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Innovative targeted therapies for chemorefractory B-cell non-Hodgkin lymphomas

Anna Esteve Arenys

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Innovative targeted therapies for chemo-refractory B-cell non-Hodgkin lymphomas

Doctoral thesis presented by:

Anna Esteve Arenys

Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)

Hematology – Oncology department

Anna Esteve Arenys	Gaël Roué	Elias Campo Güerri
Doctorand	Thesis director	Thesis tutor

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Dr. Gaël Roué

Principal investigator at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)

CERTIFIES THAT:

- The doctoral thesis entitled "Innovative targeted therapies for chemo-refractory B-cell non-Hodgkin lymphomas" submitted for the award of Doctor in Biomedicine to the University of Barcelona is a record of authentic, original research conducted by Anna Esteve Arenys under his supervision and guidance, and it is eligible to be defended.
- The two articles derived from this dissertation were published in 2017 the scientific journals "Oncogene" and "Journal of Hematology and Oncology", respectively.
- The impact factor of Oncogene was 7.519, and the impact fator of Journal of Hematology and Oncology was 6.350 in 2016.
- No part of any paper published, where Anna Esteve Arenys shares co-first authorship, has been submitted previously for the award of any other doctoral degree, and this work was conducted in equal contribution with the other first co-author.

Barcelona, October 2017

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ABBREVIATIONS

A

aaIPI: age-adjusted International Prognostic Index

ABC-DLBCL: Activated B-cell-like Diffuse Large B-Cell Lymphoma

ALL: Acute Lymphoblastic Leukemia

AP1: Activator Protein 1

APAF1: Apoptotic Protease Activating Factor 1

ARF: ADP-Ribosylation Factor 1

ASCT: Autologous Stem Cell Transplantation

Asp: Aspartate

ATM: Ataxia-Telangiectasia Mutated

B

B2M: β2-microglobulin

BCL-2: B-Cell Lymphoma-2

BCL-6: B-Cell Lymphoma-6

BCR: B-Cell Receptor

BET: Bromodomain and Extra-Terminal

BH3: BCL-2 homology domain 3

BID: BCL-2 homology 3 (BH3)-Interacting domain death agonist.

BL: Burkitt's Lymphoma

BLIMP1: B lymphocyte-induced maturation protein-1

BM: Bone Marrow

BRD4: Bromodomain-containing protein 4

BTK: Bruton's Tyrosine Kinase

C

CAR: Chimeric Antigen Receptor

CARD11: Caspase recruitment domain-containing protein 11

CDK: Cyclin-Dependent Kinase (CDK)

CHOP: Cyclophosphamide, Doxorubicin (Hydroxydaunorubicin), Vincristine (Oncovin) and Prednisolone or Prednisone

CML: Chronic Myeloid Leukemia

CNS: Central Nervous System

CR: Complete Response

CREBBP: CREB-binding protein

CSR: Class-Switch Recombination

CTL: Cytotoxic T lymphocytes

CXCL12: CXC-chemokine Ligand 12

D

DHL: high-grade B-cell lymphoma with *MYC*, *BCL2* and/or *BCL6* chromosomal rearrangements; Double Hit Lymphoma

DLBCL: Diffuse Large B-Cell Lymphoma

DNA: DeoxyriboNucleic Acid

E

EBV: Epstein-Barr virus

ECOG: Eastern Cooperative Oncology Group

EMA: European Medicines Agency

EP300: Histone acetyltransferase p300

ER: Endoplasmic Reticulum

EZH2: Enhancer of Zeste Homolog 2

F

FADD: Fas-Associated Death Domain

FC: Fludarabine and Cyclophosphamide

FCR: Rituximab plus Fludarabine and Cyclophosphamide

FDA: Food and Drug Administration

FDC: Follicular Dendritic Cells

FFPE: formalin-fixed paraffin-embedded

FFS: Failure-Free Survival

FISH: Fluorescent *In Situ* Hybridization

FL: Follicular Lymphoma

FLIPI: Follicular Lymphoma International Prognostic Index

FLIS: Follicular Lymphoma *In Situ*

G

GC: Germinal Center

GCB-DLBCL: Germinal Center B-cell like Diffuse Large B-Cell Lymphoma

GELA: Groupe d'Etudes des Lymphomes de l'Adulte

GEP: Gene Expression Profile

GSEA: Gene Set Enrichment Analysis

H

HC: Heavy-Chain

HDAC: Histone Deacetylase

HDC: High-Dose Chemotherapy

HGBL: High-grade B-cell Lymphoma

HLA-I: Major histocompatibility complex class I

HSC: Hematopoietic Stem Cell

HyperCVAD: hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone, alternating with cytarabine and methotrexate

