UNIVERSIDAD AUTÓNOMA DE MADRID
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Chronic lymphocytic leukemia: New insights into the tumor microenvironment and sensitivity to rationally selected compounds

by

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Tesis presentada en la Universidad Autónoma de Madrid para optar al grado de Doctor

Madrid 2010

This thesis, submitted for the degree of Doctor in Philosophy at the Unversidad Autónoma de Madrid, has been performed in the laboratory of Lymphoma Group at Spanish National Cancer Research Center (CNIO). The presented work was done under the guidance of Dr Miguel Ángel Piris Pinilla. This work was supported by Ministerio de Sanidad y Consumo (FIS PI05-1623) y la Red Temática de Investigación Cooperativa (FIS RD06/0020/0107).

El trabajo presentado en esta memoria para optar al grado de doctor en la Universidad Autónoma de Madrid ha sido realizado en el laboratorio de Linfomas del Centro Nacional de Investigaciones Oncológicas (CNIO) bajo la dirección del Dr Miguel Ángel Piris Pinilla. La realización de este trabajo ha sido posible gracias a la financiación del Ministerio de Sanidad y Consumo (FIS PI05-1623 y de la Red Temática de Investigación Cooperativa (FIS RD06/0020/0107).

"… Porque me gusta conversar con usted como si no fuese mi padre, me gusta hacer cuenta, como dice, de que somos dos personas que se quieren mucho, padre e hija que se quieren porque lo son, pero que igualmentese se querrían con amor de amigos si no lo fuesen…"

La Caverna
José Saramago

Dedicada a mis padres

ACKNOWLEDGEMENTS

I would like to thank so many people for their help along these five years… I feel like doing it in Spanish, but I would also like to say: THANK YOU!

Esta tesis (con sus descubrimientos y sus limitaciones) no podría haberse hecho realidad sin la ayuda, la colaboración, el interés, la motivación y el apoyo de un gran número de personas. Y por supuesto a todas ellas, quiero darles las gracias.

En primer lugar quiero agradecer a **Miguel Ángel** la maravillosa oportunidad que me brindó al entrar a formar parte de su grupo. Sin él no podría haber llegado hasta aquí. Gracias por confiar en mí y darme toda la libertad del mundo.

También quiero agradecer a **May** toda su ayuda. Porque siempre tuvo tiempo, paciencia y buenas palabras para mis problemas y mis dudas.

Parte de este trabajo habría sido imposible sin la colaboración de **María** que contestó a mis infinitas preguntas sobre la inmunohistoquímica. ¡Gracias!

También quiero agradecer a mis compañeros su ayuda y comprensión durante estos años:

Gracias a **Dani** y a **Magda** que me han acompañado desde que empecé en el CNIO en el 2005. Habéis sido los mejores compañeros que uno podía desear. Dani, por ser la mayor fuente de conocimiento y de entusiasmo que he encontrado a mi alrededor en estos años. Por las alegrías y las tristezas que hemos pasado juntos desde el principio. Por ser siempre tan crítico y a la vez saber buscar el lado positivo de las cosas, por todo lo compartido dentro y fuera del labo. A Magda por toda tu ayuda y por todo lo que hemos aprendido juntas. Y sobre todo gracias por tus risas, por el entusiasmo que pones en todo lo que haces, por tu optimismo tan contagioso y por compartir conmigo todos tus planes de futuro. Dziękuję!

A **Lina**, por tener tanta paciencia en estos últimos meses y leer y comentar toda la tesis ¡de arriba abajo! Y eso que podías haberte hecho la sueca…. Por nuestros largos paseos (con paraditas incluidas), por los sándwiches de la máquina, por las cañas y los vinos, por todas las risas compartidas, las discusiones científicas, las conversaciones acerca de la vida, del presente y del futuro. TACK!

Grazie **Pier** por la ayuda técnica y personal que siempre me has ofrecido. Por nuestras charlitas filosóficas sobre ciencia y sobre ética, sobre gastronomía ¡y por la música! Gracias por darme un empujón siempre que lo he necesitado.

Jelen, que puedo decir. ¡Gracias por ser la alegría de la huerta! Porque contigo todo parece fácil y lo duro se convierte en sencillo y lo triste en divertido. Llegaste en un momento de inflexión para muchos y sacaste lo mejor de cada uno de nosotros.

Lore, gracias por tu espíritu crítico, por tu ayuda con todo lo que haya necesitado. Gracias por dejarnos descubrir a la Lore más cariñosa y amable. Por haberme animado a salir incluso cuando no tenía fuerzas. Grazie!

Pilar y **Espe**, me habéis aportado el equilibrio perfecto entre espíritu científico y práctico. Entre focalizarse y desfocalizarse. Muchas gracias por vuestra ayuda, vuestra escucha (Espe, te debo algunas) y todos los consejos. Pilar, ¡mil gracias por tantos besos y abrazos!

Laurita, gracias por los desayunos, por los consejos, por el buen humor y por tu apoyo siempre y cada día. **MG**, gracias por tu ayuda siempre que la he necesitado (me acuerdo de aquellos EMSAS…), las

sonrisas incluso cuando las cosas no iban bien. ¡Me siento afortunada de haber compartido este tiempo con las dos!

Qué habría hecho yo sin vosotros…

Gracias a **Bea**(**triz**) Pamplona, que siempre me animó a seguir hacia adelante, a **Elena** y a **Marién** que siempre me ayudaron en todo lo que estuvo en sus manos. **Rubito**, fue un placer trabajar juntos, gracias por ayudarme a arrancar este proyecto y por todos los ánimos. Gracias a **Dani G** por tener siempre ideas, por los consejos y el cariño que siempre me has dado.

Por supuesto, gracias a todos los demás miembros de Linfomas. También a los antiguos (Abel, que fue mi salvación en los primeros tiempos, Toñi, MªCruz…) y a los nuevos que van llegando, porque éste es un trabajo en equipo y sin vosotros no podría haberse hecho realidad.

Gracias a **Gonzalo**, por todos sus consejos bioinformáticos y por las menta-poleo y las coca-colas. Gracias a **Arancha** de citometría y a **Diego** de confocal por su ayuda durante estos años y por toda su paciencia.

Además, me gustaría agradecer su apoyo incondicional a algunas personas no relacionadas directamente con este trabajo pero sin las cuales nunca podría haberlo terminado.

Nadia, ¡ya ha llegado el momento! Muchas gracias por lo que tú ya sabes, por sacarme de mi mundo y por salir del tuyo y empezar a disfrutar juntas de lo mejor de Madrid. Por seguir a mi lado incluso en la distancia y con una nueva vida. Muchas gracias a **Daniela** y a **Paola** que tan bien han cuidado de mí en estos últimos años. Gracias, chicas, por la comida, la música y la cultura italianas. Por todas las ideas y las sugerencias para mis problemas en el laboratorio, por escucharme y animarme siempre fuera de él. Por hacer que esto mereciera la pena. Vi voglio bene!

Y por supuesto, gracias a mi familia, la más maravillosa que podría desear. Gracias a mi hermana **Esther**, porque a pesar de ser tan diferentes ha creído en mí incondicionalmente. Porque yo también creo en ella incondicionalmente.

GRACIAS a **Julián** y a **Concha**, mis padres y dos PERSONAS extraordinarias. Porque me han enseñado que ser una persona coherente consigo misma es lo más importante en la vida. Que cada uno tiene que encontrar su camino para ser feliz. Gracias por los consejos, las conversaciones, las llamadas de teléfono, el apoyo, la libertad que siempre me habéis dado y por confiar en mí a pesar de los quebraderos de cabeza ¡Os quiero mucho! y a vosotros os dedico esta tesis.

Gracias a todas aquellas personas que me han ayudado antes de empezar en el CNIO, a los que me sonríen cada día por el pasillo y a todos los que no puedo mencionar por motivos de espacio, porque he llegado hasta aquí gracias a todos vosotros.

¡GRACIAS!

Beatríz

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PUBLICATIONS

ARTICLES

The following articles have been published in scientific journals:

Herreros, B., Rodriguez-Pinilla, S.M., Pajares, R., Martinez-Gonzalez, M.A., Ramos, R., Munoz, I., Montes-Moreno, S., Lozano, M., Sanchez-Verde, L., Roncador, G.*, et al.* Proliferation centers in chronic lymphocytic leukemia: the niche where NF-kappaB activation takes place. Leukemia *24*, 872-876.

Herreros, B., Sanchez-Aguilera, A., and Piris, M.A. (2008). Lymphoma microenvironment: culprit or innocent? Leukemia *22*, 49-58.

Ruiz-Vela, A., Aggarwal, M., de la Cueva, P., Treda, C., **Herreros, B**., Martin-Perez, D., Dominguez, O., and Piris, M.A. (2008). Lentiviral (HIV)-based RNA interference screen in human B-cell receptor regulatory networks reveals Mcl-1-induced oncogenic pathways. Blood *111*, 1665-1676.

Wozniak, M.B., Villuendas, R., Bischoff, J.R., Aparicio, C.B., Martinez Leal, J.F., de La Cueva, P., Rodriguez, M.E., **Herreros, B.**, Martin-Perez, D., Longo, M.I.*, et al.* Vorinostat interferes with the signaling transduction pathway of T-cell receptor and synergizes with phosphoinositide-3 kinase inhibitors in cutaneous T-cell lymphoma. Haematologica *95*, 613-621.

BOOK CHAPTERS

Herreros B. and Piris M.A.

Chapter 90: Lymphoma microenvironment

Book title: The Lymphoid Neoplasms, third edition

Hodder Arnold Publication, July 2010

SUMMARY/RESUMEN

SUMMARY

Chronic lymphocytic leukemia (CLL) shows unique features when compared with other types of malignancies. The majority of tumor cells are arrested in G0/G1 phase and only a small number of proliferating cells are observed in special anatomical structures known as proliferation centers (PCs) that can be found mainly in lymph nodes of CLL patients. This fact highlights the importance of the interactions between tumor cells and their microenvironment. Therefore, we were interesting in studying the composition of PCs as well as the nature of these interactions, in paraffin embedded samples. We described two different stroma populations that we called Actin Dendritic Cells (ADC) and STAT1 positive macrophages (STAT1 macrophages). Moreover, we identified PCs as the place where NF-κB activation takes place (indicated by nuclear localization of p50, p52 and Rel B), which is a well known altered pathway in CLL.

Although a large proportion of the patients achieve complete response with current therapies, many of them develop resistance and indeed, CLL remains an incurable disease. We have extrapolated our knowledge of the mechanisms involved in the pathogenesis of CLL to the field of new therapeutic targets and active compounds. With this purpose, we have tested a panel of four rationally selected compounds (calmidazolium, R406, TW-37 and ETP-39010) in *ex-vivo* CLL cultures. Variable sensitivity was observed, reflecting the molecular heterogeneity of the samples. In addition, we identified some candidate biomarkers and resistance mechanisms that can help to predict drug sensitivity. Briefly, our data showed that BCR signaling inhibition has a negative effect on cell viability. Moreover, samples sensitive to R406 (SYK inhibitor) showed higher expression levels of MUM1/IRF4 while resistant samples showed enrichment of pathways related to the microenvironment. We also identified Mcl-1 as a candidate biomarker for sensitivity to TW-37 (small molecule inhibitor of the Bcl-2 family) showing that sensitive samples presented higher protein levels. Finally, ETP-39010 (PIM kinase inhibitor) induced apoptosis in samples that presented markers of bad prognosis such as unmutated IGHV, ZAP70 positivity and higher expression levels of lipoprotein lipase (LPL). Inhibition of PIM kinases affected gene transcription and metabolic processes. This type of studies may contribute to the better understanding of the individual molecular features underlying drug sensitivity.

RESUMEN

La leucemia linfocítica crónica (LLC) es una enfermedad que presenta unas características únicas en comparación con otros tipos de cáncer. La mayoría de las células tumorales se encuentran arrestadas en fase G0/G1 y sólo se observa un pequeño número de células que proliferan en estructuras anatómicas especiales localizadas en los ganglios linfáticos de pacientes conocidas como centros de proliferación (CP). De ahí nuestro interés en estudiar los CPs en muestras incluidas en parafina. Así, hemos descrito por primera vez dos tipos de poblaciones del estroma dentro de los CPs que hemos denominado Células Dendríticas que expresan Actina (CDA) y macrófagos con STAT1 nuclear (macrófagos STAT1). Además, hemos identificado los CPs como el lugar donde tiene lugar la activación de NF-κB (definida por la localización nuclear de p50, p52 y Rel B), que es una de las vías de señalización comúnmente alteradas en LLC.

Por otro lado, aunque la mayoría de los pacientes alcanzan una respuesta completa con los tratamientos actuales, muchos de ellos desarrollarán resistencias, por lo que la LLC sigue siendo una enfermedad incurable. En este trabajo, hemos extrapolado nuestro conocimiento de los mecanismos implicados en la patogénesis de LLC al campo de la identificación de nuevas dianas terapéuticas. Con este objetivo, hemos probado cuatro compuestos escogidos racionalmente (calmidazolium, R406, TW-37 y ETP-39010) en cultivos de LLC *ex vivo*. Hemos observado una variabilidad en la sensibilidad a estos compuestos que refleja la heterogeneidad de las muestras. Además, hemos identificado una serie de posibles biomarcadores y mecanismos de resistencia que podrían ayudar a predecir la sensibilidad a estos compuestos. Brevemente, nuestros datos indican que la inhibición de la vía del receptor de células B tiene un efecto negativo sobre la viabilidad celular. Además, las muestras sensibles a R406 (inhibidor de SYK) presentan niveles de expresión más altos de MUM1/IRF4, mientras que las muestras resistentes tienen un enriquecimiento de las vías de señalización relacionadas con las interacciones con el entorno tumoral. También hemos identificado Mcl-1 como un posible marcador de la sensibilidad a TW-37 (inhibidor de la familia de Bcl-2) mostrando que las muestras más sensibles presentan niveles de proteína más altos. Finalmente, observamos que ETP-39010 (inhibidor de la familia de quinasas PIM) induce apoptosis en las muestras que presentaban marcadores de mal pronóstico (IGHV no mutada, ZAP70 positivas y niveles más altos de expresión de la lipoproteína lipasa). La inhibición de estas quinasas afecta a procesos relacionados con la transcripción génica y el metabolismo. En definitiva, este tipo de estudios pueden contribuir a un mejor conocimiento de las características moleculares individuales que subyacen a la sensibilidad a diferentes compuestos activos.

ABBREVIATIONS

ADC	Actin positive Dendritic Cell
BAFF	B cell Activating Factor
BCR	B Cell Receptor
CLL	Chronic Lymphocytic Leukemia
c-MAP	Connectivity MAP
CNIO	Spanish National Cancer Research Center
CR	Complete Remission
Cy3	Cyanine 3-conjugated dUTP
Cy5	Cyanine 5-conjugated dUTP
CZ	Calmidazolium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxyribonucleoside-5'-triphosphate
EC50	Effective concentration for 50% of the cells
FAM	6-carboxyfluorescein
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FDC	Follicular Dendritic Cell
FDR	False Discovery Rate
FE	Feature Extraction
GC	Germinal Center
GEP	Gene Expression Profiling
GSEA	Gene Set Enrichment Analysis
H&E	Haematoxylin and Eosin
IGHV	Variable region of the Immunoglobulin Heavy Chain
IRF4	Interferon Regulatory Factor Gene
LPL	Lipoprotein Lipase
MBL	Monoclonal B lymphocytosis
M-CLL	Mutated Chronic Lymphocytic Leukemia
MRD	Minimal Residual Disease
mRNA	Messenger RNA

MUM1	Multiple Myeloma Oncogene 1
NFκB	Nuclear Factor Kappa B
NLC	Nurse-like cells
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Proliferation Center
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
SAM	Significance Analysis of Microarrays
SLL	Small Lymphocytic Lymphoma
SDS	Sodium Dodecyl Sulfate
STAT1-Ms	STAT1 positive macrophages
TCR	T Cell Receptor
TMA	Tissue Microarrays
U-CLL	Unmutated Chronic Lymphocytic Leukemia
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
WHO	World Health Organization
ZAP70	Zeta-chain-associated Protein Kinase 70

1. INTRODUCTION

1.1. The immune system and development of mature B cells

The immune system is a bodywide network of specialized cells, tissues and organs that has evolved to protect the organism against disease caused by pathogens. It is made up of the lymphatic vessels, the spleen, the thymus, the lymph nodes and the bone marrow. The first challenge of the immune system is to identify pathogens and distinguish them from the cells of the own organism. This protection against foreign attacks is exerted primarily via an innate immune response, a first line of defense that includes physical barriers as well as cell responses exerted by neutrhophils, macrophages and granulocytes. Later in the infection process, the adaptive response mounts a more specific defense mediated mainly by lymphoid cells (B and T cells) that are assisted by other immune cells such as dendritic cells. Disorders in the immune system can lead to immunodeficiencies, autoimmune diseases or even cancer.

B cells are central players of the adaptive immune response that secrete antibodies, molecules that bind pathogens allowing their recognition by phagocytes and the activation of the complement system. Antigen binding to the B cell receptor (BCR) elicits different responses depending on the maturation stage of the B cell. In order to understand the context in which normal (and tumor) cells develop, the next paragraphs will summarize the development of B cells and the most important controls that they must undergo before becoming fully mature B cells (see Figure 1).

B cells differentiate from pluripotent cells known as **common lymphoid progenitors** in the bone marrow. Along the development, the expression of an appropriate B cell receptor, composed of a surface immunoglobulin (Ig), will play a key role in the cell fate. Ig embedded in the surface membrane has the function of recognizing and responding to exogenous antigens and is made of two heavy and two light chains, each of them presenting a constant and a variable region. Antigen recognition takes place via the variable (V) regions, which differ in sequence from one B cell to another, and provide a complete catalog of potential antigen-combining sites. The Ig locus includes several V, D and J segments. Rearrangement at the DNA level of these regions produces the extreme diversity of the BCR (Tonegawa, 1983). In fact, Ig selection takes place from the potentially functional genes of an unrearranged repertoire that includes 51 VH genes divided into 7 families (VH1–VH7), 27 D genes, and 6 JH genes. This recombinatorial process is mediated by proteins encoded by the recombination-activating

genes *RAG1* and *RAG2* (Lewis and Gellert, 1989). BCR diversity is even greater because the junctions of VH to D and D to JH are imprecise, with the deletion by exonucleases of templated nucleotides or the insertion by terminal deoxytransferase (TdT) of nontemplated nucleotides in a random manner (Desiderio et al., 1984). As a consequence, three highly variable regions known as complementarity-determining regions (CDR) are generated after VDJ recombination. The CDR3 shows the highest variability and is mainly responsible for the virtually unique antigen recognition capacity of any given lymphocyte.

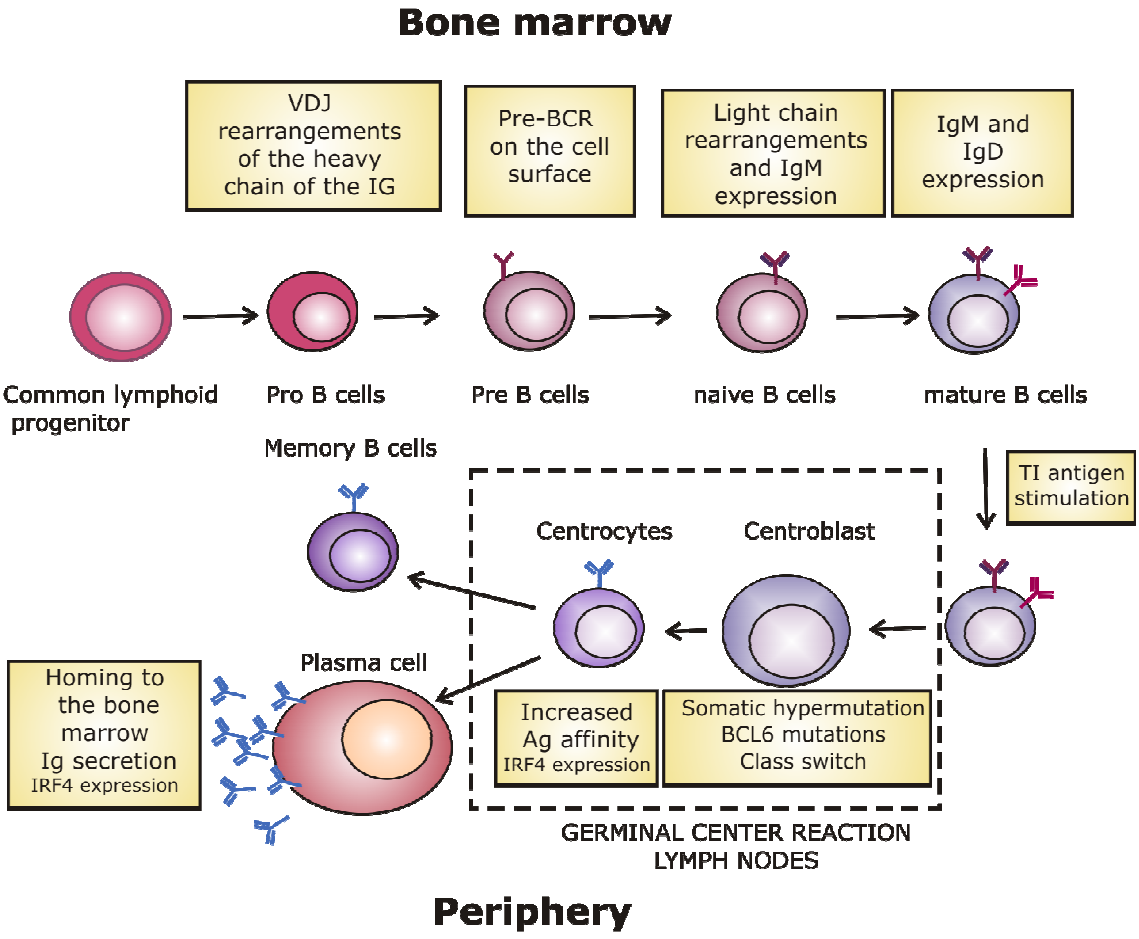


Figure 1. Normal B cell development. Hematopoietic stem cells found in the bone marrow differentiate to mature B cells that circulate in the blood until they encounter an antigen. Then, they enter the lymph nodes where they acquire increased antigen affinity and start to proliferate giving rise to long-lived memory cells that will circulate in the blood or to plasma cells, which home to the bone marrow where they produce immunoglobulins (Ag, antigen; Ig, immunoglobulin; TI, T-independent; BCR, B cell receptor).

Rearrangement of the VDJ genes of the heavy chain of the immunoglobulin takes place in the stage of **pro-B cells** (still progenitor cells but with limited self-renewal capacity). B cells express

then an incomplete BCR or pre- B cell receptor (BCR) on the surface and therefore B cells at this stage are called **pre-B cells**. The pre-BCR is made of a successfully rearranged heavy chain and a surrogate of the light chain. This pre-BCR generates signals that will stop rearrangement of the heavy chain, induce several rounds of division and allow the cell to progress to the next stage of development in which the rearrangement of the light chains of the Ig takes place. Once a light-chain gene is assembled and a complete IgM molecule is expressed on the surface, the cell is defined as an **immature B cell**. Up to this point, B cell development is antigen independent. Now, immature B cells undergo negative selection for self-tolerance and abandon the bone marrow. B cells in the periphery are known as **naïve B cells**. They will circulate in the peripheral blood until they encounter a foreign antigen. There are several checkpoints in B-cell maturation. Failure to produce a functional immunoglobulin induces apoptosis, as does an autoreactive specificity. Escape from apoptosis can occur by rearranging the other allele, most commonly the light chain. This process is known as receptor editing.

Once naïve B cells encounter an antigen that fits their BCR, they migrate into the center of primary follicles inside the lymph nodes and proliferate. At this stage they are known as **centroblasts**. Together with a network of follicular dendritic cells and CD40L+ T cells, they form the so called germinal center (GC) structures where somatic hypermutation of the Ig genes and class switch processes take place. These are mechanisms to further increase the diversity of the B-cell repertoire. The rate of introduction of base pair changes in the Ig genes during the process of somatic mutation is on the order of 10^{-4}–10^{-3} per generation. The mutations tend to cluster in the CDRs (regions of high variability found between VD and DJ segments), possibly because these regions are essential for antigen specificity. A further genetic arrangement is necessary for Ig class switching from IgM plus IgD to IgG, IgA, or IgE. The choice of isotype is cytokine determined (Stavnezer et al., 1985). Centroblasts mature to **centrocytes** that express an Ig with an increased antigen affinity. They then differentiate either into long-lived **memory B cells** that circulate in the peripheral blood or **plasma cells** that home to the bone marrow where they will secrete immunoglobulins. Naïve B cells can also mature outside of the GC as a result of a T-independent stimulation, upon binding of T-independent antigens (mainly polysachrides from bacteria or nucleic acids) that induce B cells to produce antibodies in the absence of T cell help. These centroblasts then may mature directly into short-lived plasma cells, enter a GC or mature to memory cells.

The peripheral blood B cells of normal individuals comprise 60% naïve cells with unmutated IgVH genes and 40% memory cells that carry somatically mutated IgVH genes and express surface CD27 (Klein et al., 1998). Only a small proportion of naïve cells express CD5 (Brezinschek et al., 1997), although this proportion is higher in early life.

1.2. Chronic lymphocytic leukemia

1.2.1 Lymphomas/Leukemias

B cell and T/NK cell neoplasms are clonal tumors that develop from immature or mature B, T or NK cells.The clonal expansion of tumor cells leads in the case of lymphoid cells not only to an uncontrolled proliferation of the malignant clone with the consequent invasion of healthy tissues, but also to a dysfunction of the immune system, which is accompanied by a defective protection against infections. These neoplasms form a complex group of malignancies that includes more than 30 subtypes. The World Health Organization (WHO) classifies them according to a combination of morphological and immunophenotypical characteristics, including recently also some genetic features and molecular parameters that contribute to refine this classification. According to the SEER (Surveillance and Epidemiology End Results) the incidence rate of lymphomas and leukemias in 2009 was 42.3 per 100,000 men. B cell neoplasms comprise over 90% of all lymphoid neoplasms worldwide. Precursor lymphoid neoplasms are primarily a disesase of children while mature B cell neoplasms comprise over 90% of lymphoid neoplasms worldwide and represent 4% of new cancers each year.

The distinction of the lymphoma subtype is not only relevant in terms of lymphoma pathogenesis but it also defines different clinical behaviors and therefore, determines diverse treatment strategies. This is the main reason that moves clinicians together with researchers to join efforts in order to find the most accurate lymphoma classification. B and T/NK neoplasms recapitulate the different stages of development of normal healthy immune cells, and are to some extent classified according to these stages.

1.2.2 Chronic lymphocytic leukemia: definition and immunophenotype

Chronic lymphocytic leukemia (CLL) is a malignant lymphoproliferative disorder of mature B lymphocytes, recognized as a clinical entity since the early 1900s (Turk, 1903; Osler, 1909), although cases resembling CLL had already been described by Virchow and Bennett in 1845. Based on their similar morphological and immunophenotypic features, the most recent World

Health Organization (WHO) classification scheme for hematopoietic malignancies considers CLL and Small Lymphocytic Lymphoma (SLL), a type of low grade non-Hodgkin lymphoma, to be different manifestations of the same disease and combines these entities into one disease category (CLL/SLL) (Jaffe, 2009). The term SLL is used for non-leukemic cases with the tissue morphology and immunophenotype of CLL.

CLL/SLL is characterized by a pathological accumulation of B cells within peripheral blood and lymphatic tissues. This neoplasm is composed of small, round to slightly irregular B lymphocytes in the peripheral blood (PB), bone marrow (BM), spleen and lymph nodes. The lymph nodes involved by CLL/SLL usually show a diffuse effacement of the architecture by a proliferation of small lymphocytes. A variable number of prolymphocytes with central nucleoli are always present and in some cases aggregates with larger cells called paraimmunoblasts are also found. These lymphocytes appear intermixed with accompanying cells and T cells in structures known as **proliferation centers** (PC), because proliferating CLL cells tend to accumulate in these areas.

Immunophenotypically, malignant cells express IgM/IgD, CD20, CD22, CD19, CD79a, CD5, CD43, CD11 (weak) and CD23 (which allows distinction from mantle cell lymphomas). There is also no or weak expression of FMC7, CD22, and CD79b. The accumulation of monoclonal B lymphocytes leads to leukocytosis, bone marrow failure, recurrent infection and is sometimes associated with autoimmune diseases such as hemolytic anemia.

1.2.3 Epidemiology

CLL is the most common leukemia of adults in Western countries (USA and Europe) together with acute myeloid leukemia. It shows a very low incidence rate in far Eastern countries (Japan, Singapore, China and India), even in migrant populations suggesting a role for racial differences and in favor of a genetic predisposition (Dores et al., 2007). The incidence of CLL is around 4.2 cases per 100,000 men and women per year. It increases with age reaching an incidence of 12.8/100,000 at the age of 65, which is the mean age at diagnosis. CLL/SLL has a male: female ratio of 1.5-2: 1. Mortality was 7.3 per 100,000 men and women from 2002 to 2006 (Data diagnosed between 2003 and 2007. Surveillance, Epidemiology and End Results, National Cancer Institute, http://seer.cancer.gov/statfacts/html/clyl.html).

1.2.4 Progression and transformation

The disease may be stable for a long period of time in the initial stages without a requirement for treatment. After a variable period of time the tumor may progress to stages in which patients require treatment. Clinical progression of the disease is usually associated with an increase of lymphocytes, a more diffuse pattern of infiltration in bone marrow and extramedullary tissue involvement. Transformation into a more aggressive tumor occurs only in 2-10% of the patients and is known as "Richter's syndrome". The two most common forms of transformation are diffuse large B-cell lymphoma (DLBCL) and, less frequently, Hodgkin's lymphoma.

1.2.5 Clinical management of CLL patients

CLL shows an extremely variable clinical course. Some patients have and indolent disease that may never require treatment while others present a progressive clinical course. Patients with slowly progressive disease will only start chemotherapy when the disease causes symptoms (infections, swollen lymph nodes or spleen, weight loss and tiredness among others) and they will show an average survival from diagnosis of 5 to 10 years. However, patients with a progressive clinical course are often resistant to standard treatments and show a reduced average survival of only 24 months or less. The cornerstones to predict prognosis are the clinical staging systems of Rai and Binet (see Tables 1 and 2). In general, early and intermediate stages (Rai 0-II, Binet A and B) that are characterized by lymphocytosis with or whithout lympho-and/or organomegaly can be distinguished from advanced stages (Rai III-IV, Binet C) that present anemia or thrombocytopenia additionally. Survival may range from 10 years or more in early stages to 1-2 years in advanced stages. While treatment is always recommended in this last situation, immediate therapy in patients with early stage CLL (Binet et al., 1981; Rai et al., 1975), i.e. Rai 0 or Binet A, has failed to prolong survival so far (Dighiero et al., 1998; Shustik et al., 1988).

Table 1- Rai staging system

STAGE	Lymphocytosis (>15 x 10^3/µL)	Lymphoadenopathy	Hepato-/splenomegaly	Hemoglobin	Plateletets
0	Yes	no	No	>11 g/dL	>100 x 10^3/µL
I	Yes	yes	No	>11 g/dL	>100 x 10^3/µL
II	Yes	irrelevant	Yes	>11 g/dL	>100 x 10^3/µL
III	Yes	irrelevant	irrelevant	<11 g/dL	>100 x 10^3/µL
IV	Yes	irrelevant	irrelevant	irrelevant	<100 x 10^3/µL

Table 2-Binet staging system

STAGE	Lymphocytosis (>4 x 10^3/μL)	Involved lymphoid tissue regions	Hemoglobin	Platelets
A	Yes	< 3 regions involved	>10 g/dL	>100 x 10^3/μL
B	Yes	≥3 regions involved	>10 g/dL	>100 x 10^3/μL
C	Yes	Irrelevant	<10 g/dL or	<100 x 10^3/μL

Nevertheless, these clinical stages have some limitations. For example, progressive and indolent forms of the disease cannot be predicted in early stages. This fact has become of great importance in the last years because currently, the majority of patients with CLL are diagnosed in asymptomatic, early stages as a consequence of the increasing practice of routine blood analysis. On the other hand, the mechanisms responsible for cytopenias, which define advanced stages, are not taken into consideration (cytopenias of autominue origin may have a better outcome than those due to bone marrow infiltration). Finally, these systems are not able to predict the response to the treatment.

