Leflunomide Induces Apoptosis in Fludarabine-Resistant and Clinically Refractory CLL Cells

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Abstract

Purpose: Environmental conditions in lymph node proliferation centers protect chronic lymphocytic leukemia (CLL) cells from apoptotic triggers. This situation can be mimicked by in vitro stimulation with CD40 ligand (CD40L) and interleukin 4 (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 Janus-activated kinase (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/IL-4–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 show that A771726 overcomes CD40L/IL-4–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.

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Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease, which is still considered incurable with conventional chemotherapy. Although CLL has an indolent behavior 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–anti-

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body 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; ref. 6), ofatumumab (anti-CD20; ref. 7), or allogeneic stem cell transplantation (8) are currently available therapeutic alternatives.

Predicting nonresponse 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 CD40 ligand (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.
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 signaling in chronic lymphocytic leukemia (CLL) cells. Because 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.

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 and colleagues 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 centers 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 signaling 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 signaling is induced upon binding to its ligand (CD40L and 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 tumor necrosis factor receptor–associated factors (TRAF) to the cytoplasmatic domain of CD40. The TRAF proteins activate different signaling pathways including the canonical and the noncanonical NF-κB signaling pathway, the mitogen-activated protein kinase pathway, and phosphoinositide 3-kinase pathway (23). It was also shown that CD40 is able to associate with Janus-activated kinase (JAK3) leading to STAT3 activation in B cells (24). Activation of these pathways induces expression of antiapoptotic proteins such as BCL-XL and MCL-1, both mediating resistance to purine analogues (11, 19, 25).

We have shown in antigen-presenting cells that the effects of CD40 signaling 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 because 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 prosurvival 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 dihydorootate-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 prodrug is converted into its active metabolite A771726 inside the gastrointestinal tract. Although a wide interpatient 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 signaling on proliferation and survival of CLL cells can be modulated by JAK/STAT signals and whether this breaks fludarabine resistance. Second, 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/IL-4 culture system of primary CLL cells that mimics fludarabine resistance. Our study shows that A771726 is able to block proliferation and induce apoptosis in resistant CLL cells that were activated with CD40L/IL-4. 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 tumor 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. Nine patients had received multiple lines of chemotherapy and were clinically highly resistant to either fludarabine and/or bendamustine. Classical cytogenetic analysis revealed a 17p deletion in at least 80% of interphase nuclei in 6 patients. An additional p53 mutation was identified in 4 of 6 patients.

**Cell culture**

CLL cells were purified from EDTA PB-samples of CLL patients by density-gradient separation and subsequent affinity purification with the MACS CD19 isolation Kit (Miltenyi Biotech). CLL cells were either frozen in FBS (Biochrom AG) containing 10% dimethyl sulfoxide and stored in liquid nitrogen, or cultured at a concentration of 10^6 to 3 x 10^6 cells per milliliter in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, 4 mmol/L 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 IL-4 at (50 U/mL; Sigma), or cultured in liquid nitrogen, or cultured at a concentration of 10^6 per milliliter in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, 4 mmol/L 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.

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**Western blot analysis**

CLL cells were activated as indicated, harvested, washed, resuspended, and lysed at a density of approximately 3 x 10^7 cells per milliliter in Western Blot Sample Buffer (50 mmol/L Tris, pH 7.5, 1% Brij 960, 10 mmol/L NaF, 1 mmol/L Na-orthovananadate, 1 μg/mL leupeptin, 1.5 μg/mL pepstatin, 100 mmol/L phenylmethanesulfonylfluoride) 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) with 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 Biotechnology). For enhanced chemiluminescence (ECL)-based detection, Western blots were developed with ECL plus Western blot system (Santa Cruz Biotechnology) and the secondary horseradish peroxidase–conjugated antibodies goat anti-rabbit IgG or goat anti-mouse IgG were used (Santa Cruz Biotechnology). Western blots were quantified by the ImageJ software (version 1.6.0).

**Avidin–biotin complex DNA 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-kB oligonucleotides for ABCD assays were Bio 5'-GGAAATTCGGGAAATTCGGGAAATTCGG-CCAATTCGGAAATTCGGAAATTCGG-5' (tandem repeat NF-kB consensus site) or irrelevant biotinylated sequences (mutated NF-kB sequences from the Fasl promoter).