I

IFRT: Involved-Field RadioTherapy

Ig: Immunoglobulin

IHC: ImmunoHistoChemistry

IL-1R: Interleukin-1 Receptor

IMS: Mitochondrial Intermembrane Space

IPI: International Prognostic Index

IRAK2: IL-1R-Associated Kinase 2

IRF3: Interferon Regulatory Factor 3

ISMCN: *In Situ* Mantle Cell Neoplasia

ITAMs: Immunoreceptor Tyrosine-based Activation Motifs

IWCLL: International Workshop on CLL

L

LC: Light-Chain

LDH: Lactate Dehydrogenase

M

mAbs: monoclonal Antibodies

MALT: Mucosa-Associated Lymphoid Tissue

MAPK: Mitogen-Activated Protein Kinases

MBL: Monoclonal B-cell Lymphocytosis

MCL: Mantle Cell Lymphoma

M-CLL: Mutated Chronic Lymphocytic Leukemia

MDM2: Murine Double Minute 2 homolog

MEF2B: Myocyte-specific enhancer factor 2B

MIPI: MCL International Prognostic Index

MM: Multiple Myeloma

M-MCL: Mutated Mantle Cell Lymphoma

MOMP: Mitochondrial Outer Membrane Permeabilization

MYD88: Myeloid differentiation primary response gene 88

N

NFAT: Nuclear Factor of Activated T-cells

NF-κB: Nuclear Factor-kB

NHL: Non-Hodgkin Lymphoma

NOS: Not Otherwise Specified

O

OMM: Outer Mitochondrial Membrane

ORR: Overall Response Rate

OS: Overall Survival

P

PB: Peripheral Blood

PDGFA: Platelet-Derived Growth Factor A

PDK1: Pyruvate Dehydrogenase Kinase, isozyme 1

PFL: Partial Involvement Follicular Lymphoma

PFS: Progression-Free Survival

PLCγ2: Phospholipase C-γ2

PTEN: Phosphatase and tensin homolog

R

R: Rituximab

RB1: Retinoblastoma 1

R-CHOP: Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone

R-CVP: Rituximab plus Cyclophosphamide, Vincristine, and Prednisone

R-FM: Rituximab plus Fludarabine and Mitoxantrone

R/R: Relapsed or Refractory

RS: Richter's Syndrome

S

SCT: Stem Cell Transplantation

SFK: SRC family of protein Tyrosine Kinases

SHM: Somatic Hypermutation

sIg: surface immunoglobulins

SLL: Small Lymphocytic Lymphoma

SMAC: Second Mitochondria-derived Activator of Caspase

SOX11: SRY (Sex Determining Region Y)-Box 11

SWOG: Southwest Oncology Group

SYK: Spleen Tyrosine Kinase

T

TAM: Tumor-Associated Macrophages

TFH: Follicular Helper T-cells

TI: T-cell Independent

TIR: Toll/IL-1 Receptor

TLR: Toll-like Receptor

TLS: Tumor Lysis Syndrome

TNF: Tumor Necrosis Factor

TNFR1: TNF Receptor-1

TP53: Tumor Protein p53

U

U-CLL: Unmutated Chronic Lymphocytic Leukemia

U-MCL: Unmutated Mantle Cell Lymphoma

V

VEGF: Vascular endothelial growth factor

W

WHO: World Health Organization

WHSC1: Wolf-Hirschhorn Syndrome Candidate-1/ Nuclear Receptor Binding SET Domain Protein 2 (NSD2).

WM: Walderström Macroglobulinemia

X

XIAP: X-linked Inhibitor of Apoptosis Protein

Z

ZAP70: Zeta chain of T-cell receptor Associated Protein Kinase 70

INTRODUCTION

1. B-CELL LYMPHOID NEOPLASMS

Lymphomas are a heterogeneous group of tumors characterized by the proliferation of lymphocytes predominantly in lymphoid structures but also in extranodal tissues. More than 90% of patients are afflicted by lymphomas of B-cell origin.[1]

The current World Health Organization (WHO) classification of hematopoietic and lymphoid tumors categorizes B-cell neoplasms in more than 40 distinct disease entities, according to a combination of the morphology, immunophenotype, genetic, molecular and clinical features.[2] Each entity has its own clinical course and requires specific treatments. Their diversity can often be traced to a particular stage of differentiation of the normal precursor B-cell.[1-3]

1.1 Normal B-cell development and differentiation

B-cells differentiate from hematopoietic stem cells (HSC) in the bone marrow (BM). Progenitor B (Pro-B) cells undergo a rearrangement of IGHD (diversity), IGHJ (joining) and IGHV (variable) region segments to generate heavy-chain (HC) and light-chain (LC) immunoglobulin (Ig) genes. The formed pre-B-cell receptor (BCR) is tested for functional competence, and cells that express functional (and non-auto-reactive) BCR, differentiate into mature, naïve B-cells, leave the bone marrow (BM) microenvironment and circulate as small, resting lymphocytes in peripheral blood and secondary lymphoid tissues.[4,5]

Upon contact with an antigen, naïve B-cells are activated and migrate to secondary lymphoid organs. Here, some B-cells migrate to the marginal zone of the follicle and differentiate into short-lived plasma cells. Other activated B-cells, by interaction with T-cells, aggregate into primary follicles to form germinal centers (GC). At the dark zone of the GCs, B-cells (centroblasts) experience massive proliferation as well as Ig somatic hypermutation (SHM). Centroblasts then differentiate into centrocytes and move into the light zone of the GC, where the modified antigen receptor is selected for improved binding to the immunizing antigen. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR)[6] and mutated B-cells are selected based on their ability to produce high-affinity antibodies. Positively selected cells undergo multiple rounds of proliferation, mutation, and selection, before they differentiate into plasma cells or memory B-cells and exit GC.[4,5]

Immunoglobulin somatic hypermutation (SHM). A genetic mechanism that introduces point mutations as well as small deletions, insertions, or duplications into the rearranged IGHV genes.