In order to refine clinical staging, there has been an intensive work on biological factors and their prognostic relevance. Among these, cytogenetic alterations and the mutational status of the variable region of the heavy chain of the immunoglobulins have been shown to be of great value in outcome/response to treatment predictions and have shed light into the molecular mechanisms of the disease. Other prognostic factors still under validation for the clinical practice, include ZAP70, CD38 (discussed in the next sections), CD49D (integrin alpha4) (Gattei et al., 2008), LPL (lipoprotein lipase) (Oppezzo et al., 2005) and CLLU1 (CLL upregulated gene 1) (Buhl et al., 2006).

1.2.5.1 Cytogenetic alterations

Multivariate analysis revealed an independent prognostic relevance of genomic abnormalities in CLL patients (Dohner et al., 2000). Deletion in 13q14 as a single aberration was associated with long median survival (133 months), while deletions in 11q22-q23 and particularly in 17p13 were associated with a poor prognosis (median survival time of 70 and 32 months respectively). Intermediate survival times were found for CLL cases without aberrations or with trisomy 12 (111 and 114 months respectively) (Figure 2).

Next, the most frequent genomic abnormalities will be described in more detail (for a summary, see Table 3).

13q14 deletion is the most frequently found chromosome alteration in CLL. Although several candidate tumor suppressor genes and microRNAs are located in this region (*RB1, RFP2, DLEU1, DLEU2*, miR15a, miR16-1) their possible role in CLL pathogenesis has not been fully elucidated. Recently, a mouse model lacking the miR56/16 and *DLEU2* locus has recapitulated a monoclonal B-cell lymphocytosis disorder as well as CLL and DLBCL in some cases, suggesting a role of these microRNAs and DLEU2 in the pathogenesis of the disease (Scaglione et al., 2007). In addition, DLEU7, also localized in the minimal deleted region, has been shown to inhibit NF-κB and NFAT transcription factors that are deregulated in CLL (Palamarchuk et al., 2010). Furthermore, an epigenetic suppressor mechanism has been described in this locus that could deregulate several genes simultaneously (Mertens et al., 2009). When the 13q deletion is present as a sole abnormality, however, patients show a favorable course.

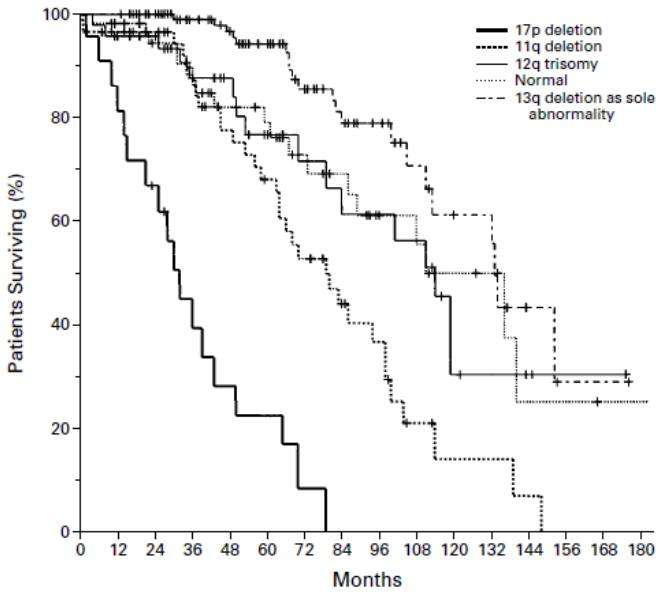


Figure 2. Survival from the date of diagnosis among the patients with CLL showing different genetic alterations. The median survival times for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as the sole abnormality were 32, 79, 114, 111, and 133 months, respectively (Taken from (Dohner et al., 2000)).

Deletions in 11q22-q32 affect the *ATM* gene (Ataxia telangiectasia mutated) in almost all cases. ATM is a master controller of cell cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability. This deletion is associated with more rapid progression (shorter treatment free intervals and survival times) and was found to be an independent adverse prognostic factor (Austen et al., 2007). ATM mutations in the residual allele have been observed in 36% of CLL cases with 11q deletion. This has been

associated with an even greater reduction in patient survival. Inactivation of ATM is likely associated to genomic instability and secondary resistance due to an impaired DNA damage response. Other genes associated with this deletion are *NPAT* (cell cycle regulation), *CUL5* (ubiquitin dependent apoptosis regulation) and *PP2R1B* (component of cell cycle and apoptosis regulating PP2A) (Kalla et al., 2007).

Trisomy 12 has been associated with an intermediate prognosis. A specific gene or genes associated with trisomy 12 has not been identified. Some candidates are *CDK4, GLI* and *MDM2* (Huang et al., 1994). However, their pathogenic role remains to be determined.

Deletion of 17p13 is found in 4 to 9% of CLL patients and always involves the *TP53* gene, which encodes for the tumor suppressor protein p53 that responds to diverse cellular stresses and induces cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. The incidence of *TP53* mutations in the remaining allele is much higher in cases with 17p deletion, where more than 90% of cases show mutations. Although the number of patients showing 17p deletion is relatively small in first-line treatment situations (4-9%), its detection is of enormous importance because these patients are unlikely to respond to conventional chemotherapy and alternative treatments should be chosen (Dohner et al., 2000).

Table 3-Genomic abnormalities found in CLL and their clinical relevance

Genomic alteration	Percentage of CLL patients	Genes affected	Prognosis	Prognosis in treatment
13q-	55%	*DLEU2 DLEU7, miR15, miR16*	-	-
13q single	36%	*DLEU2,DLEU7 miR15, miR16*	Good prognosis	-
11q-	18%	*ATM*	Rapid progression, poor prognosis	Patients are refractory to DNA-damaging drugs
+12q	10-20%	*CDK4, MDM2, GLI*	Intermediate	
17p-	4-9%	*TP53*	Rapid progression, poor prognosis	No response to conventional treatment
6q-	7%	-	Intermediate risk	-

Deletion of 6q is observed in around 7% of CLL patients. It has been described that this genomic abnormality may confer a high incidence of atypical morphology, classical immunophenotype with CD38 positivity and intermediate incidence of IGVH somatic

hypermutation. Clinicobiological features and outcome show that this cytogenetic subset of CLL should be allocated in an intermediate-risk category (Cuneo et al., 2004).

1.2.5.2 Immunoglobulin variable heavy chain gene mutational status

About 40-50% of patients with CLL present in their tumor cells somatic hypermutation of the variable genes of the heavy chain of the immunoglobulin genes (IGHV). This IGHV mutational status can differentiate two different clinical forms of the disease. If the homology with the germ line gene is higher than 98%, patients are considered to have umutated IGHV genes. This threshold, although arbitrary, was chosen because polymorphisms, which are quite common in VH genes, can account for that degree of disparity (Matsuda et al., 1993).

Patients with unmutated IGHV genes (U-CLL patients) have a more aggressive condition, including advanced, progressive disease, adverse cytogenetic features and resistance to therapy, than those with mutated IGHV genes (M-CLL patients) (Damle et al., 1999; Hamblin et al., 1999). Cases in the borderline (i.e. cases with an IGHV homology of 97-98%) seem to have an intermediate prognosis (Hamblin et al., 2008). This clinical diversity underlines some biological differences between the two groups. They show different levels of ZAP70 (see section 2.4.3), different telomere lengths and different likelihood of carrying genetic lesions. The prognosis significance of IGHV mutations is independent from other factors, particularly in early stages of the disease. Although the role of IGHV mutational status in guiding therapy should not be used as a criteria to guide treatment decisions in a routine daily setting, its use in current and future randomized clinical trials will allow its validation prospectively. However, its determination is expensive and time-consuming to be introduced in the clinical routine and therefore there have been many attempts to identify a surrogate marker.

1.2.5.3 ZAP70 expression

ZAP70 is a tyrosine kinase involved in signaling through T cell receptor. Strikingly, a group of CLL patients also expresses ZAP70. This group of patients shows a more aggressive disease. ZAP70 expression has been associated with unmutated CLL cases and has been described as a better predictor of the need of treatment (Rassenti et al., 2004). However, up to 25% of cases present discrepancy with the mutational status of IGHV, probably due to the difficulty to standardize ZAP70 measurement. Although the current available evidence is not strong

enough to incorporate this parameter in the routine practice, this marker may be helpful in providing further information and is routinely determined in prospective clinical trials

1.2.5.4 CD38 expression

Expression of CD38 on leukemic cells was the first marker to be correlated with IGHV mutations (Damle et al., 1999). Nevertheless, eventually it was found that the relationship is not absolute and according to some studies, CD38 expression may vary over the time. Consequently, this prognostic marker is considered only in the context of clinical trials but not in the clinical routine.

1.2.6 Treatment

CLL patients show an extremely variable course. While some have a life expectancy similar to that of the general population, others show a rapidly fatal course and die months after diagnosis. Up to half of all patients with early-stage disease eventually will require treatment. In the last few years, there has been a remarkable increase in the Complete Resoponse (CR) rate (assessed by physical examination and complete blood count) that has increased from less than 10% with the use of alkylators to 60-70% with modern chemoimmunotherapy regimens (Bosch et al., 2008; Tam et al., 2008). These advances are extremely relevant because CR, although not sufficient to reach disease eradication, is associated with longer survival. Patients in CR can carry up to 10^{10} malignant cells that define the so called minimal residual disease (MRD), which is the level of disease that can be detected by the most sensitive available technique. MRD status seems to predict the outcome of the disease (Bosch et al., 2008; Moreton et al., 2005) but its utility has not been yet fully validated and should not be used as a basis for treatment decisions outside clinical studies.

In spite of the development of new prognosis markers, treatment is initiated only if the disease is symptomatic or progressive, or if complications such as bone marrow failure occur. In fact, randomized trials have shown no survival advantage using chlorambucil in early stage CLL (Rai 0, Binet A) (Chemotherapeutic options in CLL: a meta-analysis of the randomized trials. CLL Triallists' Collaborative Group, J Natl Cancer Inst. 1999; 91:861-8).

DNA-damage agents such as chlorambucil were initially used to palliate symptoms in CLL patients. However, chlorambucil treatment yielded less than 10% CR and therefore purine

analogs were introduced. Fludarabine showed an increase in overall response and CR but failed to impact on overall survival. Subsequently, combinations of fludarabine and cyclophosphamide (with non-overlapping mechanisms of action) demonstrated to be more potent than fludarabine alone, although this again did not translate into improved survival. New treatment options such as chemotherapy combined with immunotherapy or stem cell transplantation (in the case of young and/or fit patients) that have been developed during the last ten years result in complete remissions in almost 50% of the patients and a treatment-free time of more than 5 years (Table 4)**.**

One of the challenges in CLL treatment is to overcome the myelosuppression and the opportunistic infections in older patients. Some alternatives showing lower toxicity are alemtuzumab (anti-CD52 antibody), bendamustine (hybrid purine analog/alkylator drug) or lenalidomide. Treatment must be tailored to the fitness level of the patient in order to tolerate more toxic combination therapies. Other of the challenges of CLL therapy is the management of relapsed CLL patients, which is greatly dependent on *TP53* and *ATM* alterations and interaction of tumor cells with the microenvironment. In these cases, alternative therapies such as glucocorticosteroids or alemtuzumab (anti-CD52) are chosen.

Table 4. Outcomes with front-line therapies in CLL (adapted from Updates in chronic lymphocytic leukemia, Michael J Keating, Emili Montserrat, 2009)

Chemotherapeutic agent	Overall response	Complete response	MRD-negative response	Remission duration
Chlorambucil (alkylating agent)	40-60%	<10%	Not expected	1-2 years
Fludarabine (purine analog)	60-80%	10-20%	<15%	1.5-3 years
Fludarabine and cyclophosphamide	80-90%	25-35%	15-25%	3-4 years
Fludarabine , cylophosphamide and rituximab	95%	72%	40-50%	6-7 years

Finally, in the absence of any other therapies capable of improving outcome, the treatment of choice for younger patients with poor-risk CLL may indeed be allogeneic stem cell transplantation.

Considering the increasing knowledge at the molecular level of the disease and the factors that may determine the response to treatment, CLL treatment is moving toward an increasingly personalized therapy (Hallek, 2009). Despite of the considerable progress that has been made

in investigating the biology and genetic alterations of the disease, treatment outcomes are still unsatisfactory and survival prolongation has not yet been achieved (Brenner et al., 2008).

1.3. Why CLL?

Considering the long periods of stable disease and the multiyear survival of the patients who eventually require treatment, it is striking that CLL still attracts a remarkable amount of interest in the scientific community. CLL accounts only for less than 1% of all new cancers and all cancer deaths in the United States per year, compared to 12.9% for lung cancer, the most lethal cancer. The interest shown by the scientific community can be underlined by the striking number of publications that are yielded after Pubmed search on "Chronic lymphocytic leukemia" (8721). Among the factors that render CLL an interesting subject for basic and clinical research, the most obvious are the ease of obtaining blood and bone marrow samples repeatedly with little discomfort or risk to patients and the possibility of achieving long-term follow-up studies due to the prolonged survival of the patients. Other factor that has contributed to the expansion of the research in the field of CLL is the development of molecular techniques such as flow cytometry and microarrays. Furthermore, the expansion of monoclonal antibodies has allowed the study and characterization of complex cellular populations,t heir differentiation and activation stages as well as their proliferation potential. This fascination at the molecular level has extended to the expansion of clinical trials in order to discover new treatment strategies that could be chosen considering the individual patient characteristics. Furthermore, CLL constitutes a model to study how genetic and epigenetic alterations, antigenic stimulation and microenvironment stimuli (see below) can cooperate in cancer pathogenesis. Finally, CLL represents a challenge in translating this knowledge at the molecular level into the clinical practice.

1.4. Biology of chronic lymphocytic leukemia

1.4.1 Cell of origin

The fundamental molecular defects and events that result in transformation of a single normal B cell into an expanded clonal population of long-lived CLL cells are largely unknown. This has made the establishment of the cellular origin of CLL difficult. Originally, it was thought that CLL cells derive from CD5+ B cells because leukemic cells always express CD5 and all mouse models that mimic human CLL show an accumulation of tumor CD5+ B cells. However, it is still unclear

whether mouse CD5+ population of B cells is analogous to human CD5+ B cells. In mouse, CD5 expression identifies a distinct lineage (B1-B cells) that produces mainly IgM of low affinity and with a broad specificity for bacterial components (T independent antigens) (Montecino-Rodriguez et al., 2006). However, CD5+ B cells in human are functionally not different from the rest of B cells (Dighiero, 1997). Moreover, CD5 expression can be modulated upon different stimuli (Caligaris-Cappio et al., 1989; Nakamura et al., 1988). These data suggest that CD5 expression might be just a reflection of the fact that CLL cells might have been activated at some point of their natural history. Thus, the immunophenotype of CLL cells (IgM+, IgD+, CD27) corresponds to the one of memory B cells that must have experienced antigen interaction (Damle et al., 2002).

In addition, either patients with mutated or unmutated *IGHV* showed a gene expression pattern that was most similar to memory B cells than to naïve CD5+ cells (Klein et al., 2001). Although these two groups of patients do not represent two different diseases, they seem to have chosen different pathways of differentiation that determine in a major way the outcome of the disease (see above) and the behavior of tumor cells themselves. Several findings suggest that mutated CLLs (M-CLL) derive from post-GC B cells. First, somatic hypermutation is a process that takes place mainly in the context of germinal center reactions. Second, one third of M-CLLs carry mutations in *BCL6*, the master regulator of GC differentiation, something that happens only in cells that have undergone GC somatic hypermutations (Pasqualucci et al., 2000). In addition, some M-CLL cases also carry IgG1 or IgG3 subtypes, typically associated with the class switching process of the GC (Hashimoto et al., 1995). On the other hand, unmutated CLL cells (U-CLL) express poly and autoreactive antibodies, suggesting that they derive from B cells that have been activated in a T independent manner or via T dependent autoantigens (Catera et al., 2008; Chu et al., 2008; Lanemo Myhrinder et al., 2008). These B cells that are chronically stimulated may be prevented from undergoing apoptosis due to the acquisition of primary transforming events and might acquire characteristics of activated memory B cells without undergoing GC reactions.

1.4.2 CLL B cell microenvironment

The importance of the microenvironment in CLL is easily appreciated by the fact that despite their longevity *in vivo*, CLL cells undergo spontaneous apoptosis under *in vitro* conditions (Collins et al., 1989). This process can be prevented by co-culture with stroma cells

(Panayiotidis et al., 1996), "nurse-like cells" (adherent cells found in peripheral blood of CLL patients that are able to provide survival cytokines -APRIL, BAFF, CXCL12- to leukemic cells) (Burger et al., 2000) or the presence of cytokines or T-cell related molecules such as CD40L (Ghia et al., 2005). These data suggest that the resistance to apoptosis and the survival advantage of CLL cells is not only an intrinsic characteristic, but it also depends on external survival stimuli. The interactions with the microenvironment take place within lymph nodes and bone marrow in the so called proliferation centers or pseudofollicles (Figure 3).

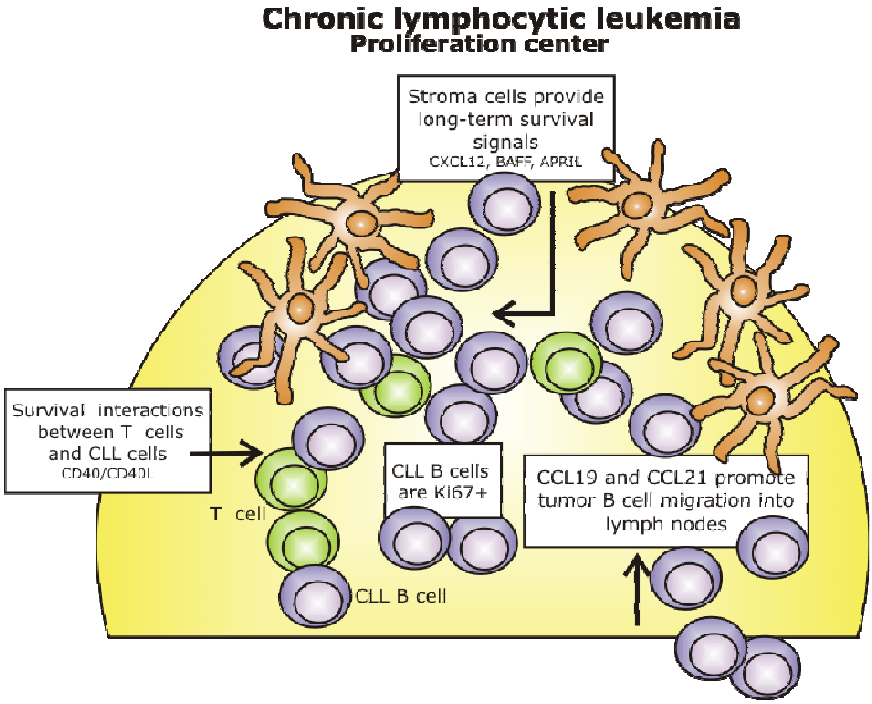


Figure 3. Scheme of a proliferation center in a lymph node. Interaction of CLL B cells with cells from the microenvironment provides survival signals

These are special anatomic structures observed in CLL but not in any other lymphoid neoplasm where leukemic cells with a blast-like phenotype show a high proliferative activity (indicated by Ki67 staining). Interestingly, proliferation centers can also be found in some systemic autoimmune diseases, supporting the importance of microenvironment interactions. Correlations between size and number of PC and the lymphocyte doubling time strongly indicate that PC are the reservoir of dividing leukaemic cells (Pileri et al., 2000), which demonstrates that CLL is not a static disease as it had been considered, but a dynamic one (Messmer et al., 2005)

CLL cells may create a microenvironment composed of T and stroma cells that supports their own survival. T cells provide short-term antiapoptotic signaling via IL10, IFNα and IFNγ production while stromal cells secrete cytokines and chemokines that attract CLL cells and help to maintain a supportive microenvironment (Ghia et al., 2005). The homing of tumor cells to PC is dependent on the expression of functional chemokine receptors on CLL cells such as CXCR4 which binds CXCL12 and CCR7 which binds CCL19 and CCL21(Burger et al., 1999; Till et al., 2002).

Considering these observations, it is hypothesized that the small lymphocytes circulating in the blood are the offspring of a proliferating pool present in the tissues.

1.4.3 Pathways involved in CLL pathogenesis

In the last years, our knowledge about the biological basis of CLL has been substantially improved and has further described CLL as a multifactorial disease that cannot be explained either by a unique genetic alteration or a well defined accumulation of pathogenic hits. On the contrary, it seems that biology is showing a complex puzzle not yet fully understood in which different signaling pathways seem to be involved. Some of them will be described in this section.

1.4.2.1 B cell receptor

BCR signaling plays two major roles in B cells: first, it is essential to decide cell fate and second, it presents the antigen to the T cells in order to allow a complete immune response. The BCR is a complex made up of two Ig heavy and two light chains coupled to two signaling components, CD79A and CD79B (also called Igα and Igβ) (Niiro and Clark, 2002). Upon antigen binding LYN and SYK kinases are activated. LYN phosphorylates the ITAM (immunoreceptor tyrosine-based activation motifs) domains in the cytoplasmic tails of CD79A and CD79B, which in turn facilitates the activation and recruitment of SYK to the signaling complex. Also FYN, LCK and FGR, members of the Src family of kinases, participate in these initial steps. Signaling continues by phosphorylation and activation of the tyrosine kinase BTK and some adaptor proteins such as VAV3 and BLNK. These events lead to activation of different signaling pathways that include:

i. Phospholipase Cγ2 (PLCγ2). It generates inositol 1, 4, 5 trisphosphate and dyacilglycerol, both second messengers that are required for the release of intracellular calcium and the activation of protein kinases C (PKC). Subsequently,

calcium flux and PKC activation lead to activation of mitogen activated protein kinases (ERK and p38) and transcription factors (NF-kB and NFAT)

ii. Phosphatidylinositol 3 kinase (PI3K). It generates phosphatidylinositol 1, 4, 5 trisphosphate and facilitates the recruitment of BTK, BAM32 and PLCγ2 to the BCR complex. PI3K also activates the protein serine/threonine kinase AKT, involved in survival signaling.

iii. RAS/RAF pathway.

In order to modulate the intensity and duration of BCR signaling, some phosphatases (SHP1 and SHIP) can be recruited to the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of several BCR co-receptors such as PIRB, FcγRIIB or CD22.

Antigenic stimulation has been recognized as a key player in the pathogenesis of CLL, as it is demonstrated by the fact that the IGHV mutational status is able to classify CLL patients in two groups with different prognosis. M-CLL cells must have encountered an antigen, as indicated by their mutational status, and appear to be unresponsive resembling a B cell that has become anergic after antigen stimulation. On the other hand, although U-CLL cells have not undergone the typical SHM process in the context of GCs, also express competent BCR that can be stimulated *in vitro*.

Moreover, CLL cells express a restricted IGHV repertoire, which is an unexpected situation considering that the chances that two independent B cell clones might carry identical Ig are virtually negligible ($1{:}10^{-9}$ to $1{:}10\text{-}^{12}$). In fact, some unrelated CLL patients share closely homologous Ig molecules that have been defined as “stereotyped receptors”. These are defined by the usage of the same IGHV/D/J germline genes, the usage of the same IGHD gene reading frame and a VH CDR3 amino acid identity of 60% or higher (Messmer et al., 2004). The third heavy chain complementary determining region (CDR3) is one of the 3 highly variable stretches which form loops in the Ig three dimensional structure and interacts directly with antigens. It is formed during the rearrangement process at the junction of the V, (D) and J genes and has the highest variability. The presence of similar stereotyped receptors argue in favor of the possibility that a limited set of discrete antigenic elements may actually be responsible for the selection and further expansion of the CLL clone.

Several studies have shown that the specificity is often autoreactive, against ssDNA, cardiolipin, myoglobin, actin or Ig themselves (rheumatic factors) (Ghiotto et al., 2004; Jang and Stollar, 2003; Stamatopoulos et al., 2007). In addition, some Igs are directed against microbial epitopes (Loomes et al., 1984). Strikingly, 26% of CLL patients carry one of more than 100 stereotyped receptors that have been described in CLL and approximately 1% carries virtually identical Ig (Murray et al., 2008; Stamatopoulos et al., 2007). Stereotypy occurs in U-CLL with a higher frequency (40% *vs* 11% in M-CLL (STAMATOPOULOS) which is in accordance with the observation that these cases usually express polyreactive BCR to autoantigens and have been stimulated in a T-independent manner. BCR sequences from M-CLL show more restricted antigen specificity. Nevertheless, in the absence of mutations, these sequences would also show autoreactivity, suggesting that both CLL groups originate from a precursor with autoreactivity (Herve et al., 2005). Furthermore, some stereotyped receptors seem to be related to prognosis. This is the case of IGHV3-21 BCR that also shares almost identical amino acid sequences of the VH CDR3 region and is biased to express lambda light chain isotype. This BCR is able to identify a subset of CLL patients with inferior outcome independently of mutational status (Lin et al., 2003; Thorselius et al., 2006).

CLL cells exhibit a functional BCR pathway (Mockridge et al., 2007) that seems to be more easily activated in the group of U-CLL, probably due to the fact that this group usually expresses also higher levels of ZAP70, an apical kinase of the pathway, and CD38. Some elements of the BCR pathway have been shown to be constitutively activated in B CLL cells: Lyn tyrosine kinase activity is high (Contri et al., 2005), levels of SYK are higher in cells with a proliferative response upon BCR stimulation and p38 is constitutively active (Sainz-Perez et al., 2006) as well as NF-kB and NFAT transcription factors (Furman et al., 2000; Schuh et al., 1996).

Taken together these observations provide a rationale for pharmacological inhibition of BCR signaling in CLL. Currently, there are more than 50 clinical trials targeting BCR regulatory molecules (Pleyer et al., 2009) that will better determine its value as therapeutic strategy.

1.4.2.2 Apoptosis

Initially, CLL was considered a static disease caused by the accumulation of lymphocytes with defective apoptotic mechanisms. Although it has been shown that there is a pool of CLL B cells that proliferate actively, defects in apoptosis still play an important role in the maintenance of the malignant cells.

Since apoptosis is essential during development and tissue homeostasis, it is a tightly regulated process that once initiated triggers permeabilization of the outer membrane of the mitochondria, caspase activation and cell death. A variety of signaling cascades regulate a complex network composed mainly by proteins of the Bcl-2 family. The core of this system is made of the proapoptotic members Bax and Bak. They are regulated by antiapoptotic proteins such as A1, Bcl-2, BCL_{XL} and Mcl-1 and also by "BH3-only" proteins. The latter group is further divided in sensitizer/derepressors (BAD, BIK, Noxa, PUMA) and direct activators (BID and BIM) (Chipuk et al., 2010). Binding of BH3-only proteins to pro-survival proteins is highly selective and varies widely in affinity (Chen et al., 2005). For example, Bim, Puma and Bid bind to all the pro-survival proteins but Bad binds only to Bcl-2, Bcl-xL and Bcl-w and Noxa binds to Mcl-1 and A1.

CLL cells constitute a paradigm of cells with deregulated apoptosis, not only at the level of Bcl-2 proteins but also upstream of Bcl-2 proteins, at the level of deregulated signaling leading to exacerbated apoptosis. In fact, one of the hallmarks of CLL is the high expression of Bcl-2. Although, the mechanisms that trigger this overexpression are not well known, different explanations have been proposed including loss of miR15 and miR16 (i.e. patients with 13q deletions) (Cimmino et al., 2005), expression of nucleolin (Otake et al., 2007) or hypomethylation of the promoter (Hanada et al., 1993). Also expression of Mcl-1, BAG-1, Bax and Bak was commonly found in circulating CLL cells (Kitada et al., 1998). Interestingly, loss of Mcl-1 results in apoptosis of CLL cells and its overexpression prolongs survival of leukemic cells treated with apoptotic stimuli (Pedersen et al., 2002). Recent data have shown a relationship between Mcl-1 expression and other commonly used prognostic markers such ZAP70, CD38 and IGHV mutational status (Pepper et al., 2008) and Mcl-1 protein levels could predict response to chemoimmunotherapy (Awan et al., 2009).

The balance between different members of the Bcl-2 family has also been shown to be altered by hyperactivation of different non-death receptors such as CD40, which is usually stimulated in the context of proliferation centers, triggering upregulation of Mcl-1, A1 and BCL XL among others (Smit et al., 2007). Moreover, signaling through BCR (see above) can induce Mcl-1, via PI3K/AKT pathway (Longo et al., 2008; Ruiz-Vela et al., 2008). Defects in death receptor sensitivity have also been described in CLL. In fact, CLL cells are resistant to CD95/Fas crosslinking, probably as a consequence of the overexpression of TOSO, a CD95 regulator (Proto-Siqueira et al., 2008; Tinhofer et al., 1998).

1.4.2.3 NF-kB pathway

The NF-kB proteins are a family of structurally related transcription factors that regulate differentiation and survival in B cells (Liou et al., 1994). In mammals this protein family includes p50 (NF-kB1), p52 (NF-kB2), p65 (Rel A), c-Rel, and Rel B. In the inactive state, NF-kB proteins occur as homodimeric or heterodimeric complexes in the cytoplasm, in some cases bound to inhibitor proteins (IkB). Upon different stimuli, including BCR signaling, IL4, BAFF or CD40 engagement, NF-kB proteins can be activated via the classical or the alternative pathways. Stimulation via the former one leads to phosphorylation of IκB, which is then ubiquinated and degraded allowing translocation of NF-kB subunits (Rel A and p50) to the nucleus and transcription of NF-kB target genes. Activation of the alternative pathway promotes processing of p100 and p105 to p52 and p50 respectively and allows their translocation into the nucleus.

Induction of NF-kB activity protects cells from apoptosis induced by a variety of stimuli including exposure to TNF-α, chemotherapy, and ionizing radiation (Beg and Baltimore, 1996), but it has also been shown to inhibit apoptosis (Furman et al., 2000). The antiapoptotic function of NF-kB resides in its capacity to activate the transcription of proteins that inhibit both the mitochondrial and the death receptor apoptosis pathways, such as Bcl-2 family of proteins (Bcl-2, Mcl-1, BCL-XL and A1/BFL1) and endogenous inhibitors of apoptosis (survivin, IAP1, IAP2, cFLIP, TRAF1 and TRAF2). NF-kB additionally promotes lymphocyte proliferation by inducing several cytokines and other ligands such as IL-2, IL-6 or CD40L.

Several studies have demonstrated that the NF-kB pathway is constitutively activated in CLL cells as compared with normal B lymphocytes (Cuni et al., 2004; Furman et al., 2000). However, the mechanisms of NF-kB activation remain uncertain and no genetic alterations have been identified. On the contrary, NF-kB activation is likely related to the interaction of leukemic cells with the tumor microenvironment. In fact, T cells found in proliferation centers in lymph nodes express CD40L that upon CD40 engagement triggers PI3K/AKT mediated NF-kB signaling (Cuni et al., 2004; Decker et al., 2000; Ghia et al., 2002). VEGF is another microenvironment factor that could mediate activation of NF-kB via CD40. CLL cells express not only VEGF receptors but also VEGF, suggesting an autocrine stimulation of the pathway (Farahani et al., 2005). Finally, BAFF and APRIL, both cytokines produced by NLC support in vitro survival o B CLL cells by activation of NF-kB (Endo et al., 2007).

Recent work has shown that p65 DNA binding ability measured in blood CLL cells is associated with advanced Binet stage and is an independent predictor marker of overall survival and time to subsequent treatment (Hewamana et al., 2008a). Moreover, ZAP70 levels correlate with p65 phosphorylation status and its DNA binding capacity (Lopez-Guerra et al., 2009), underlining the relevance of this pathway in the biology and development of treatment resistance in CLL.

