Leflunomide induces apoptosis in Fludarabine-resistant and clinically refractory CLL cells

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STATEMENT OF TRANSLATIONAL RELEVANCE:

With this study we provide evidence that the drug Leflunomide may be a promising agent for overcoming Fludarabine resistance mediated by the lymph node environment, as well as intrinsic resistance due to defective p53 signalling in CLL cells. Since Leflunomide is already approved for clinical use in rheumatologic diseases and has a well-documented safety profile, it is available for immediate clinical studies involving CLL patients.
Abstract

Purpose: Environmental conditions in lymph node proliferation centres protect chronic lymphocytic leukaemia (CLL) cells from apoptotic triggers. This situation can be mimicked by in vitro stimulation with CD40 Ligand (CD40L) and IL-4. Our study investigates the impact of the drug Leflunomide to overcome apoptosis resistance of CLL cells.

Experimental design: CLL cells were stimulated with CD40L and IL-4 and treated with Fludarabine and the Leflunomide metabolite A771726.

Results: Resistance to Fludarabine-mediated apoptosis was induced by CD40 activation alone stimulating high levels of BCL-XL and MCL1 protein expression. Apoptosis resistance was further enhanced by a complementary JAK/STAT signal induced by IL-4. In contrast, CLL proliferation required both, a CD40 and a JAK/STAT signal and could be completely blocked by pan-JAK inhibition.

Leflunomide (A771726) antagonized CD40L/IL4-induced proliferation at very low concentrations (3 µg/ml) reported to inhibit dihydroorotate dehydrogenase. At a concentration of 10 µg/ml, A771726 additionally attenuated STAT3/6 phosphorylation, whereas apoptosis of CD40L/IL-4-activated (“resistant”) CLL cells was achieved with higher concentrations (IC50: 80 µg/ml). Apoptosis was also effectively induced by A771726 in clinically refractory CLL cells with and without a defective p53 pathway. Induction of apoptosis involved inhibition of NF-κB activity and loss of BCL-XL and MCL1 expression. In combination with Fludarabine, A771726 synergistically induced apoptosis (IC50: 56 µg/ml).

Conclusion: We thus demonstrate that A771726 overcomes CD40L/IL4-mediated resistance to Fludarabine in CLL cells of untreated as well as clinically refractory CLL cells. We present a possible novel therapeutic principle for attacking chemoresistant CLL cells.
Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease, which is still considered incurable with conventional chemotherapy. Although CLL has an indolent behaviour in the majority of cases, some patients show an aggressive course and die within few years from diagnosis (1). Clinically, this “poor-risk” CLL is characterized by resistance to chemotherapy including modern purine analogue-antibody combination regimens (2). Failure to respond to an induction therapy containing purine analogues (mostly Fludarabine) can hardly be overcome by escalating cytostatic drugs (3-5). Therefore, alternative therapeutic strategies such as Alemtuzumab (anti-CD52) (6), Ofatumumab (anti-CD20) (7), or allogeneic stem cell transplantation (8) are currently available therapeutic alternatives.

Predicting non-response is a major focus of current research and poor-risk patients could be identified who have a defective p53 pathway (9). However, only about 50% of Fludarabine-refractory patients carry a p53 mutation or 17p deletion (9) raising the question of alternative resistance mechanisms in p53 wild type patients.

It is furthermore well established that chemotherapy resistance of CLL cells may not only result from CLL cell specific intrinsic defects, but may also depend to a large degree on interactions with its microenvironment (10). Interactions between CLL cells and CD40L expressing T-cells (11, 12), bone marrow stromal cells (13), and nurse-like cells producing stromal cell derived factors (14) have been shown to increase the apoptotic threshold in CLL cells.

Although CLL has been considered to be a cumulative rather than a proliferative B cell neoplasm, CLL cells have a proliferation rate that is higher than previously recognized, particularly in lymphoid tissues (15, 16). Very recently Gine et al. have shown that higher numbers of proliferation centers in lymph nodes as well as higher numbers of mitoses or percentages of KI-67 positive cells are associated with a worse prognosis (16). These proliferation centres are composed of clusters of prolymphocytes and contain numerous T-cells which are predominantly CD4 positive (17). These T-cells supply signals through their CD40L receptor as well as cytokines such as Interleukin 4 (IL-4) that stimulate CLL cells to proliferate (18).

CD40 signalling has been recognized as a strong anti-apoptotic pathway mediating Fludarabine resistance in vitro (19). All CLL cells express CD40, and in vitro resistance can be induced in 100% of patients. CD40 signalling is induced upon binding to its ligand (CD40Ligand, CD40L, CD154), a 39 kDa type II transmembrane protein member of the TNF gene superfamily (20). CD40L is expressed on activated CD4+ T-cells and activated platelets, however, a wide variety of other cells including even malignant B-cells can also express the ligand (21, 22). Binding of CD40L forms a CD40-trimer that induces the recruitment of adaptor proteins known as TNFR-associated factors (TRAFs) to the cytoplasmatic domain of CD40. The TRAF proteins activate different signalling pathways including...
the canonical and the non-canonical NFκB signalling pathway, the mitogen activated protein kinase (MAPKs) pathway and phosphoinositol 3-kinase (PI3K) pathway (23). It was also shown that CD40 is able to associate with JAK3 leading to STAT3 activation in B-cells (24). Activation of these pathways induces expression of anti-apoptotic proteins such as BCL-XL and MCL1, both mediating resistance to purine analogues (11, 19, 25).