Class-switch recombination (CSR). DNA recombination process where the constant region of the antibody heavy chain may be exchanged.

Whereas for the induction of a GC reaction, B-cells are

dependent on the interaction with T-cells, the differentiation of B-cells to plasma cells may also occur without T-cells in T-cell-independent (TI) immune responses. Plasma cells generated in TI immune responses are short-lived and unmutated.[4]

1.2 Origin of B-cell lymphoid neoplasms

B-cells are particularly prone to malignant transformation because the machinery used for antibody diversification can cause chromosomal translocations and oncogenic mutations. Additional transforming events include inactivating mutations in various tumor suppressor genes, as well as a latent infection of B-cells with viruses, such as Epstein-Barr virus (EBV). Each lymphoma subtype retains key features of their cell of origin as judged by the similarity of immunophenotype, histological appearance, and gene expression profiles.[4] The putative normal B-cell counterpart of B-cell lymphomas are shown in figure 1.

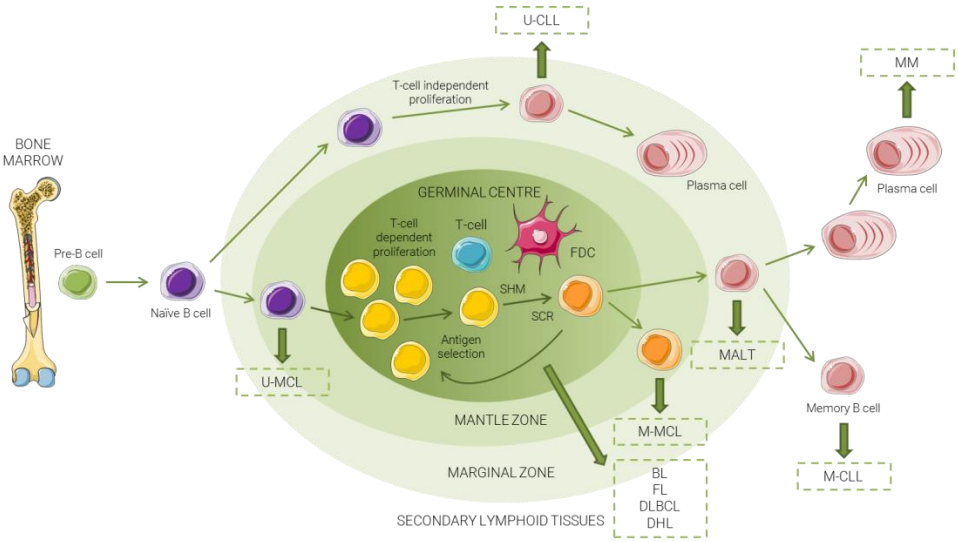

Figure 1 | **B-cell development and cellular origin of B-cell lymphomas.** Shown are the main steps in B-cell differentiation and the putative origin of human B-cell lymphomas. U-CLL, chronic lymphocytic leukemia with no IGHV mutations; U-MCL, mantle cell lymphoma with no IGHV mutations; MALT, mucosa-associated lymphoid tissue lymphoma; BL, Burkitt lymphoma; FL, follicular lymphoma; GCB-DLBCL, germinal center B-cell like diffuse large B-cell lymphoma; DHL, High-grade B-cell lymphoma with *MYC*, *BCL2* and/or *BCL6* chromosomal rearrangements or double hit lymphoma; M-MCL, mantle cell lymphoma with IGHV mutations; ABC-DLBCL, activated B-cell like diffuse large B-cell lymphoma; M-CLL, chronic lymphocytic leukemia with IGHV mutations; MM, multiple myeloma. SHM, somatic hypermutation; CSR, class-switch recombination; FDC, follicular dendritic cells.

