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A reversible carnitine palmitoyltransferase 1 (CPT1) inhibitor offsets chronic lymphocytic leukaemia cell proliferation

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Contents

Abstract

Crucial for chronic lymphocytic leukaemia (CLL) development and progression are the iterative cycles of cell re-activation and proliferation that take place in lymphoid tissues. These iterative cycles are fundamental for the development and the progression of the disease.

Since cellular fatty acid (FA) import and oxidation (FAO) were recently reported to be upregulated in CLL, compared to normal B lymphocytes, we explored the in vitro effects of ST1326, a reversible inhibitor of carnitine-palmitoyl transferase 1A (CPT1A), on leukaemic cells subject to activating microenvironment-mimicking stimuli.

ST1326 induced dose-dependent mitochondrial dysfunction and cell death, which were remarkably higher in activated/proliferating than quiescent CLL cells. Drug sensitivity was observed irrespective of the presence of *TP53* alterations or chromosomal abnormalities, which are known to cause chemoresistance in CLL patients.

The treatment of normal B lymphocytes with ST1326, at doses lethal to CLL cells, causes only a slight inhibition of cell activation/proliferation, indicating a modest cytostatic, not cytotoxic, effect.

ST1326 cytotoxicity in CLL was associated with decreased levels of intracellular Acetyl-CoA and down-regulation of signalling pathways that are crucial for leukaemic cell survival, activation and proliferation. In particular, environment-induced activation of STAT3 and STAT6 transcription factors, known to upregulate anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL, was impaired by ST1326. As a consequence, drug combination experiments with the BH3-mimetic ABT-199/Venetoclax, whose effects are counteracted by Mcl-1/Bcl-xL and cell proliferation, showed strong ST1326-mediated potentiation of ABT-199 cytotoxicity in activated/proliferating CLL cells. We also observed that ST1326 showed a synergic effect with Fludarabine in activated/proliferating CLL cells.

The data indicate that CLL cells turning to an activated/proliferating state become more dependent on FAO and more sensitive to FAO-antagonists, and pave the way for ST1326 as an adjuvant tool in anti-CLL drug-combination regimens with drugs that lose efficacy on proliferating leukaemic cells.

Introduction

Chronic lymphocytic leukaemia

Epidemiology and Incidence

Chronic lymphocytic leukaemia (CLL) is the most common B-cell malignancy in the Western world. It represents 22-30% of all leukaemia cases with a worldwide incidence between <1 and 5.5 per 100,000 people. In particular, Australia, the USA, Ireland and Italy have the highest CLL incidence rates (1).

The median age of diagnosis in the USA, Europe and Australia is approximately 70 years of age (1; 2).

Development of CLL is higher in white people than in black people and in males than in females, with an M/F sex ratio of at least 1.5 or higher (3). Additionally, males are more likely to have disease progression and require therapy (3; 4).

In Italy, the 2006 Report of the Italian Network of Cancer Registries provided evidence that, in the 1998-2002 period, CLL was the most frequently diagnosed leukaemia, representing about 33.5% of all leukaemia. It has been reported that the incidence of new cases of CLL is around 1600 for men and 1150 cases/year for women.

Diagnosis

In the presence of ≥ 5 000 monoclonal B-cells per μl in peripheral blood, over a period of more than 3 months, the diagnosis of CLL is commonly established by immunophenotyping. CLL cells co-express B and T cell surface antigens: CD19, CD20, CD23 and CD5 respectively. Furthermore, leukaemic cells derived from the same clone express the same immunoglobulin heavy chain, either kappa or lambda.

Recently, it has been confirmed that a panel of CD19, CD5, CD23, κ, λ and CD20 is sufficient for the diagnosis of CLL. In borderline cases, markers such as ROR1, CD79b, CD43, CD200, CD10 or CD81 could help the diagnosis (5).

In the presence of < 5 000 monoclonal B-cells per μl in the peripheral blood and no other features of lymphoproliferative disorder, the diagnosis is "monoclonal B-lymphocytosis" (MBL), a precursor state of chronic lymphocytic

leukaemia. Subjects with MBL, like CLL patients, show an increased risk of secondary cancers, in particular skin cancer (5).

CLL staging

The definition of the CLL stage is based on the natural history of the disease. According to the European Binet staging system (developed in 1981), CLL is divided into three stages (A, B and C) (6). This system is based on the number of areas involved. Patients in Binet stage A have 0 to 2 affected lymph node areas or organ enlargement with normal levels of haemoglobin and platelets. Binet stage B patients have peripheral and medullary lymphocytosis and 3 to 5 affected lymph node areas. Binet stage C patients have peripheral and medullary lymphocytosis with Hb <10 g/dl and/or thrombocytopenia (<100,000/mm^3).

In the USA, the most common clinical staging system used is the Rai staging system developed by Dr Kanti Rai in 1975 (7). This method is based on the concept that CLL is an accumulative pathology. The Rai staging system divides the CLL disease into five (0-IV) different stages that are described in the Kanti R. Rai et al. paper as follows:

- 0, lymphocytosis in blood as well as in bone marrow (absolute lymphocytes, 15,000/cu mm or more in blood, 40% or more lymphocytes in the marrow).
- I, lymphocytosis with enlarged lymph nodes.
- II, lymphocytosis with enlarged spleen or liver or both. Nodes may or may not be enlarged.
- III, lymphocytosis with anaemia. Nodes, spleen, or liver may or may not be enlarged.
- IV, lymphocytosis with thrombocytopenia. Anaemia and organomegaly may or may not be present.

Patients belonging to the stage Binet B or Rai stage I/II are considered as intermediate-risk groups while patients belonging to the stage Binet C or Rai stage III/IV are defined as being in the high-risk categories.

For the overall status of the patient and the disease progression these two staging systems are very helpful, but they are unfit for the prediction of disease evolution. On the contrary, molecular prognostic markers are more useful for predicting patient survival, disease progression and resistance to therapy.

Prognostic markers

In CLL, the most important prognostic factors are:

- genetic parameters, including the cytogenetic aberrations, mutational status of IGHV genes (8) and gene mutations, in particular TP53 (9);

- cell markers, including CD38 (10) and ZAP70 (11);

These parameters allow the stratification of the patients into different risk categories.

By using predominantly fluorescence in situ hybridization (FISH), different types of genomic aberrations have been identified.

The most common cytogenetic abnormality (in about 50% of CLL patients) is the deletion of the 13q14 region and subsequent loss of miRNAs (miR-15a and miR-16-1), which initiates leukaemogenesis. Usually, patients with this deletion have a good prognosis, even if, recently, it has been shown that a large deletion of 13q is associated with poor prognosis (12; 13). The other most common genetic abnormalities are represented by:

- the deletion of chromosome 11q22-q23, which is found in 10–20% of cases, causes the loss of the ataxia telangiectasia mutated (ATM) gene that encodes a DNA damage response kinase, ATM, that is very important in DNA damage detection and induction of cell cycle arrest. This alteration defines a subgroup of patients with unfavourable prognosis (13);

- trisomy 12, its prognostic relevance is still debate (14);

- deletion of chromosome 17p13 (del (17p13)), which occurs in 5–8% of cases, causes the loss of the tumour suppressor gene p53 (TP53) that is involved in the DNA damage detection pathway, cell cycle arrest, apoptosis and regulation of cellular metabolism (15). Commonly, this type of alteration is acquired at the late stages of the disease particularly after treatment. Deletion of 17p13 is associated with poor survival and high risk of resistance to chemotherapeutic agents (16). Therefore, patients that harbour this aberration are included in the highest risk prognostic category (17).

The somatic hypermutation status of the IGHV gene has a very important prognostic role.

B lymphocytes are known to recombine immunoglobulin variable (V), diversity (D), and junction (J) genes during their development, to create a unique antigen binding domain of the B cell receptor (BCR). In order to generate high-affinity antibodies, a second process namely somatic hypermutation occurs. This

process is triggered when the Ig binds the antigen and consists of the accumulation of point mutations in the V regions.

The presence or not of somatic mutations in the IGHV gene of leukemic cells divides CLL patients into two groups: unmutated CLL (U-CLL), with a germline identity ≥98%, who have an aggressive disease course, and mutated CLL (M-CLL) with a germline identity <98% who usually have a more indolent disease (8; 18).

Somatic mutations can occur in the tumour suppressor gene p53 leading to a deterioration in its function (9). It is well known that p53 exerts its tumour suppressive activities by inducing cell cycle arrest and apoptosis (9; 19). It has been reported that these functions are also related to the capability of p53 to regulate cellular metabolism and maintain cellular oxidative homeostasis (15; 20). In particular, p53 reduces lipid synthesis and promotes lipid degradation by inducing the expression of carnitine palmitoyltransferase 1 (CPT1) (20), the rate-limiting enzyme for the entry of fatty acids into mitochondria where lipid degradation occurs.

Patients harbouring these mutations are characterized by an aggressive disease course and resistance to chemotherapy and chemoimmunotherapy, as is well documented by a variety of studies (9; 16; 21), including prospective clinical trials (22; 23). It has been shown that in about 5% of untreated patients, TP53 mutations take place in the absence of del (17p13) while in about 70% of cases, TP53 mutations can occur together with del (17p13) leading to a double mechanism of inactivation (9). It has to be highlighted that TP53 represents an independent adverse prognostic marker for progression free-survival (PFS) and overall survival (OS) in CLL patients (9).

Recently, thanks to the use of next generation sequencing, new genomic abnormalities have been identified, for example i) activating mutations of *NOTCH1*, which occur in about 10% of CLL patients at diagnosis and are associated with poor prognosis, poorer responses to conventional chemoimmunotherapy and increase in the risk of transformation to diffuse large B-cell lymphoma (DLBCL) (24); and ii) dysfunctional mutations of Splicing factor 3b, subunit 1 (SF3B1), which occur in about 5%-15% of patients with CLL at diagnosis and are related to poor overall survival, faster disease progression and chemoresistance (25; 26).

CD38 is a transmembrane glycoprotein that is commonly expressed at high levels in plasma cells, germinal centres B cells, B cell precursors and, with low expression, on circulating B cells (27). The natural ligand of CD38 is CD31, an adhesion molecule expressed by different cell types in the CLL microenvironment, such as "nurse like cells" (NLCs) and T lymphocytes. The binding between CD38 and CD31 leads to survival and proliferation signals in leukaemic cells (27). CD38 is defined as a negative prognostic marker for patients with CLL (27). In the majority of studies, the threshold, that provides the best prognostic value, is $CD38^{+} \geq 30\%$ (10).

Like CD38, ZAP-70, a member of the syk tyrosine kinase family, is a negative prognostic marker for patients with CLL (11).

Biology of CLL and the microenvironment

CLL is characterized by the accumulation of CD5 and CD19 positive monoclonal B cells in the peripheral lymphoid organs, bone marrow and blood (28; 29). CLL cells are phenotypically similar to mature B cells that have encountered the antigen. Indeed, they express high levels of surface molecules (CD23, CD20 dim, CD69) and low levels of markers downregulated after cellular activation, such as CD22, receptor gamma Fc IIb and CD79b (29). Furthermore, the expression levels of surface immunoglobulin, CD20, CD79b and CD19 are usually low compared with normal B cells. Yet, they express a typical marker of the memory B-cell, CD27 (30) and also the gene expression profiles are similar.

In the past, CLL cells were considered to be quiescent apoptosis-resistant malignant B lymphocytes. This resistance was associated with the over-expression of the anti-apoptotic protein Bcl-2 (31). CLL was in fact described as a "pathology of accumulation", considering the defect in the apoptotic mechanism as one of the main causes of the disease. Over time, different studies on CLL biology revealed a new dynamic view of this disease (32). It became clear that CLL cells undergo, during their life, iterative cycles of re-activation and subsequent clonal expansion, before coming back to a quiescence state. By using a deuterium oxide 2H in vivo labelling method, in which patients consumed deuterated (heavy) water ($2H_2O$), it has been shown that the daily growth rate of CLL cells ranges from 0.08% to 1.7% (33). Furthermore, by using this same method, it has also been demonstrated that the lymph nodes are the anatomical site harbouring the largest fraction of newly born cells compared to peripheral

blood and bone marrow (34). This supports the concept that activation and clonal expansion occur in lymphoid proliferation centres within secondary lymphoid tissues, where multiple molecular interactions with antigen and microenvironment contribute to leukaemic B cell survival, chemoresistance and proliferation.

The tumour microenvironment (TME) is a very complex milieu in which signals, fundamental for the survival and accumulation of leukaemic cells, are delivered by soluble factors or direct cell contact. An important interaction that rescues CLL cells from apoptosis, both in vivo and in vitro, is represented by the binding between CD38, expressed on CLL cells, and its natural ligand CD31, expressed on NLCs (35) and T lymphocytes (29). Bone marrow stromal cells (BMSC) (36), the predominant stromal cell population in the CLL microenvironment, can also protect CLL cells from apoptosis and support their proliferation. Crucial for supporting growth and survival of leukaemic cells is the binding between CD40, expressed on CLL cells, and CD40 ligand (CD40L), expressed on activated CD4+ T helper lymphocytes (29; 37). In addition, T cells secrete different types of cytokines, including IL-4 and IL-21, that play an important role in supporting CLL cells proliferation (29; 37; 38).