We have shown in antigen-presenting cells that the effects of CD40 signalling can be modulated by complementary JAK-STAT signals (26) which are triggered through cytokines such as interferon-γ and IL-4. IL-4 seems especially important since B-CLL cells have a constitutively high expression of the IL-4 receptor (27). IL-4 is a TH2-type cytokine that signals predominantly via JAK1 and JAK3 and subsequently induces phosphorylation and activation of STAT6 (28). STAT6 itself was shown to enhance BCL-XL expression which may contribute to the pro-survival effect of IL-4 (29). Furthermore, the JAK inhibitor PF-956980 has recently been shown to effectively overcome IL4 mediated resistance to Fludarabine (30). CLL cells, especially those located in lymphoid tissues, could thus be exposed to signals that drive proliferation and raise the apoptotic threshold. These dividing and resistant cells may represent the origin of relapse. New therapeutic strategies are warranted to overcome the protective effect of these signals. Candidate targets for interventions addressing Fludarabine resistance are therefore the JAK-STAT and NF-κB pathways as they represent key players in preventing apoptosis.

An agent known to interfere with JAK-STAT (31, 32) as well as NF-κB (33) and AKT1 (34) activation is A771726, the active metabolite of Leflunomide. This agent inhibits dihydroorotate-dehydrogenase (DHODH, EC 1.3.3.1), an enzyme involved in pyrimidine synthesis. As a consequence, T- and B-cell proliferation slows down (35). Leflunomide is orally available and approved for treatment of active rheumatoid and psoriatic arthritis (35). Following intestinal resorption, this pro-drug is converted into its active metabolite A771726 inside the gastrointestinal tract. Although a wide inter-patient variability was observed, serum concentrations of A771726 up to 176 µg/ml can be achieved with a 20 mg tablet per day (36). The mode(s) of action responsible for the clinical benefits remain controversially discussed (35).

The purpose of this study was to investigate whether the stimulating effects of CD40 signalling on proliferation and survival of CLL cells can be modulated by JAK/STAT signals and whether this breaks Fludarabine resistance. Secondly, we evaluated the capacity of the JAK/STAT and NF-κB inhibitor A771726 for treating resistant CLL cells. For this purpose we used a CD40L/IL4 culture system of primary CLL cells that mimics Fludarabine resistance. Our study demonstrates that A771726 is able to block proliferation and to induce apoptosis in resistant CLL cells that were activated with CD40L/IL4. The molecular basis for this beneficial action dose-dependently involves inhibition of DHODH, suppression of the JAK/STAT pathway and a block of NF-κB-dependent MCL-1 and BCL-XL expression.
Materials and methods

Patients
Peripheral blood (PB) samples were obtained from patients with CLL after informed consent in accordance with the Declaration of Helsinki. Patient sample collection was approved by the local ethics committee of the University Hospital of Heidelberg as part of the tumour bank of the National Centre for Tumour Diseases (NCT), Heidelberg, Germany. All CLL cases used in this study matched the standard diagnostic criteria for CLL. Blood samples of 28 different patients were used. Median age was 65 years (range: 46-89). 25 patients had not received any therapy at the time of sample collection. Eighteen of these 25 patients were Binet stage A, 5 of 25 patients Binet stage B and 2 of 25 patients Binet stage C. Seven patients had a normal karyotype, 14 patients had a deletion 13q14, 2 patients had a trisomy 12q13 and 2 patients had a deletion 11q22. Three patients had received multiple lines of chemotherapy and were clinically highly resistant to either Fludarabin and/or Bendamustin. Classical cytogenetic analysis revealed a 17p deletion in at least 80% of interphase nuclei. An additional p53 mutation was identified in 2 of these 3 refractory patients.

Cell culture
CLL cells were purified from ethylene-diamine-tetraacetate (EDTA) PB-samples of CLL patients by density-gradient separation and subsequent affinity-purification using the MACS CD19 isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). CLL cells were either frozen in fetal bovine serum (FBS, Biochrom AG, Berlin Germany) containing 10% Dimethylsulfoxid (DMSO) and stored in liquid nitrogen, or cultured at a concentration of 10^6/ml in DMEM supplemented with 10% FCS, 4 mM L-Glutamine, 4.5 g/l glucose, 100 μg/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37°C in a 10% CO₂ humidified incubator.

CLL cells were activated by an irradiated baby hamster kidney (BHK) cell line stably expressing CD40L (concentration BHK/CLL 1:20) and/or IL4 at (50 U/ml) (Friesoythe, Germany, Immunotools). Expression of CD40L was confirmed by flow cytometry using an anti-CD40L mAb (BD Pharmingen), whereas the mock-transfected control BHK cell line did not express CD40L.

The pan-JAK inhibitor I (Pyridone 6, 0.15 μg/ml) (Calbiochem, Merck, Darmstadt, Germany) and Fludarabine (2-Fluoroadenine-9-β-D-arabinofuranoside: 2, 10, 25 μM; Sigma, Germany) were added to the cell cultures 60 minutes prior to stimulation. The active metabolite of Leflunomide, A771726 (Calbiochem, Merck, Darmstadt, Germany) was added to the cell cultures 90 minutes prior to stimulation and titrated at a concentration range between 1 μg/ml to 120 μg/ml.
Western blot analysis

CLL cells were activated as indicated, harvested, washed, resuspended and lysed at a density of approximately 3x10^7 cells/mL in Western Blot Sample Buffer (50 mM Tris pH 7.5, 1% BriJ 96V, 10 mM NaF, 1 mM Na-orthovanadate, 1 µg/ml leupeptin, 1.5 µg/ml pepstatin, 100 mM PMSF) and snap frozen. Protein concentrations of the lysates were determined by a modified Bradford method (Bio-Rad Laboratories). Cell lysates were analyzed by SDS-PAGE and Western blots as described (37) using antibodies specific for pSTAT1 (tyr701), pSTAT3 (tyr705) (Cell Signaling), pSTAT6 (tyr641), total STAT3, BCL-XL, and Actin (Cell Signaling) and MCL1, BCL-2 (Santa-Cruz). For ECL based detection, Western blots were developed with ECL plus Western blot system (Santa Cruz Biotechnology) and the secondary HRP-conjugated antibodies goat-anti-rabbit IgG or goat-anti-mouse IgG were used (Santa Cruz Biotechnology). Western blots were quantified using the ImageJ software version 1.6.0.