The majority of mantle cell lymphomas (MCL) derived from mantle zone B-cells, presenting unmutated IGHV regions (U-MCL). However, some MCL cases show SHM, indicating that they have undergone GC reaction (M-MCL). In the same way, the

unmutated subtype of chronic lymphocytic leukemia (U-CLL) has unmutated IGHV regions, indicating a pre-GC derivation, and the mutated CLL subtype (M-CLL) has mutated IGHV regions and may derive from a small subset of memory B-cells. Burkitt's lymphoma (BL), follicular lymphoma (FL) and germinal center B-cell-like (GCB) diffuse large B-cell lymphoma (DLBCL), show features that are consistent with GC B-cell derivation, while activated B-cell-like (ABC) subtype of DLBCL resembles post-GC plasmablasts. Mucosa-associated lymphoid tissue (MALT) lymphomas are extranodal in origin and phenotypically related to post-GC marginal zone B-cells. Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells. High-grade B-cell lymphoma with *MYC, BCL2* and/or *BCL6* chromosomal rearrangements, as known as double hit lymphomas (DHL), have features intermediate between DLBCL and BL, being consistent with a GC B-cell counterpart.[1,4,7,8] It is important to note that the actual cell of origin may be a B-cell at an earlier stage of differentiation than its normal B-cell counterpart. This can occur if the initiating oncogenic event happens early in hematopoietic development but allows further differentiation to take place before subsequent oncogenic hits are sustained.[7]

1.3 Diffuse Large B-cell Lymphoma

Diffuse Large B-cell Lymphoma (DLBCL) is the most common form of adult lymphoma, comprising 30%-40% of all new diagnoses of B-cell Non-Hodgkin Lymphoma (B-NHL) in adulthood. The annual incidence of DLBCL is estimated to be 3-6 cases per 100,000 people in Western Countries.[9,10] It includes cases that arise *de novo* as well as cases that derive from the clinical evolution of other less aggressive B-NHLs, such as FL and CLL[8].

Initial diagnosis of DLBCL is more common in the elderly, usually in the seventh decade but it may also occur in children and young adults, and it is slightly more common in males than in females.[11]

Although the course of the disease is usually aggressive, more than 50% of patients can be cured by currently available immunochemotherapy protocols.[12]

1.3.1 Origin and physiopathology of DLBCL

1.3.1.1 DLBCL subgroups

Several subtypes of DLBCL have been designated in the WHO classification, including primary DLBCL of the central nervous system; primary cutaneous DLBCL, leg type; intravascular LBCL; T-cell/histiocyte-rich LBCL; EBV-positive DLBCL; and DLBCL associated with chronic inflammation.[13] The most common subtype is diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS), a category that includes tumors, which cannot be classified in any of the other more specific entities.[14] According to the genetic similarities and the putative cell of origin, DLBCL cases can be divided into two main

groups: GCB-DLBCL, which derives from centroblasts; and ABC-DLBCL, which display several features of BCR-activated B-cells entering plasmablastic differentiation (Figure 2).[15] An additional 15%–30% of DLBCL cases cannot be classified into any of the above subgroups.[16,17] These subsets are associated with different molecular signaling pathways and clinical outcomes.[18]

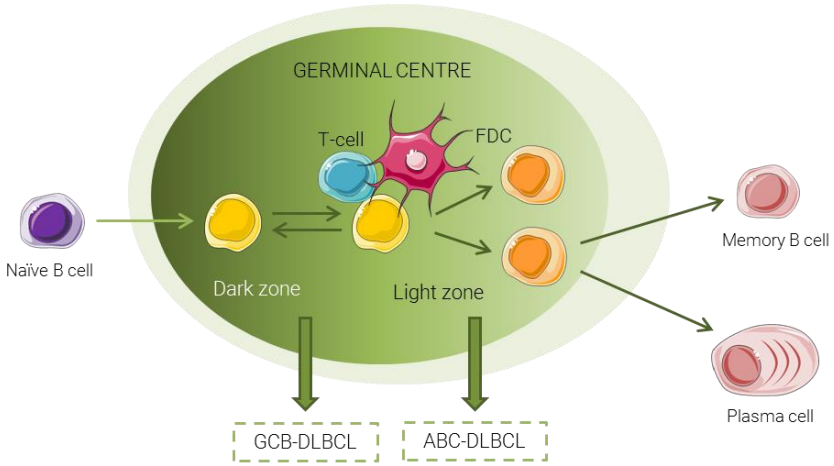

Figure 2 | **Germinal center and DLBCL pathogenesis.** Schematics of the GC reaction and its relationship with major subgroups of DLBCL. ABC-DLBCL: activated B-cell like diffuse large B-cell lymphoma; GCB-DLBCL: germinal center B-cell like diffuse large B-cell lymphoma; FDC: follicular dendritic cells. Adapted from Pasqualucci L et al., Semin Hematol, 2015.[17]

1.3.1.2 Genetic alterations in DLBCL

Compared to other B-cell malignancies, DLBCL display a significantly higher degree of genomic complexity, typically harboring between 50 and >100 lesions per case, with high variability across patients.[16] Gene expression profile (GEP) has identified the two principal molecular subtypes, the GCB and ABC forms of DLBCL,[3,19] that present different mutational landscape (Table 1) and depend on distinct oncogenic programs.[12] Genetic alterations found in DLBCL include chromosomal translocations, mutations caused by aberrant SHM, sporadic mutations and copy number alterations.[20]