**Nucleofection of MEC1 cells with NF-κB-EGFP reporter plasmid**

A total 1 x 10^6 to 3 x 10^6 cells of the CLL model cell MEC1 were transfected with the NF-κB-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) with the program U13. After nucleofection, cells were incubated in the presence of CD40L-expressing BHK cells, IL-4 (50 U/mL), and uridine (75μmol/L) with or without A771726 (80 μg/mL). Expression levels of EGFP were measured by fluorescence-activated cell sorting (FACS).

**Flow cytometry**

**Detection of apoptosis.** Apoptotic cell death was detected by flow cytometry with Annexin V–propidium iodide (PI) or 7-amino-actinomycin (7-AAD) staining. Cells were harvested and resuspended in Annexin V–binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl and 2.5 mmol/L CaCl2) containing 10% Annexin V–fluorescein isothiocyanate and 10% PI staining solution, or 10% Annexin V–PE and 10% 7-AAD staining solution (BD Biosciences). 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 mmol/L sodium azide and 1% bovine serum albumin and then stained for BCL-XL (Santa Cruz), and p-p65 (Ser529; BD Biosciences). Analyses were conducted on a double laser (488 and 637 nm) 5-color FACScan flow cytometer (BD Biosciences; upgraded by Cyteck). 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 $10^5$ per well in 96-well plates in triplicates for 96 hours and stimulated as indicated. Cells were pulsed with 0.5 μCi (0.0185 MBq) per well [3H] thymidine (Tdr; Hartmann Analytik) for the last 16 hours of culture and harvested on an semiautomatic cell harvester (Tomtec). [3H]Tdr incorporation was quantified in a TopCount Scintillation Counter (Perkin-Elmer).

**Real-time reverse transcriptase PCR quantification**

A total of $1 \times 10^6$ cells were collected in 100 μL lysis buffer from the MagnaPure mRNA Isolation Kit I (Roche Applied Science) supplemented with 1% (w/v) dithiothreitol, 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 avian myeloblastosis virus reverse transcriptase 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 carried out with the LightCycler FastStart DNA SYBR Green I kit (RAS) 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.
Figure 1. (Continued) C, Western blot analysis of BCL-XL and MCL1 in CLL cells of 4 different patients (i, ii, iii, and iv) activated for 48 hours with (1) control, (2) IL-4, (3) IL-4 + pyridone 6, (4) CD40L, (5) CD40L + pyridone 6, (6) CD40L + IL-4, (7) CD40L + IL-4 + pyridone 6, (8) CD40L + IL-4 + fludarabine. Expression levels of BCL-XL and MCL1 were quantified and normalized to Actin and their maximal expression after activation with CD40L and IL-4; *, P < 0.05. D, a total of 1 x 10^6 CLL cells were activated with IL-4, CD40L, CD40L/IL-4, and CD40L/IL-4/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.
protocol provided in the parameter specific kits. To control for specificity of the amplification products, a melting curve analysis was conducted. No amplification of unspecific products was observed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of 4 different plasmids at log dilutions to the PCR cycle number at which the detected fluorescence intensity reaches a fixed value. This approach dramatically...

Figure 2. A771726 induces apoptosis in fludarabine-resistant CLL cells. A, apoptosis of untreated and CD40L/IL-4–activated CLL cells quantified by Annexin V and 7-AAD staining. Cells were incubated with or without fludarabine (10 μmol/L) or A771726 (80 μg/mL) for 96 hours. Three representative patients (i, ii, and iii) of 9 are shown.
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 2 housekeeping genes, CD45 and β-actin. Values were thus given as input adjusted copy number per microliters of cDNA.

Statistical analysis

Differences of means were calculated by the Student t test or one-way ANOVA analysis. Statistical analyses were conducted with MedCalc (release 11.0; MedCalc Software bvba). IC50 values were calculated with GraphPad Prism software.