Another soluble factor that might support the clone expansion is represented by CXCL12 (stromal cell-derived factor 1, SDF-1), a natural ligand of the chemokine receptor CXCR4 that is overexpressed on CLL cells. CXCL12 is secreted by NLCs and, after binding CXCR4, promotes the up-regulation of anti-apoptotic genes, like Bcl-2 and Mcl-1, favouring the survival of leukaemic cells (29). Also IL-6, produced by endothelial cells, inhibits apoptosis of B-CLL cells (39).

Taken together, these results indicate a dynamic picture where CLL cells undergo iterative cycles of re-activation and subsequent clonal expansion in the lymphoid microenvironment, which crucially contribute to CLL disease progression.

CLL treatment

Since CLL disease is still an incurable disease, its pharmacological treatment remains a subject of considerable interest and relevance. Patients with CLL have a variable disease course with a third of patients never needing treatment and, on the other hand, other patients that need treatment soon after diagnosis. The

criteria for initiating treatment in CLL patients depend on the symptoms caused by the disease and also on the Binet/Rai staging systems. In particular, patients with asymptomatic, early-stage disease (Rai 0; Binet A) are usually not treated but are followed on a "watch and wait" principle (6; 7; 13). On the contrary, patients with signs of active disease (i.e. rapidly progressive lymphadenopathy) or classified as Binet stage C or Rai stage III and IV, are subjected to treatment (6; 7; 13).

For several decades, the “gold standard” treatment for CLL was represented by the alkylating agent chlorambucil (40). It was discovered in the ‘90s that the combination of cyclophosphamide, another alkylating agent, and the purine analogues Fludarabine improved the quality and duration of response in younger patients (41).

Fludarabine (FAMP) is the most studied and effective purine analogue in B-CLL. The purine analogues act by mimicking the purine’s structure and after their incorporation into DNA or RNA, they inhibit the cellular replication and transcription. Fludarabine is also able to induce cell death in quiescent cells by the activation of the mitochondrial pathway of the apoptotic cascade (42). However, a considerable clinical problem is the development of chemoresistance. This might be due to genetic alterations, like MYC overexpression (43), del(17p) (16) and/or TP53mutations (16), but is also markedly influenced by the TME, which induces the expression of anti-apoptotic Bcl-2 family proteins (44), thus contributing to chemoresistance (45; 46). As an example, the Mcl-1/Bax ratio and also Bcl-2/Bax ratio was reported to correlate with chemoresistance to Fludarabine (45; 47). Also, the induction of both Bcl-xL and A1 in CLL was associated with chemoresistance to Fludarabine in preclinical models (48).

Drugs that target two fundamental kinases involved in the transduction of signals from the BCR, namely the Bruton’s tyrosine kinase (BTK) and the phosphatidylinositol 3-kinase (PI3K) δ isoform, have been developed in recent years.

The BTK pathway is amplified in CLL, where this kinase is constitutively phosphorylated, and leads to pro-survival signals and induction of proliferation by its effect on AKT (49), nuclear factor-KB (NF-KB) (50) and extracellular signal-regulated kinase (ERK) (51).

PI3K is a family of enzymes involved in crucial cellular activities such as proliferation, survival, metabolism, migration and genomic instability (52; 53).

PI3Kδ isoform expression is restricted to leukocytes and plays a central role in the survival of normal B cells. In CLL cells the PI3K pathway is constitutively activated and dependent on PI3Kδ (54). Therefore, these two kinases represent a very good target for the CLL disease. Ibrutinib (PCI-32765), a covalent inhibitor of BTK, and Idelalisib (GS-1101 or CAL-101), a selective and reversible inhibitor of PI3Kδ, were particularly successful among drugs developed for inactivating these kinases. They are able to reduce, both in vitro and in vivo, cell survival, migration and proliferation (54; 55; 56) of CLL cells and are currently FDA approved for patients with relapsed or refractory CLL and also in patients with TP53 aberrations (57; 58).

However, recent data report the development of CLL cells resistances to Ibrutinib (59; 60). Also, immune-mediated toxicity like neutropenia and sepsis, hepatotoxicity and pneumonitis have been reported in patients on Idelalisib (61).

Another drug that showed a remarkable clinical response in CLL, independently from negative prognostic markers like TP53 aberration (62), is the B-cell lymphoma 2 (Bcl-2) Homology 3 (BH3)-mimetic Venetoclax (ABT-199).

CLL cells rely on the activity of anti-apoptotic Bcl-2 family members for their survival (63). Therefore, strategies to restore apoptosis in CLL cells by antagonizing the anti-apoptotic proteins have led to the development of BH3 mimetics as therapeutic agents (64; 65; 66; 67; 68). BH3-mimetics are small molecules modelled on the BH3 domains of BH3-only members (66). They mimic their function by binding to anti-apoptotic Bcl-2 family proteins, thus leading to the inhibition of their activity. In this way, Bax and Bak are released, causing the trigger of apoptosis (63; 67).

The prototype of these small molecules was ABT-737, which mimics the pro-apoptotic protein Bad (68). Its oral derivative is Navitoclax (ABT-263) that binds, with high affinity, the anti-apoptotic proteins Bcl-xL, Bcl-W and Bcl-2 but not Mcl-1 (67). It has shown promising results in haematological malignancies, especially in CLL (69). However, ABT-263 provoked severe thrombocytopenia due to on-target toxicity on the Bcl-xL protein, which is a fundamental survival molecule for the platelets (67; 69; 70). In order to avoid this important side effect, the second-generation compound ABT199 (called also Venetoclax) was designed for specifically binding Bcl-2 (64; 65) sparing thus Bcl-xL and platelets. In particular, ABT199 shows a subnanomolar affinity for Bcl-2, no interaction with Mcl-1 and very weak affinity for BcL-xL and Bcl-W (64). This drug has been demonstrated to be efficacious in leukaemia/lymphoma Bcl-2 dependent

cell lines and also in tumour xenograft models (64). Its effect seems superior in CLL than in other lymphoid malignancies, although evidence in mantle cell lymphoma and lymphoplasmacytic lymphoma is encouraging (71). In recent times, a phase I (65) and a phase II (62) studies have proved that Venetoclax monotherapy is active also in patients with relapsed or refractory del(17p). However, the cytotoxic activity of this BH3-only mimetic is impaired when anti-apoptotic proteins, like Mcl-1 and Bcl-xL, are overexpressed (72; 73). Recently, Tahir S.K. et al. have shown that in leukaemic and lymphoma cell lines resistant to ABT199 there is an increased expression of the anti-apoptotic proteins Mcl-1 and Bcl-xL, which are not targeted by ABT199 (73). The work of Thijssen R. et al. analysed in CLL cells to what extent microenvironmental signals can alter sensitivity to ABT-199 (72). They reported that unstimulated CLL cells are highly sensitive to ABT-199 (LC50<1nM) rather than CD40 and CD40+IL-4 stimulated CLL cells that show to be fully resistant to 10 μM ABT-199. This might be due to the over-expression, in the proliferating CLL cells, of anti-apoptotic proteins that are not targeted by ABT199, like Mcl-1 and Bcl-xL (72).

Altogether, it appears that the microenvironment and subsequent CLL cells activation/proliferation reduce CLL cell sensitivity to Venetoclax.

Following the above considerations, new therapeutic and adjuvant strategies are needed.

In recent years, an emerging role of fatty acids (FAs) metabolism in cancer cells has been recognised. During tumour progression, lipid metabolism assumes important roles by providing energy for the proliferation and macromolecules for membrane synthesis. Therefore, targeting FAs metabolism in cancer cells, including CLL, may represent a novel therapeutic strategy.

Lipid metabolism and carnitine palmitoyltransferase 1A (CPT1A) inhibitor

CLL cell energetic metabolism is nurtured not only by glycolytic mechanisms but also, unlike normal B lymphocytes, by fatty acid oxidation (FAO; known also as β-oxidation) (74).

FAO consist of a cyclical series of reactions, in the mitochondrial matrix, in which FAs are repeatedly cleaved to produce Acetyl-CoA, which then enters the Krebs cycle, and reducing equivalents, NADH and $FADH_2$, which in turn enter

oxidative phosphorylation. Since FAO takes place in the mitochondrial matrix and FAs are negatively charged, a specialized carnitine carrier system is needed to allow the entry of long-chain FAs into the mitochondria. This carrier system is composed by CPT1, carnitine/acylcarnitine translocase (CACT) and CPT2, that work together in order to transport long-chain fatty acids into the mitochondria. First of all, FAs must be activated through two reactions catalyzed by the enzyme fatty Acyl-CoA synthetase. The Acyl-CoA obtained from these reactions can be now transported into the mitochondrial matrix where FAO occurs. The first actor and rate-limiting step for the entry of FAs into the mitochondria is represented by the enzyme CPT1, which is a protein of approximately 88 kDa tightly associated with the outer membrane of mitochondria. CPT1 acts by transferring acyl-group from coenzyme A onto carnitine; its activity is physiologically inhibited by Malonyl-CoA (75), a substrate for fatty acid synthesis.

Inhibition/depletion of CPT1 causes apoptosis, the block of cancer cell proliferation and suppression of chemoresistance (76; 77), thus making this enzyme an eligible target for drugs in human cancer, including CLL. There are 3 different isoforms of CPT1: CPT1A found in liver and also in other cells except for skeletal muscle cells and brown adipose cells, CPT1B typical for the muscle cells and brown adipose cells and CPT1C that is expressed only in the neuron. The isoform CPT1A is frequently related to tumour progression in several cancers e.g. myeloma, gastric, breast, prostate, ovarian, lung, lymphoma, and leukaemia cancer (78; 79; 80; 81). In particular, the CPT1A isoform (82; 83), like other molecules implicated in the lipid metabolism such as lipoprotein lipase (LPL) (74) and nuclear receptor peroxisome proliferator-activated receptor (PPAR)-α (74), are all overexpressed in CLL cells if compared to normal B lymphocytes.

CPT1A can preserve cells from apoptosis by removing the long-chain fatty acyl-CoA from the cytoplasm avoiding the accumulation of free fatty acids that cause mitochondrial membranes damage and cell death (76; 84; 85). In particular, the FAs accumulated in the cytoplasm may activate, in virtue of their detergent properties, Bax (85) a pro-apoptotic protein that causes the trigger of apoptosis through the release of cytochrome c from the mitochondria (63; 67). Thus, CPT1 activity is extremely important in order to avoid the accumulation of free FAs and protect, in this way, the cell from apoptosis.

A selective, reversible CPT1A inhibitor, (R)-N-(tetradecylcarbamoyl)-aminocarnitine (ST1326), was recently developed (86; 87). ST1326 (Teglicar) is an aminocarnitine derivative that was initially selected by Sigma-Tau laboratories as a candidate for diabetes and ketoacidosis therapy (86). In phase 2 studies for the treatment of type 2 diabetes, it demonstrated an excellent safety profile (87). Moreover, data in the literature revealed encouraging results in the use of ST1326 against leukaemic cancer cells (81; 83).

Aim of the study

The scope of our investigation was to assess the sensitivity to ST1326 of leukemic cells derived ex-vivo from the peripheral blood of CLL patients, in an environment-mimicking context, where leukemic cells are challenged with activation stimuli that render them generally refractory to current therapeutic regimens and are responsible for disease progression.

Materials and Methods

CLL cells and drugs

CLL cells were obtained from the peripheral blood (PB) of CLL patients, following informed consent in accordance with the Declaration of Helsinki. Mononuclear cells separated by Ficoll density gradient centrifugation were assessed by flow cytometry (FACS Calibur, BD Biosciences, San Diego, CA) for standard diagnostic immunophenotyping (CD19, CD5, CD23, CD79B, CD22, CD38) and identification of heavy chain (IgM, IgG or, very rarely, IgA) and light chain (kappa or lambda) isotype. Cells were then resuspended in freezing solution (10% DMSO and 90% Fetal Bovine Serum (FBS)) and cryopreserved in liquid nitrogen. Samples from CLL patients that containing at least 95% of leukaemic cells were considered eligible for the study. Clinical and molecular characteristics of the CLL patients utilized in this study are shown in Table 1.

Cell cultures of CLL cells were performed by seeding thawed cells into RPMI (Invitrogen S.r.l., Milan, Italy) culture medium supplemented with 10% FBS (Invitrogen) at high cell density, 4×10^6/ml in 48-well plates each containing 0.5 ml of culture medium. We used two different *in vitro* stimulation models. In the first model, *in vitro* activation of CLL cells was achieved by co-culturing leukaemic cells with a stable CD40L-expressing NIH-3T3 murine fibroblast cell line (CD40L-NIH-3T3), produced in our laboratory (88), at a cell number ratio 1:100 (fibroblasts:CLL cells). Recombinant human interleukin 4 (IL-4) (PeproTech Inc.) or, in a few cases, recombinant human interleukin 21 (IL-21) (PeproTech Inc.) were added in the culture at final concentrations of 12.5 ng/ml and 25 ng/ml respectively.

In a second *in vitro* stimulation model, quiescent CLL cells were cultured in the presence of CpG DNA Toll-Like Receptor-9 (TLR-9) ligand, called ODN2006 (Invivogen), and recombinant human IL-15 (PeproTech Inc.) at final culture concentrations of 2 μg/ml and 10 ng/ml respectively (89; 90). In addition to the soluble stimuli (CpG ODN+IL-15) in a few experiments, the CLL cells were cultured in the presence of a murine line of BMSC seeded at a concentration of $1.6x10^4$ in 48 well plates.

In vitro response to activation stimuli were assessed flow cytometrically by increased expression of activation molecules (CD80, CD86, HLA-DR, CD25,

CD69, CTLA-4), increased cell size (forward light scattering) and % cells in $S+G_2M$ cell cycle phases (DNA content histograms).

