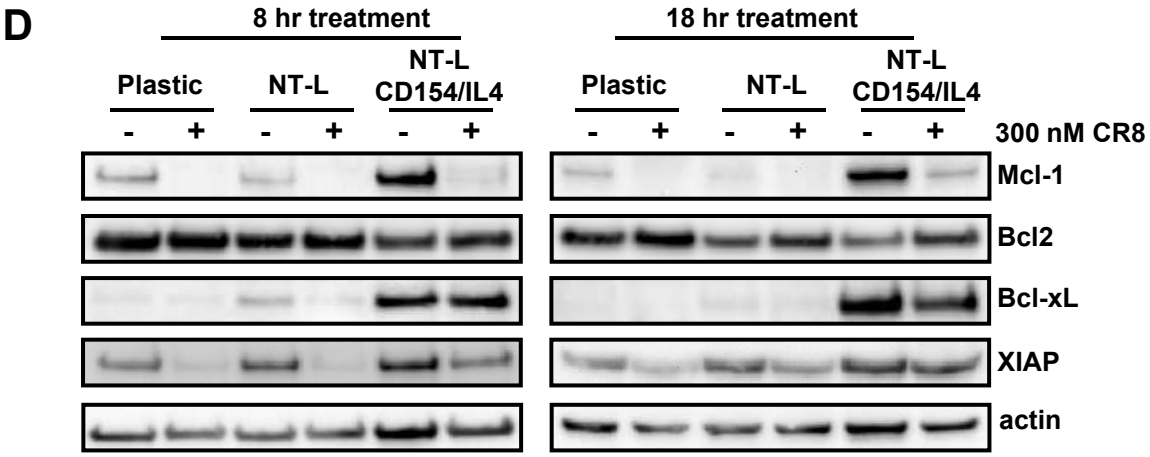


Figure 3 - Cosimo *et al.*,

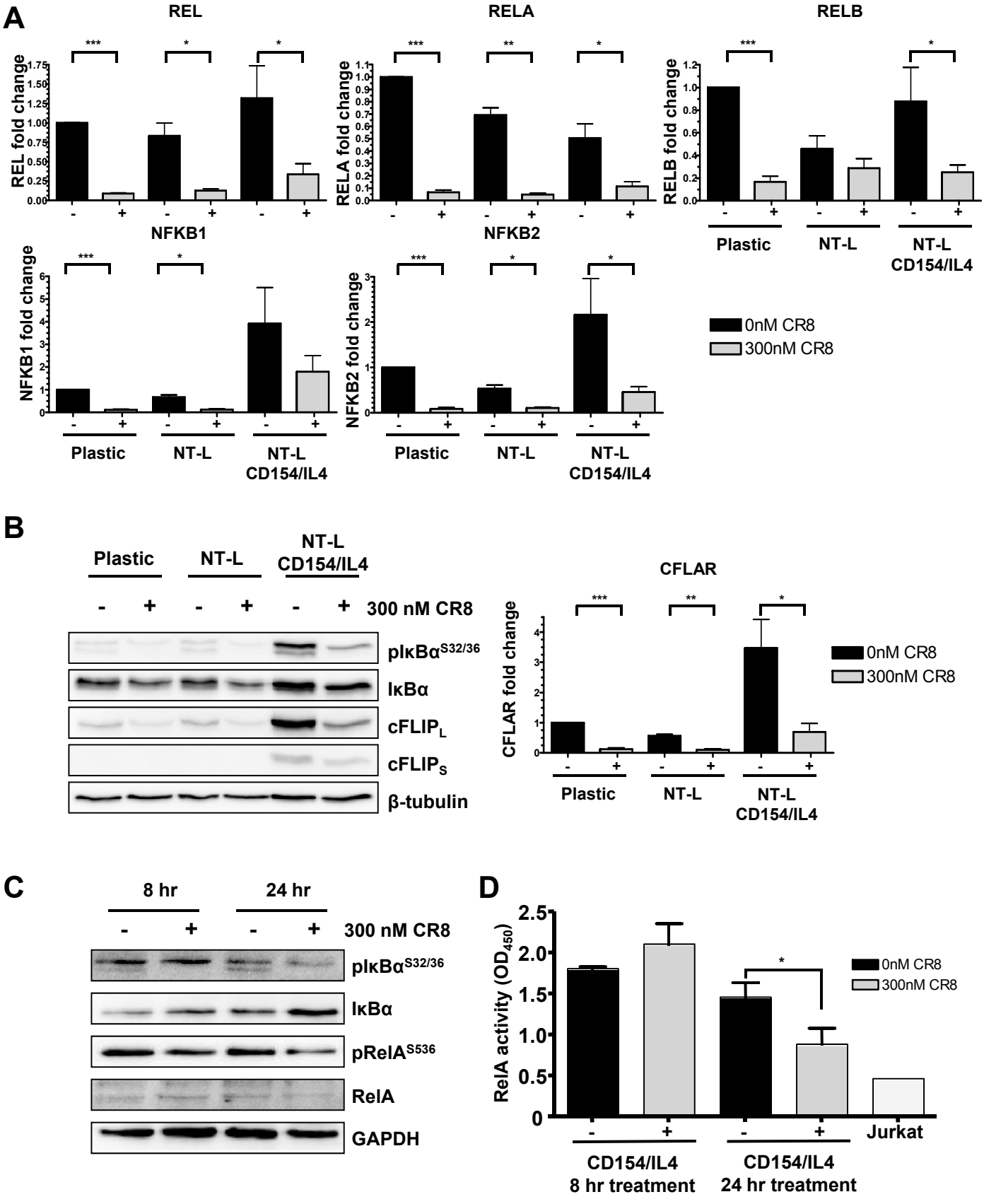