In this context, inhibition of NF-kB could be a promising therapeutic strategy, which has been shown to induce apoptosis of CLL cells (but not normal B cells) and to bypass the p53 alterations observed in patients with bad prognosis and the discovery of useful prognostic markers could help in the patient selection (Lopez-Guerra et al., 2009).

1. 5.2.4 PIM kinases

The identification of PIM1 protein (Proviral Integrations of Moloney virus) in murine lymphoma models (Cuypers et al., 1984) suggested a role of this kinase in cancer biology. Now, we know that three proteins (PIM1, PIM2 and PIM3) comprise this family of well conserved serine threonine kinases. Subsequent studies have shown that PIM kinases can cooperate with other oncogenes such as MYC and BCR/ABL, mainly in lymphomagenesis (Breuer et al., 1989; Nieborowska-Skorska et al., 2002; Zhang et al., 2008; Zippo et al., 2007) and are also involved in the development of chemotherapy resistance (Mumenthaler et al., 2009; Xie et al., 2008) Reinforcing this observation, high levels of PIM1 and PIM2 have been detected in haematological malignancies, including CLL (Amson et al., 1989; Cohen et al., 2004; Huttmann et al., 2006) and high levels of PIM3 have been described in solid tumors(Fujii et al., 2005; Li et al., 2006; Zheng et al., 2008). Compensatory effects among the different members of the family, although described have not been well characterized. They are regulated not only at the transcriptional level, but also at the post-transcriptional level. Upstream regulators, although not well characterized, include JAK/STAT, PI3K/AKT and NF-κB pathways that can be activated via growth factor receptors, IL receptors, TNFα receptors and proteins from Epstein Bar virus among others.

Their role in tumorigenesis has been related to a variety of cellular processes. PIM kinases regulate apoptotic cell death through regulation of members of the Bcl-2 family such as the proapoptotic protein BAD, which once phosphorylated is targeted for degradation. Also

$p21^{CIP/WAF1}$ and $p27^{KIP1}$ cell cycle regulators have been shown to be phosphorylated by PIM kinases allowing G1-S transition. Cooperation with MYC is mediated by modification of the Cdc25A cell cycle phosphatase and by phosphorylation of H3 that in turn allows transcription of c-MYC targeted genes. Protein-protein interaction screens have recently provided a wider list of putative PIM substrates that includes transcription regulators such as NFAT1, p65, RUNX1, RUNX3, apoptosis regulators such as NUMA, and other molecules involved in different cell processes (PTPR0, SOCS1, SCOS3 and AKTS1 among others). Taken together, these observations supported the rationale for the development of PIM kinase inhibitors that have already been proved to be effective in *in vitro* and *in vivo* models (Chen et al., 2009; Lin et al., 2010; Morwick, 2010).

1.5.4 Monoclonal B cell Lymphocytosis

Clonal B lymphocytes with CLL immunophenotype have been detected in 3.5% of the general population and their frequency increases with age, up to >7% in individuals older than 70. This situation is known as Monoclonal B cell Lymphocytosis (MBL) and is distinguished from CLL because less than 5000 monoclonal lymphocytes per mm^3 (although with the same immunophenotype) are detected for more than 3 months in the absence of enlarged lymph nodes/spleen or other lymphoproliferative disorders (Marti et al., 2005).

Higher frequencies of MBL have been detected in relatives of patients with CLL, regardless of their age (Rawstron et al., 2002), although its incidence is 100 times higher than CLL incidence (Marti et al., 2007). Moreover, MBL clones often carry genetic lesions typical of CLL such as 13q14 deletion, suggesting that MBL might be a precursor state of CLL. On the other hand, MBL clones carry almost always somatic mutations (Landgren et al., 2009). This might be related to the fact that U-CLLs that have a more aggressive behavior might not persist as a stable situation, but progress more rapidly to the stage of leukemia. Although still controversial, these observations might support MBL as the original cell carrying some sort of advantage in which other abnormalities may take place and lead to development of some cases of CLL.

1.6. Translating biological insights into treatment

Over recent years, the understanding of biological mechanisms in CLL has dramatically developed, but we are only starting to use the increasing knowledge of biological subgroups to

alter the clinical management of CLL. One disadvantage in this process is that the clinical course is generally benign in the majority of patients and therefore clinical endpoints are slowly reached. This makes the translation of biological insights into clinical practice more difficult. In spite of this, the growing number of new therapeutic approaches that act differently from classical chemotherapy shows great promise. Some examples include drugs that target different molecules of the BCR pathway (Dasatinib, Imatinib, PP2 and others), Bcl-2 family (Oblimersen, GX15-070 and ABT-263 among others (Kang and Reynolds, 2009)) or the microenvironment (thalidomide and lenalidomide) (for a review see (Pleyer et al., 2009)). In fact, there are currently 1041 clinical trials on CLL all around the world (871 in USA and 131 in Europe) that aim at demonstrating the value of new therapeutics and comparing them with the classical treatments and drug combinations. The development of new targeted treatments in the clinic must be supported by pre-clinical studies. In spite of the existing CLL animal models (Bichi et al., 2002; Planelles et al., 2004; Zapata et al., 2004) that seem to resemble CLL-like diseases, not enough preclinical *in vivo* drug studies have been carried out. Fortunately, primary CLL cells can easily be obtained from peripheral blood samples and cultured *in vitro*. Since these *in vitro* models underestimate the role of the microenvironment in the pathogenesis of the disease and in the drug sensitivity mechanisms, a lot of interest has moved to the development of xenograft models using CLL patient samples (Durig et al., 2007) and to the study of the microenvironment of CLL cells on paraffin embedded samples from CLL biopsies.

Since the development of microarray studies, it has been demonstrated that all CLL patients share a characteristic gene expression signature and therefore CLL is considered a unique entity (Rosenwald et al., 2001). However, it has also been shown that groups of patients with different characteristics from a biological point of view (i.e. M-CLL and U-CLL) have also some differences at the level of gene expression profile. Further studies have revealed that this molecular heterogeneity observed at the gene expression level can define the variable clinical course of this disease. Thus, previous work from our laboratory has shown that a specific gene signature is able to identify three groups with different treatment-free survival probabilities (Rodriguez et al., 2007). These findings are of special interest in the context of clinical settings because still, one of the main challenges in CLL management is the distinction at initial clinical stages of those patients with higher risk of progressing to a more aggressive stage. These gene expression profiling studies could also be used to identify molecular mechanisms underlying

resistance and sensitivity, being helpful in the choice of more individualized and successful treatments.

In the last decade, a strategy to link clinical characteristics of a disease, genomics (including *in vitro* and *in vivo* models) and drug development has been pursued. Some successful attempts include the development of gene-signatures that can help to predict cancer prognosis (Mamma Print, Agendia, Oncotype DX, Genomic Health, H/I test, AviaraDx) or the development of bioinformatic tools that try to connect gene expression profile (GEP) databases and drug effectiveness (Connectivity map).

The final aim in this field will be to identify CLL subgroups that are defined by specific biological characteristics with the goal of improving both response rates and overall survival.

OBJECTIVES

Chronic lymphocytic leukemia (CLL), the most frequent form of adult leukemia in western countries, is characterized by a highly variable clinical course.

Increasing evidence shows that the defective apoptosis of CLL cells can be ascribed not only to intrinsic defects of the neoplastic cells, but also to extrinsic factors found in the microenvironment that influence the behavior of the neoplastic cells.

The recognition of novel molecular variables identified through the use of high-throughput molecular analytical techniques could contribute to a better knowledge of the pathogenesis of the disease, the development of more accurate biological predictive factors and the identification of new therapeutic targets in CLL. Initial gene expression profiling analysis of CLL suggested that all CLL cases could be considered as a single entity with a homogeneous signature (Klein et al., 2001). However, the differences shown by immunoglobulin variable region (IGHV) mutational status and B cell receptor (BCR) signaling among other features suggest a level of heterogeneity that should be considered (Rodriguez et al., 2007) for the proposal of therapeutic targeting.

Hence, the objectives of this work were the following:

a. To study the microenvironment of CLL cells in lymph node samples, the interactions with other cell populations and to analyze the relevant pathways that could be involved in the pathogenesis of the disease, in particular the NF-κB pathway.

b. To select some potentially active compounds in CLL following a rational approach based on the acquired knowledge of the molecular basis of the disease, and to develop an experimental procedure to test them using *ex vivo* culture of CLL cells.

c. To investigate whether the variable sensitivity of CLL cells to these compounds can be explained by the molecular heterogeneity of the samples.

d. To propose markers for patient stratification and determination of the activity of each compound.

2. MATERIALS AND METHODS

2.1. Collection and processing of primary samples

2.1.1 Patient samples

For the first project, a group of 72 paraffin-embedded consecutive B-CLL cases submitted for diagnosis or second opinion to the CNIO pathology laboratory between 2000 and 2007 was analyzed. Forty-eight of these cases were included in a Tissue Microarray (TMA), whereas the 24 additional cases were analyzed on whole tissue-sections. The majority of CLL tissues were lymph nodes, with the exception of one bone marrow, spleen, skin and breast tissue samples. For immunofluorescence staining, frozen tissue from some of these samples was used. Criteria for the diagnosis of CLL were based on WHO recommendations (WHO 2008). For the second project, a total of 50 blood samples from CLL patients were obtained from different spanish hospitals. The median age of the series was 74, with a male: female ratio of 1.7: 1, which is representative of the disease. Sample characteristics are summarized in Table 5.

All samples were collected by the Tumor Bank at CNIO. The whole project was supervised by the Ethical Committee of the Instituto de Salud Carlos III and patients signed an informed consent.

2.1.2 Processing of blood samples and storage

All blood samples were collected in tubes containing Heparin as anticoagulant. Thirty of them were processed by gradient purification of peripheral blood mononuclear cells (PBMC) in Ficoll (GE Healthcare). Briefly, samples were laid slowly on to an equal volume of Ficoll in a falcon tube and centrifuged at 700 g for 20 to 30 minutes at room temperature (RT). Then, the ring in the interphase containing the PBMCs was washed wiht PBS. When erythrocytes were observed in the pellet, an erythrocyte lysis was performed with Buffer EL, (Quiagen, Hilden, Germany) for 10 minutes on ice. Then, the sample was centrifuged and washed again with PBS.

Twenty blood samples were processed either by Microbeads B (Miltenyi) cell negative selection kit or by B cell selection Rosette Sep kit (Stem Cell), according to manufacturer recommendations as briefly describe here. Final concentration of B cells was always >95%.

B cell isolation Kit II (human) (Miltenyi Biotec, Germany). Cells were resuspended in 40µL buffer (PBS containing 0.5% bovine serum albumin and 2mM EDTA) per 10^7 total cells. Then, 10µL of Biotin-Antibody Cocktail per 10^7 total cells were added and incubated at 4ºC 10

minutes. 30 µL of buffer per 10^7 total cells and 20µL of Anti-Biotin Microbeads were added and followed by 15 minutes incubation at 4ºC. Cells were resuspended up to 10^8 cells 500µL of buffer. This suspension was applied onto a MACS column (after it had been rinsed with buffer), properly placed in the magnetic field of a suitable MACS separator. Unlabeled cells that pass through the column were collected. Also cells recovered after some additional washes.

RosetteSep® kit for B cell negative selection. RosetteSep® Human B Cell Enrichment Cocktail was added at 50 µL/mL of whole blood to the sample and mixed well. After 20 minutes incubation at room temperature, the sample was diluted with an equal volume of PBS + 2% FBS (fetal bovine serum), mixed gently and layed on top of the density medium (Ficoll, GE Healthcare, or RosetteSep® DM-L, Stem cell Technologies Inc.). The mixture was centrifuged for 20 minutes at 1,200 x g at RT, with the brake off. The enriched cells were then removed from the plasma interface and washed with PBS. If necessary, red blood cells were lysed as described above.

Between 20 and 40 million cells were frozen per cryotube in freezing medium containing 30% of RPMI medium, 60% of fetal bovine serum (FBS) and 10% of DMSO and stored up to long periods in liquid Nitrogen. Also aliquots for DNA (kept at -20ºC) and RNA extraction (stored at -80ºC in Trizol reagent) were separated.

2.1.3 B cell purity determination after selection

Aliquots of freshly purified B cells (100 µL) were separated in cytometer tubes. Cells were labeled with CD19-PE (Miltenyi Biotec, # 130-091-247) or CD3-FITC (BD, Biosciences, San José, CA, USA) antibodies for 15 minutes at RT, washed with PBS and fixed with 2% of Paraformaldehyde (PAF) for 10 minutes at RT. After a final PBS washing, cells were resuspended in 200 µL of PBS, 10,000 events were acquired using a FACScalibur device and analyzed with CellQuest Pro software (BD Bioscience, Franklin Lakes, NJ, USA). If acquisition could not be carried out immediately after labeling, samples were kept at 4ºC up to several days until acquisition.

2.2. Immunohistochemistry

2.2.1 Tissue microarray construction

Representative areas from 48 formalin-fixed, paraffin-embedded lymph nodes infiltrated by CLL were selected on H&E-stained sections and two 1-mm-diameter tissue cores were

obtained from each specimen. The tissue cores were arrayed into a new paraffin block using a tissue microarray (TMA) workstation (Beecher Instruments, Silver Spring, MD) following described methods (Cuni et al., 2004). The TMA construction was carried on at the Immunohistochemistry Unit of CNIO

Tabla 5. Peripheral blood samples collected. ND, no data available. M, mutated; U, unmutated.

Sample code	Stage	Lymphocyte count	CD19+/ CD5+	Cytogenetics	ZAP 70	Mutational status of IGHV	CD38
70446	A0	10200	73.0%	ND	ND	U	-
70447	A0	24500	62.0%	No TP53 mutations	ND	M	-
70448	ND	ND	ND	No TP53 mutations	ND	M	ND
80002	AI	41760	66,9%	13q14 deletion	-	M	-
80003	AI	11600	32.0%	ND	+	ND	+
80005	A0	54600	90.0%	13q14 deletion	-	M	-
80047	AI	8080	25.0%	Chromosome 12 trisomy	ND	M	-
80048	AI	10500	34.0%	Normal	-	M	-
80049	AI	5300	23.0%	ND	ND	ND	-
80091	A0	ND	ND	17p deletion	ND	U	ND
80102	AI	14000	80.0%	11q and 13q deletions IGH+ rearrangement	-	ND	-
80113	ND	ND	70.0%	No TP53 mutations	-	ND	-
80117	BI	29700	25.0%	11q22 deletion	+	U	-
80138	A0	13810	ND	11q deletion	ND	M	-
80139	A0	21440	ND	13q and 17p deletions	ND	U	-
80194	A0	5600	ND	13q deletion	+	M	-
80197	A0	5880	ND	Normal	+	U	ND
80230	A0	13390	ND	Normal	+	U	ND
80231	A0	61500	ND	13q deletion	-	M	-
80232	A0	5260	ND	13q deletion	ND	M	+
80239	ND	ND	36.1%	ND	-	ND	-
80254	A0	22000	81.0%	13q14 deletion	-	U	-
90007	A0	7881	65.0%	ND	+	ND	+
90025	A0	15340	79.0%	13q14 deletion	-	M	-
90082	A0	14590	90.0%	No TP53 mutations	-	M	-
90272	A0	12880	82.0%	13q14 deletion	-	M	-
90393	A0	6714	56.0%	No TP53 mutations	-	M	-
90784	A0	ND	ND	No TP53 mutations	-	M	-
90903	AI	57000	93.0%	No TP53 mutations	ND	ND	-
90904	A	10400	33.1%	No TP53 mutations	ND	M	ND
90905	A0	ND	ND	No TP53 mutations	ND	M	-
90906	A0	180000	90.0%	IGH+ rearrangement	+	U	-
90907	AI	55000	80.0%	13q14 deletion	-	M	-
90908	A0	40000	90.0%	13q14 deletion	-	M	-
90909	BII	170000	94.0%	c-MYC trisomy, 13q deletion	+	ND	-
90921	A	11000	43.0%	No TP53 mutations	ND	M	ND
90923	A0	9300	39.0%	Normal	+	ND	+

90978	ND	ND	ND	No TP53 mutations	ND	M	ND
90994	A0	9180	35.0%	Normal	-	M	-
91066	A0	16400	60.0%	13q14 deletion	-	ND	-
91069	BII	53000	93.0%	Chromosome 12 trisomy	+	U	-
91071	A0	15400	84.0%	No TP53 mutations	ND	U	ND
91072	ND	13600	58.5%	17p deletion	ND	M	-
91103	A0	171000	96.0%	13q14 deletion	-	M	-
91207	AIII	121600	82.0%	No TP53 mutations	-	U	ND
100003	A0	ND	ND	SILENT mutation in TP53	-	M	-
100004	A0	ND	ND	No TP53 mutations	-	ND	-
100008	A0	ND	ND	No TP53 mutations	ND	M	ND
100018	BII	17900	59.3%	11q deletion	+	U	ND
100019	A0	ND	ND	No TP53 mutations	ND	M	ND
100021	A0	ND	ND	No TP53 mutations	ND	U	ND
100030	A0	ND	ND	No TP53 mutations	+	M	+

2.2.2 Immunohistochemical staining

Immunohistochemical staining of both TMA and whole tissue sections was performed by the Endvision method with a heat-induced antigen-retrieval step at the Immunohistochemistry Unit of CNIO. Sections were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker. Antibodies analyzed in this series are shown in Table 5. Reactive tonsil tissue was included as an external control. The primary antibodies were omitted to provide negative controls.

2.2.3 Immunohistochemical scoring

In this study, the immunohistochemical staining of every protein studied was analyzed in parallel with hematoxilin and eosin staining of paraffin-sections of each case to clearly identify PCs. The scoring was done by Dr Socorro María Rodríguez-Pinilla. Staining was considered positive for each marker taking into account both the percentage of positive cells as well as the cellular component where they were expressed. Cases were considered positive if distinct appreciable staining was present in the cytoplasm, membrane or nucleus (depending on the specific marker, Table 6) in a majority of the examined cells. A staining score was recognized for most of the proteins analyzed depending on the percentage of positive cells; 0=0-5%, 1=15-50% and 2=50-100%.

2.2.4 Double immunoenzymatic staining

Double immunoenzymatic labeling of paraffin sections was performed using the following protocol. In the first reaction, immunostaining was performed using the EnVision peroxidase kit (Dako, Denmark) and diaminobenzidine (DAB) chromogen-substrate (Dako K5507, Dako). In the second reaction, immunostaining was performed using the alkaline phosphatase kit (Dako

K5355, Dako) and chromogen provided therewith. Briefly, after identifying positivity for SDF-1α, actin, CD40, STAT1 and CD68 in PCs, multiple combinations between these markers were done to further characterize the positive cells.

Table 6. Primary antibodies used for the immunohistochemical study of proliferation centers.

Antibody	Description	Clone	Source	Dilution
Actin	Mesenchymal cell marker	1A4	Dako	1:1500
BAFF	Member of the TNF family of ligands, is expressed in T cells, macrophages, monocytes and dendritic cells. BAFF is involved in stimulation of B and T cell function, and is an important survival and maturation factor for peripheral B cells. BAFF signals through three different TNF receptors TACI, BCMA and BAFF-R	Rabbit polyclonal	Chemicon	1:100
Bcl-2	Anti-apoptotic member of the Bcl-2 family	124	Dako	1:50
BCL6	A sequence-specific repressor of transcription known as a maker of germinal centers	A8	Monoclonal Ab CNIO	1:300
CD10	Marker for germinal center B cells	56C6	Novocastra /DAKO	1:10 / 1:1
CD1a	Marker for Langerhans cell histiocytosis (LCH) found on interdigitating cells	010	Master Diagnostic	1:1
CD20	Commonly used marker for B cells. Initially expressed on B cells after CD19/CD10 expression and before CD21/CD22 and surface immunoglobulin expression; retained on mature B cells until plasma cell development. It delivers early signal in B cell activation, allowing resting B cells to respond to later antigens	L-26	Dako	1:300
CD21	Marker of follicular dendritic cells. Also expressed in normal B cells (particularly in marginal and mantle cells)	1F8	Dako	1:10
CD23	Also known as low affinity IgE receptor, Fc fragment of IgE receptor. After physiologic germinal cell development, the follicular dendritic cell meshwork expands and follicular dendritic cells in the light zone of the germinal center become CD23+. It is a B cell growth and activation factor, promoting differentiation into plasma cells. Used to differentiate SLL/CLL (CD23+) vs. mantle cell lymphoma or MALT lymphoma (CD23-)	MHM6	Dako	1:25
CD25	Also called IL-2 receptor alpha chain, TAG. Considered to be an activation antigen	4C9	Novocastra	1:50
CD3	Cytoplasmic expression at early T cell differentiation. Complex of delta, epsilon, gamma, zeta and eta chains of integral membrane glycoproteins that associates with TCR, and is required for TCR cell surface expression and signal transduction	SP7	Neomarkers	1:200
CD38	A type II trans-membrane glycoprotein that synthesizes cyclic ADP-ribose, a metabolite with potent calcium mobilizing properties independent of IP_3. It is also a positive and negative regulator of cell activation and proliferation, depending on the cellular environment. It is involved in adhesion between human lymphocytes and endothelial cells. Poor prognosis marker in CLL	VS38	Dako	1:100
CD4	Expressed on surface of T helper cells; it serves as co-receptor in MHC class II-restricted antigen induced T cell activation	4B12	Master Diagnostic	1:3
CD40	It plays a central role in regulating cell-mediated immunity and antibody mediated immunity. It also mediates T cell dependent immunoglobulin class switching, memory cell	Poly-rabbit	Abcam	1:2

	development and germinal center formation			
CD57	Marker for NK cells and a subgroup of follicular helper T-cell (T_{FH}). Glycoprotein with cell adhesion functions	NK-1	BD Pharmingen	1:200
CD68	A classical macrophage marker. A 110-Kd trans-membrane glycoprotein expressed by human monocytes and tissue macrophages but not restricted to the macrophage lineage (also expressed in fibroblasts). More frequently positive than the other examined macrophage markers, and proved to be almost as reliable as the recently discovered CD1a	KP1	Dako	1:2000
CD8	A transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR), usually expressed in cytotoxic T cells	C8/144B	Dako	1:25
cREL	Member of the NF-κB family of transcription factors	B6	Santa Cruz	1:200
CXCL13	B lymphocyte chemoattractant that functions in the homing of B cells into the follicles. Expressed by T_{FH} and follicular dendritic cells.	Polygoat	R&D Systems	1:25
D2-40	O-linked sialoglycoprotein found in lymphatic endothelium	D2-40	Dako	1:1
Desmin	Intermediate filament. Marker for mesenchymal cells with myogenic differentiation	D33	DAKO	1:25
FOXP3	Forkhead family transcription factor expressed by regulatory T cells	206D/67	Monoclonal Ab CNIO	1:1
Granzyme B	Serine protease that mediates apoptotic signaling in cytotoxic T lymphocytes and natural killer cells. Synthesized as inactive proenzyme, stored within cytolytic granules and released by effector cells during degranulation. It cleaves and activates caspase-3, caspase-6, caspase-7 and caspase-9	GrB-7	Dako	1:10
Ki67	Nuclear protein that is associated with and may be necessary for cellular proliferation	MIB-1	Dako	1:1000
Mcl-1	Anti-apoptotic member of the Bcl-2 family	Rabbit polyclonal	Sigma	1:500
MUM1/ IRF4	Marker expressed from centrocytes to plasma cells	Poly-goat	Santa Cruz	1:150
NFATc1	A family of transcription factors characterized by the presence of highly conserved calcineurin- and DNA-binding domains. NFAT proteins are activated in the cytoplasm by the calcium-dependent phosphatase calcineurin	7A6	BD Pharmingen	1:300
p50	Member of the NF-κB family of transcription factors	Rabbit polyclonal	GeneTex	1:1
p52	Member of the NF-κB family of transcription factors		Upstate	1:1000
p65	Member of the NF-κB family of transcription factors	F-6	Santa Cruz	1:2000
PD1	Type I trans-membrane protein that negatively regulates TCR signaling. Also expressed by B cells	NAT-105	Monoclonal Ab CNIO	1:2
Perforin	Cytolytic protein found in the granules of CD8 T-cells and NK cells. Upon degranulation, perforin inserts itself into the target cell's plasma membrane, forming a pore.	5D10	Novocastra	1:10
Rel B	Member of the NF-κB family of transcription factors	Rabbit polyclonal	GeneTex	1:1250
S100	Calcium binding protein. An marker for a subset of dendritic cells, the interdigitating reticulum cells (IDRCs), which are mainly located in T-dependent areas of lymphoid tissues	Poly-rabbit	Dako	1:2000
SDF1	Chemokine, also known as CXCL12	-	Courtesy of Dr José Alcami	1:25
STAT1	Member of the Signal Transducers and Activators of Transcription family of transcription factors. Involved in up-regulating genes due to a signal by either type I or type II interferons	C-136	Santa Cruz	1:10

Survivin	Member of the inhibitor of apoptosis (IAP) gene family, which encodes negative regulatory proteins that prevent apoptotic cell death	Rabbit polyclonal	RD Systems	1:1000
TCL1	Proto-oncogene that interacts with the Akt pleckstrin homology domain, enhancing Akt kinase activity. It enhances cell proliferation, stabilizes mitochondrial membrane potential and promotes cell survival	Poly (rabbit)	Cell Signaling	1:100
TIA1	Type I trans-membrane protein that plays a central role mediating viral immunity. Found in the granules of cytotoxic lymphocytes	2G9	Master Diagnostic	1:1
TRAF1	TRAF proteins associate with, and mediate the signal transduction from various receptors of the TNFR superfamily. This protein and TRAF2 form a heterodimeric complex, which is required for TNF-alpha-mediated activation of MAPK8/JNK and NF-kappaB. The protein complex formed by this protein and TRAF2 also interacts with inhibitor-of-apoptosis proteins (IAPs)	H-3	Santa Cruz	1:300
VEGF	Dimeric glycoprotein with a role in angiogenesis	SP28	Abcam	Prediluted
ZAP70	Tyrosine kinase usually present as part of the TCR pathway. However, CLL B cells also expressed ZAP70 aberrantly	BC.2F3.2	Biocare	1:50

2.2.5 Immunofluorescence staining

Slides from frozen samples were fixed with 4% PAF and permeabilized with 0.5% Triton. Then, they were blocked 2 minutes with FCS (Australian Foetal Calf Serum, Life Technologies Inc., USA) and incubated for 30 minutes at room temperature with primary antibodies diluted in PBS plus 10% FCS. Slides were washed in PBS and incubated 30 minutes with fluorochrome-conjugated antibodies against the corresponding Ig isotypes, (1:200 dilutions in PBS (MolecµLar Probes, Leiden Netherlands) in the dark. Following washing, antifading (Qbiogene, Illkirch, FR) and DAPI (MolecµLar Probes, Leiden Netherlands) were added. Fluorescence images were captured with an Axiocam charge-coupled device camera (Carl Zeiss, Jena, Germany) and Axiovision software (Imaging Associates, Bicester, UK), and adjusted using Photoshop software (Adobe, San Jose, CA, USA).

2.3. Statistical data analysis

For some analysis and graphics, the GraphPad software (GraphPad Software Inc., La Jolla, CA, USA) was used. To study associations between our continuous variable (EC50 values) and categorical ones (mutational status of *IGHV*, ZAP70) parametric (Student t-test) or non-parametric (Mann Whitney) methods available on the GraphPad software were used. D'Agostino method implemented in GraphPad was used to determine the normality of each series of data. The study of correlations between two continuous variables (for example, gene

expression data and EC50 values) was performed applying a Pearson correlation using Excel and SPSS (IBM Company, Chicago, Illinois, USA).

2.4. Methods for cell culture and treatments

2.4.1 Reagents for cell culture

Complete medium was prepared using commercial RPMI (GIBCO, Invitrogen, Peisley, UK) supplemented with 10% of FBS and antibiotics (100 U/mL Penicilin and 100 µg/mL of Streptomycin, GIBC, Invitrogen, Peisley, UK).

Calmidazolium was purchased from SIGMA. TW-37 was a kind gift of Dr Shaomeng Wang (Michigan University). R406 was obtained from Rigel laboratories. ETP-39010 was produced by the Therapeutics Program at CNIO. All drugs were resuspended in DMSO (XXXX) and subsequent dilutions were made in culture medium.

2.4.2 Primary samples defreezing

1mL cells were defreezed in a water bath at 37ºC. 500 µL cells were laid onto 1mL Ficoll density medium in a 2 mL eppendorf. Cells were centrifuged 10 seconds up to maximum speed. Eppendorfs were turned 180º and centrifuge 1 minute at 12,000 rpm. After being turned 180º again, eppendorfs were centrifuged an additional minute at 12,000 rpm. The ring containing the living cells was recovered, washed with PBS and resuspended in complete medium. Cells were counted and plated at an appropriate concentration, usually at 10^6/mL, for further experiments. They were allowed to stabilize 3 hours before any assay was performed.

2.4.3 Cell viability assays

Primary cells were plated at a final concentration of 10^6 cells/mL in black 96 well-plates with flat bottom, treated for tissue culture (BD Biosciences Europe, Belgium). Routinely, 10^5 cells were plated in 90 µL of complete medium. Then, 10 µL of the appropriate concentration of the drug or vehicle (DMSO) were added. One plate was usually used to assay 3 drugs per sample or alternatively, for 3 samples treated with one of the drugs. After 3 hours incubation at 37ºC to allow stabilization, cells were treated with a range of concentrations of the drug under study (Calmidazolium: 2.5 nM- 50 µM; R406: 2.5 nM-50 µM TW-37: 1 nM-20 µM; ETP-39010: 5.1 nM-100 µM). The highest dose was prepared 10 times concentrated by diluting the drug in culture medium. The subsequent concentrations were prepared by 1:3 dilutions in complete medium. The concentration of DMSO for R406 was always 0.5% to ensure solubility. Drugs were pipetted using the Laboratory Automation Workstation Biomek® NX^P (Beckman Coulter).

Controls without the drug and with the highest concentration of vehicle were also plated. After 72 hours incubation, plates were equilibrated at RT for 30 minutes. Then, 100 µL of Cell Titer Glo Reagent from the CellTiter-Glo® Luminescent assay (Promega) were added per well using a multichannel pipette. This reagent contents a stable form of luciferase and its substrate (luciferine). The reaction of luminescence depends on the availability of ATP in the medium, which reflects the presence of metabolically active cells. Luminiscence was recorded using the Envision™ 2104 Multilabel reader (Perkin Elmer, Massachusetts, USA).

Cell viability was calculated as a percentage. Cells treated with the vehicle were used as control. Some samples were especially sensitive to DMSO. In those cases, the percentage of viable cells was calculated using cells without DMSO as control and the data from the highest concentration of the drug were not used for the EC50 calculation. With these data, a sigmoidal dose-response curve (X axis: concentration in log; Y axis: cell viability in percentage) and an EC50 value were obtained using the GraphPad software. Experiments with an $r^2 < 0.9$ were not included for further analysis.

2.4.4 R406 treatment and BCR engagement

After defreezing and stabilization (3 hours at 37ºC, at a 10^6/mL concentration), CLL primary cells at a 10^7/mL concentration were treated with 5 µM R406 (i.e. 5 µL of 1 mM R406 in 1 mL of cells) or vehicle for 30 minutes on a water bath at 37ºC. Then, BCR was stimulated with 10 µg/mL of mouse F(ab')2 anti-human IgM (µ chain specific) (SouthernBiotech, Bitmingham, USA) for 5 minutes. Reaction was rapidly stopped by addition of ice cold PBS and centrifugation. After washing, pellets were frozen at -80ºC or directly lysed for protein extraction.

2.4.5 ETP-39010 treatment for gene expression studies

Cells were defreezed as previously described and a minimum of 5 x 10^6/mL cells were plated at a concentration of 10^6/mL in appropriate flasks. After 3 hours stabilization at 37ºC, paired samples were treated with 5 µM ETP-39010 or 0.1 % of vehicle alone (DMSO) for 8 hours. Then, cells were washed with PBS and pellets were resuspended in Trizol and stored at - 80ºC several days until RNA extraction was performed.