Normal B lymphocytes were obtained from the PB of healthy volunteers. Mononuclear cells were isolated by Ficoll density gradient centrifugation as a pre-separation step. Normal B cells were isolated by negative selection using a B cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. We performed an immunofluorescence staining with FITC-conjugated CD3 and PerCP-conjugated CD19 mAbs (BD Biosciences, San José, CA) to verify B lymphocytes purity.

Cell cultures of normal B lymphocytes were performed by seeding purified cells into RPMI culture medium supplemented with 10% FBS at 2×10^6/ml in 48-well plates each containing 0.5 ml of culture medium. In vitro activation of B cells was achieved by co-culturing B-lymphocytes with CD40L-NHI3T3 fibroblast and IL-4 at final culture concertation of 12.5 ng/ml.

ST1326 (Teglicar) was kindly provided by Sigma-Tau IFR S.p.A., Rome, Italy (Alfasigma S.p.A Group) and was dissolved in DMSO at the concentration of 50 mM and stored at -20°C.

Etomoxir sodium salt hydrate (Sigma, Saint Louis, Missouri, USA) was dissolved in H_2O at the concentration of 10 mM and stored at -20°C.

In drug-combination tests, ST1326 was added to the cells simultaneously to a second drug: Fludarabine monophosphate from Schering AG (Berlin, Germany), ABT-199 from LC Laboratories (Woburn, MA, USA), Ibrutinib or Idelalisib (GS-1101, CAL-101) both from PeproTech Inc.

Drug treatment on stimulated CLL cells was performed simultaneously to the exposure of CLL to microenvironment mimicking stimuli.

Flow-cytometric assays for cell viability, mitochondrial transmembrane potential (ΔΨ), proliferation and apoptosis

Cellular viability was assessed by Propidium Iodide (PI) exclusion assay. Cells were incubated with 1 μg/ml PI (Sigma Chemical Co., St Louis, MO, USA) for 5 minutes (min) at room temperature (RT). PI fluorescence was measured by flow cytometry (FACS Calibur, BD Biosciences, San Diego, CA). Cells positive for PI fluorescence were considered dead cells.

Loss of mitochondrial transmembrane potential (ΔΨ) was evaluated by decreased fluorescence of the cationic dye 3,3′-dihexyloxacarbocyanine iodide (DiOC6). Leukaemic cells were incubated for 15 min at 37°C with 40 nM DiOC6 and analysed by flow cytometry (FACS Calibur, BD Biosciences, San Diego, CA). At least 5,000 events were acquired per sample on a logarithmic scale.

Flow-cytometric analysis of DNA content was performed on cells permeabilized with 0.05% Triton X-100 and stained with 30 μg/ml PI+0.5 mg/ml RNase for 30 min at 37°C. DNA content histograms provide information on proliferation (cell-cycle-phase distributions) and apoptotic DNA fragmentation (cells in the 'sub-G_0/G_1' region, that is, cells with a DNA content lower than that of cell cycle G_0/G_1-phase cells).

Cellular externalization of phosphatidylserine molecules was assessed by flow cytometric measurements of Annexin V binding on cells stained with Annexin V-FITC, according to standard manufacturer instructions (BioVision, Mountain View, CA, USA).

Protein expression by flow cytometry

Analysis of protein levels, expressed on the plasma membrane or intracellularly, was performed by immunofluorescence and flow cytometry on a FACS Calibur.

Antibodies to surface proteins were CD80, CD69 and CD25, all from BD Pharmingen (San Diego, CA). Antibodies to intracellular proteins were rabbit polyclonal anti-Mcl-1 (H-260), anti-Bcl-xL (H-5) all from Santa Cruz Biotechnology (San Diego, CA) and used at 20 μg/ml; phospho NFKB p65 (Ser536), anti-STAT3 (pan), phospho STAT3 (Tyr705) and phospho STAT3

(Ser727) antibodies all from Cell Signaling Technology, Inc. and anti-Bax clone 3 from BD Biosciences, San José, CA.

Cells were fixed with 3% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100 for Mcl-1, Bcl-xL and Bax, or 3% PFA followed by ice-cold methanol, 8 min, for pSTATs. Secondary antibodies conjugated to Alexa fluorochrome were from Molecular Probes (Invitrogen, Eugene, OR).

For evaluation of NFKB p65 (Ser536) expression in leukaemic cells, nuclear extracts were obtained from CD40L stimulated CLL cells, either untreated or treated with 15 μM ST1326 for 24h. The nuclear pellet derived from lysates centrifugation, see paragraph Immunoblotting for more details, were fixed with 1% PFA for 10 min at RT and stained as mentioned before.

KI67 expression was assessed with FITC-conjugated KI67 mAb (BD Pharmingen, San Diego, CA). The cells were fixed with ice-cold 70% ethanol, incubate over-night at -20°C and stained for the detection of Ki-67 following manufacturer's instructions. When KI67 was analyzed in conjunction with DNA content to achieve bivariate plots of KI67 expression/cell cycle phase distributions, CLL cells were also incubated with 30 μg/ml PI and 0.5 mg/ml RNase for 30 min at room temperature in the dark.

It is important to highlight that the analysis of protein expression by flow cytometry was restricted to viable leukaemic cells (thus gating out late apoptotic cells), namely cells falling within the 'live' gate on flow cytometric FSC-SSC plots, i.e. the 'high FSC-low SSC' gate. This compartment contains CLL cells that still have intact $\Delta\Psi$, an intact plasma membrane and do not express activated caspase 3 (91). CLL cells outside the gate, with 'low FSC-high SSC' features, are mostly late apoptotic and dead cells and are not reliable for intracellular immunofluorescence assays because of unspecific staining that easily occurs on dead cells. By this approach, early changes of protein expression can be discriminated from consequences of late apoptotic steps.

Measurements of Acetyl-CoA and intracellular neutral lipids

Acetyl-CoA levels were measured by colourimetric tests using the PicoProbe Acetyl-CoA Assay Kit from AbCam (http://www.abcam.com/) following the manufacturer's instructions.

To measure intracellular levels of neutral lipids and lipid droplets, cells were stained with the lysochrome diazo dye Oil Red O (Sigma-Aldrich, http://www.sigmaaldrich.com) (working solution, 0.5g Oil Red O powder dissolved in 60% propanol) and extensively washed before measurement of fluorescence above 560 nm by flow cytometry or confocal microscopy (TCS2-AOBS, Leica Microsystems, Mannheim, Germany).

Immunoblotting

CD40L-stimulated CLL cells, either untreated or treated with 15 μM ST1326 for 24h, were washed with ice-cold PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and lysed in lysis buffer (20 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4 and protease inhibitor (Sigma Chemical Co., St Louis, MO, USA)) for 20 min on ice. Lysates were centrifuged 10 minutes at 10.000 g in a microfuge and supernatants collected. Protein concentration was assessed by the Bradford method. Samples were denaturated by the addition of Laemmli sample buffer 4x and were boiled for 5 min. 30 μg of total proteins were used for each sample and loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). Run was performed at 4°C, at 50 mA for each gel, for 120 min. Electrophoretically separated samples were transferred onto nitrocellulose (NC) membranes by electroblotting, at 400 mA, at 4°C for 90 min. NC membranes were blocked: 1h in 1X PBS, 0.1% Tween® 20 with 5% w/v Bovine serum Albumine (BSA) for the phosphorylate proteins, and 1h in 1X PBS, 0.1% Tween® 20 with 5% w/v nonfat dry milk for the total proteins; then the membranes were incubated, over-night at 4°C, with the specific primary antibodies (Abs): anti-Mcl-1 (Santa Cruz Inc, USA), anti-Bcl-xL (Santa Cruz Inc, USA), anti-phospho AKT (Cell Signaling Technology, USA), anti-AKT (pan) (Cell Signaling Technology, USA), anti-phospho-IkBα (Cell Signaling Technology, USA), anti-IkBα (pan) (Cell Signaling Technology, USA), anti-NFKB (Cell Signaling Technology, USA), anti-phospho STAT3 (Tyr705) (Cell Signaling Technology, USA), anti-phospho STAT3 (Ser727) (Cell Signaling Technology, USA), anti-STAT3 (pan) (Cell Signaling Technology, USA), anti-phospho STAT6 (Cell Signaling Technology, USA), anti-phospho JAK3 (Cell Signaling Technology, USA), anti-JAK3 (pan) (Cell Signaling Technology, USA), anti-phospho JAK2 (Cell Signaling Technology, USA), anti-JAK2 (pan) (Cell Signaling Technology, USA) and goat polyclonal anti-Actin (Santa Cruz

Inc. USA, sc-1616). Membranes were then washed for 5 times with 0.1% PBST (1X PBS, 0.1% Tween® 20) and incubated with secondary HPR-conjugated Abs (diluted at 1: 3000 for anti-mouse and 1:1000 for anti-rabbit) for 1h at RT. After extensive washing with 0.1% PBST, binding of Abs was revealed by an enhanced chemiluminescence detection system. Blots were acquired by using the instrument mini HD (Uvitec, Cambridge, UK). Each band was converted by mini HD into a densitometric trace allowing calculations of intensity and signals normalized on the signal of actin, used as the housekeeping protein, and also on the signal of total proteins, in the case of phosphorylating forms.

Combination cytotoxicity

Combination cytotoxicity of ST1326 and a second drug: Fludarabine, ABT-199, Ibrutinib or Idelalisib, was calculated by the Chou-Talalay method (92) (CalcuSyn software, Biosoft, Cambridge) with the CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI) was computed from dose-effect curves of drugs alone and in combination. CI represents a measure of the effect of drug interaction (additive effect: $0.9 \leq CI \leq 1.1$, synergism: $CI < 0.9$ and antagonism: $CI > 1.1$). CI depends on the 'fractional effect level' at which it is calculated. We considered two levels of cytotoxicity, LC75 and LC90 (concentration lethal to 75% and 90% of CLL cells).

Statistics

For statistical comparison between samples, the Mann-Whitney U test was used for unpaired sample data and the Wilcoxon signed-rank test for paired sample data. Analyses were performed using the GraphPad Prism version 5.00 statistical software (GraphPad Software Inc., La Jolla, CA).

Results

Clinical and molecular characteristics of CLL patients

Leukaemic cells derived ex-vivo from 26 patients with CLL were used to study the in vitro effects of ST1326 (Table 1). The cohort of patients is heterogeneous with regard to both disease stage and prognostic markers. It is noteworthy that several patients displayed unfavourable characteristics associated with poor prognosis and resistance to the current therapies. In particular, our cohort included 8/26 Binet C and 8/26 Binet B patients (Table 1). Likewise, patients with a more aggressive clinical behaviour included U-CLL cases (11/26), $CD38^{+}$ CLL cases (16/26), CLL patients harbouring TP53 mutations (2/26), SF3B1 mutations (1/26) and chromosomal aberrations, such as deletion 11q22 (5/26) and deletion 17p13 (6/26) (Table 1).

Pts #	Binet	IG mutations status	% CD38 (30% cut-off)	VH gene	*TP53*	*NOTCH1*	*SF3B1*	Chromosomal aberrations (FISH)
1	C	M-CLL	POS	VH3-30	wt	wt	wt	del 11q22*, del 13q14**, del 17p13***
2	B	U-CLL	POS	VH1-69	wt	wt	wt	nd
3	C	M-CLL	POS	VH3-7	nd	wt	nd	nd
4	C	U-CLL	POS	VH3-48	nd	wt	wt	nd
5	B	U-CLL	NEG	VH3-11	wt	wt	wt	del 13q14
6	B	M-CLL	POS	VH4-31	wt	wt	wt	nd
7	C	U-CLL	POS	VH5-51	nd	wt	wt	nd
8	A	M-CLL	NEG	VH3-30	nd	wt	wt	nd
9	C	U-CLL	POS	VH1-69	wt	wt	wt	del 17p13, del 13q14, trisomy 12
10	C	U-CLL	POS	VH4-74	MUT	wt	wt	del 11q22, del 13q14
11	A	M-CLL	POS	VH4-34	wt	wt	wt	nd
12	C	M-CLL	POS	VH1-2*02	nd	nd	nd	del 17p13 (5%)
13	-	U-CLL	NEG	VH1-18*04	wt	nd	nd	NEG
14	A	U-CLL	POS	VH1-69*01	wt	nd	nd	del 11q22, del 13q14, del 17p13 (5%)
15	B	M-CLL	NEG	VH4-34*01	wt	nd	wt	trisomy 12
16	A	U-CLL	NEG	VH4-39*01	MUT	nd	wt	trisomy 12
17	A	M-CLL	POS	VH3-48*02	wt	nd	nd	del 13q14
18	A	M-CLL	POS	VH1-18*01(FR 1)/JH4B	wt	nd	nd	del 11q22, del 13q14
19	B	M-CLL	POS	VH3-7*01	wt	nd	nd	trisomy 12
20	A	M-CLL	POS	VH3-72*01	wt	nd	nd	del 13q14
21	B	M-CLL	NEG	VH3-48*02	nd	nd	nd	del 13q14
22	B	U-CLL	NEG	3-23, 3-21	wt	wt	MUT	del 13q14
23	A	U-CLL	POS	VH4-34*01	wt	nd	nd	trisomy 12, del 17p13
24	C	M-CLL	NEG	VH3-72*01	wt	nd	nd	del 17p13, del 13q14
25	-	M-CLL	NEG	VH3-7*03	wt	nd	nd	del 11q22, del 13q14
26	B	M-CLL	NEG	VH3-23*01	wt	nd	nd	del13q14

- U-CLL and M-CLL : <2% and ≥2% in *IGHV* difference from germline, respectively.
- n.d. not determined
- * 27%, ** 19%, *** 11%

Table 1: *CLL patient characteristics*

In vitro cytotoxic effect of ST1326 on stimulated CLL cells

We first addressed the cytotoxic activity of ST1326 on leukaemic cells, both on quiescent and activated CLL cells. Cell activation was achieved by culturing primary CLL cells, at high cell density, in the presence of i) CD40L-expressing fibroblasts+IL-4 or IL-21, or ii) CpG ODN+IL-15, whose synergy is important

to promote CLL cells clonal expansion (89; 90), in the presence, or not, of BMSC.