Alterations Shared Across Subtypes

The most common programs affected in DLBCLs result from inactivating mutations and deletions of the histone acetyltransferases *CREBBP/EP300*, affecting ~40% of DLBCL[21,22] and of the histone methyltransferase gene *KMT2D,* in ~24-30% of cases.[8,23] These lesions may favor tumor development by reprogramming the cancer epigenome,[16] through impaired acetylation of substrates such as *BCL6* and *p53*.[17] Mutations/deletions of the

tumor suppressor *TP53* itself have also been reported[24] and *BCL6* rearrangements are detected in approximately 40% of DLBCL.[21]

B-Cell Lymphoma-6 protein (BCL-6) controls the transcription of a variety of genes involved in B-cell development, differentiation, and activation, and is required in mature B-cells during the GC reaction.[21] Deregulation of BCL-6 activity represents a key mechanism of transformation in DLBCL, achieved via direct (chromosomal translocations (40% of cases) or hypermutation of its promoter (15% of cases))[25] or indirect modalities (by enhancing the activity of its positive regulator MEF2B (~11% of cases)),[26] preventing acetylation-mediated inactivation of its function, or abrogating mechanisms controlling protein degradation (*FBXO11* mutations/deletions; 5% of cases).[16,17]

A large proportion of DLBCLs acquire the ability to escape immune surveillance, including Cytotoxic T lymphocytes (CTL)-mediated cytotoxicity, through lack of B2M (~29% of cases)[27] or HLA-I expression (~60% of cases),[17] and natural killer cell-mediated death, through genetic inactivation or defective transport of the CD58 molecule.[8]

Most DLBCLs harbor mutations in multiple genes as the result of an aberrant function of the physiologic SHM mechanism.[16] Affecting more than 50% of cases,[20] this phenomenon promotes genomic instability, favoring DNA (deoxyribonucleic acid) breaks/chromosomal translocations, and deregulating oncogenes or tumor suppressor genes such as *PIM1*, *MYC* and *PAX5*.[19,28]

GCB-DLBCL associated alterations

Chromosomal translocations involving *MYC* and *BCL2* are detected in ~10-15% and ~35-40% of GCB-DLBCL, respectively,[8,20] and deletions of the tumor suppressor *PTEN* are found in about 10% of cases.[17] *miR17-92* microRNA cluster, a negative regulator of PTEN can be also affected.[7] In addition, *EZH-2* driven epigenetic changes and B-cell migration are programs that seem to be affected with some specificity in GCB-DLBCL.[20,29] Also frequent for this subtype are mutations in genes controlling the growth and confinement of B-cells to the GC such as *GNA13* or *TNFRSF14*.[8,12,16,17,30]

ABC-DLBCL associated mutations

Constitutive activation of the nuclear factor κB (NF-κB) pathway represents a hallmark of ABC-DLBCL.[31] The underlying causes are heterogeneous and include the activation of the BCR (e.g. *CD79A, CD79B,* or *CARD11*)[12] and Toll-like receptor (TLR) (*MYD88*)[20] pathways, or loss-of-function mutations in NF-κB negative regulators *(TNFAIP3/A20*)[32].[17]

Impaired plasma cell differentiation is another major transformation mechanism in this subtype, caused by mutually exclusive lesions deregulating *BCL-6* and inactivating *PRDM1/BLIMP1*.[8,16]

Table 1 | **Genetic alterations in DLBCL.**

Gene	Class/product
DLBCL common genetic lesions	
CREBBP	CREB-binding protein
EP300	Histone acetyltransferase p300
KMT2D	Mixed-lineage leukemia protein 2
BCL6	B-Cell Lymphoma 6
MEF2B	Myocyte-specific enhancer factor 2B
FBXO11	F-box only protein 11
B2M	β2-microglobulin
HLA-I	Major histocompatibility complex class I
CD58	Cell adhesion molecule
PIM1	Pim-1 Proto-Oncogene, Serine/Threonine Kinase
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
PAX5	Paired Box 5
TP53	Tumor protein p53
GCB-DLBCL	
BCL2	B-Cell Lymphoma 2
EZH2	Enhancer of Zeste Homolog 2
PTEN	Phosphatase and tensin homolog
miR17-92	PTEN negative regulator
GNA13	G Protein Subunit Alpha 13
TNFRSF14	TNF Receptor Superfamily Member 14
ABC-DLBCL	
CD79A/B	BCR associated proteins
CARD11	Caspase recruitment domain-containing protein 11
MYD88	Myeloid differentiation primary response gene 88
TNFAIP3/A20	NF-κB negative regulator
BLIMP1	B lymphocyte-induced maturation protein-1

1.3.2 Diagnosis

Most patients are asymptomatic but when symptoms are present, they are highly dependent on the site of involvement.[15] In 40% of patients, the initial infiltrated site is extra-nodal, usually the gastrointestinal tract but also skin, central nervous system, lungs, or the bones. Bone marrow involvement is reported in about 15% of cases.[33]

A surgical excision biopsy remains the optimal method of diagnosis. This allows assessment of nodal architecture and provides adequate material for phenotypic and molecular studies.[34]