ABCD analysis

Avidin-biotin-complex-DNA (ABCD) assays were carried out as described (38, 39). Proteins recognized by specific antibodies were detected with Western blots generated with X-ray films. NF-κB oligonucleotides for ABCD assays were: Bio 5′-GGAATTTCCCGGAATTTCCCGGAATTTCCG-GGAATTTCCC; 5′-GGAATTTCCCGGAATTTCCCGGAATTTCCG (tandem repeat NF-κB consensus site) or irrelevant biotinylated sequences (mutated NF-κB sequences from the FASL promoter).

Nucleofection of MEC1 cells with NFκB-EGFP reporter plasmid

A total 1–3x10^6 cells of the CLL model cell MEC1 were transfected with the NFκB-EGFP reporter plasmid (provided by Dr. S. Drube, University Clinic of Jena) using an Amaxa Human B cell Nucleofector Kit following the instructions of the manufacturer (Amaxa, Cologne, Germany) using the program U13. After nucleofection, cells were incubated in the presence of CD40L expressing BHK cells, IL4 (50 U/ml) and Urudine (75µM) with or without A771726 (80 µg/ml). Expression levels of EGFP were measured by FACS.

Flow cytometry

Detection of apoptosis

Apoptotic cell death was detected by flow cytometry using Annexin V and propidium iodide (PI) or 7-amino-actinomycin (7-AAD) staining. Cells were harvested and resuspended in Annexin V-binding buffer (10 mM HEPES (4-(2-hydroxyethyl)-1-piperaz ineethanesulfonic acid)/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) containing 10% Annexin V-FITC and 10% PI staining solution, or 10% Annexin V-PE and 10% 7-AAD staining solution (BD Biosciences, Heidelberg, Germany). After an
incubation time of 15 minutes at 4°C, stained cells were analyzed by flow cytometry, gating on lymphocytes. Double-negative cells were counted as viable cells. The results were confirmed on the basis of changes in forward light-scattering properties of dead cells that have decreased cell size.

**Staining for intracellular proteins**

Paraformaldehyde was added to the cell culture medium at a final concentration of 4% and incubated for 15 minutes at room temperature. Cells were then harvested, washed with PBS and permeabilized by dropwise addition of ice cold ethanol (80%) to the cell pellet. During addition of ethanol the sample was vigorously vortexed. Cells were stored at -20°C for at least 2 hours. For intracellular staining cells were washed twice with PBS containing 1 mM sodium azide and 1% bovine serum albumine (BSA) and then stained for BCL-XL (Santa Cruz, Heidelberg, Germany), and p-p65 (Ser529) (BD Biosciences, Heidelberg, Germany). Analyses were performed on a double laser (488 nm and 637 nm) 5-color FACScan flow cytometer (BD Biosciences, upgraded by Cytek, Fremont, CA). Living cells were selected on the basis of their side and forward light-scattering properties.

**Proliferation assay**

CLL cells were seeded at a concentration of $10^5$/well in 96-well plates in triplicates for 96 hours and stimulated as indicated. Cells were pulsed with 0.5 µCi (0.0185 MBq)/well [3H] thymidine (TdR; Hartmann Analytik, Braunschweig, Germany) for the last 16 hours of culture and harvested on a semiautomatic cell harvester (Tomtec, Hamden, CT). [3H]TdR incorporation was quantified in a TopCount Scintillation Counter (Perkin-Elmer, Waltham, MA).

**Real-Time RT-PCR quantification**

1 x10^6 cells were collected in 100 µL lysis buffer from the MagnaPure mRNA Isolation Kit I (Roche Applied Science, Mannheim, Germany) supplemented with 1% (w/v) DTT, and mRNA was isolated with the MagnaPure-LC device using the mRNA-I standard protocol. The elution volume was set to 50 µL. An aliquot of 8.2 µL RNA was reverse transcribed using AMV-RT and oligo-(dT) as primer (First Strand cDNA synthesis kit, Roche Applied Science) according to the manufacturer’s protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 µL and stored at –20°C until PCR analysis. Primer sets specific for the sequences of BCL-XL and MCL-1 optimized for the LightCycler (RAS) were developed and provided by SEARCH-LC GmbH, Heidelberg (www.search-lc.com). The PCR was performed with the LightCycler FastStart DNA SYBR Green I kit (RAS) according to the protocol provided in the parameter specific kits. To control for specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was
observed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR cycle number (CP) at which the detected fluorescence intensity reaches a fixed value. This approach dramatically reduced variations due to handling errors over several logarithmic dilution steps.

To correct for differences in the content of mRNA, the calculated copy numbers were normalized according to the average expression of two housekeeping genes, CD45 and β-Actin. Values were thus given as input adjusted copy number per µL of cDNA.

**Statistical analysis**

Differences of means were calculated using the students t-test or one way ANOVA analysis. Statistical analyses were performed with MedCalc (release 11.0; MedCalc Software bvba, Mariakerke, Belgium). IC50 values were calculated with GraphPad Prism Software (CA, 92037, USA).
Results

CD40 signals protect CLL cells from Fludarabine-induced apoptosis – synergy with JAK/STAT

CD40L stimulation of CLL cells is known to mediate resistance to purine analogues such as Fludarabine. In order to evaluate if resistance to the pro-apoptotic activities of Fludarabine can be modulated by co-stimulation of the CD40L/CD40 and IL-4/JAK-STAT pathways, CD19 purified CLL cells were cultured in the presence of CD40L and/or IL-4 with or without of Fludarabine (10 µg/ml). Percentages of viable cells were assessed by Annexin-V and 7-AAD FACS staining 96 hours after treatment. As shown before we could confirm that CD40L+IL-4 protected primary CLL cells from Fludarabine-induced apoptosis (Figure 1a). To assess the individual contribution of IL4- and CD40-ligation to this resistance effect we have stimulated in vitro Fludarabine treated patient samples with IL-4 and CD40L alone as well as in combination (Figure 1b). CD40L stimulation rescued approximately half of the cells from apoptosis induced by Fludarabine. In contrast, IL-4 alone was not sufficient to protect a significant number of CLL cells from apoptosis induced by this drug. However, adding IL-4 to CD40L stimulation significantly augmented the percentage of viable cells almost up to levels observed without Fludarabine. The additive effect of IL-4 could be reversed by inhibiting JAK-STAT activation with the established pan-JAK inhibitor Pyridone 6 as a positive control (Figure 1b). Pyridone 6 was used at a concentration of 0.15 µg/ml and did not augment apoptosis of CLL control cells (n=3).