2.4.6 Time courses for apoptosis and cell cycle assays after drug treatment

Cells were defreezed and 10^6 cells were plated in 1mL complete medium in 24-well plates (BD Falcon). After 3 hours stabilization at 37ºC, several concentrations of the drug under study or

vehicle were added (usually 1µL of 1000 times concentrated drug). 300 µL aliquots at 24, 48 and 72 hours from the same well were taken for AnnexinV/PI analysis. When cell cycle assays were performed in parallel, 2 x 10^6 cells were plated in 2 mL medium and aliquots were taken for apoptosis and cell cycle assays from the same well.

2.5. DNA and RNA based methods

2.5.1 DNA extraction

Between 2 and 5 million cells from CLL samples were lysed using 500 µL of Tissue and Cell lysis solution (EPICENTER, Biotechnologies, Madison, WI, USA) and 7 µL of K proteinase (at 50 µL/µL concentration), EPICENTER Biotechnologies, Madison, WI, USA) by shaking at 65ºC overnight. Then, 500 µL of the Protein Precipitation reagent were added. Samples were vortexed 10 seconds and centrifuged at 10,000 rpm 10 minutes. The supernatant was recovered in a fresh eppendorf, and 1 mL of isopropanol was added to precipitate. After several inversions, samples were centrifuged at 4ºC, 10000 rpm for 10 minutes. The pellet was washed with 70% ethanol, allowed to dry at RT or at 65ºC and eluted in 50-100 µL of water. DNA was quantified with the Nanodrop 1000 (Thermos scientific, Wilmington, DE, USA) and quality was checked (260/280 and 260/230 ratios).

2.5.2 Mutational status determination of the variable region of the immunoglobulin heavy chain

The variable region of the heavy chain of the immunoglobulin was amplified by PCR using AmpliTaq Gold® DNA polymerase (Applied Biosystems, Framingham, MA, USA) following manufacturer recommendations. A multiplex PCR was set with 6 IGHVFR1 forward primers and the JH consensus reverse primer (Table 7), all recommended by the BIOMED-2 consortium (van Dongen et al., 2003). 200 ng of genomic DNA from CLL samples was used. PCR conditions were as follow: initiation step: 94ºC, 10’; denaturation step: 94ºC, 45’’; annealing step: 60ºC, 45’’; elongation step: 72ºC, 10’ (repeated 35 cycles) and final hold: 4ºC, ∞.

The products of interest showed an expected size of around 300 base pairs, depending on the rearrangement and the length of the CDR and were purified either by PCR purification kit (Quiagen, Hilden, Germany) or by 3-4% agarose gel excision with the Quiagen kit (Hilden, Germany) if two bands were amplified. In the last situation, both amplified products were further analysed. They were sequenced, first with IGHVJH consensus reverse primer and then, when the IGHV usage was determined, with the appropriate IGHVFR1 primer in order to obtain

a consensus sequence. For visualization of sequencing results, Chromas Lite software (Technelysiun Pty Ltd) was used. For the analysis of somatic hypermutations, IMGT source (http://imgt.cines.fr/) was used and the percentage of homology with the germinal IGHV as well as the rearranged V, D, J segments were obtained. Only rearranged productive sequences were considered. For further studies, samples with >98% of homology were considered unmutated and samples with <98% homology mutated.

2.5.3 *TP53* sequencing

When no cytogenetic data were available for the samples of our series (a total of 19 samples), *TP53* most frequently mutated exons were sequenced. As 90% of patients with 17p deletions also present mutations, we used these as a surrogate marker of genomic abnormalities affecting *TP53* (Dohner et al., 1995). Exons 5, 6, 7 and 8 were amplified using Platinum® Taq DNA polymerase (Invitroge, Carlsbad, CA, USA) and sequenced for detection of possible mutations. Primers are shown in table 7. PCR conditions were:

- Exon 5. Initiation step: 94ºC, 5'; denaturation step: 94ºC, 30''; annealing step: 55ºC, 30''; elongation step: 72ºC, 30'' (repeated 35 cycles) and final hold: 4ºC, ∞.
- Exons 6, 7 and 8. Iinitiation step: 94ºC, 5'; denaturation step: 94ºC, 30''; annealing step: 58ºC, 30''; elongation step: 72ºC, 30'' (repeated 35 cycles) and final hold: 4ºC, ∞.

Table 7. Primers used for the determination of IGHV mutational status and sequencing of exons 5, 6, 7, and 8 of TP53 gene in DNA samples from CLL patients (fw, forward primer; rev, reverse primer).

Name	Sequence	Application
VH1-FR1	GGCCTCAGTGAAGGTCTCCTGCAAG	IGHV mutational status
VH2-FR1	GTCTGGTCCTACGCTGGTGAAACCC	IGHV mutational status
VH3-FR1	CTGGGGGGTCCCTGAGACTCTCCTG	IGHV mutational status
VH4-FR1	CTTCGGAGACCCTGTCCCTCACCTG	IGHV mutational status
VH5-FR1	CGGGGAGTCTCTGAAGATCTCCTGT	IGHV mutational status
VH6-FR1	TCGCAGACCCTCTCACTCACCTGTG	IGHV mutational status
JH consensus	CTTACCTGAGGAGACGGTGACC	IGHV mutational status
E5fw	TCCTCCCTCTTCCTACAG	Exon 5 of TP53
E5rev	ACCCTGGGCAACCAGCCCTGT	Exon 5 of TP53
E6fw	ACAGGGCTGGTTGCCCAGGGT	Exon 6 of TP53
E6rev	AGTTGCAAACCAGACCTCAGGCG	Exon 6 of TP53
E7fw	TCCTAGGTTGGCTCTGACTGT	Exon 7 of TP53
E7rev	AGTGGCTCCTGACCTGGAGTCT	Exon 7 of TP53
E8fw	GGGACAGGTAGGACCTGATTTCCTT	Exon 8 of TP53
E8rev	ATCTGAGGCATAACTGCACCCTTGG	Exon 8 of TP53

PCR products were purified using a kit from (Quiagen, Hilden Germany). Exon sequences were compared with those available at Ensembl using the CLC sequence viewer (CLC bio company, Aarhus, Denkmark). If necessary, products were sequenced with forward and reverse primers. Only one nucleotide change was found in sample 10003 and its relevance was checked at the International agency for research on cancer (IARC) TP53 data base (http://www-p53.iarc.fr/). It corresponded to a silent change that did not represent a change of aminoacid and has not been described as affecting protein function.

2.5.4 RNA extraction

Five to 10 million cells were stored in 1mL Tri®reagent (SIGMA-ALDRICH, Steinheim, Germany) at -80ºC. After defreezing, they were disaggregated using a 1mL syringe and a needle. The suspension was kept 10 minutes at RT and then, 200 µL of chloroform were added. Samples were mixed thoroughly 15 seconds and maintained 10 additional minutes at RT. After 15 minutes of centrifugation at 8000g, the aqueous phase was recovered in a fresh tube. The same volume of isopropanol and 2 µL of lineal acrilamide were added and samples were left at -20ºC overnight. Next day, samples were centrifuged at 12,000 g, 15 minutes, at 4ºC. Pellets were washed with 70% cold ethanol and resuspended in RNAase free water. For a higher purity, the RNeasy® Mini Kit from Quiagen (Hilden, Germany) was used following manufacturer instructions, including the DNAase treatment. Finally, RNA was resuspended in 20-35 µL.

If necessary, RNA was precipitated again with 1 µL lineal acrilamide, 0.5 volumes of AcNH4 7.5M and 2.5 volumes of ethanol overnight. After 20 minutes centrifugation, the pellet was washed with 70% ethanol, allowed to dry and resuspended in an appropriate volume. RNA was quantified using the Nanodrop 1000 device (Thermos scientific, Wilmington, DE, USA) and visualized on a 1% agarose gel.

2.5.4. Microarray hybridization

2.5.4.1 cDNA synthesis from total RNA

2 µg of total RNA was mixed with 2 µl of a 5,000-fold dilution of Agilent's Two-Color Spike-in RNA control and amplified using Agilent Low RNA Input Fluorescent Amplification Kit (Agilent Technologies, Inc., Santa Clara, CA). The mixture in a final volume of 6.5 µl (total concentration at least 5 ng/µl) was mixed with 5µl of T7 promoter primer. The primer and the template were denatured by incubating the reaction at 65^{O}C for 10min and placing on ice for 5min. Following,

8.5 µl of cDNA Master Mix was added and the samples were incubated first at 40^{O}C in a circulating water bath for 2 hours and then at 65^{O}C in a heating block for 15 minutes to inactivate MMLV-RT. Following that time, the samples were incubated on ice for 5 minutes.

cDNA Master Mix composition: 4 µl of 5X First strand buffer, 0.1M DTT 2 µl, 10 mM dNTP mix 1 µl, 1 µl of MMLV-RT and 0.5 µl of RNaseOUT.

2.5.4.2 Fluorescent cRNA synthesis: in vitro trasnscription and incorporation of fluorochromes

To each sample tube, either 2.4 µl of 10 mM cyanine 3-CTP (sample) or 2.4 µl of 10 mM cyanine 5-CTP (Stratagene Universal Human Reference RNA) was added and mixed. Following, to each sample, 57.6 µl of Transcription Master Mix was added an incubated in a circulated water bath at 40^{O}C for 2 hours. Following amplification and labeling, each sample was assessed on the Nanodrop ND-1000 to measure yield and specific activity.

Transcription Master Mix composition*:* 15.3 µl of Nuclease-free water, 20 µl of 4X Transcription buffer, 0.1 M DTT 6 µl, 8 µl of NTP mix, 50% PEG 6.4 µl, 0.5 µl of RNase OUT, 0.6 µl of Inorganic pyrophosphatase and 0.8 µl T7 RNA Polymerase.

2.5.4.3 Hybridization

cRNA target was prepared as follows: 0.75 µg cyanine 3-labeled, linearly amplified sample cRNA was mixed with 0.75 µg cyanine 5-labeled, linearly amplified reference pool cRNA, 50 µl of 10X control targets and Nuclease-free water to final volume of 240 µl. The hybridization solution was prepared by adding 240 µl of 2X target solution to 10 µl of 25X fragmentation buffer. The mixture was incubated at 60^{O}C in the heating block for 30min. Following, 250 µl of 2X hybridization buffer (from In situ Hybridization kit) to the final volume of 500 µl, mixed, spinned and 490 µl of the hybridization solution was applied to 60-mer Agilent 4X 44K Human Whole Genome oligonucleotide microarrays or 8x 15K CLL-specific oligonucleotide microarrays and assembled in microarray hybridization chamber (G2534A). Once fully assembled, the chambers were loaded into the hybridization rotator rack and set to rotate at 4 rpm. The hybridization was performed in a rotating oven at 60^{O}C for 17 hours.

All the washing steps were performed at room temperature. First the sandwiched slides were submerged in Wash Solution 1 to remove oligo microarray slide. The slides were washed for 1min in the Wash Solution 1 with the magnetic stir. The slides were then transferred to the

staining dish containing Wash Solution 2 and washed for 1 minute. Following, the slides were transferred to the staining dish containing the Wash Solution 3 and washed for 30 seconds. All steps were performed in the dark. The dried slides were scanned with a G2565BA Microarray Scanner System (Agilent Technologies, Palo Alto, CA).

Wash solution 1 composition*:* 6X SSPE, 0.005% N-Lauroylsarcosine, deionized nuclease free water.
Wash solution 2 composition*:* 0.06X SSPE, 0.005% N-Lauroylsarcosine, deionized nuclease free water. The buffers 1 and 2 are passed through a 0.2 µm sterile filtration unit before use.
Wash solution 3 composition*:* The Agilent Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile.

2.5.4.4 Data normalization and preprocessing

Data were extracted with the use of Feature Extraction (v.10) software (Agilent Technologies, Santa Clara, CA, USA). Arrays were normalized using global loess method implemented in GEPAS (Gene Expression Pattern Analysis Suite), available at http://gepas.bioinfo.cipf.es/. Preprocessing was also performed using the bioinformatics tools provided by GEPAS. Inconsistent replicates were eliminated, replicates were merged and genes with less than 70% of the values were filtered. For some analysis, missing values were input using the K-Nearest Neighbors method (K>15).

2.6 Protein based methods

2.6.1 Total protein extraction

Total protein extracts were prepared by lysing cells in RIPA lysis buffer (Sigma-Aldrich) containing protease inhibitor cocktail set III (Calbiochem, San Diego, CA) plus protease inhibitor cocktail set VIII (Calbiochem) for 20 minutes on ice. Cell debris was removed by centrifugation (10,000 x g, 10 minutes, 4^{O}C). The supernatant (protein extract) concentration was measured using Protein assays reagents A, B, and S (BioRad, Hercules, CA) following the manufacturer's instructions using bovine serum albumin (BSA) as a standard. Briefly, 1 ml of Agent A was mixed with 20 µl of Agent S. First, the adequate amount of either standard (BSA) or sample was applied to each well of 96-well plate, following 25 µl of previously prepared mixture was added. Finally, 200 µl of Agent B was added and the whole preparation was incubated for 15 minutes at RT and measured at a wavelength of 750 nm.

Composition of RIPA buffer: 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0. Just before use the protease inhibitor cocktail was added: 0.1 mM sodium vanadate, 5 μg/ml leupeptine, 5 μg/ml aprotinine, 1 mM phenylmethylsulphonyl fluoride (PMSF).

2.6.2 Western blotting

Following primary antibodies have been used for Western Blot (WB) analysis: Mcl-1 (S-19, Santa Cruz Biotechnology), Bcl-2 (235J/E1, produced in the Monoclonal Antibodies Unit at CNIO), p-AKT (p-Ser 473, Cell Signaling), p-BLNK (p-Tyr 84, MBL, Woburn, MA, USA), total AKT total (#9272, Cell Signaling), BLNK (Sc-8003, Santa Cruz Biotechnology), PIM2 (H-73, Santa Cruz Biotechnology). As loading control an antibody to GAPDH (clon 273A/B9, produced in the Monoclonal Antibodies Unit at CNIO) was used.

Western Blotting was performed according to standard protocols. First, the protein extracts were subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels using the acrylamide concentration adequate for the size of the detected proteins (10-15%) using Mini-Protean 3 system (BioRad).

Following electrophoresis, the proteins were wet-transferred onto nitrocellulose membranes (Whatman, Dassel, DE) using Mini Trans-Blot Cell equipment (BioRad). Transference was performed at 40 mA during 12-20 hours at room temperature or 400 mA during 1.5 hour at $4^{O}C$.

Membranes were blocked with 5% BSA or Milk in PBS-T (phosphate-buffered saline with 0.1% Tween-20) during 1 hour with shaking and sequentially immunoprobed with primary antibodies at adequate dilution. Primary antibodies were diluted in 5% BSA in PBS-T. Antibody detection was performed using fluorescent-labeled secondary antibodies (Alexa 680λm and Alexa 800λm, Rockland, Gilbertsville, PA, USA) and scanned with Odyssey Infrared System Scanner (LI-COR Biosciences, Lincoln, NE, USA). Band intensities were quantified using the ImageJ 1.34S software (National Institute of Health, Bethesda, MD, USA).

Electrophoresis buffer 5X composition*:* TrisHCl 0.13 M, glycine 0.95 M, SDS 0.5%.
Sample buffer (Laemmli buffer) 4X composition*:* 62.5 mM TrisHCl pH 6.8, glycerol 20%, SDS 2%, 2-mercaptoethanol 5%, bromophenol blue 0.025%.
Transference buffer 10X composition*:* TrisHCl 0.025M, glycine 0.2 M, 20% of methanol

2. 7 Cell biology methods

2.7.1 Apoptosis measurement: Annexin V/Propidium iodide assays

Routinely, 3 x 10^5 cells were collected, washed with PBS and resuspended in 200 µL of Binding Buffer 1X (diluted with water from Binding Buffer 10X, BD Biosciences, Franklin Lakes, NJ, USA). Then, 4 µL of APC-labeled Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and 0.5 µL of propidium iodide (PI) (from a 1 mg/mL PI solution, SIGMA-ALDRICH, Steinheim, Germany) were added and samples were incubated 10 minutes at RT, in the dark. FACSCalibur or FACSCanto devices (BD Biosciences, Franklin Lakes, NJ, USA) were used for acquisition of 10,000 events. Data were analyzed using the CellQuest Pro software (Becton Dickinson). For apoptosis analysis, Annexin V positive/PI negative cells were considered early apoptotic cells whereas Annexin V positive /PI positive cells were considered late apoptotic cells. In some experiments, only Annexin V staining was used. In all time courses, the percentage of death was calculated considering the basal death of each sample as follows: (% death - % basal death)/ (100 - % basal death)

2.7.2 Cell cycle analysis

Between 3 and 5 x 10^5 cells were washed with PBS in a falcon tube and were resuspended in 330 µL of cold PBS. Cells were permeabilized by addition of 670µL of cold 100% ethanol (stored at -20ºC) drop by drop against the wall of the flacon tube. Samples were kept at least 30 minutes at 4ºC or up to one week at -20ºC. After washing with PBS, samples were incubated at 37ºC for 30 minutes with 500 µL of PBS containing 1 µL of Dnase-free Rnase A (Quiagen, Inc., Valencia, CA) and 50 µg/mL of PI. 10000 events were acquired using a FACSCalibur device (BD Biosciences, Franklin Lakes, NJ, USA). Parameters were set up to detect G1 phase at 200 and G2/M phase at 400 units in the FL-3 channel. Data were analyzed using the CellQuest Pro software (Becton Dickinson).

2.8 Bioinformatics methods

2.8.1 Connectivity Map

This bioinformatic tool was used to generate new hypothesis of candidate drugs that could reverse the gene expression profile of CLL cases with bad prognosis (Lamb et al., 2006). Two lists of genes differentially expressed (up and down regulated genes) between two groups of CLL samples (either with short or long time of treatment free survival) were applied to the c-Map (versions .01 and .02). The c-Map relies on a data base of gene expression values from

previous experiments that include 6 -hour treatments of several cell lines with a panel of up to 1500 drugs (for version 0.2) ever licensed for human use by the FDA This tool is able to look for connections between the gene expression data base and the gene-query.

2.8.2 Differentially expressed genes

The following bioinformatic tools were used to look for genes differentially expressed between two conditions (for example, treated *versus* non-treated samples), depending on the number of samples and genes to be analyzed. Student t-test implemented in the T-rex tool of GEPAS was used to study genes differentially expressed between control and ETP-39010 treated samples (hybridized on a 4X 44K platform). These genes were further included in functional groups using the FatiGO tool implemented in Babelomics (http://babelomics.bioinfo.cipf.es/).

For analysis of samples hybridized on the 8X 15K platform, t-test limma (not permutation based) available at ASTERIAS (http://www.bioinformatics.org/asterias/wiki/Main/HomePage) was applied. In some cases, Significant Analysis of Microarrays tool developed at the University of Stanford was chosen.

2.8.3 Sample clustering

In order to cluster samples on a gene base, two different tools were employed. First, CLUSTER software (University of Stanford) was used to hierarchically cluster the samples (using the average linkage method). Genes were adjusted to the mean, in order to emphasized differences between different samples. For data representation, the Tree View tool (University of Stanford) was employed. Second, consensus Clustering tool (Broad Institute) available at http://www.broadinstitute.org/cancer/software/genepattern/index.html was used in order to confirm clustering.

2.8.4 Gene set enrichment

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether an a priori defined set of genes (i.e. genes grouped in pathways) shows statistically significant, concordant differences between two biological states (in our study, between sensitive and resistant samples or treated and control samples). The metric used in all analysis was t-test and the number of permutations depended on the microarray platform employed (200 permutations for 8-pack arrays and 1,000 permutations for 4x44K arrays).

3. RESULTS I

Proliferation centers in chronic lymphocytic leukemia: the niche where NF-κB activation takes place

3.1 Identification of Proliferation Centers and morphological description

CLL/SLL lymph node infiltration has as distinctive characteristic the presence of the so called pseudofollicles or proliferation centers (PCs). They are more rarely described in the white pulp of the spleen and bone marrow. PCs are newly formed structures, and have not been described in reactive lymphadenitis or other lymphoproliferative conditions.

We found PCs in all 72 cases analyzed, except for one bone marrow sample, although their size and number varied from sample to sample. They can be easily identified using the H&E staining (see Figure 4) because they appear as vaguely nodular areas that can be recognized against a monotonous background of small mature-looking lymphocytes. Therefore, they are clearly visible at low magnification (Schmid and Isaacson, 1994). PCs contain a continuum of small, medium and large cells consisting of prolymphocytes, which are small to medium-sized cells with relatively clumped chromatin and small nucleoli, and paraimmunoblasts, which are larger cells with round to oval nuclei, dispersed chromatin, central eosinophilic nucleoli and slightly basophilic cytoplasm. These morphological differences have allowed us to identify which was the cell type that expressed a specific cell marker without the need of double staining.

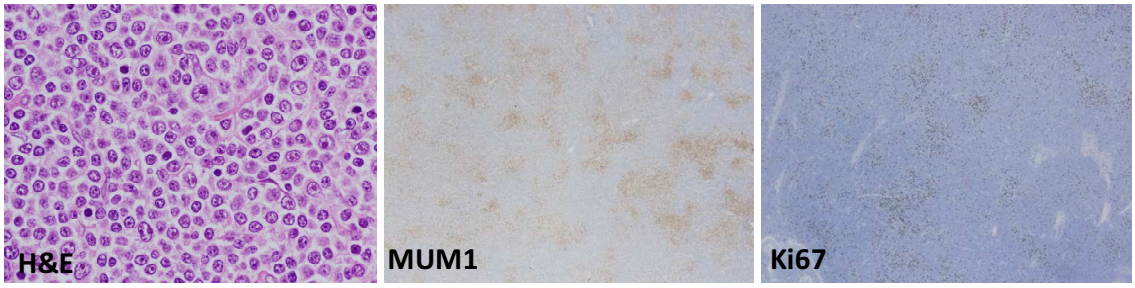


Figure 4. Description of PCs. H&E staining identifies several types of tumor and accompanying cells. All PCs were stained with MUM1/IRF4 and Ki67 antibodies

As two major SLL/CLL subtypes showing different clinical outcomes and somehow biological behavior have been described (U-CLL and M-CLL), we asked whether PCs were different in both groups. Data of IGHV mutational status were available only for 40 samples included in the TMA. No differences either in PC number, size or cell composition could be found in our series.

All PCs showed Ki67 staining, revealing a pool of proliferating cells made of the large B cell population. Moreover, MUM1/IRF4 expression was also found in the large B cells of all PCs, confirming the findings of previous studies (Soma et al., 2006).

3.2 Description of cell composition of the microenvironment

The study in depth of the PCs showed the presence of specific cellular subpopulations inside these anatomical structures. We identified several types of accompanying cells including reactive small T cells, dendritic cells, macrophages and endothelial cells in addition to tumor B prolymphocytes.

3.2.1 Stroma cells: macrophages and dendritic cells

Double immunohistochemistry combinations using a large panel of markers for dendritic cells and macrophages revealed the presence of at least two different populations of stroma cells (Table 6 for marker description). We could identify two groups of stroma cells. The first one expressed Actin and SDF1 (also known as CXCL12). We have named this type of cells as actin-positive dendritic cells (ADCs). The second group of stroma cells identified, herein called STAT1-positive macrophages (STAT1-Ms) was composed by macrophages positive for STAT1, CD68 and CD40 (Figure 5). Some ADCs were also positive for D2-40 and CD23, but not for CD21 (follicular dendritic markers). No immunoreactivity was found for STAT3, STAT4, CD1a (dendritic marker), S100 protein (marker for interdigitating dendritic cells), desmin or TRAF1. Electron microscopy studies confirmed our findings and showed the presence of at least these two types of non-lymphoid cells in PC.

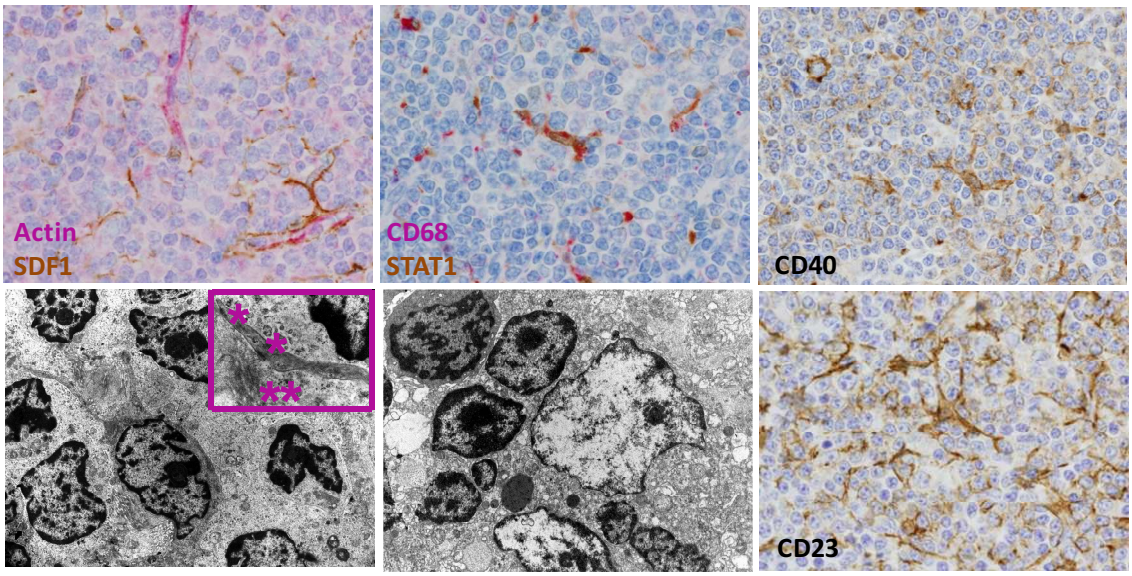


Figure 5. Stroma cells found inside PCs. Upper panel shows double immunohistochemical staining of ADCs and STAT1-Ms. CD40 is expressed by ADCs (morphologically different).Lower panel shows electron microscopy images of ADCs (left) and STAT1-Ms. Inset (x 25000) of ADCs depicts a detail of the main cell showing a cytoplasmic process with desmosomes (one asterisk) and thin filaments (two asterisks). Histiocyte-like cell presented an organelle-rich cytoplasm and some lysosomes. Some ADCs, distinguished by the long dendritic processes, expressed also CD23.

The percentage of ADCs was stable among all cases while the percentage of STAT1-Ms seemed to be higher in some specific samples.

3.2.2 T cells

When compared with the surrounding tissue, it was apparent that PCs contained an increased number of T cells. Accordingly, small CD3-positive cells were found intermixed among the CD20-positive proliferating prolymphocytes. Bystander T cells inside PCs were mostly CD4 positive, while CD8 cells were found equally distributed (Figure 6). CD3-positive T cells inside PCs expressed CD40L (Figure 9). Markers for different T cell subpopulations were observed in the T cell population found inside the proliferation centers including FOXP3, CD25, PD1, CD57 and CXCL13 (Figure 6), confirming a great immunophenotypical complexity. Neither positive cells for CD10, BCL6 nor cytotoxic markers (TIA1, granzyme B and perforin) were found.

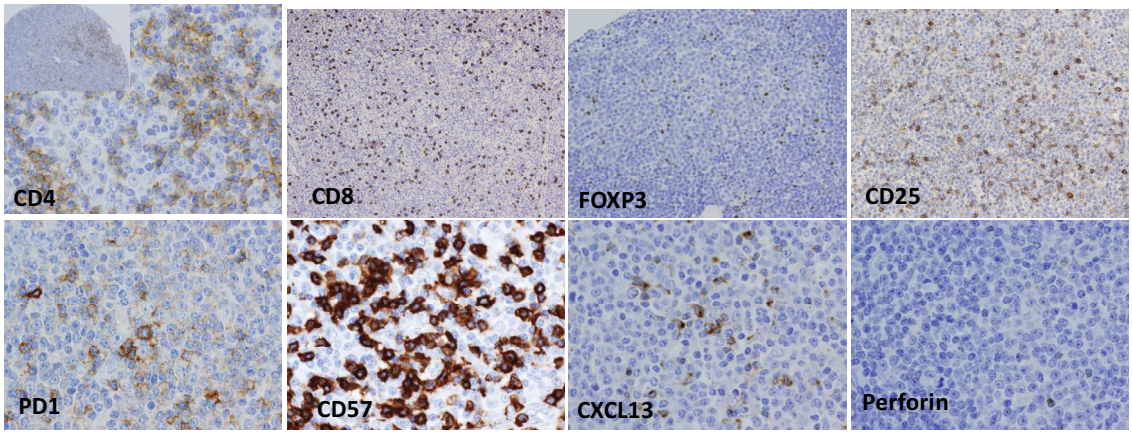


Figure 6. Markers for T cells in lymph node samples of CLL patients. Higher proportion of CD4-positive T cells was observed inside PCs (see also lower magnification, on the left corner of CD4 panel). T cells of PCs are positive for several markers of different CD4 phenotypes and negative for CD8 markers such as perforin, TIA1 and granzyme B (not shown).

3.3 Relevant pathways found activated in PCs that could be involved in the maintenance and proliferation of tumor cells

3.3.1 NF-κB pathway

Since it has been described that CLL cells have constitutively activated NF-κB, which appears to enhance leukemia cell survival, we decided to study the expression of the different NF-κB subunits in our series. Interestingly, we could identify nuclear expression of any of the members of the NF-κB family in all PCs. p50, p52 and Rel B were found exclusively in the nucleus of B prolymphocytes that were identified according to their morphology (Figure 7).

Nuclear p50 was found in the B prolymphoctyes of nearly all cases and those negative for nuclear p50 expressed either p52 or Rel B (percentages of positive cases for each marker in Figure 8). Although in most cell types and conditions the p50 subunit forms a dimer with p65, it is well known that other subunit complexes also participate in the regulation of cellular processes and tumorigenesis.

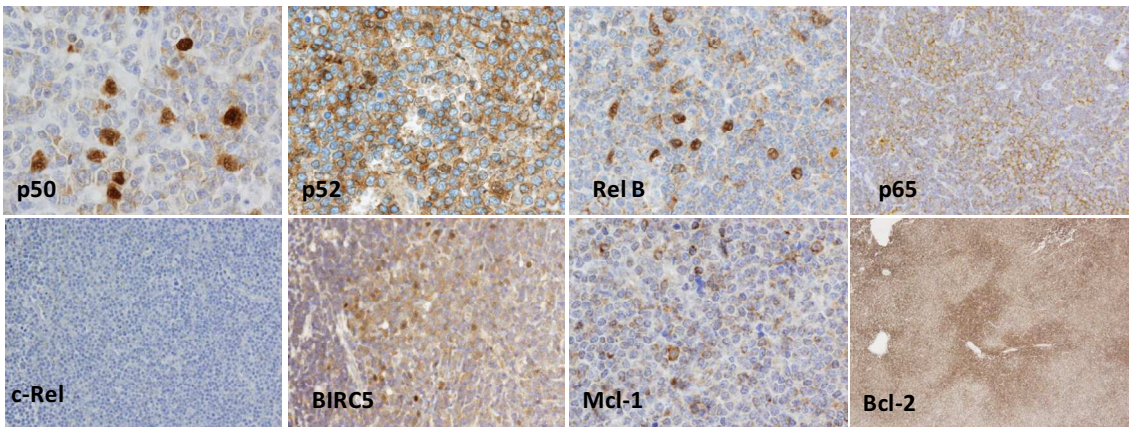


Figure 7. NF-κB status in CLL cells inside PCs. p50, p52 and Rel B were observed in the nucleus of CLL cells, indicating activation of this family of transcription factors. However, p65 showed cytoplasmic localization and c-Rel was not expressed in the majority of the cases.

In order to confirm the activation of the pathway, also the expression of some NF-κB targets was investigated. We found that Mcl-1 and BIRC5 (also known as Survivin), survival and antiapoptotic mediators, were also expressed exclusively in malignant cells found in the PCs (and not outside) in the majority of the cases (91.94% and 100% respectively). Bcl-2 was also found in PCs, but not in an exclusive manner as it was also seen outside of the PCs.