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. To evaluate whether resistance to the proapoptotic activities of fludarabine can be modulated by costimulation 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 (Fig. 1A). To assess the individual contribution of IL-4- 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 (Fig. 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 (Fig. 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 antiapoptotic 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. The enhancing effect of IL-4 on CD40L-induced BCL-XL and MCL1 expression was reversed by pyridone 6 (Fig. 1B). Of note, fludarabine had no effect on BCL-XL and MCL1 expression in the

CD40L + IL-4

CD40L + IL-4 + A771726

p-p65 (p-REL-A)

FasL

NF-κB

p65/RelA

RelB

CD40L + IL4

A77 1726

Kb-EGFP + CD40 + IL-4

Kb-EGFP + CD40L + IL-4 + A771726

PSC

EGFP

Figure 2. (Continued) E, CLL cells were activated with CD40L/IL-4 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, and 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. ABCD assay was used to visualize the binding of NF-κB p65 and RELB to a κB consensus site. CD40L-induced p65 and RELB DNA binding were reduced after A771726 treatment. One representative out of 2 experiments is shown. Three of 5 individual patients (i, ii, and iii) are shown. G, A771726 inhibits NF-κB-dependent EGFP expression in MEC1 cells. MEC1 cells were transfected with an NF-κB-dependent EGFP plasmid and incubated with IL-4, CD40L, and uridine (75 μmol/L)—with and without A771726 (80 μg/mL). EGFP expression was quantified by FACS. One representative out of 2 experiments is shown.
presence of CD40L/IL-4 costimulation. Real-time PCR analyses revealed that upregulation of BCL-XL and MCL1 protein levels appears due to a strong transcriptional upregulation of BCL-XL and MCL1 mRNA levels in CLL cells (Fig. 1D).

Our results show 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 Fig. 1B.

**The active metabolite A771726 of leflunomide induces apoptosis in CD40L/IL-4–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 IL-4) and the NF-kB pathway (induced by CD40L; refs. 31, 33), we have tested whether A771726 is able to overcome CD40L/IL-4–mediated fludarabine resistance in CLL cells. Figure 2A shows 3 representative out of 9 experiments carried out with CLL samples from 9 individual patients, illustrating that fludarabine as well as A771726 induce apoptosis in unstimulated CLL cells. Simultaneous exposure to CD40L and IL-4 reproducibly caused resistance to fludarabine, but not to A771726 (Fig. 2A). To determine the effective concentration range for the proapoptotic effect of A771726, CD40L/IL-4–activated CLL cells were incubated with various concentrations of A771726 yielding an IC50 of 80 \( \mu \)g/ml for A771726 alone, however, nearly all cells were apoptotic already at the next concentration step (100 \( \mu \)g/ml; Fig. 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 \( \mu \)g/ml; Fig. 2B). Induction of apoptosis by A771726 at 80 \( \mu \)g/ml correlated with strongly reduced BCL-XL and MCL1 mRNA expression levels, which translated into lower protein levels of these antiapoptotic factors (Fig. 2C and 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-kB (33). Accordingly, 80 \( \mu \)g/ml leflunomide completely blocked phosphorylation of the NF-kB family member p65 (p-p65; Fig. 2E), diminished expression levels of its target genes BCL-XL and MCL1, and caused cell death (Fig. 2A–C). In parallel, A771726 inhibited DNA-binding activity of the NF-kB transactivation factors p65 (RELA) and RELB in ABCD assays (Fig. 2F). Finally, we transfected the model CLL cell line MEC1 with an NF-kB-EGF response plasmid and incubated these cells in the presence of CD40L-expressing BHK cells, IL-4 with and without A771726. A771726 (80 \( \mu \)g/ml) was able to almost completely block NF-kB–dependent EGF expression after 12 hours (Fig. 2G). Therefore, we conclude that A771726 causes NF-kB inhibition and apoptosis of CLL cells by concentrations of 80 \( \mu \)g/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 whether A771726 also induced apoptosis in CLL cells of clinically refractory patients. We incubated unstimulated and CD40L/IL-4–stimulated CLL cells of 9 patients who were clinically refractory to either fludarabine and/or bendamustine with A771726 in vitro. Representative Annexin V/7-AAD FACS plots of 3 different patients are shown in Fig. 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 6 patients had 17p deletions in more than 80% of evaluated interphase nuclei. Four 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 (Fig. 3B). In 3 out of the 9 patients who have been clinically refractory according to inCLL criteria (41), no p53 alteration could be detected. A771726 was equally an effective inducer of apoptosis in CLL cells of such patients (Fig. 3C).

Refractory CLL cells were also simulated with CD40L and IL-4 and incubated with fludarabine or A771726. CD40L/IL-4 stimulation protected CLL cells of clinically refractory patients from spontaneous apoptosis in vitro (Fig. 3B and C). More importantly, A771726 effectively induced apoptosis in CD40/IL-4 activated and clinically refractory CLL cells. In line with previous results, BCL-XL expression was also inhibited in CLL cells of clinically refractory patients (Fig. 3D).