The anti-apoptotic proteins BCL-XL and MCL1 are known to mediate Fludarabine resistance (11, 19, 25). Expression of BCL-XL and MCL1 was therefore measured in CLL cells by quantitative Western blotting. CD40L induced expression of BCL-XL and MCL1 (but not of BCL-2, n=3, data not shown). IL-4 alone induced MCL1 but did not cause accumulation of significant levels of BCL-XL (Figure 1c). Of note, combined IL-4 and CD40 ligation synergistically enhanced BCL-XL and MCL1 expression (Figure 1c). The enhancing effect of IL-4 on CD40L-induced BCL-XL and MCL1 expression was reversed by Pyridone 6 (Figure 1c). Of note, Fludarabine had no effect on BCL-XL and MCL1 expression in the presence of CD40L/IL-4 co-stimulation. Real-time PCR analyses revealed that up-regulation of BCL-XL and MCL1 protein levels appears due to a strong transcriptional up-regulation of BCL-XL and MCL1 mRNA levels in CLL cells (Figure 1d).

Our results demonstrate that JAK/STAT enhances CD40-induced BCL-XL and MCL1 protein expression levels, and this correlates with apoptosis rates measured by FACS as shown in Figure 1b.
The active metabolite A771726 of Leflunomide induces apoptosis in CD40L/IL4 activated Fludarabine-resistant CLL cells at clinically achievable concentrations

Because A771726 (the active metabolite of Leflunomide) is known to interfere with the JAK-STAT pathway (induced by IL4) and the NF-κB pathway (induced by CD40L) (31, 33), we have tested if A771726 is able to overcome CD40L-IL4 mediated Fludarabine resistance in CLL cells. Figure 2a shows three representative out of nine experiments performed with CLL samples from nine individual patients, illustrating that Fludarabine as well as A771726 induce apoptosis in unstimulated CLL cells. Simultaneous exposure to CD40L and IL4 reproducibly caused resistance to Fludarabine, but not to A771726 (Figure 2a). In order to determine the effective concentration range for the pro-apoptotic effect of A771726, CD40L/IL-4-activated CLL cells were incubated with various concentrations of A771726 yielding an IC50 of 80 µg/ml for A771726 alone, however, nearly all cells were apoptotic already at the next concentration step (100 µg/ml) (Figure 2b). In combination with Fludarabine, A771726 was significantly more effective than on its own in lower concentrations (IC50 for A771726 in combination with Fludarabine: 56 µg/ml) (Figure 2b). Induction of apoptosis by A771726 at 80 µg/mL correlated with strongly reduced BCL-XL and MCL1 mRNA expression levels, which translated into lower protein levels of these anti-apoptotic factors (Figures 2c, d). BCL-2 expression was lost in response to CD40L activation as shown by others (40), and no additional effects of A771726 were observed on BCL-2 expression (data not shown).

A771726 was previously shown to inhibit NF-κB (33). Accordingly, 80 µg/ml Leflunomide completely blocked phosphorylation of the NF-κB family member p65 (p-p65) (Figure 2e), diminished expression levels of its target genes BCL-XL and MCL1 and caused cell death (Figures 2a-c). In parallel, A771726 inhibited DNA-binding activity of the NF-κB transcription factors p65 (RELA) and RELB in Avidin-biotin-complex-DNA (ABCD) assays (Figure 2f). Finally, we transfected the model CLL cell line MEC1 with a NFκB-EGFP response plasmid and incubated these cells in the presence of CD40L-expressing BHK cells, IL4 with and without A771726. A771726 (80 µg/ml) was able to almost completely block NFκB dependent EGFP expression after 12 hours (Figure 2g). Therefore, we conclude that A771726 causes NFκB inhibition and apoptosis of CLL cells by concentrations of 80 µg/mL.

The active metabolite A771726 of Leflunomide induces apoptosis in clinically refractory CLL cells and CLL cells with a defective p53 pathway

Next, we evaluated if A771726 also induced apoptosis in CLL cells of clinically refractory patients. We incubated unstimulated and CD40L/IL4 stimulated CLL cells of nine patients who were clinically refractory to either Fludarabine and/or Bendamustine with A771726 in vitro. Representative Annexin-V/7-AAD FACS plots of three different patients are shown in Figure 3a. Refractoriness to
Fludarabine could be confirmed in vitro, whereas in contrast A771726 induced apoptosis in a dose dependent manner.

P53 alteration analyses revealed that six patients had 17p deletions in more than 80% of evaluated interphase nuclei. 4 of the 6 refractory patients harbored additional p53 mutations in the remaining alleles. A771726 also effectively induced apoptosis of CLL cells with p53-defective pathways (Figure 3b). In three out of the nine patients who have been clinically refractory according to iwCLL criteria (41), no p53 alteration could be detected. A771726 was equally an effective inducer of apoptosis in CLL cells of such patients (Figure 3c).

Refractory CLL cells were also simulated with CD40L and IL4 and incubated with Fludarabine or A771726. CD40L/IL4 stimulation protected CLL cells of clinically refractory patients from spontaneous apoptosis in vitro (Figure 3b, c). More importantly, A771726 effectively induced apoptosis in CD40/IL4 activated and clinically refractory CLL cells. In line with previous results BCL-XL expression was also inhibited in CLL cells of clinically refractory patients (Figure 3d).