The response to microenvironment mimicking stimuli was followed by monitoring cellular parameters associated with CLL activation phenotype and increased in cell size (forward light scattering) (not shown). ST1326 was added at the beginning of the stimulation.

Multiparameter flow cytometry was used to measure cell death (damage of cell plasma membrane by PI uptake) and loss of mitochondrial transmembrane potential (decreased fluorescence of the cationic voltage-dependent fluorescent probe $DiOC_6$).

We observed a significant decrease in the viability of in vitro stimulated CLL cells compared to quiescent ones (Figures 1A, B). Cell death was preceded by mitochondrial transmembrane potential ($\Delta\Psi$) dissipation, as demonstrated by ST1326-induced $\Delta\Psi$ decrease observed in cells with an intact plasma membrane (Figures 1B, C).

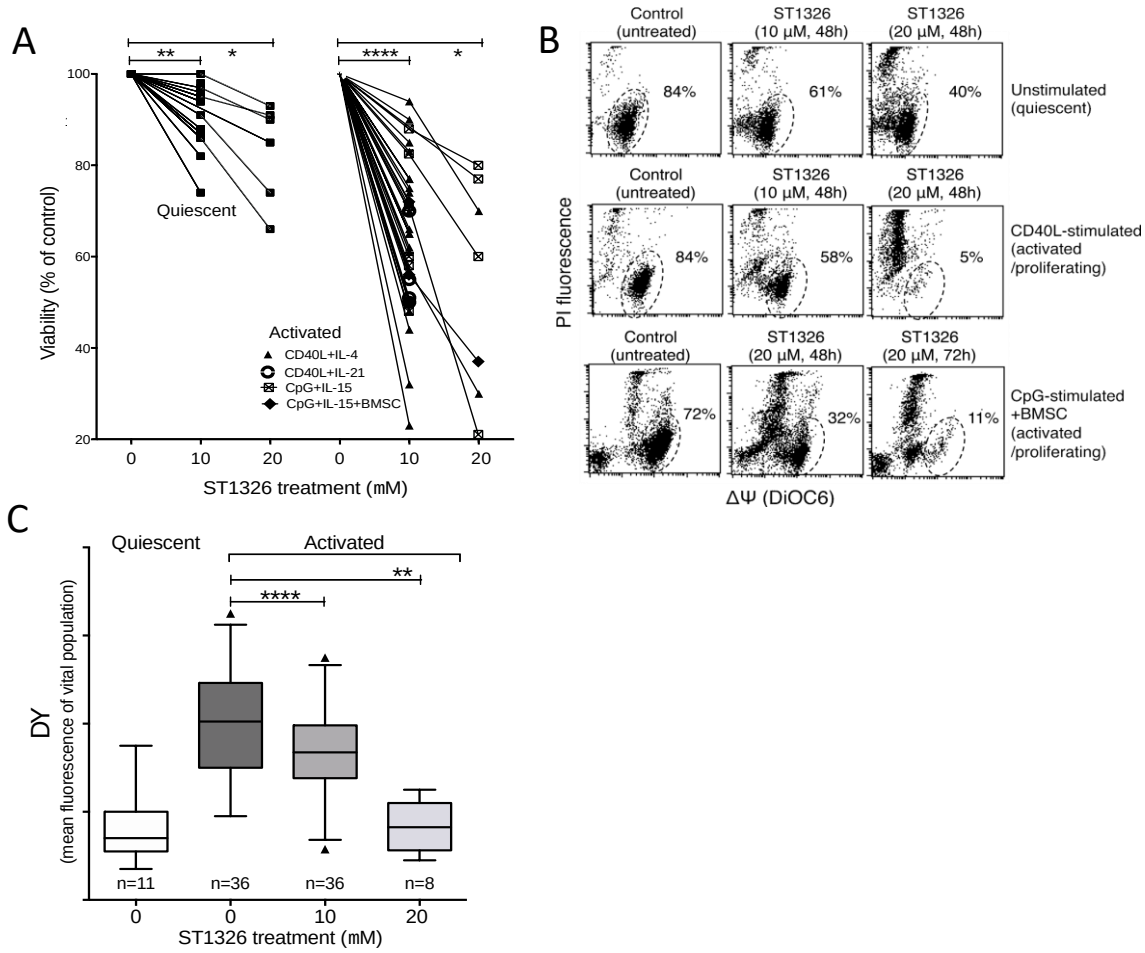

Figure 1- *Sensitivity to ST1326 of quiescent and activated/proliferating CLL cells.*

A) Cell viability assessed by flow cytometric PI exclusion tests on quiescent or CD40L-stimulated cultures of leukaemic cells from 26 independent CLL patients untreated or treated with ST1326 (48 hours). Cell activation was achieved either through CD40L-NIH-3T3+IL-4 (number of independent experiments n=22) or IL-21 (n=4), or by CpG/ODN2006+IL-15, in the absence (n=7) and the presence (n=3) of BMSC. Mean (± range) of 10 µM ST1326-induced viability reduction (% control) were: 64.8±(23-94), 56.5 (50-70), 68 (48-88) and 61 (55-72), for the four different activation systems, respectively. Statistical significance of the difference is evaluated by two-sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001;***p 0.001;****p 0.0001. **B**) Flow cytometric bivariate plots of ΔΨ as measured by DiOC6 fluorescence, and cell viability, as assessed by PI fluorescence, of leukaemic cells from one representative CLL patient. Cells were non-stimulated or stimulated either with CD40L-NIH-3T3+IL-4 or CpG+IL-15 (in this sample also in the presence of BMSC), and exposed to ST1326 for the times indicated. All controls were measured 48 hours after culture start. Events in the dotted gate are cells with functional ΔΨ and intact plasma membrane. This CLL patient harbors 17p13 deletion. **C**) ΔΨ gated on the vital cell population, as measured by DiOC6 uptake and expressed as flow cytometric fluorescence intensity (MFI) of the PI-excluding cell population (with intact plasma membrane), on quiescent or CD40L-stimulated cultures of leukaemic cells from 26 CLL patients untreated or treated with ST1326 from the beginning of stimulation (48 hours). Number of samples for each condition is indicated. Statistical significance of the difference is analysed by t-test two- sided Wilcoxon signed rank test. *p 0.05; **p 0.01; ***p 0.001; ***p 0.001; ****p 0.0001.

Notably, ST1326 was effective independently from stimulation and even when proliferation was induced in the presence of BMSC (Figures 1A, B), known to provide a protective microenvironment. Furthermore, the CPT1A inhibitor showed to be effective also in CLL samples harbouring mutated TP53, mutated SF3B1 or chromosomal abnormalities, known to confer chemoresistance (Table 1 and Figure 1B).

Interestingly, cytotoxicity was achieved at drug concentrations 10 times lower than concentrations needed for the more common CPT1 inhibitor Etomoxir to reach similar effects (Figure 2).

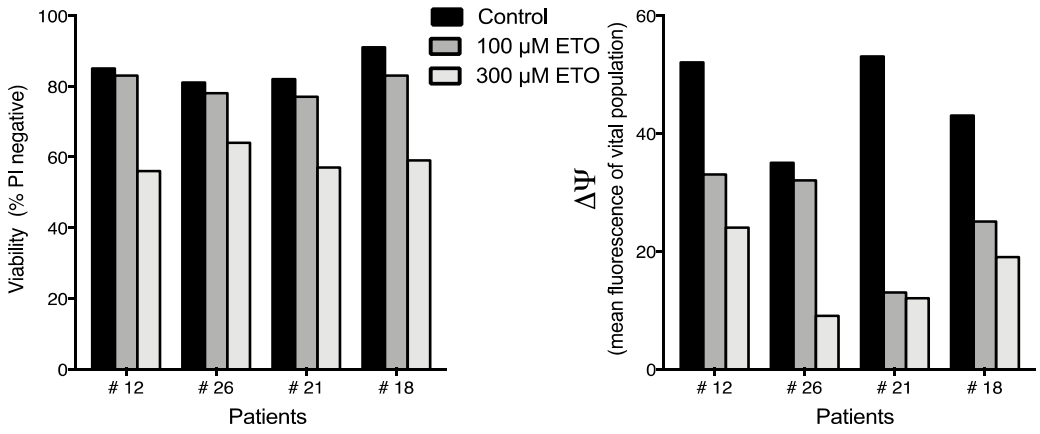

Figure 2- *Etomoxir is cytotoxic in vitro at ten times higher molarity if compared to ST1326.*

Left: Cell viability assessed flow cytometrically PI exclusion tests on CD40L+IL4-stimulated cultures of leukaemic cells from 4 CLL patients untreated or treated with Etomoxir (48 hours). *Right*: Mitochondrial transmembrane potential (ΔΨ) gated on the vital cell population, as measured by DiOC6 uptake and expressed as flow cytometric fluorescence intensity (MFI) of the PI-excluding cell population (with intact plasma membrane), on same sample as 'left'.

Proliferating/activated CLL cells are more sensitive to ST1326 treatment

In order to deepen the understanding of the differential effect of ST1326 on quiescent and proliferating/activated CLL cells, we analysed the subpopulation of i) activated/proliferating cells, ii) quiescent cells and iii) death cells, within the same sample. In particular, we compared the percentage of the cells present in these areas between the control and treated sample.

We were able to discriminate the live cells from the dead cells by staining with PI, which is not able to cross the cell membrane and is therefore taken up

only by dead cells (data not shown). In order to distinguish the quiescent cells from the activated ones, we relied on morphological aspects; resting cells are characterized by a lower front scatter value compared to the activated/proliferating cells (data not shown).

Comparing over the course of the days the percentage of cells present in each compartment, in the control (stimulated cells) and in the treated cells (stimulated in the presence of ST1326), we observed a reduction in the percentage of cells present in the quiescent compartment with a similar trend between control and treated cells. Instead, if we consider the population of activated cells, after an initial increase in the percentage of cells present in this compartment, there is a sharp reduction only in the case of the treated sample. In conjunction to this depletion in the treated sample, an increase of death cell fraction was observed, suggesting that dead cells arose preferentially from the activated compartment rather than from the quiescent one (Figure 3).

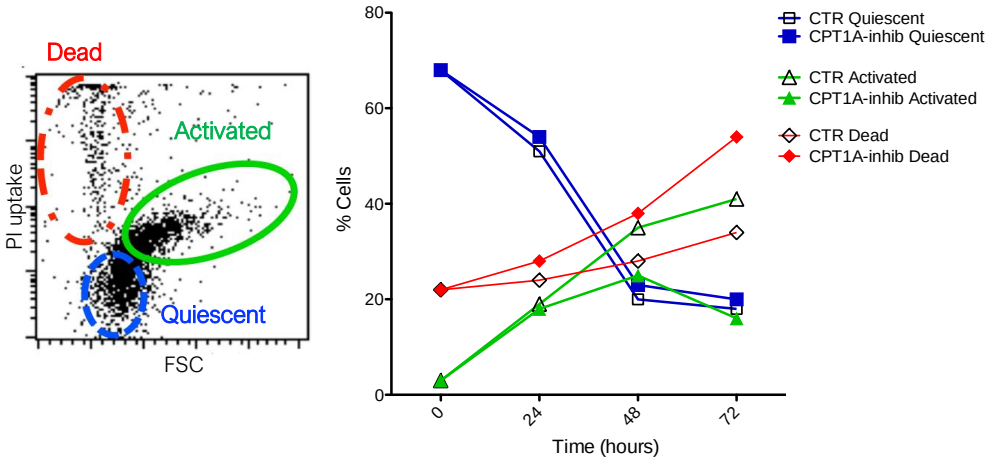

Figure 3- *Intra-patient analysis: differential effect of ST1326 on quiescent and activated/proliferating CLL cell compartment.*

CLL cells from one patient stimulated with fibroblast-CD40L+IL-4 and treated, or not, with ST1326 10 µM, were analyzed with flow cytometry for the determination of cells size and cell mortality by using the PI exclusion assay. The graph on the right shows the analysis of the percentage of cells present in the quiescent, activated/proliferating and death area during the days. 'CPT1A-inhib' indicates ST1326.

We focused on this behaviour by KI67/DNA measurements. KI67 expression rose after stimulation in a fraction of CLL cells, as expected (Figure 4 left). However, the presence of ST1326 induced the disappearance of KI67+ cells and the concomitant increase of sub-G1 events (Figure 4 left), indicating that cycling cells were more sensitive to ST1326 than quiescent ones. Results of overall KI67 expression are displayed for 8 CLL patients (Figure 4 right).