Morphologically, three common variants have been recognized: the centroblastic, the immunoblastic and the anaplastic, being the first the most common variant (Figure 3).[15]

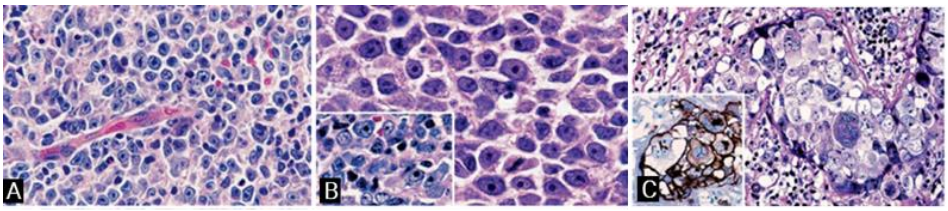

Figure 3. | **Diffuse large B-cell lymphoma.** (A) Centroblastic variant. (B) Immunoblastic variant. (C) Anaplastic variant. Swerdlow SH et al., WHO Classification of Tumors, 2008.[15]

DLBCL cells express pan B-cell markers such as CD19, CD20, CD22, and CD79a, but there may be a lack of staining with one or more of these. Surface and/or cytoplasmic Ig (usually IgM) can be demonstrated in 50-75% of cases. CD30 may be expressed especially in the anaplastic variant, and there are about 10% of cases that express CD5 and 30%, CD10. The proliferation fraction as detected by cells expressing the proliferation-associated antigen, Ki67 is usually high (more than 40% of neoplastic cells) and p53 is expressed in 20-60% of cases.[11]

GEP studies are needed to identify the DLBCL subtype.[35] However, GEP techniques are not applicable to the routine clinical practice, and different approaches using immunophenotypic algorithms with small panels of biomarkers were developed.[35] Different immunohistochemical staining (IHC) algorithms for the classification of GCB and non-GCB DLBCL subtypes are being used. The most common is the Hans decision tree, which uses the expression pattern of CD10, BCL-6 and IRF4/MUM1.[36] Other ICH algorithms for DLBCL classification include Colomo,[37] Muris,[38] Choi,[39] and Tally.[40] However, it must be taken into account that they are not reliable techniques for the classification of DLBCL subtypes, as they can deliver discordant results.

Recent improvements in technology have provided the opportunity to use formalin-fixed paraffin-embedded (FFPE) tissue biopsies for reliable GEP.[41,42] For example, the use of NanoString® gene expression platform permits an investigational *in vitro* diagnostic assay using the GEP of DLBCL cells for the classification of its subtype. The investigational test is performed using RNA extracted from FFPE tumor tissue diagnosed by pathology as DLBCL. These methods may represent a promising alternative to the ICH-based algorithms, providing a robust clinical tissue subtype characterization, and are being implemented in several hospitals.

1.3.3 Prognostic factors

The International Prognostic Index (IPI) originally proposed in 1993, remains the routinely used tool to classify and predict the outcome for DLBCL patients.[33] The IPI is a clinical and biological score that takes into account factors that are linked to the patient's characteristics, disease extension and tumor growth, such as Eastern Cooperative Oncology Group (ECOG) performance status[43] and Ann Arbor stage,[44] to segregate DLBCL patients into four prognostic groups with distinct median overall survival (OS).[45] Additionally, another scoring system was generated to classify young patients with aggressive lymphomas: the age-adjusted international prognostic index (aaIPI).[46]

Stromal gene signatures in DLBCL are prognostically relevant.[47] The presence of host infiltrates in the tumors, such as T-cells,[48] masts cells[49] or tumor-associated macrophages,[50] have been shown to be related to a better outcome. Moreover, it has been established that differences in the clinical features and treatment responses are also dependent on genetic and molecular factors.[33,51] In fact, patients in the GCB subgroup have a higher 5-year survival rate after chemotherapy than the ABC subgroup (~75-60% vs ~35%).[13,18]

1.3.4 Treatments

Treatment of DLBCL has undergone notable advances in the last 50 years. The current standard regimen of DLBCL is R-CHOP, a combination of rituximab with cyclophosphamide (an alkylating agent), doxorubicin (an intercalating agent), vincristine (which prevents cells from duplicating by binding to the protein tubulin), and prednisone (a corticosteroid), which achieves a cure in many patients. In clinical practice, the treatment decision is determined based on the individual IPI score and age.[10,34,52]

1.3.4.1 Current standard therapy (first-line treatment)

CHOP followed by involved-field radiotherapy (IFRT)[53] was first established as the standard therapy for DLBCL patients, but the introduction of monoclonal anti-CD20 antibody rituximab improved the outcome of DLBCL patients and set up R-CHOP as the standard treatment for this malignancy. The benefits of immunochemotherapy were confirmed in different clinical trials.[54-59] Reduced-intensity immunochemotherapy is used as a strategy to reduce toxicities in elderly and unfit patients.[60]

Although treatment with immunochemotherapy has significantly improved the outcome of DLBCL patients, there is still one-third of patients that do not respond to treatment,[61] and around 30-40% of young patients with high-risk IPI develop a refractory disease.[10,62] Additional cycles of R-CHOP[57,63,64] or maintenance with rituximab[59] were not shown to offer any therapeutic benefit.