**Proliferation of CLL cells requires complementary CD40 and JAK-STAT signals**

The proliferating compartment of CLL is located in lymphoid tissue (16), the anatomical site where CD40/IL4 stimulation of CLL cells in vivo appears most likely. CD40L and IL4 stimulation of CLL cells is an established model to mimic the microenvironment within lymph nodes.

To assess the individual contribution of CD40- and/or IL-4 ligation to induce proliferation of primary CLL cells, we stimulated CD19 purified CLL cells for 96 hours with CD40L and/or IL-4. Proliferation was quantified by thymidine incorporation in cultures of 6 different patients (Figure 4a). CD40L but not IL4 induced proliferation of CLL cells. In contrast to the CD40L-mediated anti-apoptotic effects, CD40L-induced proliferation could be completely blocked by Pyridone 6, a specific pan-JAK inhibitor (Figure 4a). Thymidine incorporation could be further enhanced by combining CD40L and IL-4. Proliferation of CLL cells induced by these stimuli was also inhibited effectively by Pyridone 6 (Figure 4a). Absolute counts of incorporated thymidine varied widely between individual cultures. Therefore we normalized counts to the protocol achieving maximal proliferation (CD40L+IL-4, range 11590-61373 cpm). Taken together, a complementary CD40 and JAK-STAT signalling is required for the induction of proliferation. Interfering with only one of the pathways could completely inhibit proliferation.

KI-67 staining measured by FACS analysis was used to verify proliferation induction by CD40L and IL-4 with or without JAK inhibition. KI-67 FACS plots of 3 representative patient (n=6) are shown in Figure 4b. Percentage of KI-67 positive cells induced by CD40L and IL-4 also varied between individual patients (median 8.5 %, range: 3.5 – 17.8%). Incubation with Pyridone 6 significantly reduced percentages of KI-67 positive cells (median: 0.6%, range: 0.1-1.5%, p=0.03). These
observations reveal that both, CD40 signalling and the JAK-STAT pathways, are critically involved in the CD40L inducible growth of primary CLL cells.

**The active metabolite A771726 of Leflunomide effectively blocks CD40L/IL-4-induced proliferation of CLL cells by two different mechanisms**

Leflunomide (A771726) is known to inhibit lymphocyte proliferation and is therefore used to treat rheumatologic diseases. The drug is well tolerated by patients for periods of several years. A long term exposure of CLL patients to A771726 could therefore be used to inhibit CLL cell proliferation and progression of the disease. Thus, we have tested if A771726 is able to inhibit CD40L/IL4 induced CLL cell proliferation already at lower concentrations not inducing apoptosis. We therefore co-stimulated CLL cells of six different patients with CD40L and IL-4 and added different concentrations of A771726 for 96 hours (*Figure 5a*). Proliferation of CLL cells was effectively blocked by 3 µg/ml of this drug. A771726 blocks dihydroorotate dehydrogenase generating orotate, which is metabolized to uridine triphosphate (UTP) in following steps. CTP-Synthase (EC 6.3.4.2) generates CTP from UTP. Thus, supplementing cells with uridine can antagonise biological effects of A771726 on DHODH. Indeed, addition of uridine (75 µM) restored proliferation of CLL cells incubated with 3 and 5 µg/ml of A771726. Thus, these cells are growth-arrested but not fully apoptotic.

At A771726 concentrations ≥10 µg/ml, cell growth could no longer be completely rescued by uridine, suggesting that alternative inhibitory mechanisms are operational. Accordingly, STAT3 and STAT6 phosphorylation was already inhibited by 10 µg/ml A771726, and higher concentrations of this agent (80 µg/ml) completely blocked phosphorylation of these transcription factors (*Figure 5b*). Similarly, the pan-JAK inhibitor Pyridone 6 induced growth arrest of CLL cells that could not be reversed by the nucleoside uridine (*Figure 5a*). In view of the results that CLL cell proliferation requires both, CD40 and JAK/STAT signalling, we conclude that inhibition of STAT3/6 phosphorylation represents a second inhibitory mechanism of A771726 on proliferation of CLL cells. Taken together, CLL cell proliferation is inhibited by A771726 at much lower concentrations than CD40L/IL4 mediated apoptosis resistance.
Discussion

CD40 signaling stays at the interface of various vital functions of CLL cells, such as proliferation, survival, and immunogenicity. Accordingly, numerous attempts are undertaken to use CD40 as therapeutic target for CLL. These strategies aim for apoptosis induction, opsonization, or immune modulation applying various biological strategies including antibody-based and gene-therapeutic approaches (42-44). However, none of these modalities has left the experimental stage to date.

Our group has recently reported that CD40 and cytokine-mediated JAK/STAT pathways provide strictly complementary signals for the induction of IL-12p70 in human dendritic cells (26). Despite the complexity of intracellular signalling networks, cells are likely to use and re-use functional motives in a modular way (45). We have therefore investigated if CD40 and JAK/STAT signals exert similar complementary effects in human CLL cells using proliferation and apoptosis resistance as functional endpoints. Indeed, we could show that IL-4 and CD40 had an additive anti-apoptotic effect rescuing more than 90% of CLL from undergoing Fludarabine-induced apoptosis after 96 hours. In contrast to CD40 ligation alone, which rescued approximately half of the CLL cells, IL-4 had no independent effect on Fludarabine resistance on its own.

The anti-apoptotic effect of CD40L relies on the induction of apoptosis inhibitors such as BCL-XL and MCL1 (11, 40). We show here that IL-4 in combination with CD40L augments BCL-XL and MCL1 levels in comparison to CD40L alone. In contrast, IL-4 alone, although inducing phospho-STAT6 and low MCL1 levels, did not alter BCL-XL expression nor enhance CLL cell survival in the context of Fludarabine.