3.3.2 BAFF signaling

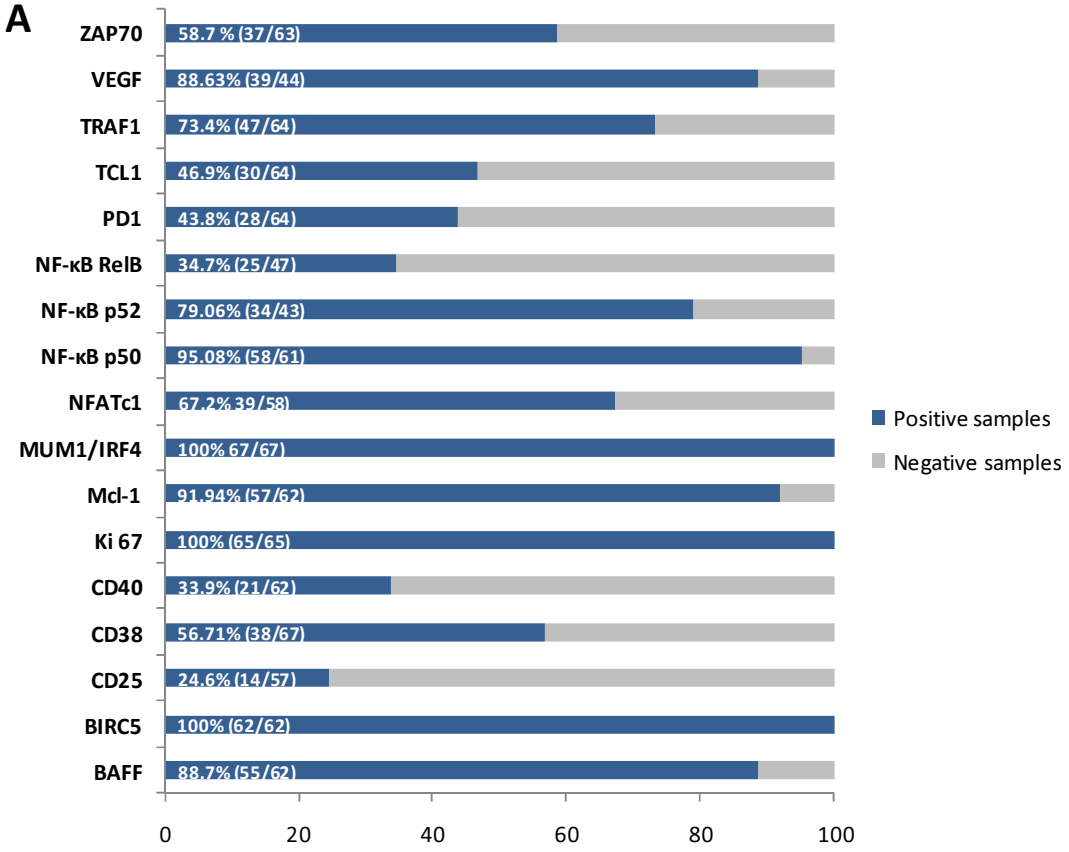
We were also interested in the upstream pathways that could be responsible for the NF-κB activation. For that reason, we studied the expression of the cytokine BAFF, which has been shown to present an NF-κB consensus sequence in its promoter and is known to be important for CLL cell survival. We observed BAFF expression in B lymphocytes only inside the PCs (88.7% of all cases) (Figure 8). Surprisingly, stroma cells did not express BAFF.

3.3.3 CD40 pathway

However, BAFF expression alone could not explain the NF-κB activation in all cases. Therefore, we investigated the role of CD40, a member of the TNF receptor family that activates both the classical and alternative NF-κB pathways in normal lymphocyte development. Thus, it has been

shown that blocking the CD40 pathway through anti CD40L mAb *in vitro* results in NF-κB inhibition and increases cell death of CLL cells (Furman et al., 2000). One third of all cases from our series (33.9%) showed expression of CD40 exlclusively in B prolymphocytes of PCs (Figure 8).

A



B

B-prolymphocytes (neoplastic cells)				
Positive markers				**Negative markers**
1	**2**	**3**	**4**	**Proliferation Centers**
TRAF1	Bcl-2	CD40	NF-κB1 (p50)	c-Rel
BAFF	TCL1	SYK	NF-κB2 (p52)	Rel A (p65)
IRF4/MUM1		VEGF	Rel B	CD10
MCL1		ZAP70	NFATc1	GCET1
PD1		CD23		CD30
Ki67		CD38		BCL6

Figure 8. Expression of different markers in PCs. A) Percentage of positive cases in B-prolymphocytes for each marker analyzed. In brackets, the number of positive cases out of the total of valorable samples. B) Different patterns of staining were observed and are summarized here. 1, positive markers exclusively in PCs. 2, positive markers in PCs and in small non-proliferating neoplastic cells outside PCs with lower intensity in PCs. 3, positive markers in PCs and in small non-proliferating neoplastic cells outside PCs. 4, localization change, from cytoplasm to the nucleus in PCs.

In order to confirm the activation of the pathway, we studied by immunofluorescence the expression of its natural ligand (CD40L, also known as CD154) in T cells. The double labeling (CD3, CD40L) showed that T cells surrounding B prolymphocytes actually expressed CD40L (Figure 9).

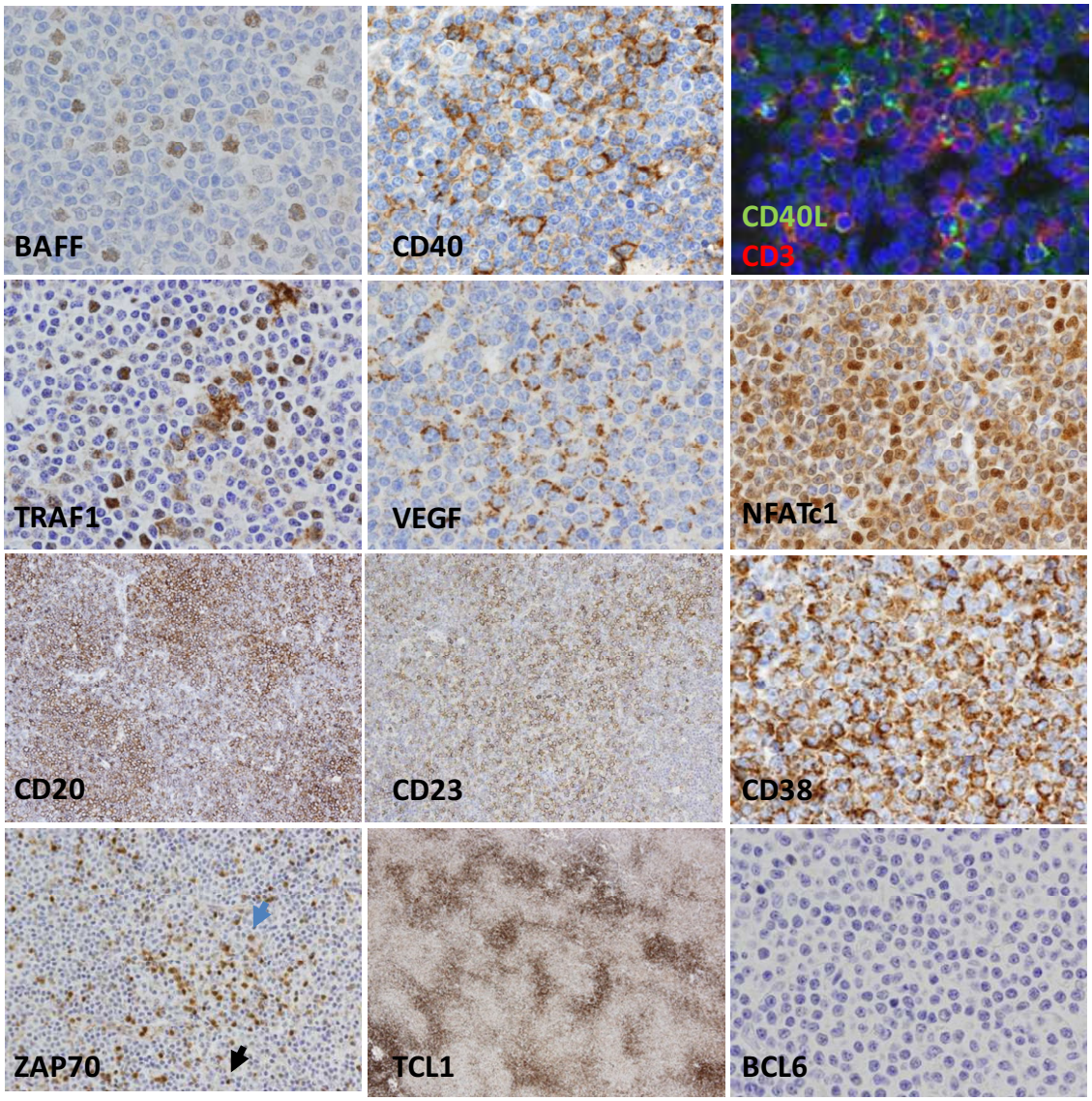


Figure 9. Activated pathways, prognostic and germinal center markers inside proliferation centers are shown. Immunofluorescence was performed on frozen tissue. Arrows indicate ZAP70-positive B-prolymphocytes (blue) or T cells (black).

The antiapoptotic effect of CD40 ligation in CLL is known to be mediated by VEGF. Both VEGF and CD40 engagement are essential for NF-κB activation in B CLL cells (Farahani et al., 2005). In agreement with this, we found that VEGF was overexpressed in the cytoplasm of B prolymphocytes of PCs in 88.63% of the cases of our series

Our observations were consistent with a previous work (Basso et al., 2004), in which B cells, upon CD40 ligation, acquired a specific signature that included genes such as MUM1/IRF4, p50 and TRAF1. Interestingly, transgenic mouse overexpressing TRAF2DN and Bcl-2 in B cells develop a CLL-like lymphoproliferative disease. TRAF2DN mutant lacks the N-terminal RING and zinc finger domains and therefore, it mimics TRAF1. For these reasons, we decided to study the expression of TRAF1 in our series. We observed TRAF1 only in B prolymphocytes of the PCs. The staining was positive in the nucleus and cytoplasm of 34.4% of the cases, exclusively in the nucleus of 28.1% of the cases and only in the cytoplasm of 10.9% of them, making a total of 73.4% positive cases (TRAF1). The presence of nuclear TRAF1 expression was demostrated using both immunohistochemistry on paraffin-embedded tissues and immunofluorescence on frozen samples (data not shown).

3.4 Other findings

We also studied other proteins that could play a role in the proliferation of CLL/SLL cells exclusively inside the PCs and we found overexpression of PD1 (43.8%) and NFATc1 (67.2%) in tumor cells.

On the other hand both PCs and non-proliferating small B cells of CLL samples were positive for some markers such as CD20, SYK, Bcl-2, TCL1, CD38, CD23 and ZAP70 (Figure 9). PCs in most cases showed a weaker staining for Bcl-2 and TCL1 (shown to be reduced in proliferating compartments). No immunoreactivity was found for germinal center markers such as BCL6 (Figure 9), GCET1 and CD10 or other markers such as CD30 (data not shown).

4. RESULTS II

New insights into the relationship between molecular features of chronic lymphocytic leukemia and sensitivity to rationally selected compounds (calmidazolium, R406, TW-37 and ETP-39010): an *ex vivo* approach

4.1 Rational selection of compounds to be tested on primary samples

Since it has been shown that antigen selection and BCR signaling are important in the pathophysiology of CLL (Caligaris-Cappio and Ghia, 2008), we decided to functionally explore this pathway at different levels. For that, we collected a variety of gene expression data and analyzed them with the BCR pathway as the main focus. Our main objective was to study the molecular heterogeneity of CLL patients and how this could influence the response to some selected compounds.

First, we used an *in silico* approach by means of the connectivity Map (c-Map) developed by the Broad institute and available at: http://www.broadinstitute.org/science/projects/connectivity-map/connectivity-map.

This bioinformatic tool is based on the creation of a large reference catalogue of gene expression data from cultured human cells perturbed with many chemicals. The c-Map aims at connecting gene patterns that characterize a disease with gene patterns produced by drugs or genetic approaches.

Gene expression profiles from 160 CLL patients generated previously in our group were used (Rodriguez et al., 2007). These patients were classified into two groups, according to long or short treatment free survival. Genes differentially expressed between these groups were obtained using the public available tool T-REX (GEPAS). Two lists of genes upregulated (74 genes) or downregulated (43 genes) in the group of poor prognosis were generated and applied to the c-Map version 0.1. This version included data for 164 distinct bioactive small molecule compounds and 564 gene expression profiles. Table 9 shows the compounds retrieved by the programme. A negative score indicates that the drug is able to reverse the gene profile that has been inquired. Thus, compounds with scores close to the highest negative score (-1) were selected. Interestingly, the mechanisms of action of several of these drugs were related to calcium signaling. Calmidazolium inhibits calmodulin-dependent phosphodiesterase and Ca^{2+}-transporting ATPase but it can also cause elevation of intracellular calcium independent of calmodulin inhibition. Felodipine is a calcium channel blocker that decreases the intracellular levels of calcium inhibiting many cellular processes. Other compounds obtained in the c-Map analysis act through dopaminergic, adrenergic, cholinergic or histaminic receptors triggering calcium signaling upon receptor engagement.

Table 9. c-Map results obtained with version 0.1. Score range is from -1 to 1. Negative scores indicate that the drug reverses the gene signature provided. HL60, acute myeloid leukemia cell line; MCF7, human breast cancer cell line; PC3, human prostate cancer cell line. HDAC, histone deacetylase. Sources used were DrugBank and PubMed

c-Map name	Description	Dose	Cell line	Score
calmidazolium	Inhibitor of calmodulin	5 µM	MCF7	-1
trifluoperazine	Antiadrenergic, antidopaminergic, and minimal anticholinergic effects. Protein binding is dependent on calcium	10 µM	MCF7	-0,904
felodipine	Calcium channel blocker used to control hypertension	10 µM	MCF7	-0,889
thioridazine	Central adrenergic-blocking, dopamine-blocking and minor anticholinergic activity	10 µM	MCF7	-0,878
resveratrol	Suppresses NF-kB. Anti-inflammatory and antioxidant effects. Potential anticancer properties	10 µM	MCF7	-0,871
prochlorperazine	It blocks D2 dopamine receptors, anticholinergic and alpha-adrenergic receptors	10 µM	MCF7	-0,845
felodipine	calcium channel blocker used to control hypertension	10 µM	MCF7	-0,762
prochlorperazine	It blocks D2 dopamine receptors, anticholinergic and alpha-adrenergic receptors	10 µM	MCF7	-0,744
resveratrol	It suppresses NF-kB. Anti-inflammatory and antioxidant effects. Potential anticancer properties	10 µM	MCF7	-0,725
sirolimus	Immunosuppressant . It binds the cytosolic protein *FK-binding protein 12* (FKBP12) and inhibits the mTOR Complex1	100 nM	MCF7	-0,654
rottlerin	It inhibits protein kinases with some specificity for PKC	10 µM	MCF7	-0,682
sirolimus	Immunosuppressant. It binds the cytosolic protein *FK-binding protein 12* (FKBP12) and inhibits the mTOR Complex1	100 nM	HL60	-0,6
trichostatin A	HDAC inhibitor	1 µM	MCF7	-0,588
wortmannin	Inhibitor of phosphoinositide 3-kinases (PI3Ks)	1 µM	MCF7	-0,567
trichostatin A	HDAC inhibitor	100 nM	MCF7	-0,562
trichostatin A	HDAC inhibitor	1 µM	MCF7	-0,562
trichostatin A	HDAC inhibitor	100 nM	PC3	-0,536
wortmannin	Inhibitor of phosphoinositide 3-kinases	10 nM	HL60	-0,536
trichostatin A	HDAC inhibitor	1 µM	MCF7	-0,529
trichostatin A	HDAC inhibitor	100 nM	MCF7	-0,522
sirolimus	Immunosuppressant . It binds the cytosolic protein *FK-binding protein 12* (FKBP12) and inhibits the mTOR Complex1	100 nM	MCF7	-0,513
sirolimus	Immunosuppressant . It binds the cytosolic protein *FK-binding protein 12* (FKBP12) and inhibits the mTOR Complex1	100 nM	MCF7	-0,508

These results suggested that a way to reverse the bad prognosis gene signature/phenotype might be the modulation of the calcium mediated pathways. In fact, calcium is an essential mediator in the signaling through the BCR. Levels of calcium can be sensed by calmodulin, which interacts with protein kinase C and in turn activates NF-kB as well as the transcription factor NFATc (Beals et al., 1997). Therefore, the well known calmodulin inhibitor calmidazolium was chosen to be tested in our study.

Other drugs of interest obtained by the c-Map analysis were resveratrol, siroliums and rottlerin. Especially interesting for us was resveratrol, a phytostrogen found in red wine and a variety of plants that is known by its anti-inflammatory and antiproliferative effects mediated by the modulation of cyclooxigenase and the transcription factor NF-kB, respectively. The impact of the latter one in the maintenance of CLL cells is well described and resveratrol was therefore considered a reasonable candidate to be used in our study. However, this compound exerts many effects and since we wanted to focus on the BCR pathway, we decided to choose compounds that were structurally closely related to resveratrol but have a more restrictive effect, such as piceatannol. This compound is a potent but not specific inhibitor of the apical Syk kinase (Bullington et al., 1998), whose inactivation in turn inhibits NF-kB activation in B cells. The inhibition of NF-kB *via* BCR signaling inhibition fits with previous data of the group that show that CLL patients with a poor prognosis show an increased expression of a BCR signature (Rodriguez et al., 2007). At this time, a new specific Syk kinase inhibitor known as R406 was generated by Rigel Pharmaceuticals Inc (South San Francisco, California) and therefore this compound was finally selected to be tested in our series of patients. As it has been already suggested, SYK kinase might be the bottle neck of the signaling through the BCR signaling, and R406 offered a good opportunity to further dissect this pathway (Baudot et al., 2009; Gobessi et al., 2009).

Recently, a new expanded version of c-Map has been released (c-Map v.02). This new version includes 7,000 gene expression profiles representing 1309 compounds and now also some genetic reagents. When our CLL signature was applied to the v.02, the drugs obtained were different from those proposed in the c-Map v. 01, which was expected considering the increase in the data catalogue. However, among the compounds with highest negative scores, we could again find drugs whose mechanism of action affects calcium trafficking (lebovulonol -score -0.9-, apomorphine -score -0.8-, maprotiline -score -0.8-, among others) and drugs that are direct calcium blockers (prenylamine, nitrendipine and nifendipine), confirming our initial

hypothesis. Moreover, the majority of the compounds retrieved by the previous version were also obtained with the actualised version (wortmanin, resveratrol, trichostatin A, etc.)

On the other hand, two additional drugs were selected for our study following a more classical analytical approach. One of the main features of CLL cells is that they have impaired apoptosis (Hamblin and Oscier, 1997). In fact, several pathways, including BCR cascade but also CD40 signaling, CD95 and TRAIL receptors, contribute to modify the balance of pro and antiapoptotic molecules. Several drugs and antisense molecules that target different members of the Bcl-2 family are already at clinical trials. Many of them target antiapoptotic members of this network such as Bcl-2 or are BH3 mimetics that block Bcl-2. However, several lines of evidence show that another antiapoptotic member of this family, Mcl-1, may have a greater relevance in the regulation of the balance between survival and death signals in CLL cells (Ruiz-Vela et al., 2008). Interestingly, when the gene expression profile of 16 CLL cases was investigated for several members of the Bcl-2 family we observed a variability in the levels of the different members of the family and that cases with higher expression of antiapoptotic proteins (Mcl-1, Bcl-2, Bcl-x_L) also displayed a lower expression of proapoptotic proteins (Bax, Bak). Nevertheless, Bcl-2 was highly expressed in all samples (Figure 10A). Taking this data into account, we decided to test a new compound known as TW-37 that derives from the natural compound gossypol (Wang et al., 2006). It is a nonpeptidic small-molecule inhibitor that binds to the BH3-binding groove of Bcl-2, Bcl-x_L and also Mcl-1 interacting with the same amino acid side chains as the natural Bim. Inhibition of this network allows us the interference of one of the latest events in the survival signals delivered in B cells.

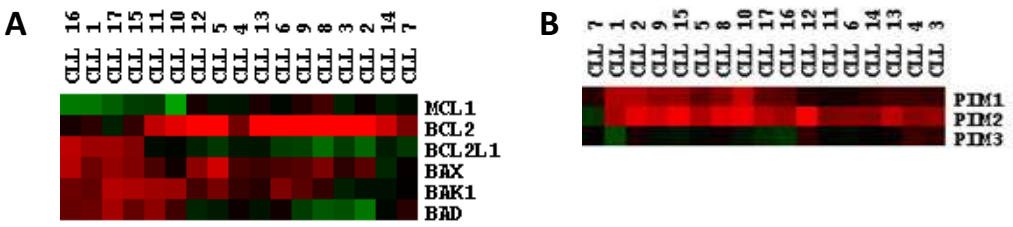


Figure 10. Gene expression profile of CLL samples. Data represented were normalized with lymph nodes and tonsils (Agilent platform, 4x44K). A) Heterogeneous expression of proapototic (Bax and Bak) and antiapoptotic members (Mcl-1, Bcl-2 and Bcl-x_L, also known as BCL2L1) was observed. B) PIM1 and PIM2 variability in expression levels was observed in CLL cases.

Finally, the examination of gene expression profiles from CLL samples confirmed previous data that indicated that PIM kinases might be deregulated in this malignancy. Our data showed that

PIM2 kinase is overexpressed in practically all CLL samples when compared with normal tissues (lymph nodes and tonsils), though at different levels (Figure 10B). PIM1 kinase showed even a greater variability among different samples. These kinases have been extensively related to B cell development and tumorigenesis and represented a novel therapeutic target in CLL. Also recent data from others suggest that inhibition of PIM kinases might be of therapeutic interest in CLL (Chen et al., 2009). In order to further explore this pathway, we decided to test a new pan-Pim kinase inhibitor that has been developed at the Experimental Therapeutics Programme of CNIO.

4.2 Measurement of compound sensitivity on CLL samples

Our main interest at this point was to study the drug sensitivity of every primary sample, in order to obtain a standard value that could be used to compare sensitivity of different samples to every compound. Before starting the screening, we decided to carry out some pilot experiments. To look for the best conditions for further assays, initial tests of basal cell viability were performed. Seven samples of PBMCs from CLL patients were assayed for spontaneous apoptosis (using Annexin V staining) at several time points after plating the cells. A considerable increase in cell death along time was observed (data not shown). Since we wanted to measure the decrease of cell viability upon drug treatment, it was important to maintain the lowest levels of basal cell death. Taking this and previous reports into consideration (Krutzik et al., 2008), we decided to plate the cells and let them rest only 2-3 hours at 37ºC before addition of the active compounds.

Another factor to consider was the time of incubation after drug dispensation. Preliminary experiments with two of the drugs (calmidazolium and R406) showed that a time and dose dependent increase in apoptosis could be observed for the sensitive samples, while resistant samples presented only a small induction of apoptosis (data not shown). However, Annexin V assays are time consuming and demand relatively high number of cells. Therefore we decided to use a commercial cell viability assay (Cell Titer GLO, Promega) that allowed us to minimize the number of cells required per experiment and therefore the use of a wider range of concentrations. Taking into account these factors, we decided to perform the cell viability assays using the following procedure: we let cells stabilize 2-3 hours after defreezing, we added the active compound using an automated dispensation protocol and measured cell viability at 72 hours.

4.3 Study of sensitivity to calmidazolium (inhibitor of calmodulin) in primary CLL cells

4.3.1 Effect of calmidazolium on cell viability

A first series of 21 samples was used to establish calmidazolium (CZ) sensitivity. This series included samples of PBMCs from CLL patients. EC50 values obtained were between 909 µM and 6,779 µM (summarized in Table 10) and revealed variability in drug sensitivity up to 6 fold. We were interested in knowing if this variability could correlate with some clinical parameters that are related to patient prognosis, being the mutational status of *IGHV* one of the most robust ones. However, t-test comparison between M-CLL and U-CLL showed no statistically significant differences between these groups (data not shown).

As PBMCs samples presented a variable percentage of tumor cells that could somehow masked the EC50 values, a second series of 14 purified B cells was used to assay calmidazolium sensitivity (Table 11). In spite of the heterogeneous drug response observed (with EC50 values between 650 nM and 28555 nM) no statistical difference was found between M-CLL and U-CLL samples.

4.3.2 Gene expression patterns define response to calmidazolium in CLL cells.

If these parameters could not explain drug response variability, we asked whether more complex features could explain sensitivity/resistance to calmidazolium. To explore this possibility, we used gene expression profiling (using a custom 8-pack array platform) of 12 purified B cell samples for which we had also calculated EC50 values. As an approximation, we considered that samples with EC50 values above the median (2,777 nM) of the series were resistant (6 samples), while those below the median were sensitive (6 samples) (Figure 11).

Since the number of genes (around 900) and samples (12) is limited, t-test analysis did not retrieve genes statistically different between both groups. Therefore, we decided to analyze gene expression patterns rather than single gene expression values. For that, we studied if there were positive or negative correlations between EC50 values and gene expression values. This means, if increasing EC50 values correlated with increasing or decreasing values of gene expression among the samples.

Table 10. Series of PBMC from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. % of IGHV homology data provided by some hospitals was not available. No *TP53* mutations indicates that there were not mutations in exons 5, 6, 7 or 8, but no FISH data are available.

Sample code	EC50 (nM) CZ	CD19	SHM	% IGHV homology	FISH/*TP53mut*	ZAP70
80232	909	ND	M	ND	13q deletion	ND
80113	956	70%	ND	ND	No *TP53* mutations	-
80091	1,395	ND	U	99.6	17p deletion	ND
90921	1,397	43%	M	96.34	No *TP53* mutations	ND
80102	1,511	80%	ND	ND	11q and 13q deletions. IGH+ rearrangement	-
80005	1,586	90%	M	86	13q14 deletion	-
80117	1,781	25%	U	99.6	11q22 deletion	+
90082	1,833	90%	M	93.17	No *TP53* mutations	-
80230	1,938	ND	U	ND	Normal	+
90533	2,220	ND	ND	ND	ND	ND
90905	2,423	ND	M	96.73	No *TP53* mutations	ND
70446	2,605	73%	U	100	ND	ND
90978	2,732	ND	M	96.02	No *TP53* mutations	ND
90025	2,924	79%	M	93.63	13q14 deletion	-
80002	3,102	66.9%	M	94.74	13q14 deletion	-
80048	3,178	34%	M	90.65	Normal	-
80139	3,187	ND	U	99.6	13q and 17p deletions	ND
90393	3,371	56%	M	90.76	No *TP53* mutations	-
80254	3,398	81%	U	100	13q14 deletion	-
80047	3,786	25%	M	95.53	Chromosome 12 trisomy	ND
80197	5,046	ND	U	ND	Normal	+
80194	5,267	ND	M	ND	13q deletion	+
70448	5,614	ND	M	87.9	No *TP53* mutations	ND
80231	5,812	ND	M	ND	13q deletion	-
80138	6,032	ND	M	93.55	11q deletion	ND
70447	6,779	62%	M	93	No *TP53* mutations	ND

Table 11. Series of purified B cell samples from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. CD19 a. Purif, indicates CD19 purity of samples after B cell selection. No *TP53* mutations indicates that there were no mutations in exons 5, 6, 7 or 8, but no FISH data are available. MR, gene expression microarray (8 x 15K).

Sample code	EC50 CZ	CD19	CD19 a. Purif	SHM	% IGHV homology	FISH/p53mut	ZAP70	Assays
90903	650.3	ND	99.8%	ND	ND	No *TP53* mutations	ND	MR
100008	666.1	ND	99.7%	M	89.16	No *TP53* mutations	ND	MR
91103	873.8	96%	97.7%	M	92.77	13q14 deletion	-	MR
100018	1,277	59.3%	96%	U	99.18	11q deletion	+	MR
91066	1,796	60%	99.8%	ND	ND	13q14 deletion	-	MR
91072	2,750	58.5%	97%	M	93.95	17p deletion	ND	MR
91207	2,777	82%	97%	U	99.59	No *TP53* mutations	-	MR
90908	3,131	90%	99.5%	M	96.4	13q14 deletion	-	MR
90994	3,619	35%	99.7%	M	ND	Normal	-	MR
90909	3,678	94%	98.4%	ND	ND	c-MYC trisomy, 13q deletion	+	MR
90906	3,759	90%	99.8%	U	99.6	IGH+ rearrangement	+	MR
91069	4,346	93%	96.3%	U	99.18	Chromosome 12 trisomy	+	MR
90907	4,817	80%	99.5%	M	94.3	13q14 deletion	-	MR
100004	28,555	ND	99.5%	ND	ND	No *TP53* mutations	-	-

Using this approach, we obtained 46 genes with Pearson coefficient >0.5 or <-0.5 and p values <0.05. These genes were able to cluster (CLUSTER, Stanford University) our samples into two groups that corresponded to the ones previously defined as resistant or sensitive. Then, we compared gene expression of these 46 genes between the two groups (t-test Limma, Asterias, CNIO) and 14 were differentially expressed (FDR-independent <0.05). These genes were also able to classify our samples in sensitive and resistant (Figure 12B). Moreover, consensus Clustering (Gene pattern, Broad Institute) confirmed these results (Figure 12C). The 14-gene signature included the following genes: *BMP7, DIAPH2, XIAP, ADAM17, MALAT1, MOSPD2, RPL23, UPK2, RPL4, TLE4, PRKCE, BIRC6, MBTD1* and *ZSCAN29*

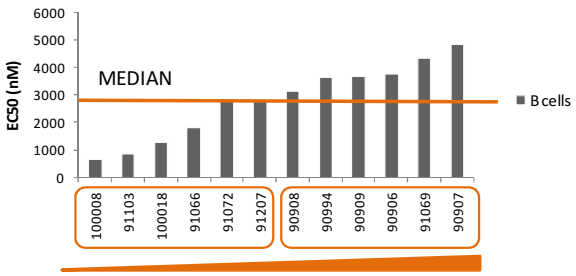


Figure 11. EC50 values for calmidazolium of B-CLL cells. Purified CLL samples that had been hybridized in gene expression microarrays were divided into sensitive or resistant cells considering the median of the series (2,777 nM).

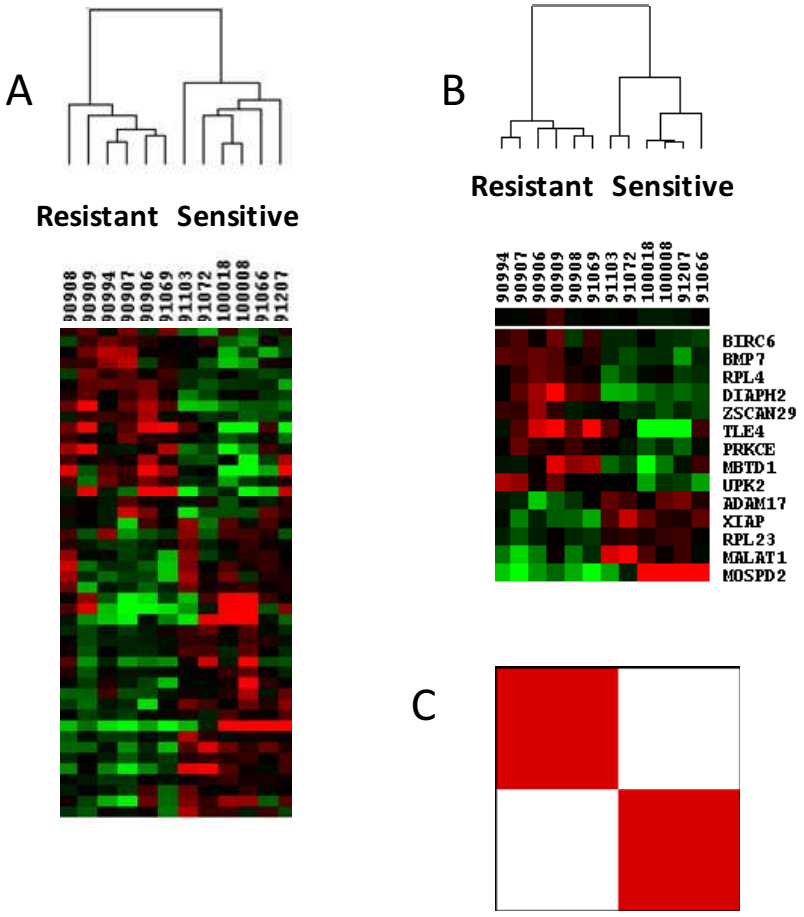


Figure 12. A 14-gene signature defines response to calmidazolium. A) Hierarchical cluster of CLL samples using the initial 46-gene signature. B) Hierarchical cluster of CLL samples using the 14-gene signature. C) Consensus Clustering with the same genes as in B) defines the same two groups of samples.