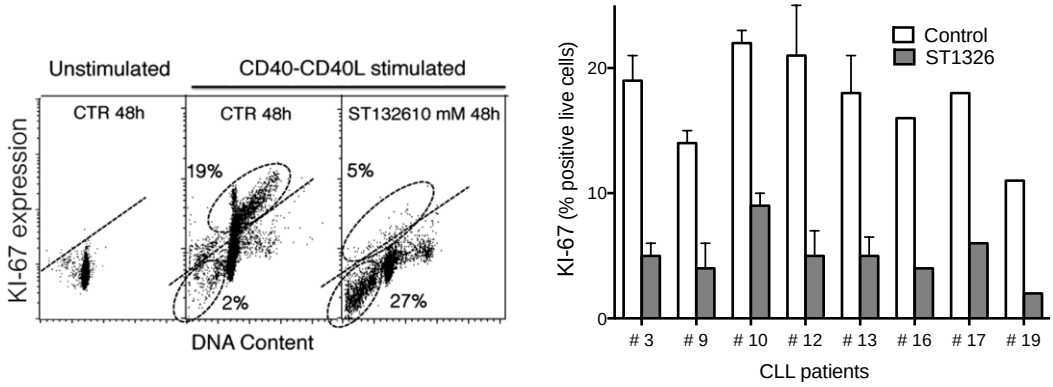

Figure 4- *ST1326 determines a reduction in the percentage of Ki-67 positive cells and an increase in the cells present in the sub-G1 compartment.*

Left: Flow cytometric distributions of DNA content/KI67 expression for one representative CLL sample. The CD40L-stimulated cells were tested in the absence or presence of ST1326. Gates identify KI67+ cells (above the negative-threshold dotted line) and sub-G1 events (apoptotic cells and bodies, below). *Right*: %KI-67-positive cells from CLL samples of eight CLL patients, tested with or without addition of ST1326.

Taken together, these results confirm the observation that activated and/or proliferating CLL cells are more sensitive to ST1326 than quiescent ones.

The proof of the different sensitivity of the non-stimulated and stimulated cell cultures came also from the observation that a 40-hours pulse of ST1326 given at later stages after stimulation (i.e. when the cells already express activation markers and start progressing through the G1/S checkpoint) was more cytotoxic than when drug was administered at the beginning of stimulation. This is at variance with other drugs (Fludarabine and ABT-199) that lost their effectiveness if added to overtly activated CLL cells (Figure 5).

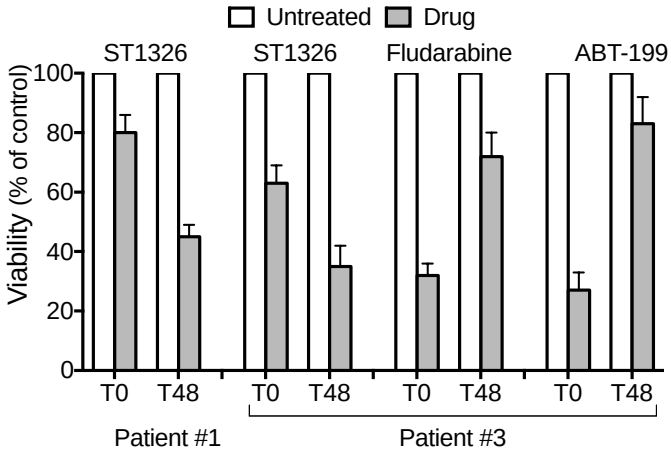

Figure 5- *Increased sensitivity to ST1326 in CLL cells that are in advanced stages of their activation.*

Cell viability in cells from two CLL patients exposed to a 40-hours drug 'pulse', either concomitantly with CD40L-stimulation (T0) or after 48-hours (T48). The different ST1326 sensitivity of CLL cells at T0 or T48 is evident. In contrast, the effect of Fludarabine (10 μM) and ABT-199 (5 nM) was remarkably impaired if the cells were exposed to the drugs at T48.

ST1326 cytotoxicity effect correlates with the in vitro proliferative response to activation stimuli

We analyzed the correlation between the cytotoxic response to the drug and i) clinical parameters (Binet disease stage), ii) molecular parameters (mutational status of the variable region of the IGV immunoglobulin and expression of CD38) or iii) in vitro cellular parameters (proliferative potential).

We found a strong relationship of ST1326 cytotoxicity with the in vitro CLL proliferative response to activation stimulus (Figure 6), which further corroborates the notion that CLL cells in an activated/proliferating state are more sensitive to the inhibition of CPT1A compared to the quiescent ones. Conversely, cytotoxicity was apparently independent from all other clinical and molecular parameters (Figure 6).

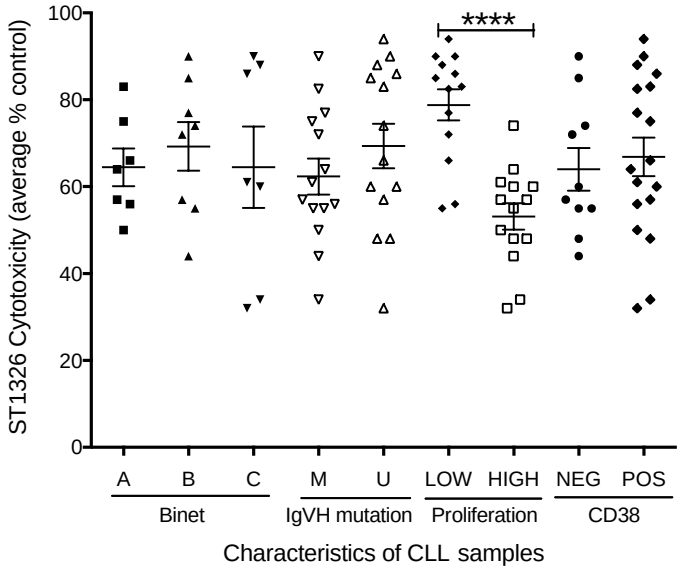

Figure 6- *Correlation between cytotoxic effect of ST1326 and in vitro proliferative response to microenvironment stimuli*.

Effect of ST1326 on average cell viability of CD40L-stimulated CLL cells. The 26 patients were grouped according to disease stage (Binet A, B or C), Ig mutational status (U-CLL, <2% mutations in IGVH) and mutated (M-CLL, ≥2% mutations in IGVH genes), proliferative response to CD40L-stimulation ('low' or 'high' if % cells in S+G2M cell cycle phase after 72h stimulation was ≤ or > 8%, respectively), and CD38 expression (cut-off: 30%). Statistical significance of the difference is assessed by two-sided Mann-Whitney test. The difference is not significant where no indication is reported. *p 0.05; **p 0.01; ***p 0.001; ***p 0.001; ****p 0.0001.

CLL cells are more sensitive to ST1326 than healthy peripheral blood lymphocytes

We explored a possible therapeutic window between CLL cells and normal B lymphocytes. To this end, experiments on purified B cells obtained from the peripheral blood of healthy donors, activated by CD40L-expressing fibroblasts+IL-4, were conducted. The response to the stimulation was followed by monitoring cellular parameters associated with B activation phenotype and increased in cell size (forward light scattering) (not shown). ST1326 was added simultaneously to the activating stimuli.

We found that normal B lymphocytes were less sensitive to ST1326 cytotoxicity, at the doses lethal for CLL cells (Figure 7 for CD40L+IL-4 stimulated B cells, not shown for unstimulated B cells). Instead, cell proliferation was partly inhibited, indicating a cytostatic, rather than cytotoxic, effect of ST1326 on normal B cells (Figure 7).

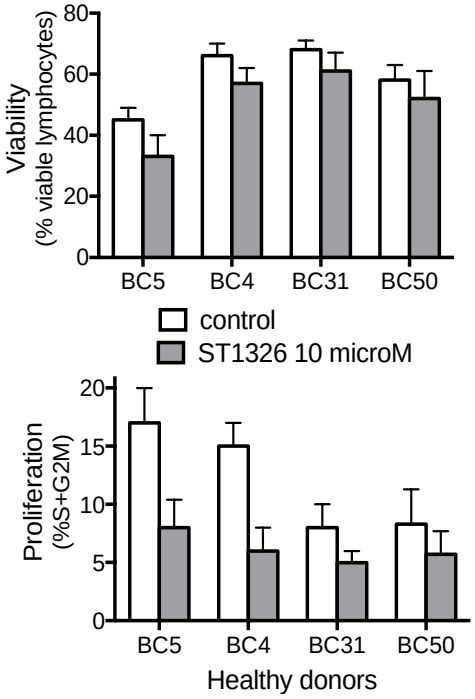

Figure 7- *Healthy peripheral blood lymphocytes show a partly inhibition of cell proliferation after the treatment with ST1326.*

Normal B cells from healthy volunteers were purified by magnetic beads (negative selection), activated by co-culturing with CD40L+IL-4 and treated for 48h with 10 μM ST1326. Cell death and proliferation were measured by flow cytometry. Viability was unaffected, while cell proliferation (% cells in S+G2M phase) is reduced in two samples.

ST1326 cytotoxicity was associated with decreased levels of intracellular Acetyl-CoA

Important for cell proliferation is the production of the Acetyl-CoA (93), generated by the degradation of FAs via β-oxidation. Since the CPT1A inhibitor prevents the entry of FAs into the mitochondria, we decided to verify if the treatment with ST1326 causes a reduction in Acetyl-CoA production.

We stimulated the CLL cells for 24 hours, a sufficient time in order to induce the cells to become activated blasts, and then we added ST1326. We decided to add drugs after 24 hours of stimulation because the treatment with ST1326 is stronger when it is administered at the beginning of stimulation, as demonstrated before. We measured the cytoplasmic level of Acetyl-CoA at different time points (0, 8, 15, 24, 30 hours) after the treatments. As a positive control, 2-Deoxy-

D-glucose (2-DG) was used. 2-DG is a stable glucose analogue that is actively taken up by the hexose transporters and phosphorylated, but it cannot be fully metabolized (94). The treatment with 2-DG decreases the amount of substrates available for aerobic respiration and, as a result, this causes a reduction in Acetyl-CoA production.

We observed that the administration of ST1326 decreased intracellular levels of Acetyl-CoA already after 15 hours of treatment (Figure 8), further corroborating the notion that ST1326 is able to block the degradation of FAs in mitochondria, thus reducing the production of Acetyl-CoA.

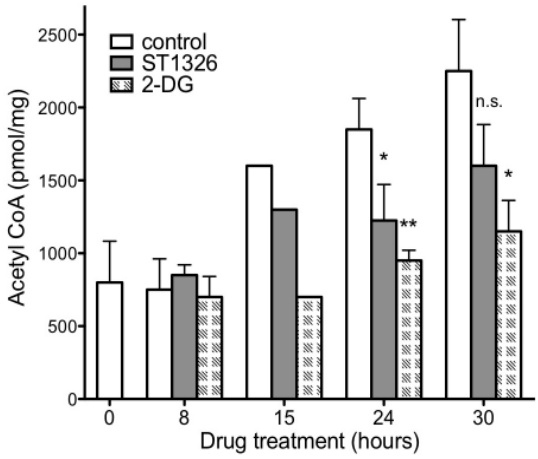

Figure 8- *Effect of ST1326 on Acetyl-CoA production.*

Acetyl-CoA levels in leukemic cells from one CLL patient stimulated for 24 hours and then exposed to ST1326 (10 μM) or 2-DG (5 mM). Data obtained by colorimetric tests using the PicoProbe Acetyl-CoA Assay Kit, are reported as mean ± SD of three experiments (two settings with CpG- and one with CD40L-stimulus). The levels of intracellular Acetyl-CoA increase during CLL cell activation, and ST1326 affects the increase rate. 2-DG was used as control. *p 0.05; **p 0.01; ***p 0.001; ***p 0.001; ****p 0.0001.

Stimulated CLL cells die by apoptosis after ST1326 treatment

We verified if the treatment with ST1326 causes apoptotic cell death in stimulated CLL cells, by using an Annexin V+PI labelling. Annexin V is a protein that binds phosphatidylserines, which are normally found in the inner membrane but become flipped to the outer during the early stages of apoptosis. Cells that are positive for Annexin V and negative for PI are defined as early apoptotic cells while cells that are positive for both Annexin V and PI are defined as late apoptotic cells.

We observed that ST1326 determined an increase in the percentage of activated cells that undergo apoptosis (Figure 9). The percentage of Annexin V-positive cells was reduced when ST1326 was administered together with L-carnitine and oleic acid (Figure 9). This is in line with described target competition of the drug (86), and possibly due to fatty acid-promoted activation of PPAR-α that in turn upregulates CPT1A (95), thus counteracting the cytotoxic effects of ST1326.

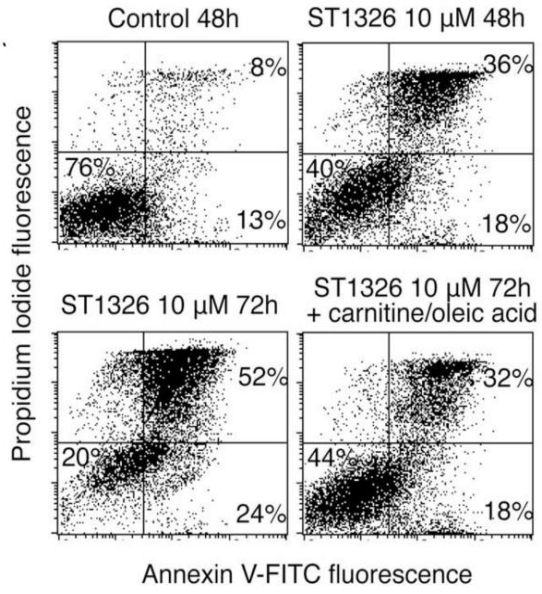

Figure 9- *Cytotoxic effect of ST1326 in the absence and presence of oleic acid + carnitine.*

Annexin V-FITC/PI cytometric distributions of one CLL CD40L stimulated sample, untreated or treated with ST1326 (± oleic acid (300 µM)/BSA (50 µM) + L-carnitine (100 µM) for 48h and 72h. The graph shows the percentage of AnnexinV^{neg} cells (viable cells), AnnexinV^{pos} / PI^{neg} (early apoptotic cells) and AnnexinV^{pos} / PI^{pos} (dead cells).