Fludarabine mediates apoptosis of CLL cells via induction of the pro-apoptotic transcription factor p53 (46). However, CD40L inhibits the pro-apoptotic effects of this agent without reducing p53 levels (own unpublished results). As CLL cells resistant to Fludarabine express high levels of BCL-XL, direct interaction between BCL-XL and p53 might be a potential mechanism of CD40L-induced resistance to p53-mediated apoptosis (47). BCL-XL is an NF-κB target gene (48), and increased p65 phosphorylation coinciding with increased p65 DNA binding could indeed be shown by us in CD40L-stimulated cells.

In contrast to anti-apoptotic effects, stimulating induction of measurable in vitro growth of CLL cells strictly required the presence of both, CD40 and JAK/STAT signalling. IL4 alone or CD40L in the presence of a pan-JAK-inhibitor could not induce proliferation. This suggests that it should be possible to block CLL proliferation if only one of the two complementary signalling pathways is impeded. It is not clear which JAK/STAT family members are involved in the growth-promoting effects of IL-4 in CLL. As siRNA studies in human CLL cells are lacking, the problem cannot be finally resolved at the time being.
Given the synergistic interactions of NF-κB and JAK/STAT in CLL apoptosis and proliferation resistance, a drug that interferes with both signalling pathways represents a promising candidate to overcome survival signals provided by the microenvironment. We have therefore investigated the anti-rheumatic drug A771726 known to have inhibitory effects on T-lymphocytes, B-lymphocytes (35), CLL cells (49) and Myeloma cell lines (50). We could demonstrate that this drug induces apoptosis in CD40L/IL4–activated, resistant CLL cells. This apoptosis induction coincided with reduced BCL-XL and MCL1 expression. This seems especially important since MCL1 and BCL-XL expression levels have been associated with resistance to standard treatments such as Fludarabine (51) in vivo or novel approaches such as BH3 mimetics (ABT-737, ABT-263) (52). Most interestingly, we could also show that clinically refractory, p53 defective CLL cells remain sensitive to A771726, supporting a p53 independent mechanism of apoptosis induction by A771726.

Transcriptional regulation of BCL-XL is complex but has been shown to depend on NF-κB as well as STAT activity (48). Accordingly we could show that A771726 effectively reduces both, NF-κB activation as well as phosphorylation of STAT3 and STAT6. Such a finding is especially important since STAT3 and NF-κB collaborate in oncogenesis and apoptosis resistance (53). Moreover, CLL cells need NF-κB signalling for their survival and the NF-κB subunit p65/RelA was shown to be associated with CLL cell progression (54). Accordingly we could show that p65/RelA phosphorylation can be inhibited by A771726 and consequently does not bind to cognate DNA.

The concentration of A771726 required to induce apoptosis of CD40L/IL-4-activated CLL cells was significantly lower in the presence of Fludarabine (IC50 56 µg/ml), arguing for a synergistic effect of both drugs on CLL apoptosis. These concentrations are achievable by an oral intake of 20 mg A771726 per day in patients treated for active rheumatoid or psoriatic arthritis (36). A combination of methotrexate and Leflunomide (A771726) is already a clinical routine in rheumatology which suggests that clinical experience of combination treatment should rapidly become attainable.

The anti-proliferative effect of A771726 in vivo is primarily thought to be attributable to its ability to block the enzyme DHODH. This results in depletion of intracellular pyrimidines which can be reversed by addition of uridine. However, it has also been reported that A771726 can inhibit the JAK-STAT pathway induced by cytokines (31, 32, 55). Accordingly in our hands, this compound induced uridine-reversible inhibition of CD40L/IL4-stimulated CLL proliferation at low levels (3-5µg/ml) without interfering with STAT phosphorylation. At intermediate levels (>10 µg/ml), however, A771726 inhibited STAT3 and 6 phosphorylation. This coincided with a reduced sensitivity to uridine rescue. Therefore this agent affects proliferation of CLL cells by blocking DHODH at very low concentrations and by additionally inhibiting JAK/STAT at intermediate concentrations.

Our results advocate the exploration of Leflunomide (A771726) in patients with CLL. In comparison to other novel drugs currently being studied in this condition, this compound has remarkable advantages: On the one hand, the drug seems to be highly effective in the setting of Fludarabine
resistance, although this still has to be confirmed in vivo. On the other hand, translation into clinical application in the field of CLL appears to be readily achievable since the drug has already been approved for clinical use for over ten years. Leflunomide (A771726) is an oral drug that can be taken over prolonged periods of time. It is known that serum levels can differ strongly after intake of a 20 mg tablet a day. Therefore, monitoring serum levels will be important in order to achieve apoptosis-inducing concentrations in all patients. However, in a significant proportion of patients with rheumatologic diseases these dosages are well tolerated (36). In addition, lower concentrations should still be capable of inhibiting proliferation of CLL. Therefore, clinical evidence should quickly be attainable with this promising drug that inhibits both, proliferation and apoptosis-resistance by blocking JAK/STAT and NF-κB activation.

In conclusion, complementary JAK/STAT signalling is required for CD40-mediated proliferation of CLL cells and strongly augments CD40-mediated apoptosis resistance of CLL cells. Accordingly, pan-JAK inhibition can block proliferation and reduces the apoptosis resistance of CLL cells conditioned with CD40L and IL-4. However, a much stronger abrogation of chemoresistance can be achieved with therapeutic doses of the clinically approved DHODH blocker Leflunomide (A771726). This drug affects both, JAK-STAT and NF-κB signalling, resulting in reduced BCL-XL and MCL1 expression. In particular the potent inhibition of CD40L-induced Fludarabine resistance raises hopes that Leflunomide (A771726) might set the stage for a novel therapeutic principle complementing our growing armature against chemotherapy resistant malignancies.

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References


Figure legends

Figure 1: CD40L and JAK-STAT signals synergize to induce resistance to Fludarabine in primary CLL cells.

(A) Apoptosis of CLL cells quantified by AnnexinV and 7-AAD staining. Cells were activated with CD40L and IL4 with and without Fludarabine (10 µg/ml). Induction of apoptosis was quantified after 96 hours. Double negative cells were considered as viable. FACS plots of 3 representative patients (i, ii, iii ) out of 11 are shown.