4.4 Study of sensitivity to R406 (SYK inhibitor) in primary CLL cells

4.4.1 R406 effect on cell viability

R406 cytotoxic effect was measured in a series of 22 PBMC CLL samples (Table 12). Unfortunately, the response to the drug did not adjust to a sigmoidal curve, as it is necessary for EC50 calculations. However, two groups of samples could be distinguished. The first one showed no effect on cell viability upon drug treatment while the second one showed a reduction in cell viability after R406 treatment. As R406 is a compound that targets the SYK kinase involved in BCR signaling, it could be especially active in the B-cell population. Therefore, we decided to assess cell viability in a new series of 9 purified B CLL samples. We

again observed two groups of samples with different sensitivity (Table 13). One group included 4 samples that were very sensitive to R406, showing EC50 values ranging from 270 nM to 1498 nM while a second group was made up of 5 samples that were resistant and did not show a measurable reduction of cell viability, even at the higher dose used (50 µM).

Table 12. Series of PBMC from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. No *TP53* mutations indicates that there were not mutations in exons 5, 6, 7 or 8, but no FISH data are available. R indicates, resistant sample with no measurable decrease in cell viability. D*, effect on cell viability but not adjusted to a sigmoidal curve.

Sample code	R406 EC50 (nM)	CD19	SHM	%IGHV homology	FISH/TP53mut	ZAP70
70447	R	62.0%	M	93	No *TP53* mutations	ND
70448	R	ND	M	87.9	No *TP53* mutations	ND
80002	R	66.9%	M	94.74	13q14 deletion	-
80003	D*	32.0%	ND	ND	ND	+
80005	R	90.0%	M	86	13q14 deletion	-
80047	D*	25.0%	M	95.53	Chromosome 12 trisomy	ND
80048	D*	34.0%	M	90.65	Normal	-
80091	D*	ND	U	99.6	17p deletion	ND
80113	D*	70.0%	ND	ND	No *TP53* mutations	-
80138	D*	ND	M	93.55	11q deletion	ND
80139	D*	ND	U	99.6	13q and 17p deletions	ND
80194	R	ND	M	ND	13q deletion	+
80197	D*	ND	U	ND	Normal	+
80230	D*	ND	U	ND	Normal	+
80231	R	ND	M	ND	13q deletion	-
80232	D*	ND	M	ND	13q deletion	ND
80254	R	81.0%	U	100	13q14 deletion	-
90025	R	79.0%	M	93.63	13q14 deletion	-
80117	751.9	25.0%	U	99.6	11q22 deletion	+
70446	D*	73.0%	U	100	ND	ND
80102	D*	80.0%	ND	ND	11q and 13q deletions. IGH+ rearrangement	-
90393	D*	56.0%	M	90.76	No *TP53* mutations	-

Table 13. Series of B cell purified samples from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. CD19 a. Purif, indicates CD19 purity of samples after B cell selection. No *TP53* mutations indicates that there were not mutations in exons 5, 6, 7 or 8, but no FISH data are available. R, resistant sample. MR, gene expression microarray (8 x 15K); F, Western Blot in F(ab')2 stimulated cells.

Sample code	R406 EC50 (nM)	CD19	CD19 a. Purif.	SHM	%IGHV homology	FISH/p53mut	ZAP70	Asssays
90906	270.8	90%	99.8%	U	99.6	IGH+ rearrangement	+	MR, F
90909	R	94%	98.4%	ND	ND	c-MYC trisomy, 13q deletion	+	MR
91072	R	58%	97%	M	93.95	17p deletion	ND	MR
91103	R	96%	97.7%	M	92.77	13q14 deletion	-	MR, F
90903	257.8	ND	99.8%	ND	ND	No *TP53* mutations	ND	-
91207	R	82%	97%	U	99.59	No *TP53* mutations	-	MR
100004	R	ND	99.5%	ND	ND	No *TP53* mutations	-	F
100008	600.1	ND	99.7%	M	89.16	No *TP53* mutations	ND	MR
100018	1,428	59.3%	96%	U	99.18	11q deletion	+	MR, F

4.4.2 R406 inhibits BCR signaling in primary CLL samples and induces apoptosis

It has been well described, that *in vitro* stimulation of primary B-CLL cells with anti-IgM is able to initiate BCR signal transduction. R406 has been described to inhibit SYK, one of the apical kinases of the pathway. Therefore, we decided to check the activity of this compound by monitoring the phosphorylation of some of its direct (BLNK) or indirect (AKT) targets in a set of B-CLL samples. Cells were plated, incubated 3 hours after defreezing and treated with 5 µM R406 (Chen et al., 2008) or vehicle (DMSO) for 30 minutes. Then, cells were stimulated with F(ab')2 for 5 minutes. Reaction was rapidly stopped with ice cold PBS and cells were centrifuged and washed. Surprisingly, BLNK phosphorylation showed neither substantial induction after BCR stimulation or inhibition upon R406 treatment (Figure 13). On the contrary, AKT phosphorylation was induced after F(ab')2 treatment in all tested samples, although phosphorylation induction was minimal in one of the resistant samples. Interestingly, sensitive samples (90906 and 100018) already showed phosphorylated AKT in basal conditions, indicating that the pathway may be already activated. Moreover, these samples were ZAP70 positive, which is in agreement with published data that show that these CLL cells were more

sensitive to ligation of sIgM than leukemic cells that lacked ZAP70 expression (Chen et al., 2002). R406 treatment was able to completely inhibit AKT phosphorylation in all samples, even the basal phosphorylation levels (see sample 90906). We then decided to test R406 induced apoptosis in one representative sample of each group (sensitive or resistant). R406 was able to induce apoptosis in the sensitive sample that presented increased BCR signaling (sample 100018) while the resistant one (sample 100004) showed no substantial induction of apoptosis.

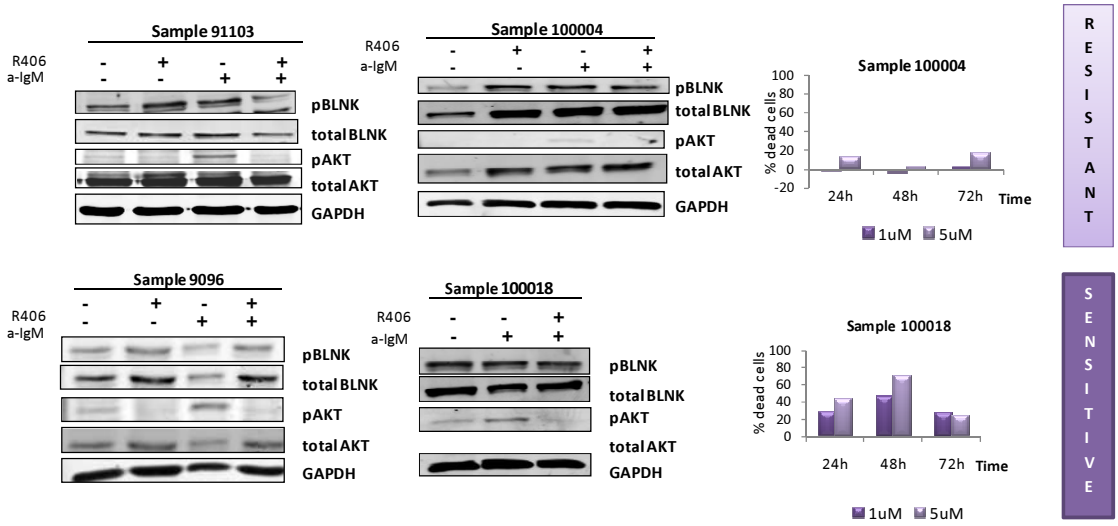


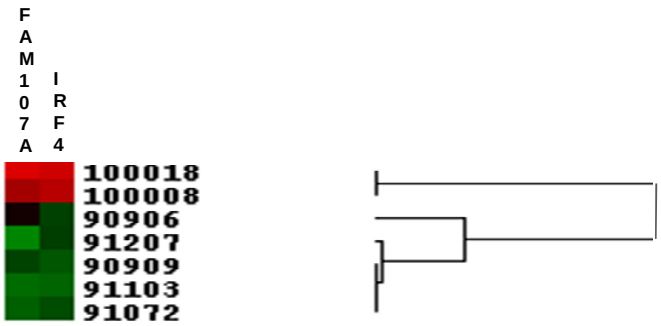
Figure 13. R406 effect on BCR signaling and apoptosis. Inhibition of SYK in resistant or sensitive samples inhibits BCR signaling. Cells were treated with R406 at 5 µM concentrations for 30 minutes. Stimulation was performed with 10 µg/mL F(ab')2, 5 minutes (a-IgM, anti-IgM).

4.4.3 Gene expression signature of R406 resistant and sensitive samples

In order to further investigate the molecular differences between resistant (90909, 91072, 91207, 91103) and sensitive (90906, 100008, 100018) samples, we performed microarray analysis of 7 B-CLL samples using 8-pack custom microarrays. SAM analysis showed two differentially expressed genes ($p<0.01$) between both subgroups, MUM1/IRF4 and FAM107A, which presented higher levels in the group of sensitive samples (Figure 14A). We also performed Gene Set Enrichment Analysis (GSEA) in order to generate hypothesis that could explain drug resistance (Figure 14B). For that, we used Biocarta pathways that have been curated for pathways especially relevant in lymphomas (Aggarwal et al., 2009). Interestingly, the two significant pathways (FDR<0.25) were chemokine and cytokine signaling pathways.

Although with a lower significance, other pathways found to be enriched in resistant samples were VEGF and TNF pathways.

A



B

Pathway	Size	Genes enriched
Chemokine signaling	18	*PLCG2, PI3CG, MAPK11, MAPK14, MAPK3, MAPK13, MAP2K2, CCL7, MAPK8, JUN, CAMK2B*
Cytokine-Cytokine receptor interaction	21	*LTA, TNFSF13B, TNFSF10, TNFSF11, IL2RA, CCL7, TNFRSF4, IL2, IL1A*
VEGF signaling pathway	15	*SHC1, PLCG2, PIK3CG, MAPK11, MAPK3, PIK3R2, MAPK13, MAP2K2, PXN*
TNFR pathway	38	*MAP3K14, LTA, IKBKB, TNFAIP1, TNFRESF1A, RELA, TRAF2, TRAF5, MAPK8, JUN, MADD*

Figure 14. Gene expression analysis of R406 sensitive and resistant CLL samples. A) *FAM107A* and *MUM1/IRF4* distinguished sensitive from resistant samples. B) Pathways enriched in resistant CLL samples (GSEA).

4.5 Study of sensitivity to TW-37 (small molecule inhibitor of Bcl-2 family) in primary CLL cells

4.5.1 TW-37 effect on cell viability

Following a similar approach to the one described in previous sections, 29 PBMC CLL samples were used to calculate EC50 for the Bcl-2 family inhibitor. The range obtained was between 32.82 nM and 753.1 nM, being sample 80113 an outlier with an EC50 value of 24,191 nM (Table 14). When *IGHV* mutational status was used to classify samples, no difference in EC50 values was found (Figure 15A). However, when samples with alterations affecting *TP53*, i.e. samples with 17p deletion, were excluded from the analysis (samples 80139 y 80091), a

statistical tendency (p value 0.07) was observed showing that U-CLL cases have lower EC50 and in consequence were more sensitive to the drug (Figure 15B).

Table 14. Series of PBMC from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. No TP53 mutations indicates that there were no mutations in exons 5, 6, 7 or 8, but no FISH data are available.

Sample code	TW-37 EC50 (nM)	CD19	SHM	% IGHV homology	FISH/p53mut	ZAP70
70446	32.82	73%	U	100	ND	ND
90978	70.35	ND	M	96.02	No *TP53* mutations	ND
80002	73.56	66.9%	M	94.74	13q14 deletion	-
90082	77.84	90%	M	93.17	No *TP53* mutations	-
80230	87.04	ND	U	ND	Normal	+
80003	101.3	32%	ND	ND	ND	+
80117	129.8	25%	U	99.6	11q22 deletion	+
80231	130.9	ND	M	ND	13q deletion	-
90921	131.4	43%	M	96.34	No *TP53* mutations	ND
91071	142.1	73%	U	99.6	No *TP53* mutations	ND
80138	168.5	ND	M	93.55	11q deletion	ND
80197	169.9	ND	U	ND	Normal	+
8000537	192.3	90%	M	86	13q14 deletion	-
80194	204.1	ND	M	ND	13q deletion	+
80254	222.8	81%	U	100	13q14 deletion	-
80139	236.4	ND	U	99.6	13q and 17p deletions	ND
80047	251.9	25%	M	95.53	Chromosome 12 trisomy	ND
70447	262.4	62%	M	93	No *TP53* mutations	ND
90533	278.2	ND	ND	ND	ND	ND
70448	292.7	ND	M	87.9	No *TP53* mutations	ND
90923	329.1	39%	ND	ND	Normal	+
90025	395.1	79%	M	93.63	13q14 deletion	-
80232	397.5	ND	M	ND	13q deletion	ND
80102	417.2	80%	ND	ND	11q and 13q deletions. IGH+ rearrangement	-
80048	476	34%	M	90.65	Normal	-
90393	556	56%	M	90.76	No *TP53* mutations	-
90904	705.5	33.13%	M	91.27	No *TP53* mutations	ND
90905	705.5	ND	M	96.73	No *TP53* mutations	ND
80091	740.6	ND	U	99.6	17p deletion	ND
90903	753.1	ND	ND	ND	No *TP53* mutations	ND
80113	24,191	70%	ND	ND	No *TP53* mutations	-

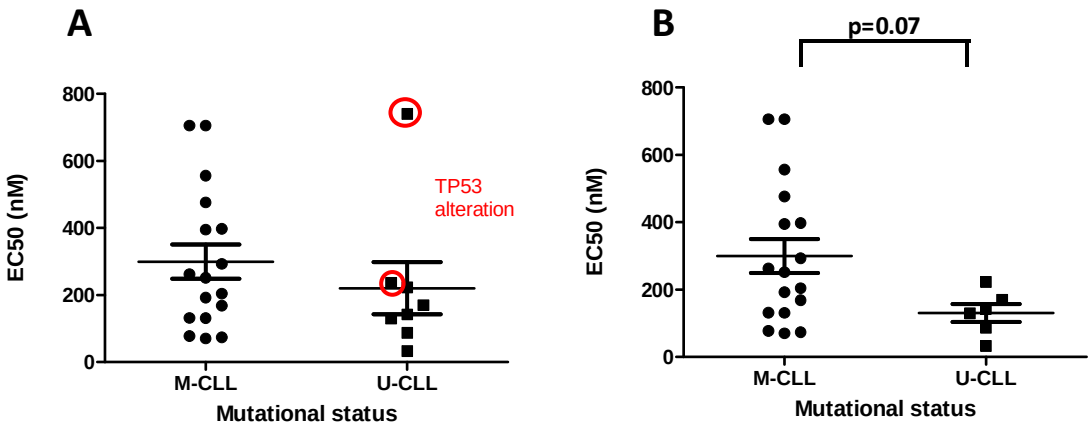


Figure 15. TW-37 sensitivity is associated with mutational status of PBMCs CLL samples. A) Sensitivity to TW-37 in U-CLL samples versus M-CLL (including all samples with cytogenetic data available). No significant difference was observed. B) Samples with alterations affecting *TP53* were excluded from the analysis. A tendency (t-test, p: 0.07) of U-CLL samples to present lower values of EC50, being more sensitive to the drug, was observed.

Table 15. Series of purified B cell samples from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. CD19 a. Purif, indicates CD19 purity of samples after B cell selection. No *TP53* mutations indicates that there were no mutations in exons 5, 6, 7 or 8, but no FISH data are available. MR, gene expression microarray (8 x 15K); W, Western Blot (basal protein levels); A, apoptosis time course; C, cell cycle time course.

Sample	TW-37 EC50 (nM)	CD19	CD19 a. Purif	SHM	% IGHV homology	FISH/p53mut	ZAP70	Assays
91066	310.5	60%	99.8%	ND	ND	13q14 deletion	-	MR, W
100018	381.8	59.3%		U	99.18	11q deletion	+	MR, W, A
90784	459.4	ND	95.2%	M	96.75	No *TP53* mutations	-	-
91103	495.6	96%	97.7%	M	92.77	13q14 deletion	-	MR, W, A, C
90994	544.1	35%	99.7%	M	ND	Normal	-	MR
90906	650	90%	99.8%	U	99.6	IGH+ rearrangement+	+	MR, W, A
91072	1,496	58.5%	97%	M	93.95	17p deletion	ND	MR, W
100008	2,422	ND	99.7%	M	89.16	No *TP53* mutations	ND	MR, W, A
90909	2,489	94%	98.4%	ND	0	c-MYC trisomy, 13q deletion	+	MR, W, A
100004	2,846	ND	99.5%	ND	ND	No *TP53* mutations	-	W, A
91207	8,620	82%	97%	U	99.59	No *TP53* mutations	-	MR, W, C
90907	9,741	80%	99.5%	M	94.3	13q14 deletion	-	MR, W
90908	9,760	90%	99.5%	M	96.4	13q14 deletion	-	MR, W

These results suggested that samples from patients with unmutated *IGVH* genes (with a more aggressive clinical course) are the most sensitive to the inhibitor TW-37 and that the sensitivity to the drug was p53 dependent (Figure 15).

These results prompted us to expand our study. Therefore, we evaluated cell viability in a series of 14 purified B cell samples (see Table 15). However, differences in EC50 values between U-CLL and M-CLL samples could not be confirmed (data not shown), probably due to the small size of the series (only 9 samples had available *IGHV* mutational status data).

4.5.2 TW-37 induces apoptosis in CLL cells and has no effect on cell cycle

TW-37 has been shown to induce apoptosis mediated by caspases 3 and 9 (Mohammad et al., 2007) and to mediate S-phase cell cycle arrest (Ashimori et al., 2009). Therefore, we decided to study both cellular mechanisms in our purified CLL samples. Apoptosis was measured by Annexin V/PI staining. Cells were defreezed, plated at a concentration of 10^6/mL in 24-well plates and allowed to stabilize for 3 hours before addition of the drug. Considering previous EC50 data, we decided to monitor apoptosis at 24h, 48h and 72h using four different drug concentrations (100 nM, 500 nM, 1 µM and 10 µM), except for samples 90906 and 90909 that were treated only with 100 nM, 500 nM and 1 µM concentrations due to sample limitations. As shown in Figure 16A, those samples with EC50 below the median of the series (1496 nM) and therefore considered sensitive (90906, 91103 and 100018) presented an increase in apoptosis that was time and dose dependent. However, those samples with higher EC50 values (90909, 100004 and 100008) presented no apoptosis (samples 90909 and 100008) or a limited apoptosis induction reached only at higher doses and longer time exposure (sample 100004).

Cell cycle analysis was performed in parallel, using the same drug concentrations (100 nM, 500 nM, 1 µM and 10 µM). As expected, CLL cells in basal conditions were arrested in G0/G1 phase and showed a striking reduced S-phase. Upon TW-37 treatment, no remarkable effects on cell cycle were observed. In figure 16B, control (DMSO) and the drug concentration corresponding approximately to the median EC50 of the series are shown. Reduction of G0/G1 phase in the sensitive sample (91103) at 72 hours was the consequence of the increase in sub G0 phase, which represents an increase in cell death. Therefore, we concluded that TW-37 exerts its antitumor activity by apoptosis induction.

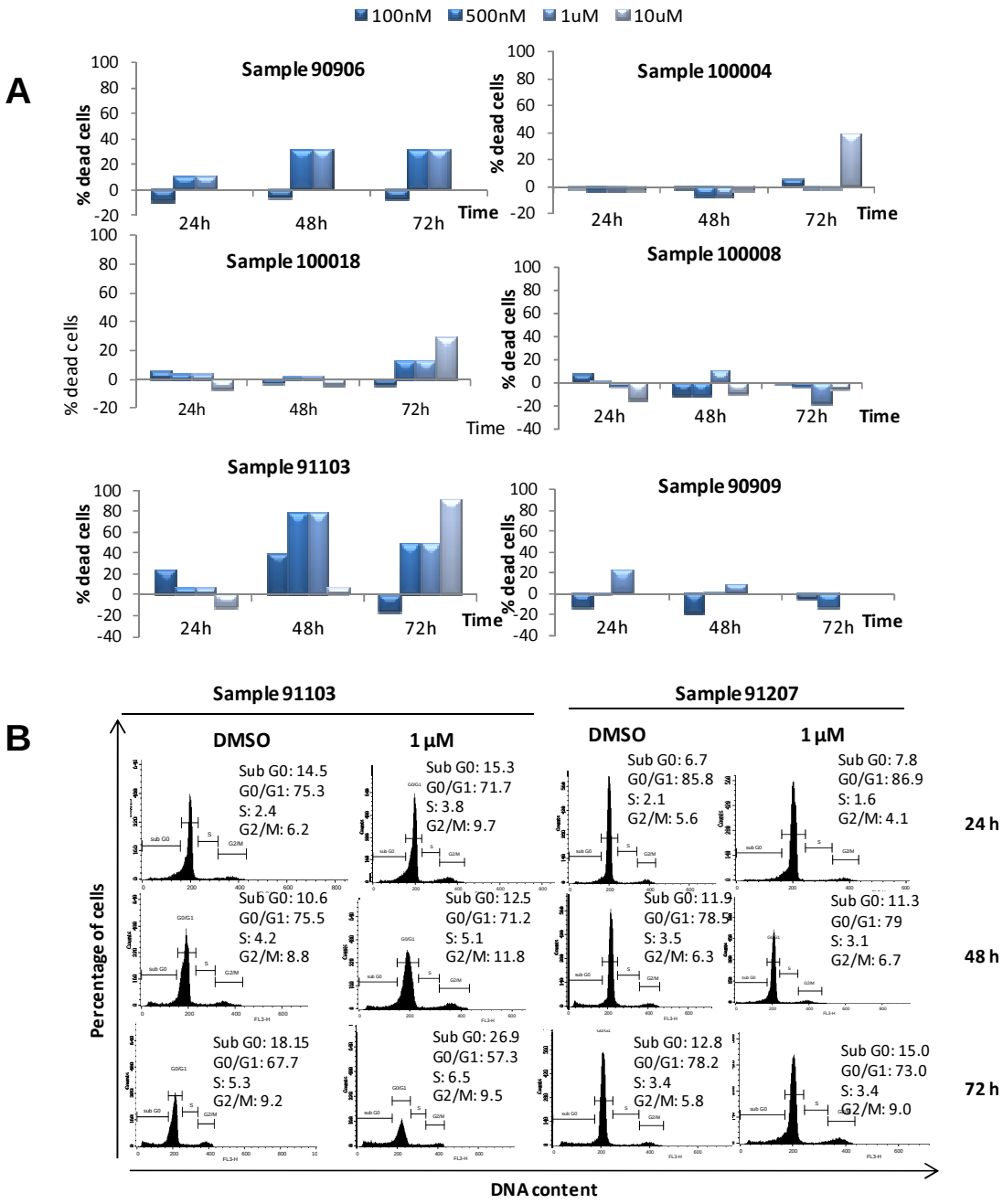


Figure 16. Effect of TW-37 in apoptosis and cell cycle.

A) Apoptosis time course with different doses and time points. All values were normalized with DMSO (see Material and Methods). Left panel shows more sensitive samples while right panel shows samples with very high EC50 in which apoptosis is not induced or induced at high concentrations.

B) Cell cycle analysis of a sensitive (91103) and a resistant (91207) sample.

4.5.3 TW-37 candidate biomarkers

TW-37 has been described to bind to Bcl-2, Bcl-x_L but also to Mcl-1 (which distinguishes this small molecule inhibitor from other members of this drug family). This binding blocks their heterodimerization with the propapoptotic members of the Bcl-2 family and expands the pool of proapototic effectors allowing induction of apoptosis.

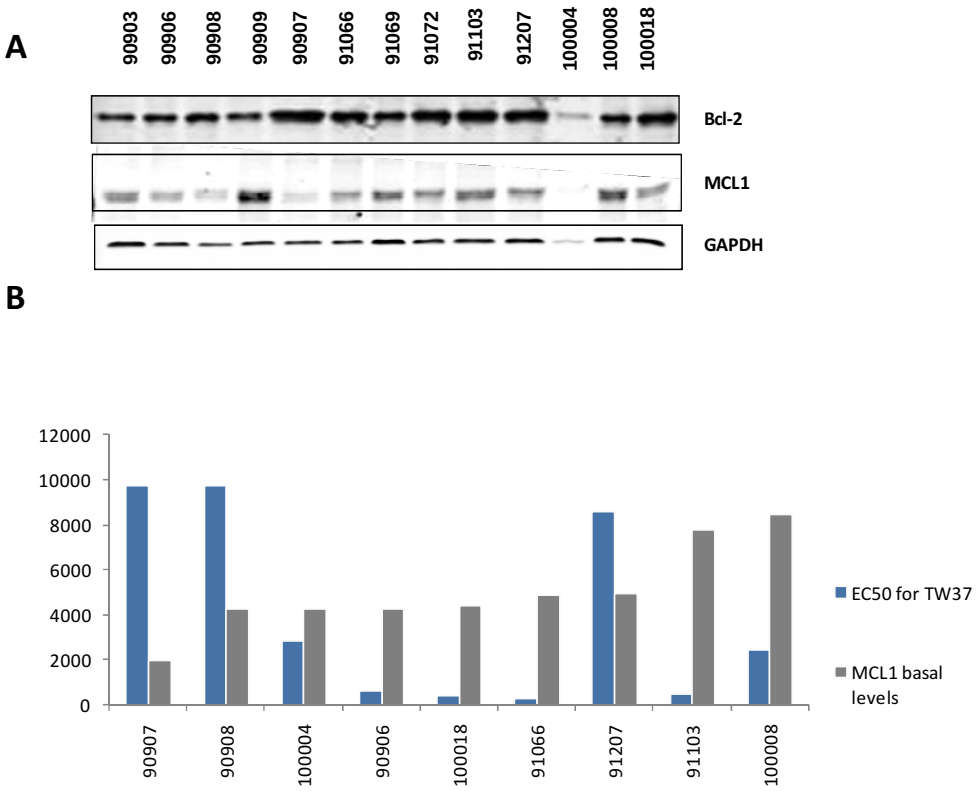


Figure 17. Inverse correlation between Mcl-1 protein levels and response to TW-37. A) Mcl-1 and Bcl-2 basal levels measured by WB in CLL samples. B) Correlation between EC50 values (nM) and Mcl-1 levels (represented as arbitrary units) normalized with GAPDH levels.

Bcl-2 levels were very high in all CLL samples studied (Figure 17A) and could not explain why some samples were more resistant, requiring doses up to 9 µM to reach the 50% reduction in cell viability. The use of TW-37 was especially interesting in our series because it also inhibits Mcl-1 binding. Since patients with higher levels of Mcl-1 present a worse clinical course (Pepper et al., 2008) this compound could potentially be useful in the context of CLL. Considering this, we hypothesized that Mcl-1 levels could explain TW-37 response. We performed Western Blot of 11 purified B-CLL samples and quantified basal Mcl-1 levels normalizing with GAPDH levels (Figure 17B). As patients with mutations and/or deletions in

TP53 show decreased survival and clinical resistance to chemotherapeutic treatment (Dohner et al., 1995) and our previous data suggested that TW-37 acts on a p53 dependent manner, we excluded those samples of further analysis. A Pearson correlation between EC50 values in our TW-37 and basal Mcl-1 protein levels in B-CLL samples showed a coefficient of -0.5, indicating that samples with higher Mcl-1 levels showed lower EC50 and therefore were the most sensitive ones (Figure 17A).

4.5.4 TW-37 gene signature for resistant B-CLL samples

Furthermore, we were interested in studying if molecular differences in gene expression could define sensitive (6 samples) and resistant samples (5 samples). The two groups of samples were defined by the median EC50 of the whole B-CLL series (1,496nM).

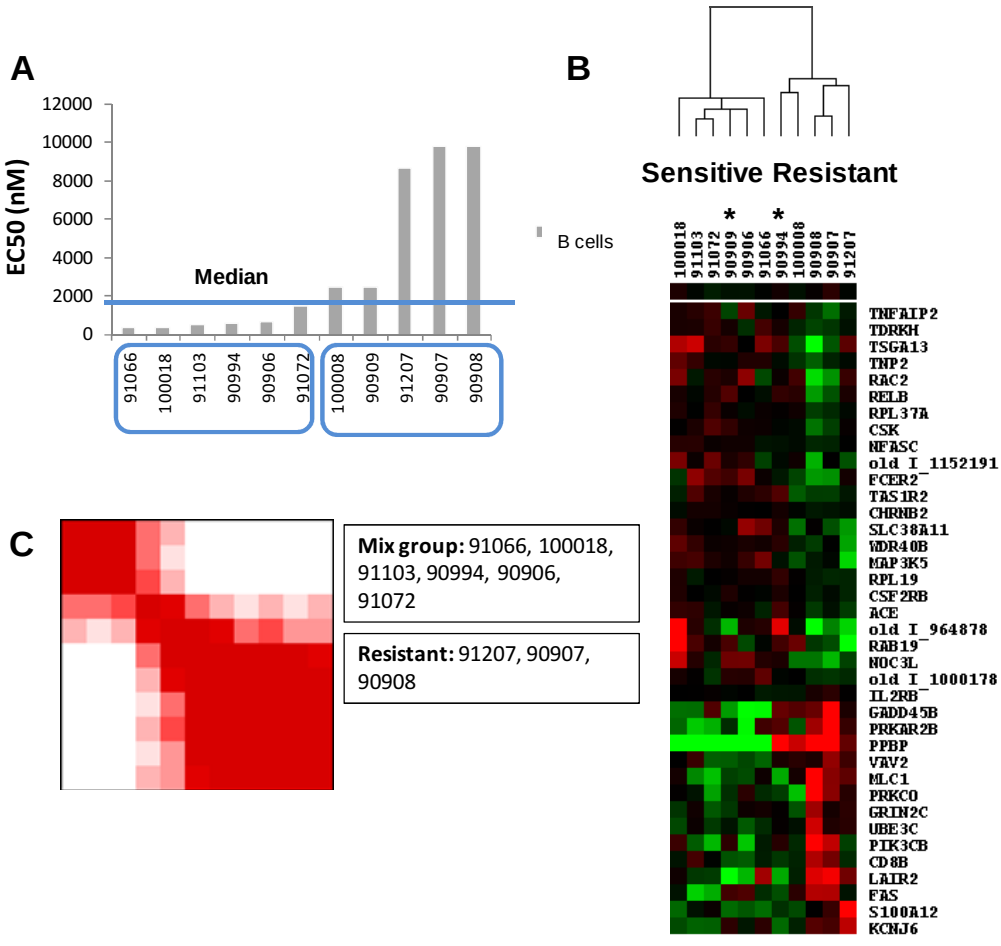


Figure 18. Analysis of gene expression profile of TW-37 sensitive and resistant CLL samples. A) Classification of samples in sensitive or resistant according to the median EC50 of the series (1,496 nM). B) Hierarchical cluster of the samples using the set of 38 genes obtained as described in the text. Asterisks indicate samples that do not belong to the group, as it had been defined in A). C) Consensus clustering using the same 38-gene signature.

Samples presenting EC50 values below the median were considered to be sensitive while samples with EC50 values above the median were considered to be resistant (Figure 18A).