1.3.4.2 Second-line therapy

Patients with DLBCL who experience relapse or fail to achieve complete response (CR) after first-line therapy, are treated with high-dose chemotherapy with Autologous Stem Cell Transplantation (HDC-ASCT)[65,66] or with a combination of rituximab with a salvage chemotherapy regiment.[67,68] However, patients with treatment failure within 6-12 months have a poor prognosis.[65] Therefore, the efficacy of these therapies should be improved with new active drugs.[10]

1.3.4.3 Novel agents

The identification of ABC- and GCB-DLBCL by GEP, with different prognosis, permitted the design of different therapeutic approaches for each subgroup.[3,52] ABC-DLBCL patients are less likely to respond well to CHOP-based regimens than those with GCB disease, therefore the exploration of new therapeutic targets is particularly important for this subgroup. Research of new therapies is focused on gene mutations and signaling pathways involved in each subgroup.[31,69] Several novel agents are undergoing evaluation in DLBCL, as both single agents or in combination with standard chemotherapy R-CHOP.

Immunomodulatory drugs

Immunomodulating agents inhibit angiogenesis, stimulate immune responses, alter cytokines and induce apoptosis.[10] Lenalidomide, for example, inhibits NF-κB activity *in vitro* and also exerts significant anti-tumor activity.[52] This drug has been evaluated as a single agent in relapsed/refractory (R/R) DLBCL with a CR of 29.4% vs 4.3% for ABC-DLBCL and GCB-DLBCL respectively.[70] Some clinical trials are ongoing in order to evaluate efficacy and safety of treatment with lenalidomide plus R-CHOP as a first-line therapy (NCT03003520, NCT02285062, NCT01856192, NCT02636322).

Proteasome inhibitors

Bortezomib is a proteasome inhibitor approved for use in MCL.[71] It inhibits NF-κB through blocking IkBα degradation and enhances the activity of chemotherapy in ABC-DLBCL.[72] In addition, in contrast to the adverse outcome associated with ABC subtype after treatment with R-CHOP, no survival differences are observed between GCB and ABC subtypes following R-CHOP plus bortezomib.[73]

Histone deacetylase inhibitors

Histone deacetylase (HDAC) inhibitors are potent antiproliferative agents, which cause cell-cycle arrest, apoptosis, cell differentiation, and in some cases, autophagy.[74] A large number of clinical trials, testing this group of drugs, are ongoing in both solid tumors and hematological malignancies. In particular, the efficacy and safety of vorinostat,[75,76] mocetinostat[77] and abexinostat[78] are being investigated in DLBCL and other lymphomas.

The most probable use of these inhibitors will be in combination with other biological agents.

Bruton's tyrosine kinase (BTK) inhibitors

Mutations affecting CD79B and CD79A are found frequently in ABC DLBCL and result in a chronic activation of BCR signaling and consequent NF-κB activation.[79] Therefore, kinase inhibitors that interfere with BCR signaling (e.g., BTK inhibitors) are emerging as a new treatment paradigm for ABC-DLBCL.[16] The BTK inhibitor ibrutinib has been shown to be effective in several types of B-cell lymphomas including ABC-DLBCL. As a single agent, in R/R DLBCL, ibrutinib has shown an RR of 37% in ABC-DLBCL patients compared to 5% in the GCB subtype.[80] In combination with R-CHOP, encouraging clinical trial results are being published in R/R non-GCB DLBCL.[81,82] Currently, this agent is being investigated in several clinical trials (NCT02636322, NCT02077166, and NCT02142049 among others).

CAR-T cells

Modified autologous T-cells genetically engineered to express anti-CD19 specificity, also known as chimeric antigen receptor T-cells (CAR-T cells), have shown promise in clinical trials of R/R DLBCL (CR of 53%)[83] and are one of the most exciting recent advances in the management of B-cell malignancies.

1.4. High-grade B-cell lymphoma with rearrangements of *MYC* and *BCL2* and or/ *BCL6* (so-called double-hit lymphoma)

Recent genetic and proteomics studies identified a prognostic role for *MYC* and *BCL2* genetic translocations. *MYC* is rearranged in 5% to 15% of DLBCL[84] and, in some cases *MYC* rearrangements are associated with *BCL2* and/or, to a lesser extent, *BCL6* rearrangements, with even less common lymphomas bearing all 3 rearrangements in the so-called double hit (DHL) or triple hit lymphomas.[85] These are now recognized as **high-grade B-cell lymphoma (HGBL), with rearrangements of *MYC* and *BCL2* and/or *BCL6*** in the updated WHO classification.[2]