(B) Mean percentages of viable CLL cells after activation with CD40L/IL4 with and without Pyridone 6 (0.15 µg/ml) and/or Fludarabine (10 µg/ml). Activation by CD40L but not by IL4 alone protects CLL cells from apoptosis induced by Fludarabine. Combined stimulation of CLL cells with CD40L and IL4 significantly increased survival. Means±SEM of 11 individual patients are shown, ** p<0.01.

(C) Western blot analysis of BCL-XL, MCL1, in CLL cells of 4 different patients (i, ii, iii, iv) activated for 48 hour with 1) Control, 2) IL4, 3) IL4+Pyridone 6, 4) CD40L, 5) CD40L+Pyridone 6, 6) CD40L+IL4, 7) CD40L+IL4+Pyridone 6, 8) CD40L+IL4+Fludarabine. Expression levels of BCL-XL and MCL-1 were quantified and normalized to Actin and their maximal expression after activation with CD40L and IL4, * p<0.05.

(D) 1x10^6 CLL cells were activated with IL4, CD40L, CD40L/IL4 and CD40L/IL4/Pyridone 6. mRNA levels of BCL-XL and MCL1 were quantified by real time PCR and normalized to mRNA levels of CD45. Means±SEM of 4 individual patients are shown, * p<0.05.

Figure 2: A771726 induces apoptosis in Fludarabine resistant CLL cells

(A) Apoptosis of untreated and CD40L/IL4 activated CLL cells quantified by AnnexinV and 7-AAD staining. Cells were incubated with or without Fludarabine (10 µM) or A771726 (80 µg/ml) for 96 hours. Three representative patients (i, ii, iii) of 9 are shown.

(B) Mean percentages±SEM of viable CLL cells after activation with CD40L/IL4 and exposure to different concentrations of A771726 (0-120 µg/ml). Means±SEM of 9 patients are shown. The IC50 for single agent A771726 was 80 µg/ml. The IC50 for A771726 in combination with Fludarabine was 56 µg/ml.

(C) Western blot analysis of BCL-XL and MCL1 expression in CLL cells activated with CD40L/IL4 and different concentrations of A771726. A771726 strongly reduces expression of BCL-XL and MCL-1. Western lysates were prepared after 48h, when BCL-XL and MCL-1 expression was maximally induced and significant numbers of cells treated with A771726 were still alive. Pyridone 6 partially inhibits BCL-XL and MCL1 expression induced by CD40L/IL4.
(D) 1x10^6 CLL cells were activated with CD40L/IL4 with and without A771726 (80µg/ml). mRNA levels of BCL-XL and MCL1 were quantified by real time PCR and normalized to mRNA levels of CD45. Means±SEM of 8 individual patients are shown, * p<0.05.

(E) CLL cells were activated with CD40L/IL4 with and without A771726. After 48 hours, cells were harvested, fixed with formaldehyde, permeabilized and stained for phospho-p65. Experiments of 4 different patients (i, ii, iii, iv) are shown. Based on forward and side light-scattering properties of CLL lymphocytes only living cells were evaluated. Higher concentrations of A771726 (80 µg/ml) blocked phosphorylation of RELA (p65).

(F) A771726 inhibits p65 and RELB DNA-binding. Avidin-biotin-complex-DNA (ABCD)-assay was used to visualize the binding of NF-κB p65 and RELB to a kappa-B consensus site. CD40L-induced p65 and RELB DNA binding were reduced after A771726 treatment. One representative out of 2 experiments is shown. 3 of 5 individual patients (i, ii, iii) are shown.

(F) A771726 inhibits NF-κB dependent EGFP expression in MEC1 cells. MEC1 cells were transfected with an NFκB-dependent EGFP plasmid and incubated with IL4, CD40L and uridine (75µM) - with and without A771726 (80 µg/ml). EGFP-expression was quantified by FACS. One representative out of 2 experiments is shown.

Figure 3: The active metabolite A771726 of Leflunomide induces apoptosis in clinically refractory CLL cells with defective p53 pathway

(A) Apoptosis of clinically refractory CLL cells were quantified by AnnexinV and 7-AAD staining. Cells were incubated with or without Fludarabine (10 µM) or A771726 (40, 80, 120 µg/ml). 3 representative patients of 9 (i, ii, iii) are shown.

(B) Viability of p53 defective CLL cells that were unstimulated as well as CD40L/IL4 activated and exposed to different concentrations of A771726 (0-120 µg/ml). Means±SEM of six patients are shown.

(C) Viability of refractory CLL patients without p53 alterations that were unstimulated as well as CD40L/IL4 activated and exposed to different concentrations of A771726 (0-120 µg/ml). Means±SEM of three patients are shown.

(D) Purified CLL cells of 2 clinically refractory CLL patients were activated with CD40L/IL4 in the presence of A771726 (80µg/ml). After 48 hours, cells were harvested, fixed with formaldehyde, permeabilized and stained for BCL-XL. Expression of BCL-XL was quantified by FACS.
Figure 4: Complementary CD40L and JAK-STAT signaling induces proliferation of B-CLL cells.

(A) Proliferation of B-CLL cells measured by \(^3\)H-thymidine incorporation. Cells were activated with CD40L and IL-4 with and without the pan-JAK inhibitor Pyridone 6 for 96 hours. \(^3\)H-thymidine was added for the last 16 hours. Each data set was normalized to its maximum count rate at stimulation with CD40L plus IL4. Means±SEM of 7 individual patients are shown; ** p<0.01.

(B) After activation with the indicated protocols for 96 hours, CLL cells were stained for KI-67 expression and DNA content and analysed by flow cytometry. FACS plots of 3 representative patients (i, ii, iii) out of 6 are shown. Please note that due to the fixation procedure all cells are 7-AAD-positive.