We correlated patterns of expression of every gene among the different samples with EC50 values and obtained 38 statistically significant ($p<0.05$) genes with a Pearson coefficient higher that 0.5 or lower than -0.5. These genes were used to cluster our samples (CLUSTER, Stanford). We obtained two groups, corresponding to sensitive and resistant samples (Figure 18B). However, some of the samples showing intermediate EC50 were not classified as expected. Analysis using consensus Clustering (Gene Pattern) confirmed these results, showing two defined groups (Figure 18C). The first included the most resistant samples (90907, 90908 and 91207) while the second was made up of a mixture of sensitive samples and samples with intermediate sensitivity to the drug. This analysis revealed that the threshold used to define sensitivity to TW-37 was not the most appropriate. In fact, the mean value of EC50 (3,093 nM) could better stratify samples into sensitive and resistant ones. Genes highly expressed in the resistant samples included *GADD45B, PRKAK2B, PP3P, MLC1, PRKCO, GRIN 2C, PIK3CB, CD8 B, LAIR 2, FAS AND KCNJ6.* On the other hand, some genes such as *TNFIAP2, TDRKH, TNP2, RAC2, REL B, CSK, NFASC, FCER 2, TAS1R2, SLC 3811, MDR4, MAP3K5, RAB19, NOC3L and IL2RB* were less expressed in resistant samples compared to sensitive ones. Taken all together, these data show that TW-37-resistant samples can be defined by a 38-gene specific signature.

4.6 Study of sensitivity to ETP-39010 (PIM kinases inhibitor) in primary CLL cells

4.6.1 ETP-39010 effect on cell viability in primary CLL cells

As previously shown, heterogeneity in the PIM gene expression levels has been observed in CLL samples. Accordingly, variability in drug sensitivity was also evident when we used the PIM inhibitor ETP-39010 in 15 PBMCs CLL samples, ranging from less than 1 µM to 13.6 µM (Table 16). As patients with unmutated IGHV show a worse clinical course, we were interested in investigating differences between M-CLL and U-CLL; however, no statistically significant difference was observed.

Due to the background provided by non-tumor cells, we decided to expand our study including 16 CLL samples of purified B cells (Table 17). The calculated EC50 values ranged from 1,276 nM

to 22,648 nM, showing a slight increase in absolute values compared with our previous PBMCs series but revealing also wider variability (up to twenty times).

Table 16. Series of PBMC from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. CD19 a. No *TP53* mutations indicates that there were no mutations in exons 5, 6, 7 or 8, but no FISH data are available.

Sample code	ETP-39010 EC50 (nM)	CD19	SHM	% IGHV homology	FISH/*TP53mut*	ZAP70
90903	765.2	ND	ND	ND	No *TP53* mutations	ND
80138	1,120	ND	M	93.55	11q deletion	ND
70446	1,503	73%	U	100	ND	ND
90978	1,536	ND	M	96.02	No *TP53* mutations	ND
91071	1,734	73%	U	99.6	No *TP53* mutations	ND
80002	1,828	66.9%	M	94.74	13q14 deletion	-
90082	2,790	90%	M	93.17	No *TP53* mutations	-
90025	2,794	79%	M	93.63	13q14 deletion	-
90923	2,911	39%	ND	ND	Normal	+
90921	3,076	43%	M	96.34	No *TP53* mutations	ND
80091	3,348	ND	U	99.6	17p deletion	ND
80139	4,404	ND	U	99.6	13q and 17p deletions	ND
80197	6,484	ND	U	ND	Normal	+
90905	8,375	ND	M	96.73	No *TP53* mutations	ND
90904	13,607	33.1%	M	91.27	No *TP53* mutations	ND

When we studied whether the differences in sensitivity correlated with prognostic parameters, we observed that samples from U-CLL showed a tendency to be more sensitive to ETP-39010 (p value 0.09). Moreover, ZAP70 positive samples (also a bad prognosis parameter, related with the absence of somatic IgVH mutations), were also more sensitive to ETP-39010 inhibitor (p value < 0.05) (Figure 19).

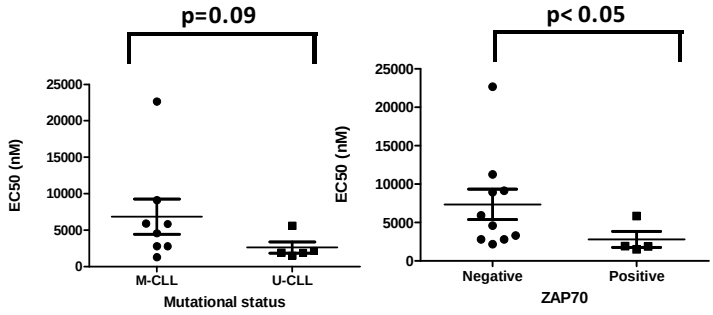


Figure 19. Sensitivity to ETP-39010 and prognostic parameters. U-CLL and ZAP70 positive samples are more sensitive to the PIM inhibitor. For this analysis a series of purified B CLL samples and PBMC samples containing more than 80% tumor cells was used. Mann-Whitney t-test was applied.

Table 17. Series of purified B cell samples from CLL patients. EC50 values and clinical parameters are shown. ND indicates no available data. M, indicates IGHV with <98% homology with germinal gene; U, indicates IGHV with >98% homology with germinal gene. CD19 a. Purif, indicates CD19 purity of samples after B cell selection. No *TP53* mutations indicates that there were not mutations in exons 5, 6, 7 or 8, but no FISH data are available. MR, gene expression microarray (8 x 15K); M_{44}, gene expression microarray (4 x 44K);W, Western Blot (basal levels); A, apoptosis time course; C, cell cycle time course.

Sample code	ETP-39010 EC50 (nM)	CD19	CD19 a. Purif	SHM	% IGHV homology	FISH/*TP53* mut	ZAP70	Assays
100008	1,276	ND	99.7%	M	89.16	No *TP53* mutations	ND	MR, $M_{44,}$ W
100018	1,484	59%	-	U	99.18	11q deletion	+	MR, $M_{44,}$ W, A
90906	1,890	90%	99.8%	U	99.6	IGH+ rearrangement	+	MR, W, A
91069	1,931	93%	96.3%	U	99.18	Chromosome 12 trisomy	+	MR, W
91207	2,151	82%	97%	U	99.59	No *TP53* mutations	-	MR, $M_{44,}$ W, A, C
100004	3,304	ND	99.5%	ND	ND	No *TP53* mutations	-	$M_{44,}$W, A
90908	4,583	90%	99.5%	M	96.4	13q14 deletion	-	MR, W
100021	5,585	ND	99%	U	99.19	No *TP53* mutations	ND	MR, M_{44}
90909	5,605	94%	98.4%	ND	ND	c-MYC trisomy, 13q deletion	+	MR, W, A
100030	5,854	ND	-	M	93.83	No *TP53* mutations	+	-
91103	5,896	96%	97.7%	M	92.77	13q14 deletion	-	MR, W, A, C
91072	6,400	58.5%	97%	M	93.95	17p deletion	ND	MR, $M_{44,}$ W
91066	8,926	60%	99.8%	ND	ND	13q14 deletion	-	MR, W
90784	9,108	ND	95.2%	M	96.75	No *TP53* mutations	-	-
90994	11,245	35%	99.7%	M	ND	Normal	-	MR
90907	22,648	80%	99.5%	M	94.3	13q14 deletion	-	MR, W

4.6.2 ETP-39010 induces apoptosis and has no effect on cell cycle

Other PIM inhibitors have been recently tested in *in vitro* studies using primary CLL samples. It is well known that tumor CLL cells found in the blood are arrested in G0/G1 phase and therefore, the effect of the drug is probably not exerted via cell cycle regulation. Recent

studies using another inhibitor of PIM kinases have demonstrated that inhibition of these kinases can induce apoptosis and decrease new RNA synthesis (Chen et al., 2009). In order to further investigate whether ETP-39010 works in a similar manner, we have assessed apoptosis by Annexin V/ PI in a panel of purified B-CLL samples.

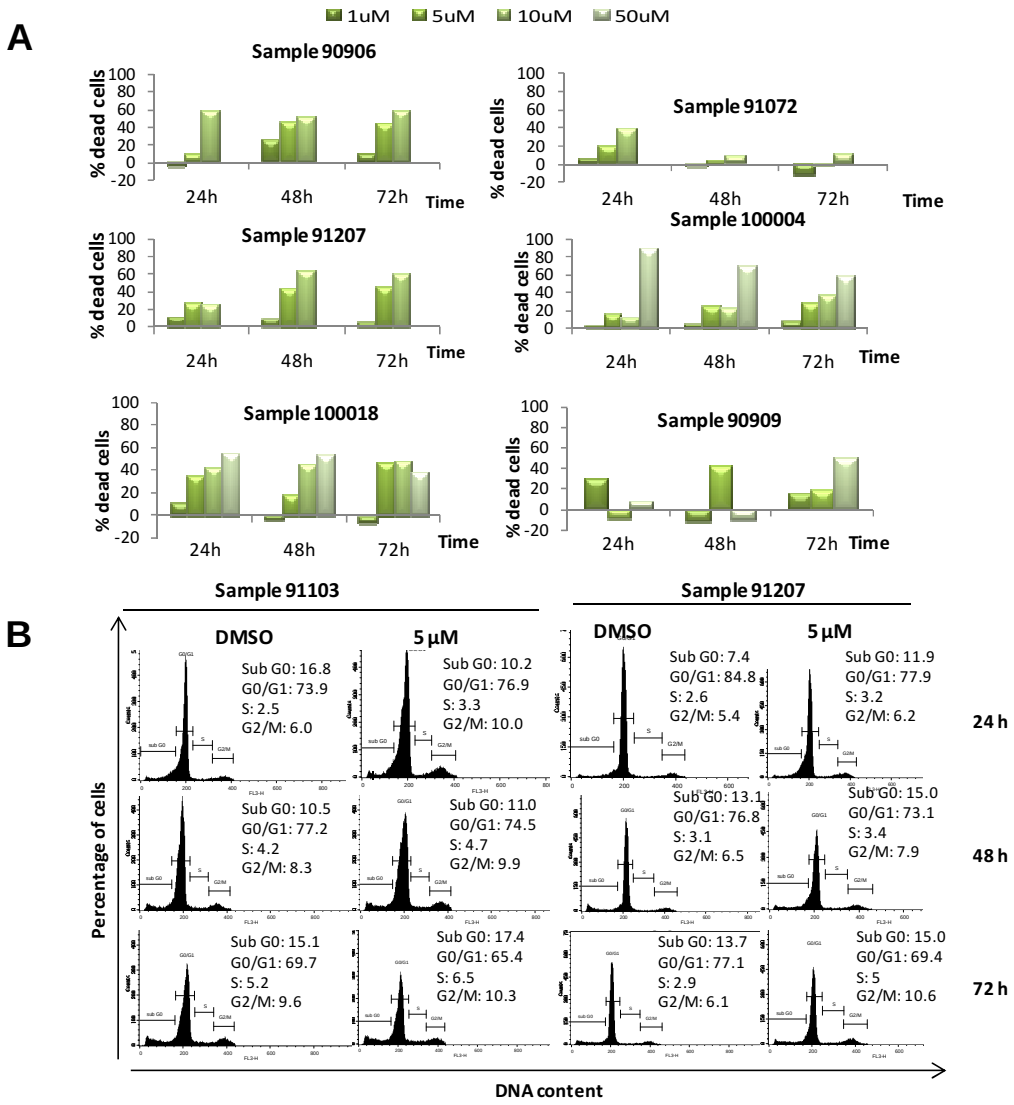


Figure 20. Effect of ETP-39010 on apoptosis and cell cycle. A) Apoptosis time course with different doses and time points. B) Cell cycle analysis of a sensitive (91207) and a resistant (91103) sample.

A time course assay was designed including different drug concentrations (1 µM, 5 µM, 10 µM and 50 µM) and three time points (24h, 48h and 72h). Two samples (90906 and 91207) were treated only with 1 µM, 5 µM and 10 µM concentrations due to sample limitations. As shown in Figure 20A, an increase in apoptosis that was time and dose dependent was observed in all

assayed samples, except in 90909 and 91072 samples (that showed EC50 values higher than the median EC50 of the series). This confirmed that inhibition of PIM kinases is able to induce apoptosis in B-CLL samples.

On the other hand, we analysed the cell cycle in two CLL samples at 1 µM, 5µM, 10 µM and 50 µM concentrations and 24, 48 and 72 hours. Figure 20B shows representative histograms of control and CLL samples treated with 5 µM concentration of ETP-39010 (median EC50 of the series). As primary CLL samples are already arrested in G0/G1 phase, we did not observe any consistent effect of ETP-39010 on cell cycle.

4.6.3 PIM2 levels positively correlate with sensitivity to ETP-39010

PIM2 is the member of the family with the highest expression levels in CLL samples (see Figure 8)and therefore, we wondered whether basal PIM2 protein levels could explain drug sensitivity. We performed Western Blot analysis, quantified PIM2 protein levels and studied a possible relationship with EC50 values, excluding again those samples with alterations in *TP53* (Figure 21A).

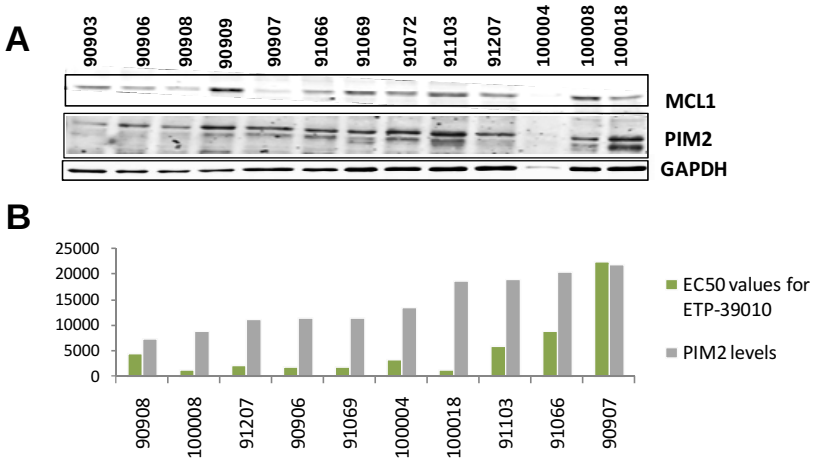


Figure 21. Direct correlation between PIM2 protein levels and response to TW-37. A) Mcl-1 and PIM2 basal levels measured by WB in B-CLL samples. B) Correlation between EC50 values (nM) and PIM2 levels (represented as arbitrary units) normalized with GAPDH levels.

A positive correlation (Pearson coefficient 0.7) between both was found, indicating that higher levels of drug might be necessary to inhibit the pathway in those cells expressing higher PIM2 protein levels (Figure 21B). When the whole series (Figure 21A) was considered, samples with higher PIM2 levels seemed to have also higher Mcl-1 levels.

4.6.4 Gene signature of sensitive and resistant B-CLL samples.

In order to study the gene expression signature of the ETP-39010-sensitive (EC50 < Median EC50) and ETP-39010-resistant CLL samples (EC50 > Median EC50), we studied the correlation between gene expression patterns and EC50 values. We obtained 55 genes with statistically significant ($p<0.05$) Pearson values (higher than 0.5 or lower than -0.5).

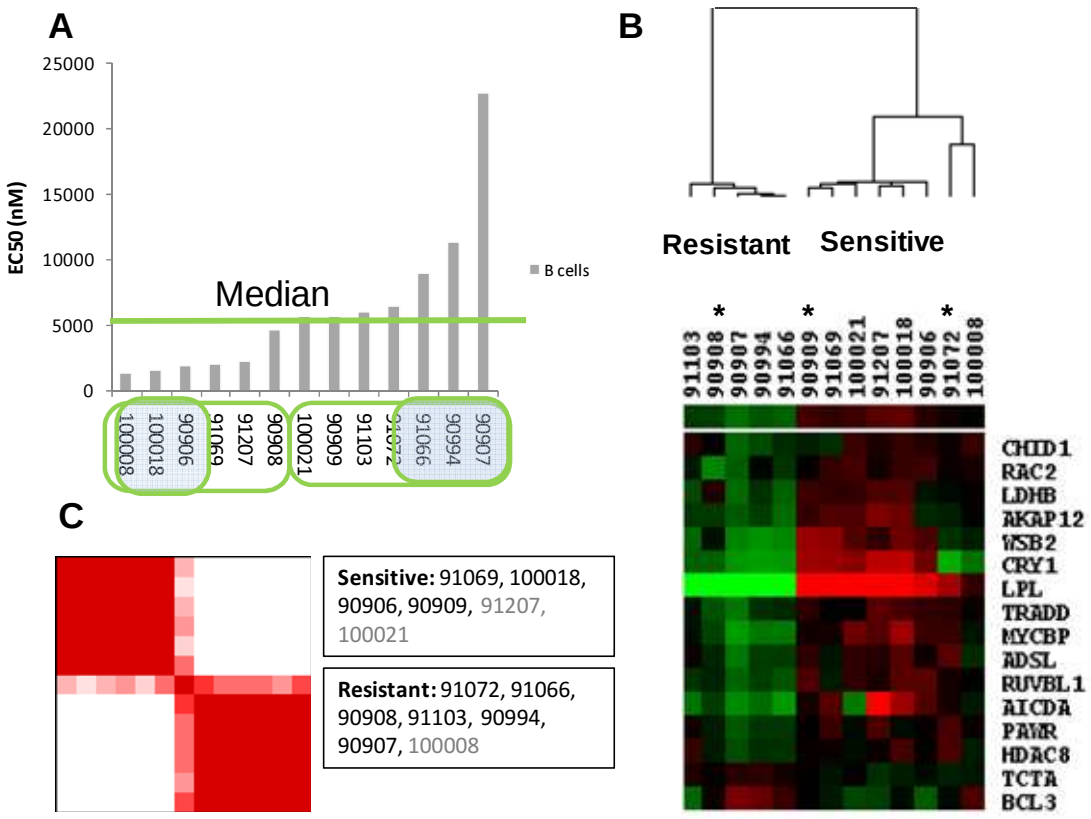


Figure 22. Analysis of gene expression profile of ETP-39010 sensitive and resistant B-CLL samples. A) Classification of samples in sensitive or resistant according to the median EC50 of the series (5585 nM). B) Hierarchical cluster of the samples using the set of 15 genes obtained as described in the text. Asterisks indicate samples that do not belong to the group, as it had been defined in A). C) Consensus clustering using the same 15-gene signature. Samples in grey were not considered to be in the expected group, according to the definition set in A).

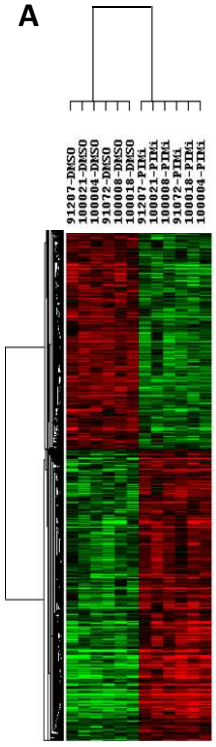
Then, we performed t-test (Limma, Asterias) of these genes between the three most sensitive and the three most resistant samples (in blue in Figure 22A) and 15 of them were found to be differentially expressed (FDR_ind < 0.05). This set of genes was able to distinguish sensitive and resistant samples, with some exceptions that corresponded to samples with EC50 values around the median (Figure 22B). These genes were: *LPL, WSB2, MYCBP, CRY1, LDHB, PAWR,*

RAC2, RUVBL1, TCTA, TRADD, AICDA, AKAP12, CHID1, BCL3, HDAC8 and *ADSL*. Consensus clustering verified the existence of two groups including mainly sensitive or resistant samples, but again showing some exceptions (Figure 22C).

4.6.5 ETP-39010 mechanism of action in B-CLL cells

The physiological function of PIM kinases is still under study. It has been shown that they play an important role in cell growth and survival, as well as in maintenance of transformed phenotypes (Brault et al., 2010). Nevertheless, the upstream events leading to their activation are not so well established and neither are the downstream targets of this family of kinases.

A



B

Pathway	Percentage	Genes	
Regulation of transcription, DNA.dependent	21.2	*MYBBP1A, TBX3, UBP1, MCM7, FOXD4, CDCA7, TFB2M, FOXE3, FOXJ3, FOXM1, GATA1TBX19*	Up
Transcription from RNA polymerase II promoter	10.1	*GATA1, BRD8, UBP1, FOXE3, TBX3, TBX19, ELF3, POLR2B, DDX20*	
Apoptosis	8.7	*CLU, CLDN15, EI24, TNFRSF21, DDX20, STK17A, FADD, LRDD, DFFA, CCAR1*	
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	36.45	*NAP1L1, PARP15, GATAD1 , BRD7, PARN, EXOSC1, MYCBP2, IMP4, TARBP1, JMJD2C, RAD18, ATP5G1, INTS7, POLR3D, SNAPC5, PHB, ZBTB26, FOXK2, POP4, NDUFA10, ADSL, TFB1M SFRS7 EXOSC3 RCOR3*	Down
Protein metabolic process	30.84	*PFTK1 , KCTD7, AP2A2, PDK3, HPRT1, PIGP, FBXL20, LAP3, IDE, HDAC3, EPRS, MTIF3, MAPKAPK3, TLR6, TRIAD3, CDC16, STAMBP, JAK3, DNAJC19, DNAJC16, UQCRC2, VRK3, LCMT1, STK10, SCYL3, VPS16, NCSTN, CCT7, DARS2, PTEN, PARP15, TRIM5*	
Transport	18.69	*KHDRBS1, NUP155, COPE, PCM1, CHMP4A, CLTB, RUFY1, DYNC1I2, SEH1L, SNUPN, ACTR1A, NFKBIE, VPS4B, BSPRY, GHRL, CBLL1, SNX1, KCTD7, AP2A2, ABCD2, ATP11C, SFT2D2, ATP5D, SLC33A1, SEC23IP, DNAJC19, UQCRC2, VPS16, SNX14, FXC1, KCNAB3, RER1, SNX2, ATP5G1, VTI1B, ABCB4 SLC30A6, TCIRG1, ELMO1, VPS29, ATP5S, AQP11, NUPL2, SFXN2*	
Signal transduction	12.46	*NPY5R, SH3BP1, PRKAA1, IL9R, FIBP, IFNAR1, RAB28, ARL10, MLLT7, KHDRBS1, BTLA, FGD3, GUCY2C, MICAL1, IL18BP, GHRL, RHOQ, LTB, IDE, RFXANK, SPRY3, MAPKAPK3, TLR6, STAMBP, JAK3, NCSTN, INVS, SNX14, PTEN, BRD7, ELMO1, TLR1, RIPK1, CD19, PHB, CSNK1A1L, BTRC*	
Cellular biosynthetic process	10.59	*MTIF3, TLR6, AGPAT1, ASL, MAT2A, DARS2, ATP5G1, GALNT7, TLR1, ADSL, ZDHHC2, ADK, MTR, TMLHE, ATP5G2, MRPL30, RABGGTA, EIF3S1, EIF3S12, PFKFB1, PRKAA1, IFNAR1, MTIF2, GUCY2C, NCOA5, COQ7, HPRT1*	
Cell cycle process	5.3	*HDAC3, NBN, RAD9B, CDC16, CCT7, PTEN, PPP6C, POLR3D, SUPT3H, PCAF, RBM5, CDC27, MLLT7, CLASP2, KHDRBS1, RBL1*	

Figure 23. Gene expression profile induced by ETP-39010 treatment. A) Mcl-1 gene expression levels in treated and control cells. B) 1420 genes were differentially expressed between not treated and treated samples. C) Gene Ontology functions in which these genes were grouped.

We were interested in revealing the mechanism of action of our ETP-39010 in the context of CLL, considering that PIM kinases activity seems to be tissue dependent.

For that, 12 CLL samples were cultured at a 10^6/mL concentration and treated with a 5 µM concentration of the ETP-39010 compound (which was approximately the median of the whole series) for 8 hours. This early time point was chosen in order to investigate the direct targets

altered upon inhibition of PIM kinases. Controls were treated with the corresponding concentration of vehicle (DMSO). At the end, RNA from 6 pairs of samples (DMSO and ETP-39010 treated) reached the high quality criteria for microarray hybridization (Agilent, 44K) and were further analysed. Around 28,000 genes were left after normalization and preprocessing of the data. We then assessed comparison of controls and ETP-39010 treated samples using the t-test implemented in the T-REX bioinformatic tool (GEPAS). A list of 1,420 genes was differentially expressed ($p<0.01$), being 819 overexpressed and 601 downregulated upon ETP-39010 treatment (Figure 23A). These genes were submitted to FatiGo (Babelomics) for a description of gene function and the results are shown in figure 23B.

Pathway	Size	Genes	
Complement and coagulation cascades	63	*KLK3, F3, A2M, SERPINF2, C1QB, PROC, FGB, C4BPB, CFI, FGG, PLAT, F2, KNG1, C3, C4BPA, TFPI, CFH, SERPINE1, THBD, SERPING1, SERPINA5, PLAUR, PLAU, F2R, CPB2, BDKRB1, F5, FGA, BL2, SERPINA1, C1QC, F7, C1R, C4B, F10, MASP1, SERPIND1, CD59, C8B, CFB, C2, KLKB1, SERPINC1, C1S*	Up
ECM receptro interaction	75	*ITGA1, COL2A1, VWF, SDC2, THBS1, DAG1, ITGA8, ITGA2, LAMB1, ITGA6, FN1, COL5A1, COL4A2, COL5A3, LAMA1, LAMA4, TNN, SV2B, ITGA11, LAMC2, COL1A2, ITGB3, LAMC2, SDC4, TNC, SDC3, IBSP, VTN, ITGA3, COL6A2, SDC1, LAMB2, ITGA7, GP1BB, HSPG2, SPP1, LAMB3, RELN, COL6A1, ITGA2B, COL11A1, COL11A2, TNXB, COL5Q2, FNDC4, ITGB4, COL1A1, ITGA9, SV2A, THBS4, THBS3*	
Oxidative-phosphorylation	106	*NDUFB2, UQRC2, TCIRG1, NDUFA10, ATP5D, LHPP, ATP5G2, ATP5G1, NDUFB1, NDUFB5, NDUFS7, NDUFA3, NDUFB3, COX17, UQCRC1, NDUFB8, ATP5E, ATP5I, NDUFA13, NDUFS2, SDUFA1, ATP5J2, NDUFA11, NDUFS1, NDUFB10, NDUFA2, NDUFA9, ATP5A1, UQCRQ, COX7C*	Down
Pyruvate metabolism	37	*ME2, PDHB, ACYP1, PDHA1, DLD, ALDH9A1, ACSS2, ACACB, ACAT2*	
Aminosugars metabolism	27	*NAGK, RENBP, LHPP, CYB5R1, MTMR1, NPL, HEXA, AMDHD2, GNPDA1*	
Propanoate metabolism	30	*ACADM, PCCB, MLYCD, ECHS1, ALDH9A1, SUCLG2, HIBCH, ACSS2, PCCA, ACACB, ACAT2*	
Valine, Leucine and Isoleucine degradation	43	*ACADM, PCCB, ACAA1, DLD, ECHS1, OXCT1, HADHSC, HADHB, ALDH9A1, BCKDHB, HIBCH, BCAT2, PCCA, MCCC1, ACAT2*	
Aminoacyl-tRNA biosynthesis	35	*EPRS, DARS2, LARS, YARS2, MARS2, GARS, TARSL1, AARS, VARS, IARS, AQRS, CARS, HARS, TRAS, MTFMT*	
Toll-like receptor signaling	93	*IFNAR1, TLR1, TLR6, LY96, RIPK1, IRAK4, MAPK13, CCL3, CCL4, MAP3K7, TRAF6, PIK3CG, STAT1, TIRAP, TLR2, TLR7, TLR9, MAPK1, MAP2K2, TNF, MAP2K6, IFNA10, MAP2K7, IKBKE, LBP*	
Fatty acid metabolism	42	*ACOX3, ACSL5, ACADM, ACAA1, ECHS1, HADHSC, HADHB, ACADSB*	

Figure 24. GSEA pathways related to chemical inhibition of PIM. Pathways significantly (FDR< 0.25) up or downregulated upon ETP-39010 treatment, indicating genes in which the pathway is enriched.

We were also interested in analyzing which were the changes induced by ETP-39010 in a pathway-related manner. We submitted our data to GSEA (including 28,346 genes) together with the gene sets obtained from KEGG data base (Figure 24). Among the pathways found to be downregulated after ETP-39010 treatment (FDR<0.25) there were several related with aminoacid, lipid and energetic metabolism as well as antigen processing and presentation. Interestingly also the proteasome and the ubiquitin mediated proteolysis pathways were

downregulated upon treatment. Significant upregulated pathways were complement and coagulation cascades and ECM-receptor interactions.

5. DISCUSSION

5.1 Proliferation centers in chronic lymphocytic leukemia: the niche where NF-κB activation takes place.

5.1.1 Proliferation centers in CLL

Tumor promoting signals coming from the microenvironment are known to play a very important role in a number of lymphoid malignancies (Herreros et al., 2008) and CLL is a paradigm of the dependence of tumor cells on external stimuli. PCs are a hallmark of CLL and cannot be found in any other lymphoid malignancy but in some autoimmune diseases (Corcione et al., 2005; Takemura et al., 2001), suggesting that these structures could be the place where (auto)antigen stimulation takes place. Previous studies of PCs had proposed that the immunophenotype of the large CLL cells seemed to mimic activated of B lymphocytes as shown by Ki67, HLA-DR, BIRC5 and MUM1/IRF4 expression, among other markers (Soma et al., 2006; Swerdlow et al., 1984). However, this is the first work that includes a large series of samples and markers, in an attempt to better characterize PCs.

The presence of numerous and prominent PCs has been related with atypical CLL cases, which have been associated with poor prognosis (Bonato et al., 1998). However, we did not find any relation between number or size of PCs and IGHV mutational status (also related to poor prognosis) in our series. One possible explanation for this could be that the samples analyzed had been submitted to the Molecular Pathology Program at the CNIO for diagnosis, being probably biased towards more severe or atypical cases. On the other hand, mutational status of IGHV was available only for a limited number of samples that were included in the TMA, and therefore, the size of the series was relatively small.

Proliferating large B cells (Ki67 positive) were observed mainly inside PCs where the complex microenvironment might induce proliferation and inhibit apoptosis (see next sections). PCs are also characterized by the expression of MUM1/IRF4, a transcriptional regulator that is induced by NF-κB and that plays a role in response to B cell signaling or CD40 engagement (Shaffer et al., 2009). Its expression in B cells of all PCs has been previously reported (Soma et al., 2006) and could again be an indication of BCR activation. Moreover, no differences were found in the expression of either the prognostic marker ZAP70 or SYK between large cells inside PCs and small cells surrounding PCs. Determination of BCR activated status in patient samples is a difficult issue that has not been solved yet due to the difficulties to detect phosphorylated proteins in paraffin embedded patient samples. Around the proliferating B cell population, an

accumulation of small B cells with more condensed chromatin that might represent the offspring of the proliferation compartment was observed, as it had been previously described (Swerdlow et al., 1984).

5.1.2 Microenvironment composition of CLL cells inside PCs

CLL cells undergo spontaneous apoptosis when cultured *in vitro,* highlighting the importance of the microenvironment for survival of leukemic cells. However, the exact composition of these interactions is not well known and the possibilities of studying it using *in vitro* models are limited. Furthermore, none of the *in vivo* CLL mouse models developed (Bichi et al., 2002; Planelles et al., 2004; Zapata et al., 2004) presents PCs in the lymphoid tissue or bone marrow and therefore, its functional relevance cannot be easily addressed. Interesting attempts to characterize the non-tumor populations that contribute to CLL survival have been carried out. Tsukada et al. described the existence of some stroma cells that could differentiate *in vitro* from the peripheral blood of CLL patients in the so called "nurse-like cells" (NLC) (Burger et al., 2000; Tsukada et al., 2002). These cells were able to provide survival signals mediated mainly by secretion of cytokines such as SDF1 (Burger et al., 2000), BAFF and APRIL (Nishio et al., 2005). Nevertheless, NLC present in the peripheral blood were functionally immature, as they had to differentiate *in vitro* in order to support CLL cells. This may indicate that NLC must exert their protective role after differentiation in a distinct compartment, as PCs found in lymphoid tissues or bone marrow could be.