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

The proliferating compartment of CLL is located in lymphoid tissue (16), the anatomic site where CD40/IL-4 stimulation of CLL cells in vivo appears most likely. CD40L and IL-4 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 (Fig. 4A). CD40L but not IL-4 induced proliferation of CLL cells. In contrast to the CD40L-mediated antiapoptotic effects, CD40L-induced proliferation could be completely blocked by pyridone 6, a specific pan-JAK inhibitor (Fig. 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 (Fig. 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: 11,590–61,373 cpm). Taken together, a complementary CD40 and JAK/STAT signaling is required for the induction of proliferation. Interfering with only one of the pathways could completely inhibit proliferation.
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 was quantified by Annexin V and 7-AAD staining. Cells were incubated with or without fludarabine (10 µmol/L) or A771726 (40, 80, and 120 µg/mL). Three representative patients of 9 (i, ii, and iii) are shown. B, viability of p53-defective CLL cells that were unstimulated as well as CD40L/IL-4 activated and exposed to different concentrations of A771726 (0–120 µg/mL). Means ± SEM of 6 patients are shown. C, viability of refractory CLL patients without p53 alterations that were unstimulated as well as CD40L/IL-4 activated and exposed to different concentrations of A771726 (0–120 µg/mL). Means ± SEM of 3 patients are shown. D, purified CLL cells of 2 clinically refractory CLL patients were activated with CD40L/IL-4 in the presence of A771726 (80 µg/mL). After 48 hours, cells were harvested, fixed with formaldehyde, permeabilized, and stained for BCL-XL. Expression of BCL-XL was quantified by FACS.

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 Fig. 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 signaling 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 whether A771726 is able to inhibit CD40L/IL-4-induced CLL cell proliferation already at lower concentrations not inducing apoptosis. We therefore costimulated CLL cells of 6 different patients with CD40L and IL-4 and added different concentrations of A771726 for 96 hours (Fig. 5A). Proliferation of CLL cells was effectively blocked by 3 \( \mu \)g/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 cytidine triphosphate from UTP. Thus, supplementing cells with uridine can antagonize biological effects of A771726 on DHODH. Indeed, addition of uridine (75 \( \mu \)mol/L) restored proliferation of CLL cells incubated with 3 and 5 \( \mu \)g/mL of A771726. Thus, these cells are growth arrested but not fully apoptotic.

At A771726 concentrations of 10 \( \mu \)g/mL or more, 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 \( \mu \)g/mL A771726, and higher concentrations of this agent (80 \( \mu \)g/mL) completely blocked phosphorylation of these transcription factors (Fig. 5B). Similarly, the pan-JAK inhibitor pyridone 6 induced growth arrest of CLL cells that could not be reversed by the nucleoside uridine (Fig. 5A). In view of the results that CLL cell proliferation requires both, CD40 and JAK/STAT signaling, 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/IL-4–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 signaling networks, cells are likely to use and reuse functional motives in a modular way (45). We have therefore investigated whether 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 antiapoptotic 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 antiapoptotic 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 with 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 proapoptotic transcription factor p53 (46). However, CD40L inhibits the proapoptotic 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 antiapoptotic effects, stimulating induction of measurable in vitro growth of CLL cells strictly required the presence of both CD40 and JAK/STAT signaling. IL-4 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 2 complementary signaling 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 signaling pathways represents a promising candidate to overcome survival signals provided by the microenvironment. We have therefore investigated the antirheumatic drug A771726 known to have inhibitory effects on T lymphocytes, B lymphocytes (35), CLL cells (49) and myeloma cell lines (50). We could show that this drug induces apoptosis in CD40L/IL-4–activated, resistant CLL cells. This apoptosis induction coincided with reduced BCL-XL and MCL1 expression. This seems especially important because 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 and ABT-263; ref. 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 because STAT3 and NF-κB collaborate in oncogenesis and apoptosis resistance (53). Moreover, CLL cells need NF-κB signaling 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 antiproliferative 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/IL-4–stimulated CLL proliferation at low levels (3–5 μg/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 with other novel drugs currently being studied in this condition, this compound has remarkable advantages: on 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 seems to be readily achievable because the drug has already been approved for clinical use for more than 10 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 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 signaling 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 signaling, 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 armament against chemotherapy resistant malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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