Figure 5: A771726 blocks proliferation induced by CD40L and IL-4 by two different mechanisms.

(A) Proliferation of CLL cells quantified by \(^3\)H-thymidine incorporation. Cells were activated by CD40L and IL4 and incubated for 96 hours with different A771726 concentrations. \(^3\)H-thymidine was added for the last 16 hours, counts were normalized to the CD40L plus IL4 protocol. Proliferation was effectively blocked by A771726 starting at a concentration of 3 µg/ml (black bars). Addition of uridine (75 µM) restored proliferation of CLL cells incubated with 3 and 5 µg/ml of A771726. At A771726-concentrations of ≥10 µg/ml, proliferation could not be completely restored by uridine. The inhibitory effect of Pyridone 6 could also not be overcome by uridine. Means of 7 individual patients are shown, * p>0.05.

(B) Western blot analyses of CLL cells activated with CD40L and IL4 for 48 hours with and without A771726. A771726 inhibits STAT phosphorylation at a minimal concentration of 10 µg/ml. Pyridone 6 (0.15 µg/ml) inhibits STAT phosphorylation. 3 representative western blots (i, ii, iii) out of 4 are shown.
Figure 1a

**Fludarabine 10µM**

### Untreated control

#### i)

![FL2-H](image1) ![FL3-H](image2)

**7-AAD**

Annexin-V

#### ii)

![FL2-H](image3) ![FL3-H](image4)

**7-AAD**

Annexin-V

#### iii)

![FL2-H](image5) ![FL3-H](image6)

**7-AAD**

Annexin-V

### Figure 1b

- **Control**
- **Fludarabine**
- **Fludarabine+Pyridone6**

![Graph](image7)
Figure 1c

i) ii) BCL-XL MCL-1 Actin

iii) iv) BCL-XL MCL-1 Actin

** relative BCL-XL/Actin

** relative MCL-1/Actin

![Bar chart showing relative expression levels of BCL-XL and MCL-1 compared to Actin in different conditions.](#)
Figure 1d
Figure 2a

i) Untreated control  Fludarabine  A771726

7-AAD  

CD40L + IL4

Annexin-V

ii) Untreated control  Fludarabine  A771726

7-AAD  

CD40L + IL4

Annexin-V

iii) Untreated control  Fludarabine  A771726

7-AAD  

CD40L + IL4

Annexin-V
**Figure 2b**

![Graph showing Annexin-V-7AAD negative cells against A771726 (µg/ml). The graph compares CD40L+IL4 and CD40L+IL4+Fludarabine (10µM).](image)

**Figure 2c**

![Image showing Western blot analysis against MCL-1, BCL-XL, and Actin with A771726 (µg/ml) and Pyridone-6 (µg/ml).](image)

**Figure 2d**

![Graphs showing mRNA expression of MCL1 and BCL-XL against Control, CD40L+IL4, CD40L+IL4+AT771726 for 8h and 24h.](image)
**Figure 2e**

- CD40L+IL4
- CD40L+IL4+A771726

**Figure 2f**

<table>
<thead>
<tr>
<th>FasL</th>
<th>NF-κB</th>
</tr>
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<tbody>
<tr>
<td>i)</td>
<td></td>
</tr>
<tr>
<td>ii)</td>
<td></td>
</tr>
<tr>
<td>iii)</td>
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</tbody>
</table>

- p-p65 (p-REL-A)

**Figure 2g**

- Kb-EGFP+ CD40+IL4
- Kb-EGFP+ CD40L+IL4+A771726

- 28.0%
- 2.2%
**Figure 3a**

<table>
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<tr>
<th>Untreated control</th>
<th>Fludarabine (10µM)</th>
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<tbody>
<tr>
<td>i)</td>
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<tr>
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<td>7-AAD</td>
<td>Annexin-V</td>
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<tr>
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<td>3% 11%</td>
<td>4% 19%</td>
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<td></td>
<td>10^4</td>
<td>10^4</td>
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<tr>
<td>ii)</td>
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<tr>
<td></td>
<td>10^4</td>
<td>10^4</td>
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<td>iii)</td>
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<td>10^4</td>
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**Figure 3b**

![Graph showing the percentage of Annexin-V/7-AAD negative cells for different conditions.]

- No activation
- CD40L+IL4

% of Annexin-V/7-AAD negative cells

- Control
- Flu
- 40
- 80
- 120

A771726 (µg/ml)
**Figure 3c**

![Bar chart](image)

% Annexin-V/7-AAD negative cells

- Control
- Flu
- 40
- 80
- 120

Legend:
- No activation
- CD40L+IL4

**Figure 3d**

![Histograms](image)

- Untreated control
- CD40L+IL4
- CD40L+IL4+A771726

**BCL-XL**

![Graphs](image)
Figure 4a

![Graph showing normalized [3H]-Tdr incorporation](image)

- **No Pyridone6**
- **Pyridone6 0.15 µg/ml**

![Bar chart](image)

Figure 4b

![Flow cytometry plots](image)

- **i)** 7-AAD vs. CD40L+IL4 vs. CD40L+IL4 + Pyridone6
- **ii)** KI-67 vs. CD40L+IL4 vs. CD40L+IL4 + Pyridone6
- **iii)** KI-67 vs. CD40L+IL4 vs. CD40L+IL4 + Pyridone6

![Graph showing percentage of KI-67 positive cells](image)
**Figure 5a**

![Graph showing % normalized [3H] TdR (cpm) against A771726 (μg/ml) and Pyridone 6 (μg/ml). The x-axis represents different concentrations of A771726 and Pyridone 6, while the y-axis shows % normalized [3H] TdR (cpm). The graph includes error bars indicating variability.](image)

**Figure 5b**

![Western blot images showing protein expressions of P-STAT3, STAT3, P-STAT6, STAT6, and Actin.](image)
Clinical Cancer Research

Leflunomide induces apoptosis in Fludarabine-resistant and clinically refractory CLL cells

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