Herein, we have defined two different stroma populations that were immunophenotypically distinct from the NLC and may represent the functional accompanying cell types of PCs *in vivo*. They did not show the typical immunophenotype of either follicular dendritic cells or other types of dendritic cells. The ADC population that expressed SDF1 could be responsible of the migration of circulating CLL cells into lymph nodes via CXCR4, its receptor that is expressed in leukemic cells (Burger et al., 1999). Once inside the proliferation centers, NF-κB activation may induce BAFF secretion that can favor survival of CLL cells. Surprisingly, no BAFF expression was observed in the stroma component, as it has been described for the NLC. On the other hand, we could speculate that STAT1-Ms could activate T cells found in the surroundings and play a role in the presentation of autoantigens to CLL cells. STAT1 translocation to the nucleus with its consequent activation can be induced upon cytokines such as VEGF, which binds VEGFR2 and activates JAK proteins and STAT transcription factors (Bartoli et al., 2000). Interestingly, VEGF was expressed by prolymphocytes in PCs of almost all cases analyzed. Therefore, it is

possible that not only stroma cells may have an impact on tumor cells, but also CLL cells could attract and activate macrophages and other accompanying cells in order to create a supportive environment.

Additionally, B cell-T cell interactions seem to be essential for CLL cells survival (Ghia et al., 2005). We demonstrated that T cells in close contact with tumor cells, expressed CD40L. Moreover, CD4 T cells found intermixed with CLL cells inside PCs expressed a variety of markers characteristic of different subpopulations of T cells, suggesting that the environment is able to modulate the plasticity of T cells. These T cells showed an activated phenotype according to the expression of CD25 and PD1.

5.1.3 Proliferation centers are the niche where NF-κB activation takes place

CLL cells from peripheral blood samples express abundant levels of nuclear NF-κB DNA-binding activity, often elevated compared to normal B cells (Furman et al., 2000; Romano et al., 1998).

Moreover, variable levels of the phosphorylated IκB inhibitor have been observed in involved lymph node samples of CLL patients (Rodriguez et al., 2004). This constitutive activation seems to be linked to environmental signaling such as CD40L stimulation (Cuni et al., 2004) or BCR engagement (Bernal et al., 2001). In fact, NF-κB enhances survival of leukemia cells and chemical inhibition of the pathway at different levels induces apoptosis (Hewamana et al., 2008b; Pickering et al., 2007) and may represent a therapeutic alternative, even for patients resistant to conventional treatments such as fludarabine (Lopez-Guerra et al., 2009).

However, the place and mechanisms that trigger NF-KB activation *in vivo* are not well understood. We hypothesized that interactions occurring inside PCs could lead to activation of NF-κB and enhance survival/proliferation. In fact, we observed nuclear localization of p50 (a well stablished marker for NF-κB activation) in CLL cells inside PCs but not in the other lymph node compartments. Other NF-κB family members found in the nucleus of CLL cells of PCs were p52 and Rel B. Although in most cell types and conditions p50 –p65 heterodimers have been described, NF-κB family members are promiscuous and other subunit complexes can participate in the regulation of cellular processes and tumorigenesis (Budunova et al., 1999). Recently, nuclear p65 expression has been reported in circulating CLL cells (Hewamana et al., 2009). In that report, a different method for NF-κB subunits detection was used and this could explain the differences with our study. As stimuli leading to NF-κB activation in PCs may not be present in peripheral blood, it is also possible that the NF-κB activation intensity or pattern

could change in circulating CLL cells, an issue that remains unknown but deserves further investigation. Mcl-1, and Survivin (both known NF-κB targets) have been shown to be upregulated upon BCR engagement (Bernal et al., 2001; Gobessi et al., 2009) and were found highly expressed in this pool of CLL cells inside PCs. Moreover, Mcl-1 has been shown to be an essential inhibitor of BCR mediated apoptosis (Ruiz-Vela et al., 2008). All these data confirm the idea that antigen stimulation can lead to NF-κB activation and enhanced survival inside a specific microenvironment as PCs.

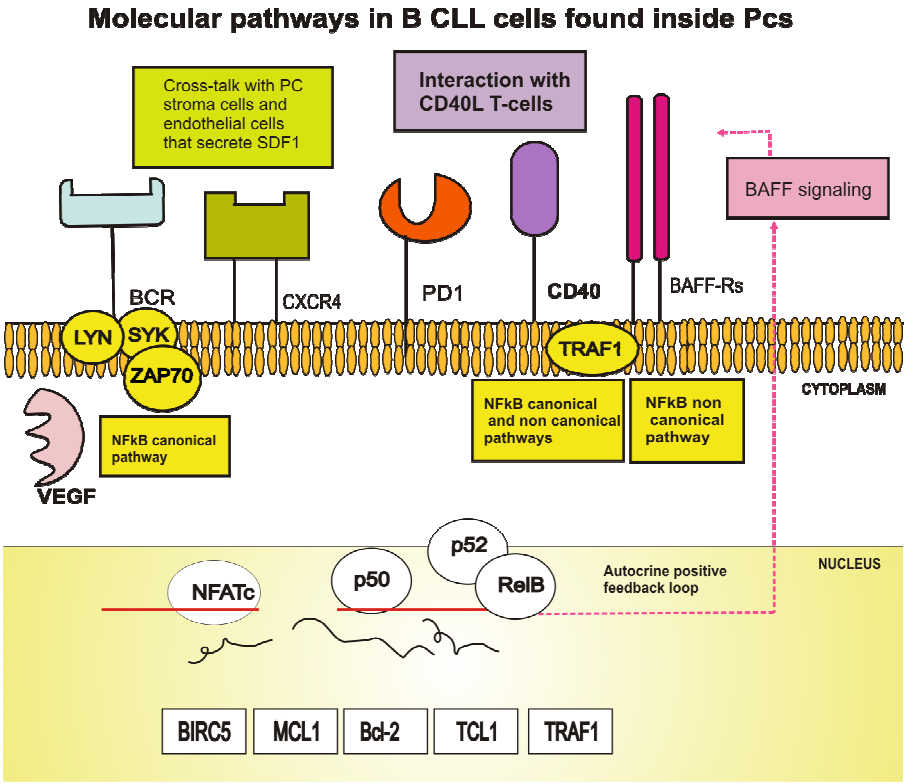


Figure 24. Relevant pathways in CLL cells inside PCs. NF-κB seems to be the common target of a variety of signaling cascades that are activated in the microenvironment of the PC. Some of these are BCR, CD40 and BAFFR.

In addition, other stimuli can trigger NF-κB activation such as the cytokines BAFF and CD40L. BAFF belongs to the tumor necrosis factor (TNF) ligand family and plays crucial roles in homeostasis of B cells, tolerance, and malignancy. We observed BAFF staining only in prolymphocytes of PCs. This finding is in agreement with previous reports on circulating CLL cells in which leukemia cells themselves are reported to produce BAFF that can enhance survival (Bojarska-Junak et al., 2009; Kern et al., 2004). Furthermore, NF-κB and NFAT

transcription factors (the latter one also observed only in prolymphocytes inside PCs) can induce BAFF expression creating a positive feed-back loop (Fu et al., 2006).

CD40 signaling is known to activate both the canonical and non-canonical NF-κB pathways (Siebenlist et al., 2005) in normal lymphocyte development and CLL cells. Our findings suggest that PCs are the place where engagement of CD40 receptor by CD40L-expressing T cells takes place, triggering NF-κB activation. Interestingly, many of our observations confirmed gene expression data obtained upon CD40 activation of B cells. In fact, CD40 stimulation generated a specific gene signature that included MUM1/IRF4, p50 and TRAF1 (Basso et al., 2004; Saito et al., 2007), all of them specifically expressed in PCs. High levels of TRAF1 in CLL and other lymphoid malignancies had already been observed and have also been related to NF-κB activation (Munzert et al., 2002). TRAF1 nuclear localization was striking, a phenomenon that so far has only been described in Hodgkin Reed-Sternberg cells (Izban et al., 2000). However, its biological meaning remains unknown and deserves further investigation.

5.1.4 Similarities and differences with normal germinal centers

Germinal centers are the physiological structures inside lymph nodes where B-cell proliferation takes place upon antigen encounter. In this sense, PCs may resemble GCs. Moreover, Ki67, BAFF, CD40, VEGF, Mcl-1 and BIRC5 are expressed both in GCs and PCs. On the contrary, Bcl-2 and TCL1 are downregulated in both contexts, as it has been described in proliferating cells (Herling et al., 2007; Herling et al., 2006).

However, NF-κB activation occurs only in a minority of B cells inside GCs, while it seems to be a general phenomenon in prolymphocytes of PCs. Also MUM1/IRF4 and TRAF1 expression is relatively specific of PCs, as they are only expressed by a small population of B cells in GCs.

Regarding morphology, PCs are not compartmentalized in two different zones (dark and light) as GCs and they lack CD21+ follicular dendritic cells and the characteristic rim of follicular T helper cells that occupy the outer zone of the GC. On the contrary, we observed specific subpopulations of SDF1 ADCs and STAT1-Ms. Dendritic cells in GCs also express SDF1, which attracts normal lymphocytes into the lymph node. GC B cell markers (CD10, GCET1 and BCL6) were not expressed by B cells of PCs.

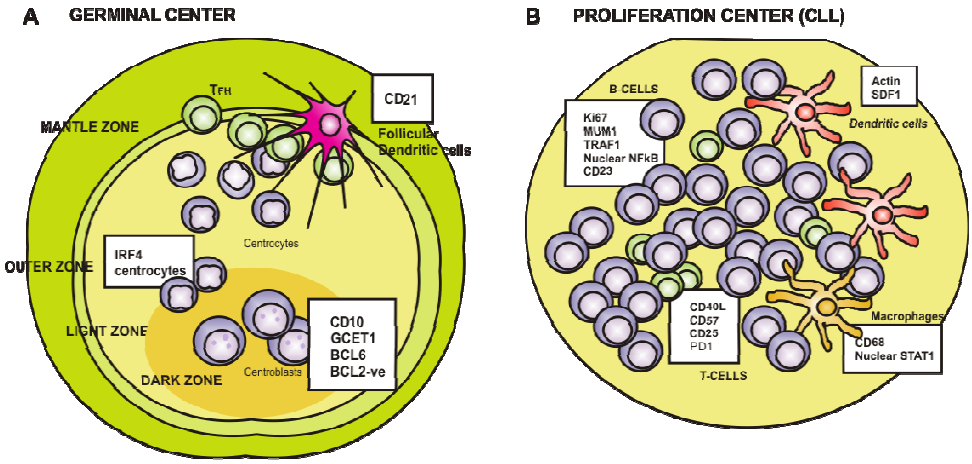


Figure 24. Schematic representation of a physiologic germinal center in A) and a proliferation center typically found in CLL lymph nodes in B). Anatomical differences, as well as differences in cell populations and activation status of B cells are observed.

In summary, we believe that PCs represent the supportive environment to which CLL cells are attracted and where they proliferate via multiple interactions that trigger NF-κB activation. PCs are different from GCs as tumor cells seem to modulate cellular interactions creating a new favorable microenvironment that avoids apoptosis controls.

5.2 New insights into the relationship between molecular features of chronic lymphocytic Leukaemia and sensitivity to rationally selected compounds (calmidazolium, R406, TW-37 and ETP-39010): an *ex vivo* approach

5.2.1 Rational selection and investigation of new agents for CLL treatment

CLL presents an extremely variable clinical course with a survival time that can range from months to years after diagnosis. Although 80-90% of CLL patients achieve overall response and up to 70% complete response, the vast majority of patients are prone to relapse after primary treatment. Moreover, those patients who relapse after first-line treatment with purine analogs and rituximab or those carrying high-risk genomic abnormalities such as del(11q22) and del(17p13) usually show bad responses to alternative therapies and have a median survival of less than a year. CLL is a disease of elderly and treatment should be tailored in an attempt to obtain maximum efficacy with minimum toxicity. Taken together, these data suggest that preclinical studies on new therapeutic strategies are warranted.

In this work we were interested in analyzing how sensitivity to rationally selected compounds can be determined by molecular characteristics. The successful development of targeted

therapies in cancer is best illustrated by antibody-based therapies for the treatment of ErbB-2-/Her2 positive breast cancer (Slamon et al., 2001), and the use of Gleevec (STI571/imatinib) as a kinase inhibitor for the treatment of Bcr-Abl-positive chronic myeloid leukemias (Druker, 2002). In this work, we have used a double approach to rationally select the compounds to test. First, with the use of connectivity Map (Lamb et al., 2006) we have looked for connections between a characteristic gene signature of CLL patients with poor prognosis and small molecules that could potentially revert this signature. From this analysis we have chosen calmidazolium and R406. Second, based on previous experimental evidences, we have selected two compounds that interfere with Bcl-2 family members (TW-37) and with PIM kinases (ETP-39010) respectively.

Unfortunately, animal models or cell lines do not fully recapitulate the complexity of the disease and consequently, have not been routinely applied to investigate the utility of new compounds in CLL. Therefore, we have chosen an *ex vivo* model using primary CLL cells from blood specimens in order to test the selected active compounds. The employment of xenograft models has been limited due to inefficient or short-term engraftment. However, recent studies (Durig et al., 2007) have reported successful xenografts using CLL samples that could be useful for further studies.

5.2.2 B cell receptor signaling

In order to dissect the BCR pathway, we assayed two compounds that exert their effects at different levels of the signaling cascade: calmidazolium and R406.

Calmidazolium is a well known calmodulin inhibitor. Calmodulin is known to mediate many cellular processes by sensing calcium presence. One of these processes is BCR signaling via interaction with calcineurin phosphatase and calmodulin dependent protein kinase II that trigger NFATc translocation to the nucleus and activation of NF-κB translocation by activation of IKK. The fact that CLL samples showed a variable sensitivity to calmidazolium may reflect differences in the status of calcium dependent pathways.

We did not observe differences between subgroups of samples defined by mutational status of IGHV or ZAP70 expression. There are several possible explanations for this. First, the presence of non-tumor cells in the PBMC samples could mask the results on cell viability. Nevertheless, when we analyzed data from a new series of purified B-CLL cells, again no differences could be found between subgroups of samples. On the other hand, calmodulin is involved in many

cellular processes in addition to BCR signaling, which could also be essential for CLL cells and that are not reflected in this classification.

Microarray analysis revealed that sensitive and resistant samples (defined by the median EC50) could be differentiated using a small group of genes. Among the genes found to have higher expression levels in resistant samples were *BIRC6*, which inhibits apoptosis by facilitating the degradation of apoptotic proteins by ubiquitination and *PRKCE* that is able to activate NF-κB transcription factor. In fact, PRKC can be activated by second messengers, calcium and diacylglycerol, without the requirement of calmodulin explaining the compensation mechanism that can lead to activation of survival pathways independently of calmodulin and conferring resistance to calmidazolium treatment.

R406 is a SYK kinase inhibitor (Braselmann et al., 2006), developed by Rigel. It was initially used to inhibit this kinase in activated mast cells, macrophages and B cells in rheumatoid arthritis and a new derivative known as R788 is already in phase II clinical trials. R788 is also starting phase II clinical trials for immune thrombocytopenic purpura as well as B and T cell lymphomas. Surprisingly, R406 was not able to induce 100% cell death in most of the PBMCs samples even at the highest concentration of 50 μM, although some samples were indeed sensitive to the drug. However, purified B cells were clearly more sensitive to the drug showing EC50 values around 1μM or lower, which is in accordance with recent published work (Gobessi et al., 2009). Several explanations for this are possible. As R406 is directed against SYK kinase, non-tumor cells for which SYK may not be essential for survival would not undergo cell death. On the other hand, the non-tumor component of the sample might be delivering survival signals independent of the BCR to CLL cells. Our data indicated that samples with increased BCR signaling, even in absence of F(ab')2 stimulation, were sensitive to R406 while samples with no basal BCR activity were resistant to R406. As measurement of BCR signaling we used phosphorylated AKT because, surprisingly, no consistent phosphorylation of BLNK was observed in our experiments. This could be due to the experimental conditions used (as BLNK is the first substrate of SYK, a stimulation of 5 minutes might be too long). AKT kinase can be activated via other receptors apart from BCR (growth factor and cytokine receptors among others). However, in our experimental setting phosphorylation of AKT was induced only upon BCR engagement. Some recent data (Davis et al., 2010) have claimed that R406 compound could also inhibit other kinases apart from SYK. In fact, initial reports on R406 already indicated partial effect on other kinases such as Lck or Lyn, though with a lower specificity compared to

SYK kinase (Cha et al., 2006). However, the relevance of this inhibition has not been fully addressed in CLL cells.

At the gene expression level, sensitive samples could be distinguished from resistant samples by the higher expression of *MUM1/IRF4* and *FAM107A*. Interestingly, MUM1/IRF4 was also found expressed specifically in CLL cells inside PC, where antigenic stimulation and NF-κB seems to take place. Furthermore, MUM1/IRF4 is essential for B cell activation (Mittrucker et al., 1997), indicating that blood CLL cells with high MUM1/IRF4 levels might be constitutively activated and consequently were sensitive to R406, which confirms our previous data on basal phosphorylated AKT levels. Considering this, the value of MUM1/IRF4 as a surrogate marker of B cell activation and sensitivity to R406 should be further investigated. On the other hand, although the exact function of FAM107A is not completely understood, it has been shown to be lost in several types of cancer indicating a role as tumor suppressor (Wang et al., 2000). CLL samples with higher FAM107A might be therefore, more prone to respond to R406 treatment.

Another evidence of the importance of the interactions with the non-tumor cells was that R406 resistant samples showed an enrichment of chemokine and cytokine pathways. This seems to indicate that the resistance mechanism might be related to an increased response to microenvironmental stimuli that were able to compensate for the interference of the survival signals coming from BCR.

Considering calmidazolium and R406 together, approximately half of the samples (4 out of 7) could be assigned to the same group of resistant or sensitive samples, indicating that the efficacy of these compounds did not overlay. Genomic abnormalities, the lack of specificity of calmidazolium and the fact that it only inhibits the calcium dependent events occurring after BCR engagement could explain the discordance in the rest of the samples.

5.2.3 Bcl-2 family

Bcl-2 proteins play a critical role in the regulation of apoptosis in CLL. Accordingly, Bcl-2 and Mcl-1 overexpression has been widely described and related not only to the etiology of CLL but also with resistance to treatment. Several strategies have been used to interfere with Bcl-2 family members and some are already in clinical trials. For example, Oblimersen is an antisense oligodeoxynucleotide that targets Bcl-2 mRNA. Phase III clinical trial using oblimersen in combination with fludarabine/cyclophosphamide have shown an increase in 5-year survival in CLL patients (O'Brien et al., 2009). Moreover, several small molecule inhibitors that directly

interact with antiapoptotic Bcl-2 proteins have been developed and are already in phase I or II clinical trials for CLL patients (ATB-737, Gossypol, FX15-070) (for a review see (Kang and Reynolds, 2009)). In this work, we have tested the TW-37 compound, a new pan-Bcl-2 inhibitor that binds at the BH3 binding groove of Bcl-2, Bcl-x_L and with higher affinity at Mcl-1. In doing so, TW-37 is able to block their heterodimerization with proapoptotic proteins such as Bax, Bid and Bak (Wang et al., 2006). Mcl-1 expression has recently been shown to correlate with stage of disease, lymphocyte doubling time, IGHV mutational status, CD38 and ZAP70 expression. Moreover, high Mcl-1 expression correlated with in vitro fludarabine resistance (Pepper et al., 2008). Since TW-37 also targets Mcl-1, this compound was especially interesting to be tested in CLL samples. Actually, PBMCs samples were all sensitive to TW-37 with EC50 values lower than 1μM. In accordance with the role of Mcl-1 in the poor prognosis group, we observed that U-CLL samples were more sensitive to TW-37 than M-CLL. Interestingly, those samples with defective p53 pathway (17p deletion) showed higher EC50 values. This seems to indicate that TW-37 may induce a p53 dependent cell death mechanism.

Purified B-CLL samples showed an increase in the median EC50 compared to PBMCs samples (from 236 nM to 1496 nM). This could support the idea that some non-tumor cells present in the sample are providing alternative survival signals. However, the heterogeneity of the PBMC series regarding the proportion of tumor cells does not definitively allow confirming this idea. Furthermore, we observed a greater variability in TW-37 sensitivity when CLL cells were used (from 310.5 nM to 9760 nM), indicating that maybe other factors related to the sample composition could mask drug sensitivity of tumor cells. As expected, TW-37 was able to induce apoptosis in a time and dose dependent manner. On the other hand, TW-37 did not affect cell cycle progression as it had been observed in other models (Ashimori et al., 2009), probably because CLL cells found in the peripheral blood are known to be quiescent in the absence of external stimuli (Liu et al., 2010).

We observed that samples with higher levels of Mcl-1 protein but not Bcl-2 protein were more sensitive to TW-37. This is in accordance with the mechanism of action of the compound that shows high affinity for Mcl-1 and displaces Bak from the Mcl-1-Bak heterodimer (Mohammad et al., 2007). Therefore, Mcl-1 protein levels could be used as a biomarker of TW-37 sensitivity, although this observation should be further validated in an independent series of samples. Finally, we identified a group of genes that could distinguish TW-37-resistant samples. Although the median of the series was used initially to divide the series in resistant

and sensitive samples, it became clear that only the three samples with the highest EC50 values were actually resistant (shown by consensus clustering). These samples were defined by a higher expression of genes involved in several survival pathways. So, GADD45B is activated upon DNA damage and can trigger activation of p38/JNK pathway; PPBP (also known as CXCL7) is a chemokine that can modulate the composition of the microenvironment; VAV2 has been related to angiogenesis and promotes calcium flux as part of the BCR pathway inducing activation of the transcription factor NFAT; PKCO (also known as PKCQ) activates NF-κB upon BCR signaling; PIK3CB (p100 β) has a well known role in cell growth, proliferation and survival. Finally, FAS induces apoptosis and can simultaneously activate NF-κB via FLIP. In summary, the cooperation of several pathways seems to confer a resistant phenotype to TW-37 treatment.

5.2.4 PIM kinases

Chemical inhibition of PIM kinases has been recently shown to be effective inducing apoptosis in T cell leukemia cell lines and xenograft models and CLL samples (Chen et al., 2009; Lin et al., 2010). The rationale for the use of PIM kinase inhibitors in CLL is based on the observation that elevated PIM1 and PIM2 mRNA levels can be observed in CLL samples compared with lymph nodes and tonsils (Cohen et al., 2004). Pim kinases have been related to apoptosis, cell cycle progression, transcription and survival pathways. Its role as oncogenes and the resolution of their tridimensional structure have made them candidate druggable targets.

We have tested the ETP-39010 compound developed at the Experimental Therapeutics Program of CNIO that inhibits the three members of the family, though with different IC50 values (0.024 mM for PIM1, 0.089 mM for PIM2 and 0.025 mM for PIM3). Either PBMCs (median EC50 2794 nM) or purified B-CLL samples (median EC50 5595 nM) were sensitive to the compound at the low μM range. CLL samples presenting parameters of bad prognosis (unmutated IGHV and ZAP70 expression) had lower EC50 values, indicating that they were more sensitive to the compound.

As previously described, we observed induction of apoptosis upon ET-39010 treatment that was time and concentration dependent. However, no changes in cell cycle were detected (Chen et al., 2009; Lin et al., 2010).

Surprisingly, PIM2 levels were positively correlated with ETP-39010 EC50 values. Our data indicated that samples with bad prognosis markers were more sensitive to the drug and recent published work has shown that CLL patients with poor prognosis present higher levels of PIM2

(Huttmann et al., 2006). At least, two possible explanations are possible. First, as the PIM inhibitor is an ATP competitive molecule, it could be that higher PIM levels would require higher concentrations of the compound to be inhibited. Second, IC50 values for PIM2 are higher than IC50 values for other PIM isoforms. Thus, it is possible that the heterogeneity in drug sensitivity is due to PIM1 levels or that CLL cells are more dependent on PIM1 than PIM2. However, due to sample limitations and the difficulty to detect PIM1 by WB, we could not correlate PIM1 protein levels with EC50 values.

Gene expression analysis revealed a gene signature of ETP-39010 sensitivity. One of the most interesting genes included in this signature was LPL. Its expression has been previously described as a bad prognostic indicator in CLL providing even better prognostic assessment than ZAP70 at advance stages of the disease (Oppezzo et al., 2005). Interestingly, LPL was found to be more expressed in the group of ETP-39010 sensitive samples, which also showed other bad prognostic factors such as ZAP70 expression and unmutated IGHV. Other genes showing higher levels in sensitive samples were TRADD, which is an adaptor molecule of TNF signaling that induces cell death, PAWR, a transcriptional repressor that can inhibit NF-κB and downregulate Bcl-2 and HDAC8, a histone deacetylase related to epigenetic repression and regulation of transcription.

Some of the genes regulated after ETP-39010 treatment were related with gene transcription and its regulation. In fact, PIM kinases have been shown to interact with several transcription factors inducing transcriptional activation (MYC, NF-κB) (Hammerman et al., 2004; Zippo et al., 2007) or repression (FoxO1a and FoxO3a) (Zhang et al., 2007). The balance between both actions leads to a controlled growth and survival. Moreover, interference of PIM kinases leads to inhibition of phosphorylation of Bad (blocking the degradation of this proapoptotic molecule) and FoxO3a (which in turn, upregulates proapoptotic Bim and PUMA) inducing apoptosis (Fox et al., 2003; Yan et al., 2003). On the other hand, transcription of PIM kinases is induced upon cytokine activation via JAK/STAT mediators triggering cell growth and survival (Hammerman et al., 2005). Therefore, PIM inhibition in our series of samples leaded to a downregulation of genes involved in processes of protein metabolism, byosinthetic processes (Babelomics analysis) and other energetic and metabolic pathways (GSEA analysis).

Further studies in an extended panel of samples could reveal more hints about mechanisms of resistance, providing a rationale for more effective drug combinations.

5.2.5 Overview and open questions

Our study showed the molecular heterogeneity underlying differences in drug sensitivity. It sheds light into possible resistance mechanisms and therefore, could be the rational base for studies of drug combinations and targeted therapies in order to improve treatment outcome in CLL. The integration of gene expression and chemical compound activity data could facilitate the identification of useful agents for cancer treatment in a more individualized manner.

One fundamental issue to consider when new compounds are intended to be introduced in clinical trials is the selection of biomarkers. *Ex vivo* approaches are a useful tool for preclinical studies that is facilitating the identification and validation of new therapeutic targets before entering expensive and long-term clinical trials. Herein, we propose some biomarkers that could help in the patient stratification (Mcl-1, MUM1/IRF4, LPL, p-AKT) or monitorization of the activity of the active compounds (p-AKT). Nevertheless, their utility in a clinical setting should be further validated.

In this field, one important question to address is the relevance of the interactions between CLL cells and the microenvironment. As the pool of proliferating cells seems to localize inside the lymph tissues, it would be interesting to study the effect of new therapeutic agents on tumor cells in this context. The inefficacy to target tumor cells in these niches could explain the discordance observed between preclinical studies and clinical trials.

The final aim of studies like the one presented here will be to shift from the administration of widely cytotoxic drugs towards a more personalized approach, in which each patient is treated according to the specific molecular alterations of the tumor (based on gene mutations, gene expression data, proteomic profiles or deregulated pathways). Individualized treatments based on the knowledge about molecular pathogenesis of cancer may be the best approach to treat cancer in the future.

CONCLUSIONS/ CONCLUSIONES

Conclusions

1. Proliferation centers observed in lymph node samples of patients with chronic lymphocytic leukemia (CLL) showed a special cell composition distinct from the physiological germinal centers. We identified two different populations of stroma cells that we called Actin Dendritic Cells and STAT1 macrophages. They may contribute to the homing and survival of leukemic cells.

2. Proliferation centers are the niche where NF-kB activation takes place. NF-κB pathway was activated exclusively in CLL cells found inside the proliferation centers, as demonstrated by nuclear localization of p50, p52 and Rel B subunits.

3. Activation of NF-κB inside proliferation centers appeared to be associated with changes in the microenvironment, including CD40, BAFF and VEGF signaling.

4. Molecular signatures of the neoplastic cells in CLL suggested multiple potential therapeutic targets that could be tested in *ex vivo* culture of primary cells.

5. CLL samples presented a variable sensitivity to calmidazolium, a calmodulin inhibitor. This variability was underline by a molecular heterogeneity as more resistant samples showed a differential expression of some genes such as BIRC6 and PKCE.

6. Sensitivity to SYK inhibition using the compound R406 was associated with increased basal levels of phosphorylated AKT and MUM1/IRF4 gene, a well-established NF-kB target. R406-resistant samples were enriched in pathways related to microenvironment interactions such as cytokine and chemokine pathways.

7. Sensitivitity to Bcl-2-family inhibition using the TW-37 compound was associated with higher levels of MCL1 proteína, a potential candidate biomarker of TW-37 sensitivity. Moreover, resistance to this compound seemed to be related to differential expression of GADD45B, VAV2, PKCO, PIK3CB and FAS.

8. Inhibition of PIM kinases by ETP-39010 induced apoptosis and had no effect on cell cycle in CLL samples. Sensitive samples presented unfavorable prognosis markers such as unmutated immunoglobulin heavy chain (IGHV) as well as increased expression of ZAP70 and LPL.

Conclusiones

1. Los centros de proliferación observados en ganglios linfáticos de pacientes con leucemia linfocítica crónica (LLC) presentan una composición especial y diferente de los centros germinales fisiológicos. En estos centros de proliferación hemos identificado dos poblaciones diferentes de células del estroma que hemos denominado Células Dendríticas que expresan Actina y Macrófagos STAT1. Ambos tipos celulares parecen contribuir a la atracción y supervivencia de las células tumorales.

2. Los centros de proliferación son el nicho dónde tiene lugar la activación de NF-kB. La vía de señalización de NF-kB aparece activada exclusivamente en las células de LLC que se encuentran en dichos centros de proliferación, como demuestra la localización nuclear de las subunidades p50, p52 y Rel B.

3. La activación de NF-kB en los centros de proliferación parece estar relacionada con eventos del microambiente tumoral, como la señalización vía CD40, BAFF y VEGF.

4. La firma molecular de las células tumorales en LLC sugiere varias dianas terapeúticas potenciales cuyo valor puede ser investigado mediante cultivos *ex vivo* de células primarias.

5. Las muestras de LLC presentan una sensibilidad variable a calmidazolium, un inhibidor de calmodulina. Esta variabilidad se explica por la heterogeneidad molecular observada, de modo que las muestras más resistentes a calmidazolium se diferencian del resto por la expresión de algunos genes como BIRC6 y PKCE.

6. La sensibilidad de las muestras primarias a la inhibición de SYK mediante el compuesto R406 se asocia con niveles basales más altos de AKT fosforilado y una mayor expresión de MUM1/IRF4, un conocido gen diana de NF-kB. Las muestras resistentes a R406 presentan un enriquecimiento de vías de señalización relacionadas con el microambiente, como las relacionadas con citoquinas y quimiocinas.

7. La sensibilidad a la inhibición de la familia de Bcl-2 mediante el compuesto TW-37 se está asociada con niveles más altos de la proteína Mcl-1, que proponemos como candidato a biomarcador de la sensibilidad a dicho compuesto. Además, la resistencia

a TW-37 está relacionada con la expresión diferencial de GADD45B, VAV2, PKCO, PI3KCB y FAS.

8. La inhibición de las quinasas de la familia PIM mediante ETP-39010 induce apoptosis en células primarias de LLC pero no ejerce ningún efecto sobre el ciclo celular. Las muestras sensibles presentan marcadores de prognosis no favorable como la presencia de mutaciones en la región variable de la cadena pesada de las inmunoglobulinas (*IGHV*), la alta expresión de ZAP70 y una mayor expresión de LPL.

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APPENDIX