Apoptosis of ST1326-treated CLL cells was associated with Bax activation and intracellular accumulation of lipids

One of the most important executors of apoptosis is represented by the activated form of Bax. After various type of apoptotic-stimuli, Bax triggers the apoptotic cascade by inducing the release of cytochrome c from mitochondria (31; 63).

We evaluated by flow cytometric analysis the expression of the activated form of Bax by specific antibody, after ST1326 treatment of both proliferating

CLL cells and proliferating normal B lymphocytes. We observed a strong increase, in a time-dependent manner, in the expression of active-Bax in proliferating CLL cells (Figure 10A). On the contrary, proliferating normal B lymphocytes showed only a slight increase in the expression of active-Bax (Figure 10A). This confirms that normal B lymphocytes, at the doses lethal for CLL cells, were less sensitive to ST1326 cytotoxicity.

By blocking CPT1A, ST1326 treatment prevents the entry of FAs into the mitochondria. One consequence of this blocking may be an intracellular accumulation of lipids that, in virtue of their detergent properties, may induce Bax activation (85). Experiments with Oil red O (ORO) staining, which binds neutral triglycerides and lipids, showed ST1326-mediated intracellular accumulation of lipids (Figure 10B), which was in part inhibited by supplementation of L-carnitine (Figure 10B).

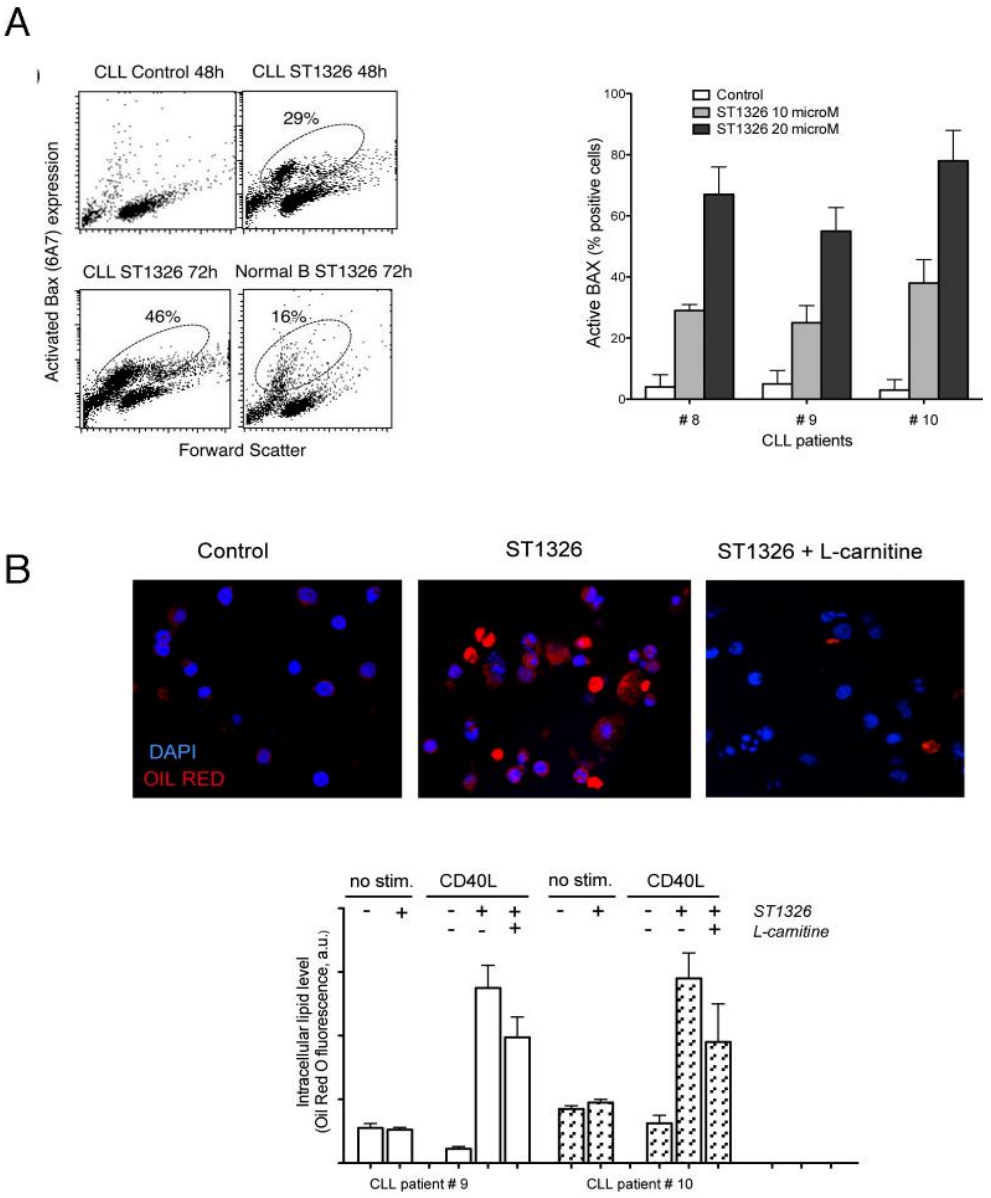

Figure 10- *ST1326 induces Bax activation and intracellular accumulation of lipids that is impaired by exogenous L-carnitine.*

A) *Left*: Flow cytometric distributions of Forward Light Scatter (FSC)/active-Bax expression, of CD40L+IL-4-stimulated B lymphocytes from one CLL patient and one healthy donor, untreated or treated with ST1326 for the reported times. The percentage of cells expressing the active form of Bax (recognized by the 6A7 antibody) is indicated. *Right*: data from CLL samples of three CLL patients. **B)** *Upper*: Confocal microscopy images of CLL cells stimulated with CD40L+IL-4-fibroblasts in the absence (left) or presence of ST1326 (10 μM, 48 hours), without (middle) or with L-carnitine addition (100 μM). Intracellular neutral triglycerides and lipids are in red (Oil Red O staining) and nuclei in blue (DAPI staining). *Lower*: intracellular lipid levels as evaluated by fluorescence intensity of Oil Red O-stained cells from two CLL patients. Stimulated CLL cells displayed lower intracellular lipid levels than quiescent cells, possibly reflecting the fatty acid use for de novo membrane synthesis during proliferation.

ST1326 impairs stimulation-induced activation of Mcl-1 and Bcl-xL in CLL cells

The Bcl-2 family members are key regulators of the intrinsic apoptotic pathway. Especially relevant are Mcl-1 (45) and Bcl-xL (44), that are associated with high risk of CLL disease progression and poor response to chemotherapeutic agents and BH-3 mimetics treatment (46; 72; 73). We investigated the effect of ST1326 on the expression of these two important anti-apoptotic proteins.

Flow cytometric immunofluorescence measurements and western blots were performed altogether on 14 samples from 7 different CLL patients, stimulated with CD40L+IL-4 or quiescent (the latter only in the case of immunofluorescence). The results indicated, in all samples, a consistent decrease of both anti-apoptotic proteins expression after 24 hours of treatment with ST1326 (Figure 11), when overt cell death is not observed yet. Also, the analysis was performed on viable cells only (as defined by flow cytometric scatter signals). Thus, reduction of anti-apoptotic protein expression anticipated cell death and is not due to cell death-associated proteolytic mechanisms.

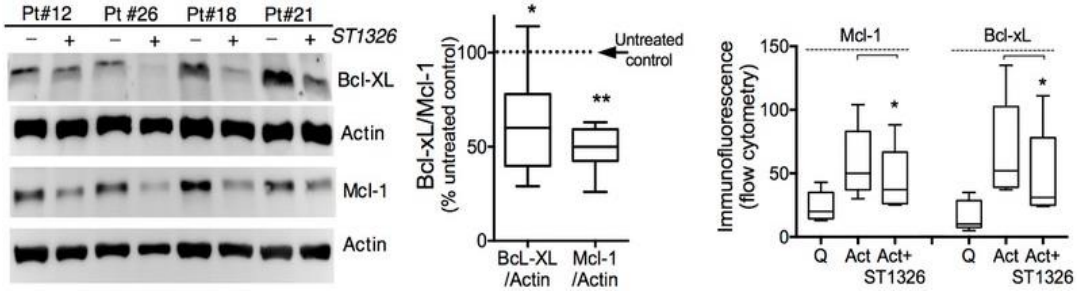

Figure 11- *ST1326 impairs stimulus-induced Mcl-1 and Bcl-xL up-regulation.*
Left: Expression of the Bcl-2-family anti-apoptotic members Mcl-1 and Bcl-xL, in leukaemic cells from 4 CLL patients, stimulated with CD40L+IL-4, and treated with or without ST1326 24 hours after stimulation. *Middle*: Cumulative data for 24 and 42 hours treatment are represented in the box/whiskers graph (5-95 percentile), where protein level in ST1326-treated samples (divided by housekeeper) is calculated as % untreated controls.
Right: Protein expression evaluated by intracellular immunofluorescence and flow cytometry (background-subtracted fluorescence) on samples from 5 CLL patients is reported as cumulative graph. Quiescent (Q= non-stimulated), stimulated (Act) in the absence or presence of ST1326. Non-parametric t-test was used to assess statistical significance of the difference. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.

ST1326 impairs stimulation-induced activation of STAT3 and STAT6 in CLL cells

In CLL, Mcl-1 and Bcl-xL expression are under the control of signalling pathways that include AKT (96), NF-KB/STAT3 (97) and STAT6 (98).

Transcription factors STAT3, NF-KB and AKT are aberrantly activated in many cancer cells. These tumour-associated transcription factors co-regulate gene expression, resulting in significant upregulation of genes involved in tumour cell survival, proliferation and immunosuppression. Specifically in CLL cells, NF-KB is activated also by the unphosphorylated form of STAT-3 (99), which is highly expressed in CLL cells (100). STAT3 exhibits the peculiarity of being activated not only through the more common (Tyr705)-phosphorylation but also through the phosphorylation on Ser727, which is constitutively phosphorylated in CLL cells (100).

Accordingly, we analysed by western blot the effects of ST1326 on the expression of Akt (pan), IkBα (pan), NF-KB (pan), JAK3 (pan), JAK2 (pan), STAT3 (pan), and the phosphorylated forms: p-STAT3 (Tyr705), p-STAT3 (Ser727), p-STAT6, p-JAK3, p-JAK2, p-Akt, and p-IkBα. Only NF-KB (pan), STAT3 (pan), p-STAT3 (Tyr705) and p-STAT3 (Ser727) were analysed also by flow cytometry.

AKT did not display a decrease pattern similar to Mcl-1/Bcl-xL. Its phosphorylation was only mildly decreased (Figure 12), in spite of marked Mcl-1/Bcl-xL reduction. Likewise, no clear-cut association was evident between the drop of anti-apoptotic proteins expression and NF-KB modulation, as indicated by the expression of NF-KB regulators p-IKBα/IKBα, and by cytoplasmic (WB) and nuclear (flow cytometry) NF-KB levels (Figure 12). The changes of NF-KB expression in the nucleus after ST1326 treatment were very variable across patients (Figure 12) and did not correlate with Mcl-1/Bcl-xL drop intensity or with apoptotic response to the drug.

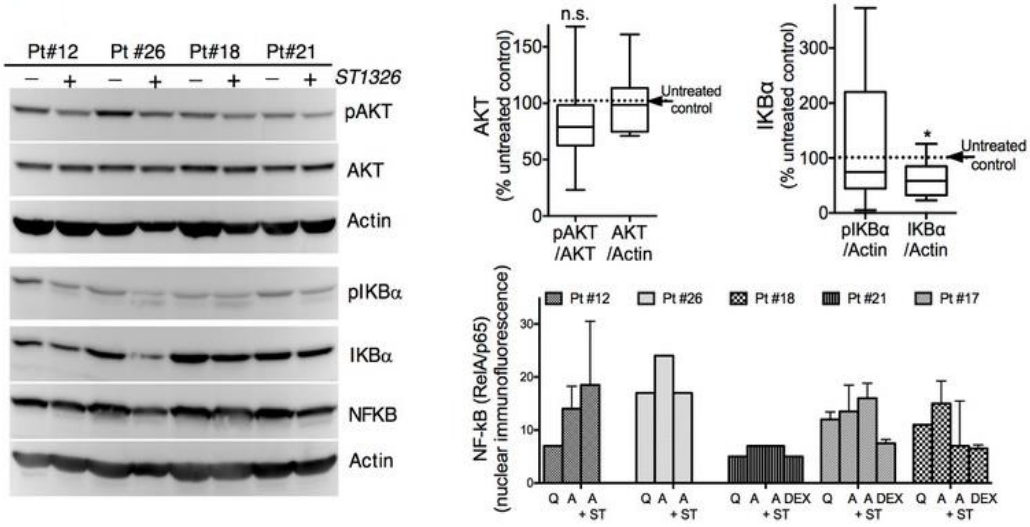

Figure 12- *Effects of ST1326 on AKT and NF-kB activity.*

Left: Western blots of total and phosphorylated (p) AKT (Ser473) and IKBα (Ser32/36) and NF-kB (RelA/p65) in 4 CLL cell samples stimulated with CD40L+IL-4 for 1 day and treated with ST1326 for another 24 hours. Different signalling pathways were examined in parallel gels and blots were stripped and re-probed with the indicated antibodies.