Figure 4 - Cosimo *et al.*,

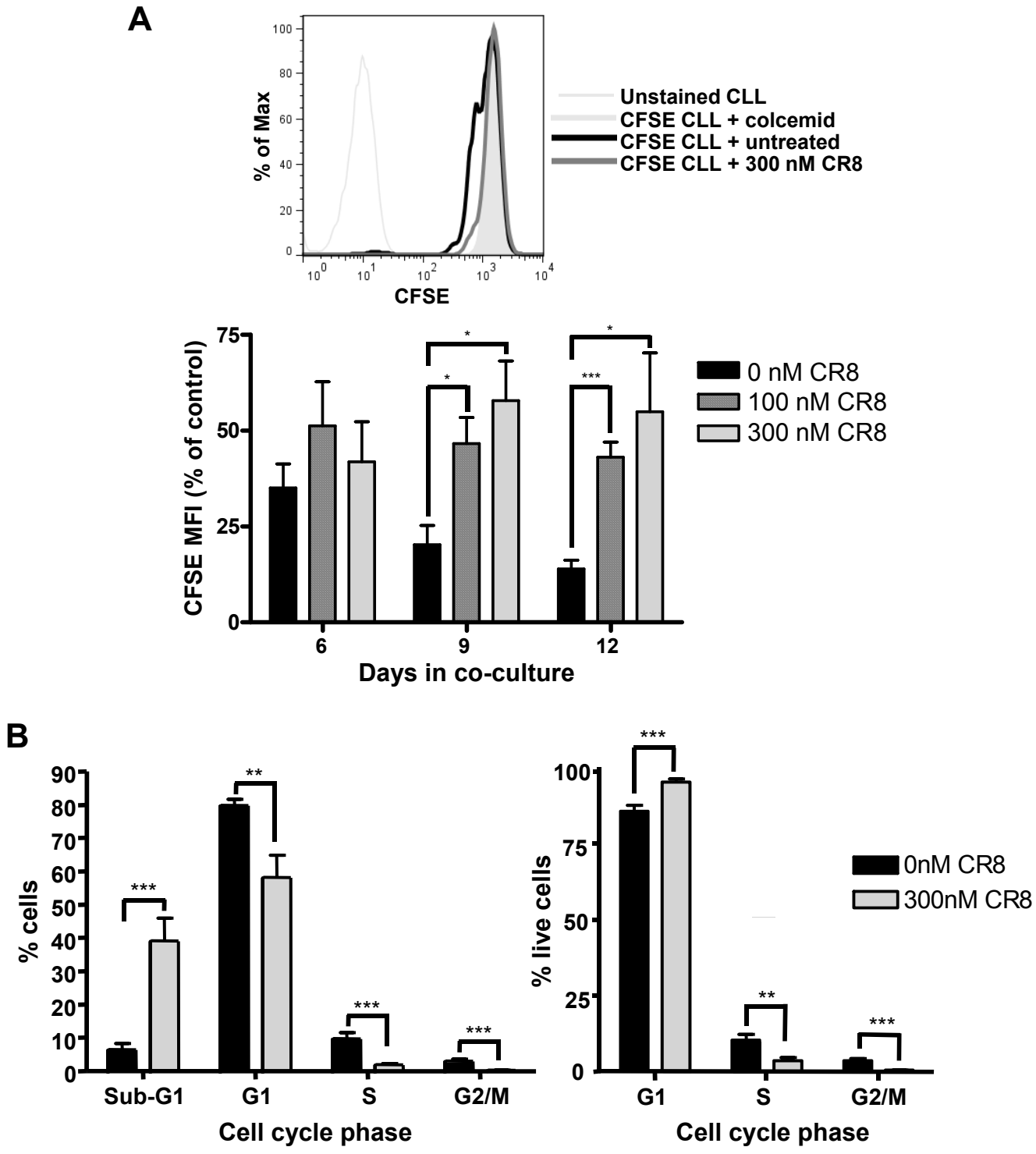


Figure 5 - Cosimo *et al.*,