Right upper panel: Cumulative data calculated as in figure 11. Statistical significance of the difference is analysed by non-parametric t-test. *p 0.05; **p 0.01; ***p 0.001;****p 0.0001.
Right lower panel: NF-kB (RelA/p65) expression in isolated nuclei from CLL cells treated in culture as above, stained with anti-NF-kB (RelA/p65) antibody and measured by flow cytometry. Cells were non-stimulated (Q), CD40L+IL-4 stimulated without (A) or with ST1326 (A+ST). Dexamethasone (DEX) treatment for 24 hours (100 nM) was used as positive control for NF-kB down-regulation.

Instead, STAT3 phosphorylation was profoundly affected by ST1326 (Figure 13). Expression of both phospho (Tyr705)- and phospho (Ser727)-STAT3, displayed substantial reduction, observed well before overt apoptosis (Figure 13). Interestingly, total STAT3 (U-STAT3) expression was diminished as well. Also, STAT6 phosphorylation was remarkably reduced after treatment with the aminocarnitine-derivative (Figure 13).

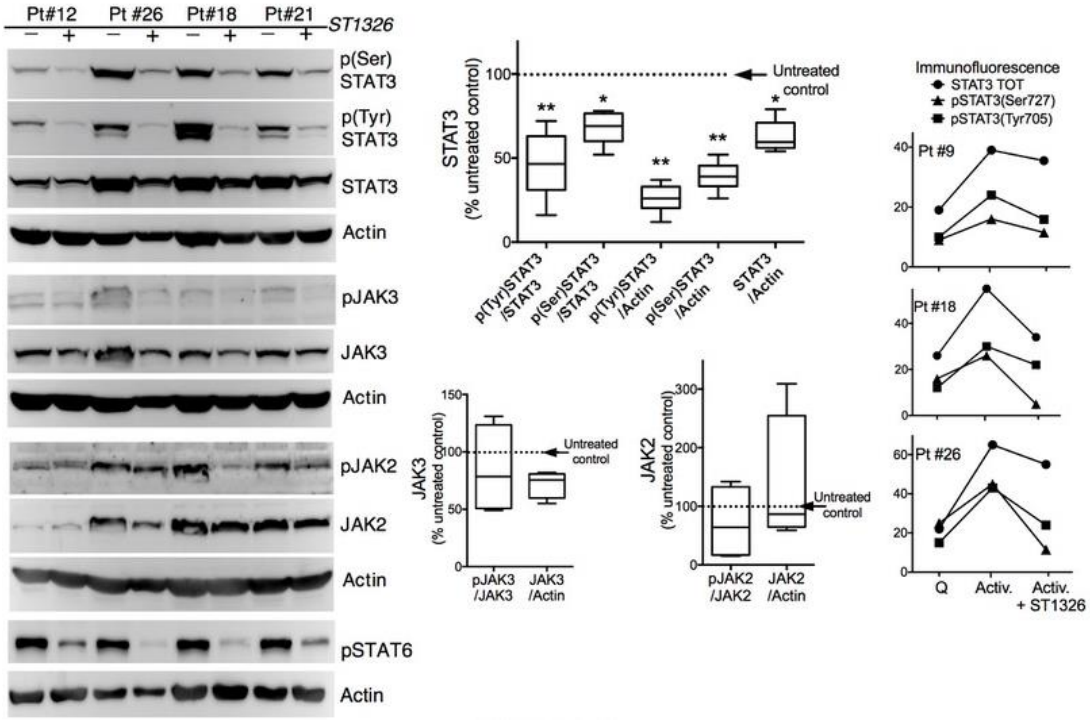

Figure 13- *ST1326 induces remarkable STAT3 and STAT6 down-regulation.*

Left: Western blots of STAT3 (pan) and its phosphorylated forms pSTAT3(Tyr705) and pSTAT3(Ser727), of total and phosphorylated JAK2 (Tyr1008) and JAK3 (Tyr980/981), and pSTAT6, in leukaemic cells from 4 CLL patients stimulated with CD40L+IL-4 for 1 day and treated with ST1326 for another 24 hours. Different signalling pathways were examined in parallel gels and blots were stripped and re-probed with the indicated antibodies.

Middle: Cumulative data for STAT3 are calculated from western blots of 8 samples from 6 CLL patients: the average ratio of STAT3 (unphosphorylated, phospho-Tyr705 and phospho-Ser727 STAT3) versus either unphosphorylated protein level or the housekeeper are displayed as relative decrease of protein levels in ST1326-treated compared to untreated samples (set to 100). Cumulative data for JAK2 and JAK3 are calculated from 4 CLL patients. Non-parametric t-test was used to assess statistical significance of the difference. *p 0.05; **p 0.01; ***p 0.001; ****p 0.0001.

Right: expression of STAT3 and its phosphorylated forms as evaluated by intracellular immunofluorescence and flow cytometry on leukemic cells from 5 CLL patients, either non-stimulated (Q) or stimulated (Activ.) in the absence or presence of ST1326 (24-30 hours). Importantly, background-subtracted data are restricted to viable cells.

In the face of the striking reduction of STAT3 and STAT6 phosphorylation in all CLL patients, the phosphorylation of JAK2 and JAK3, which are involved in STATs regulation (101; 102), displayed very modest and occasional reduction (Figure 13).

Combination cytotoxicity of ST1326 with ABT-199

ABT-199 (Venetoclax) is a promising BH3 mimetic that shows a selective affinity for Bcl-2. However, due to its selectivity for Bcl-2, ABT-199 has limited efficacy when Bcl-xL and Mcl-1 are overexpressed, which is the case of proliferating CLL cells (72; 73). Accordingly, we explored whether resistance to the BH3-mimetic could be overcome by ST1326 that down-regulates Bcl-xL and Mcl-1 in stimulated CLL cells.

Combined cytotoxicity of ST1326 administered together with other drugs was calculated by the Chou–Talalay method (103). This method is based on physico-chemical algorithms of the mass-action law, specifically with the unified theory of the median-effect equation and its combination index theorem for drug combinations (92).

The activated/proliferating CLL cells are much more resistant to ABT-199 than quiescent cells are (72). Thus, CD40L-stimulated CLL cell cultures were treated in our dose-response experiments with 10-fold higher doses of ABT-199 compared to unstimulated CLL cell cultures (that is, 5 or 50 nM for unstimulated or stimulated CLL cells, respectively). We observed that the co-administration of ST1326 with ABT-199 significantly increased the cytotoxicity of sub-lethal doses of ABT-199 (Figure 14 upper).

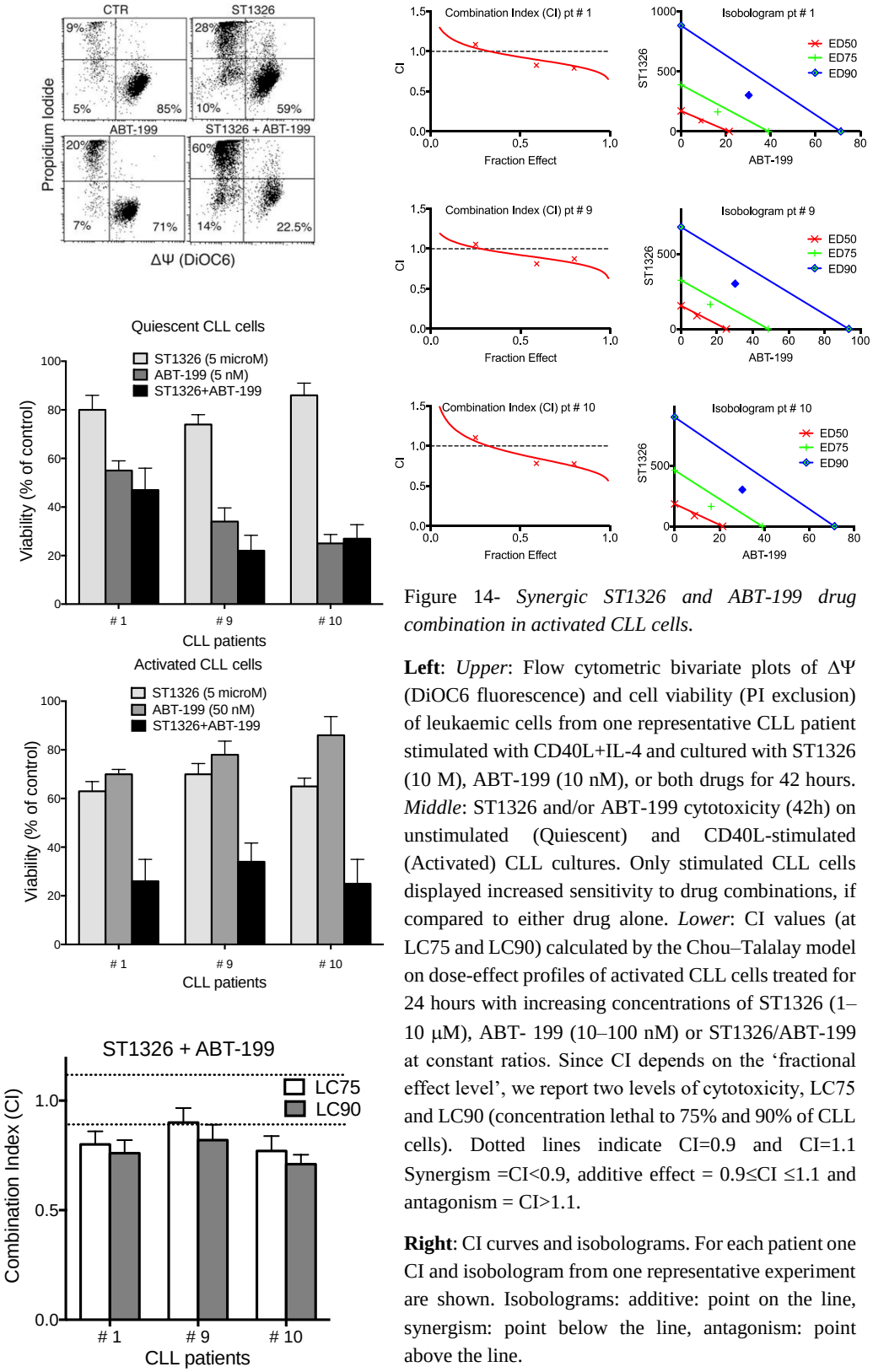

Figure 14- *Synergic ST1326 and ABT-199 drug combination in activated CLL cells.*

Left: *Upper*: Flow cytometric bivariate plots of ΔΨ (DiOC6 fluorescence) and cell viability (PI exclusion) of leukaemic cells from one representative CLL patient stimulated with CD40L+IL-4 and cultured with ST1326 (10 M), ABT-199 (10 nM), or both drugs for 42 hours. *Middle*: ST1326 and/or ABT-199 cytotoxicity (42h) on unstimulated (Quiescent) and CD40L-stimulated (Activated) CLL cultures. Only stimulated CLL cells displayed increased sensitivity to drug combinations, if compared to either drug alone. *Lower*: CI values (at LC75 and LC90) calculated by the Chou–Talalay model on dose-effect profiles of activated CLL cells treated for 24 hours with increasing concentrations of ST1326 (1–10 μM), ABT- 199 (10–100 nM) or ST1326/ABT-199 at constant ratios. Since CI depends on the 'fractional effect level', we report two levels of cytotoxicity, LC75 and LC90 (concentration lethal to 75% and 90% of CLL cells). Dotted lines indicate CI=0.9 and CI=1.1 Synergism =CI<0.9, additive effect = 0.9≤CI ≤1.1 and antagonism = CI>1.1.

Right: CI curves and isobolograms. For each patient one CI and isobologram from one representative experiment are shown. Isobolograms: additive: point on the line, synergism: point below the line, antagonism: point above the line.

Since the combination effect also depends on the 'fractional effect level' at which it is calculated, we considered two levels of cytotoxicity, LC75 and LC90 (concentration lethal to 75% and 90% of CLL cells). CI values and isobolograms demonstrate a 'more-than-additive' effect for ST1326/ABT-199 drug combination (Figures 14 lower and right).

Combination cytotoxicity of ST1326 with Fludarabine

Fludarabine is a chemotherapeutic agent with a remarkable activity in B-CLL (41), whose cytotoxicity is hampered by high expression of Mcl-1 and Bcl-xL (45; 46). Our drug combination experiments showed that the combined treatment of CLL cells with Fludarabine and ST1326 induced higher cytotoxicity if compared to each drug alone in activated conditions (Figure 15 upper).

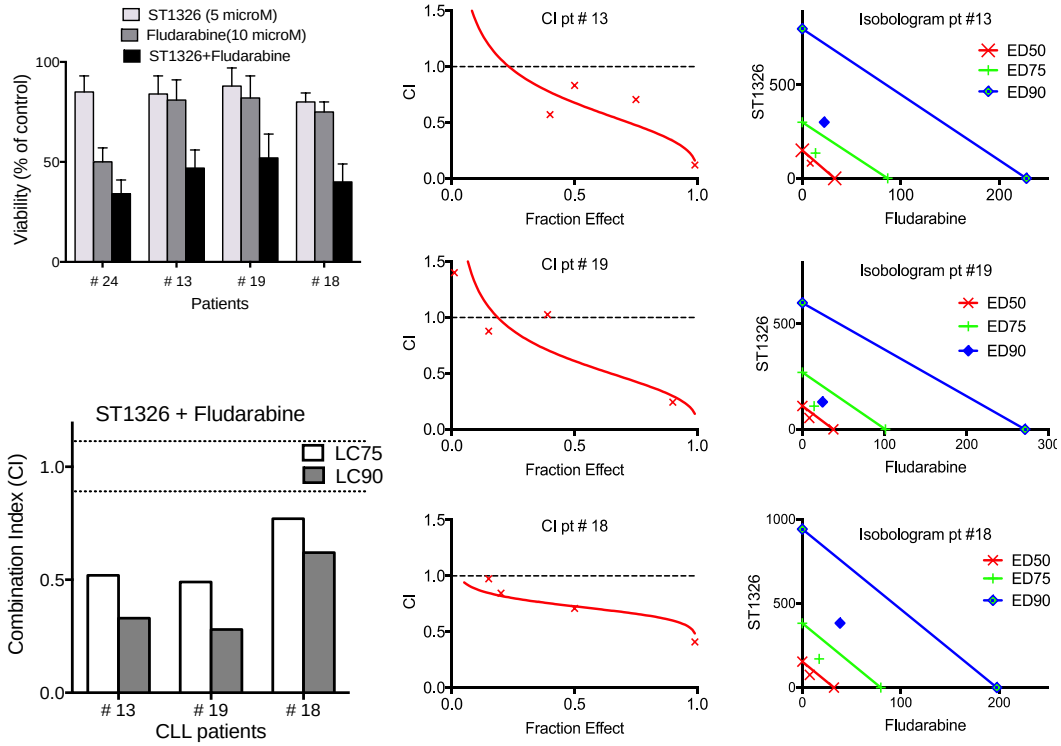

Figure 15- *Synergic ST1326 and Fludarabine drug combination in activated CLL cells.*

Left: *Upper*: Combination cytotoxicity of ST1326 (5 μM) and Fludarabine (10 μM), administered for 24 hours to activated CLL samples. *Lower*: CI values (at LC75 and LC90) calculated by the Chou–Talalay model on dose-effect profiles of activated CLL cells treated for 24 hours with increasing concentrations of ST1326 (1–20 μM), Fludarabine (1–30 μM) or ST1326/Fludarabine at constant ratios.
Right: CI curves and isobolograms. For each patient, CI and isobologram from one representative experiment are shown, at LC75 and LC90 level of cytotoxicity.

In particular, isobolograms and CI calculated from cytotoxicity profiles showed a remarkable level of synergy for ST1326/Fludarabine, in most CLL samples (Figures 15 lower and right).

Combination cytotoxicity of ST1326 with Idelalisib and Ibrutinib

A major advancement in therapy for CLL has been the development of two BCR signalling inhibitors: Ibrutinib and Idelalisib. However, recent data have reported the development of resistances to Ibrutinib (59; 60), and the need to reduce the Idelalisib treatment dose due to relevant adverse events (61). Therefore, it would be interesting to evaluate if the combined treatment of these drugs with ST1326, can improve their efficacy.

Data from drugs combination experiments showed that ST1326 did not potentiate the effects of either Ibrutinib or Idelalisib (Figure 16).

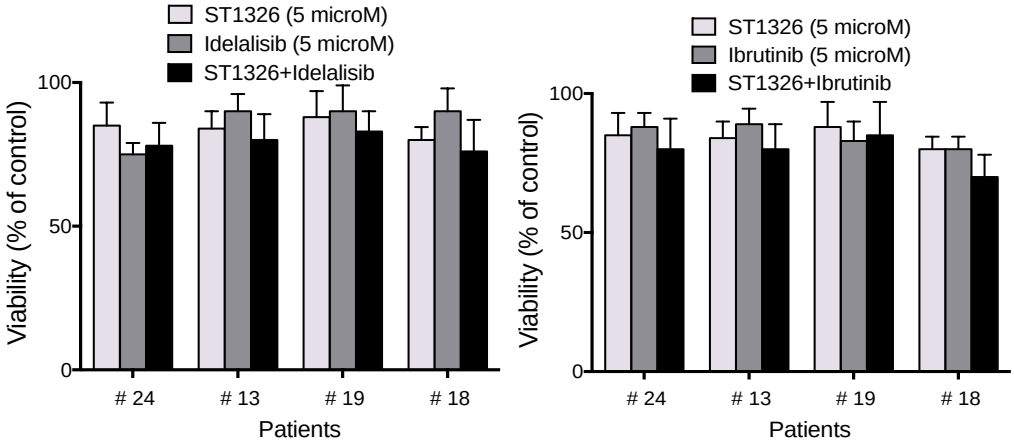

Figure 16- *ST1326 did not potentiated the effects of Ibrutinib or Idelalisib.*

Combination cytotoxicity of ST1326 (5 μM) and Ibrutinib (5 μM) or Idelalisib (5 μM) administered for 24 hours to activated CLL samples.

Discussion

Is becoming increasingly clear that lipid metabolism has a crucial role in cancer cells. A recent study showed that CLL cells display an altered lipid metabolism if compared to normal B lymphocytes (74). In particular, the leukaemic cells show an up-regulation of factors involved in FAs metabolism such as LPL, CPT1A and PPAR-α (74). Thus, lipid metabolism can represent another important source of energy, in addition to glucose metabolism, able to satisfy the energetic needs of proliferating leukaemic cells. ST1326 is a therapeutic agent that acts by blocking FAO metabolism, through the reversible and selective inhibition of CPT1A (86; 87), the rate-limiting enzyme of FAO.

In this study, we addressed the in vitro effects of ST1326 on CLL cells either quiescent or stimulated to become activated blasts that enter the cell cycle.

We demonstrated, for the first time, that ST1326 exerts in vitro, at doses that are clinically achievable, a remarkable apoptotic activity towards activated and proliferating CLL cells, irrespective of p53/SF3B1 mutational status or chromosomal aberrations, known to confer chemoresistance. Interestingly, normal B lymphocytes were more resistant to the cytotoxic activity of the drug. At the doses that are lethal to CLL cells, no cytotoxic, though slightly cytostatic, effect of ST1326 was observed.

The cytotoxic effects of ST1326 in CLL cells were associated with a reduction of intracellular Acetyl-CoA levels and the downregulation of signalling pathways that are crucial for survival, activation and proliferation of leukaemic cells. In particular, a reduction in Mcl-1 and Bcl-xL expression after ST1326 treatment accounted for the potentiation of Venetoclax and Fludarabine, whose cytotoxic efficacy is hindered when cells enter proliferation and upregulate expression of anti-apoptotic proteins. These data indicate that FAO inhibition could represent a suitable target for CLL treatment.

Treatment of CLL cells with ST1326 induced dose-dependent decrease of cell viability. Our results are in line with data previously reported by Pacilli et al., which demonstrated a potent cytotoxic effect of ST1326 on Burkitt's lymphoma cells (81). As well, Ricciardi et al. showed a cytotoxic activity of ST1326 on leukaemia cell lines and primary hematopoietic malignant cells, including cells from CLL patients (83). However, Ricciardi et al. did not evaluate the case in which the CLL cells are subjected to activating microenvironment-

mimicking stimuli. In our experimental setting, we administered ST1326 also on stimulated CLL cultures and we demonstrated, for the first time, that activated/proliferating CLL cells are more sensitive to ST1326 treatment than quiescent ones. This is a very important result because these cells significantly contribute to CLL disease progression.

We found that ST1326 treatment determined a reduction in the levels of intracellular Acetyl-CoA. The depletion of cytosolic Acetyl-CoA may be a consequence of CPT1A-blocking activity of ST1326 (81; 86; 87). In addition, it has been demonstrated, in a work by Pacilli et al., that ST1326 is able to inhibit also the activity of CACT (81), an enzyme responsible for the transport of acetyl-carnitine in the cytoplasm, where it regenerates Acetyl-CoA and carnitine. As a consequence, also the inhibition of CACT causes a reduction in the intracellular level of Acetyl-CoA. Finally, given that Acetyl-CoA is also required for de novo synthesis of FAs, needed for the formation of new membranes, its reduction could result in a decrease of FAs production, which are very important for proliferating cells (93). Altogether, we may hypothesize that the depletion of cytosolic Acetyl-CoA induces a cellular energetic downturn that might account for the growth inhibition effects of ST1326.

We observed an intracellular accumulation of lipid that is probably due to the CPT1A-blocking activity (86; 87), in line with previous observations on lymphoma and leukaemia cell lines (81; 83). This could account for the observed ST1326-induced activation of Bax if we consider the detergent properties of the accumulated lipids (85).

The results of the present study showed that CPT1A inhibitor strongly affects STAT3 and STAT6 activation in stimulated CLL cells independently, at least in part, from the modulation of JAK2/3 and NF-KB. Interestingly, STAT3 total expression was diminished as well after ST1326 administration, probably because its expression is also regulated by p-STAT3 (100; 101). It remains to be elucidated how this drug down-regulates STAT3 and STAT6 activity. It is important to stress that STATs have a crucial role in CLL pathogenesis (99; 100). Thus, the data of our study may advocate a potential benefit of ST1326 as a STAT3/STAT6-modulator.

Peripheral blood B lymphocytes from healthy donors, were less sensitive to the cytotoxic effect of ST1326, at the doses that were effective on CLL cells, in line with the previous observation of no cytotoxic effect of ST1326 on normal bone marrow CD34+ cells (83). Instead, we observed a cytostatic effect after the treatment with the CPT1A inhibitor.

We hypothesized that higher cytotoxicity of ST1326 in CLL cells may be due to the observation that cancer cells appear to be more addicted to the protective activity of Bcl-2 anti-apoptotic members compared to normal cells, possibly because these anti-apoptotic proteins have allowed the tumour cells to survive to deadly insults such as genomic instability, oncogene activation and cell cycle checkpoint violation (63).

Induction of cell death was associated with the down-modulation of Mcl-1 and Bcl-xL expression, two important anti-apoptotic proteins for CLL cells (44; 45), that are correlated with the development of resistance against current therapies (45; 46; 72).

In particular, the cytotoxic activity of the Bcl-2 selective inhibitor ABT-199 (64), a novel promising antileukaemic agent, is impaired when Mcl-1 and Bcl-xL are overexpressed (72; 73). In our experimental setting ST1326 potentiated the action of ABT-199 in stimulated CLL cells, and the combination of the two drugs turned out to be synergic.

Another considerable clinical problem in CLL is the development of resistance against Fludarabine (16; 43; 45; 46). ST1326, by reducing Mcl-1 and Bcl-xL expression, could make proliferating CLL cells more sensitive to this chemotherapeutic agent. Our results demonstrated that ST1326 shows a synergic effect with Fludarabine in stimulated CLL cells.

Finally, we evaluated the combination of ST1326 with novel BCR inhibitors: Ibrutinib and Idelalisib. Also in this case, chemoresistance and systemic side effects advocate therapies capable of reducing the administered dose (59; 60; 61). Our results show that ST1326 did not potentiate the effects of both drugs. A possible explanation could be that Ibrutinib and Idelalisib inhibit proliferation of leukaemic cells and this may hinder CLL cells sensitivity to ST1326 (55).

The proliferative compartment in CLL is responsible for disease persistence after treatment (104; 105). Therefore, targeting this crucial compartment with ST1326 in combination with standard therapies, such as ABT-199 and Fludarabine, could be an efficient approach to try to eradicate both proliferative and non-proliferative compartments of CLL cells.

ST1326 is a well-tolerated drug and is used in clinics for diabetes treatment. Data of the literature also show an antiproliferative effect by two other drugs, Etomoxir and Perhexiline which, unlike ST1326, are irreversible inhibitors of CPT1 (82; 106). However, the use of these irreversible CPT1 inhibitors has been discouraged in the clinical practice because of excessive liver (107) and cardiac toxicity (108). Interestingly, in our in vitro study a ten times lower dose was needed for ST1326 to reach a cytotoxicity level similar to that obtained with Etomoxir. ST1326, possibly due to its reversibility and high selectivity for the CPT1A isoform, displays in vivo only low and transient liver toxicity (87). The doses used in our study are clinically achievable. Investigations on the pharmacokinetics of this aminocarnitine derivative, performed by Sigma-Tau, indicated that plasma levels reach drug concentrations in the high nanomolar/low micromolar range, particularly under fasting conditions (ST1326, Sigma-Tau Investigator's Brochure, Edition no. 3, Nov 5, 2007). Notably, autoradiography on rats and monkeys documented drug distribution in the spleen (unpublished data from Sigma-Tau), one of the lymphoid tissues where CLL cells reside and might receive activation stimuli. Thus, the drug concentration used in our experiments is not far from plasma levels and could be even lower than drug concentration in lymphoid organs.

These results demonstrate that sensitivity of CLL cells to inhibition of beta-oxidation is much stronger in cells activated by microenvironment stimuli and entering proliferation than in quiescent ones, suggesting that activated leukaemic cells are more dependent on FAO for survival and proliferation. This notion is particularly important because CLL disease development and progression are strictly linked to the iterative re-activation cycles that occur in lymphoid tissues. Therefore, we feel that our results pave the way for ST1326 as a novel tool to be proposed in anti-CLL drug-combination therapeutic regimens, particularly with drugs whose efficacy is offset by cell activation and proliferation.

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