DHL is characterized by highly aggressive clinical behavior, complex karyotypes,[86] and a broad morphologic, immunophenotypic and genetic spectrum that overlaps with BL and DLBCL, or less frequently, with FL with blastoid morphology.[87]

The median age of onset of DHL is approximately 60 years and it is more commonly observed in males, with a male to female ratio of 2:1.[88]

1.4.1 Origin and physiopathology of DHL

DHL comprises a subset of aggressive lymphomas in elderly patients, previously often diagnosed mostly as DLBCL but also as a Burkitt-like lymphoma.[89]

1.4.1.1 Genetic alterations in DHL

DHL cases harbor a chromosomal breakpoint at the *MYC*/8q24 locus together with another recurrent rearrangement, typically t(14;18)(q32;q21) involving *BCL2* gene (62% of cases) (Figure 4). Far less common are DHL with concurrent rearrangements of *MYC* and *BCL6* (8% of cases), or triple hit lymphomas (16% of cases), involving all three genes.[89,90]

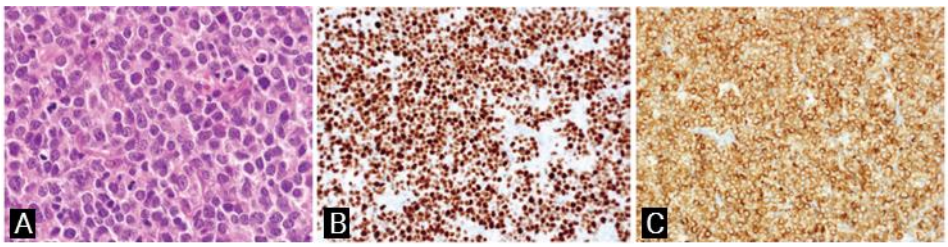

Figure 4. | **High-grade B-cell lymphoma with rearrangements of *MYC* and *BCL2*.** (A) DHL cells with somewhat blastoid nuclei and a high mitotic rate. Note the extensive nuclear MYC (B) and cytoplasmic BCL-2 (C) expression. Swerdlow SH et al., Hematology Am Soc Hematol Educ Program, 2014.[85]

In cases of concurrent rearrangement of *MYC* and *BCL2*, the predominant phenotype is GCB-DLBCL while the DHL composed by concurrent *BCL6* and *MYC* rearrangement have more frequently an ABC phenotype.[89,91,92]

MYC

MYC is a transcription factor that binds to promoter regions of target genes and modulates their expression by the recruitment of specific coactivators and repressors. The transcriptional program regulated by MYC includes 10% to 15% of all human genes.[93] The main cell functions and pathways under control of MYC are cell proliferation and growth, apoptosis, DNA replication, metabolism and energy regulation, and protein and nucleotide biosynthesis.[94]

MYC plays a crucial role in the lymphoid follicle formation, where it is transitory expressed in different subsets of cells. MYC is initially expressed in B-cells after interaction with antigens and T-cells.[93] The subsequent upregulation of BCL-6 represses *MYC* and initiates the formation of the GC dark zone. *MYC* is again upregulated in some cells of the light zone, which will reenter into the dark zone and undergo further cycles of proliferation and SHM. MYC-negative cells in the light zone will exit the GC to differentiate into memory B-

cells or plasma cells. BCL-6 and BLIMP1 (B lymphocyte-induced maturation protein-1) directly repress *MYC* in GC B and plasma cells, respectively.[91]

MYC alterations have been frequently detected in aggressive B-cell lymphomas,[95] including the t(8;14) translocation, characteristic of BL (80–90% of cases).[86] The Ig genes are the most frequent *MYC* translocation partner, leading to constitutive *MYC* expression[91] and a significant negative impact on the patient outcome.[96]

BCL-2

BCL-2 (B-cell lymphoma 2) is the founding member of the BCL-2-family proteins that play a crucial role in the regulation of apoptosis through their ability to regulate mitochondrial cytochrome release. BCL-2 is an anti-apoptotic protein that promotes the survival of cells upon major stressors such as DNA-damage and growth factors deprivation.[97,98]

The overexpression of BCL-2 does not promote cell proliferation as most previously discovered oncogenes do; rather, overexpression of BCL-2 inhibits cell death. It promotes cancer cell accumulation by opposing cell death.[99,100]

BCL2 t(14;18) chromosome translocation breakpoint is found in nearly all cases of FL (80-90% of cases) and in 20-30% of cases of DLBCL,[86] more specifically GCB-DLBCL.[101] This translocation juxtaposes *BCL2* to the IGH gene, resulting in BCL-2 protein overexpression and inhibition of apoptosis.[102]

BCL-6

BCL-6 is a transcriptional repressor expressed in many tissues but in B-cells is mostly restricted to GC B-cells, where is required for GC formation in response to antigen stimulation.[45] It represses many target genes involved in apoptosis, DNA damage response, cell cycle control, proliferation, and differentiation.[89]

As we saw in the previous section, BCL-6 is involved in B-cell development, differentiation, and activation. Multiple mechanisms act coordinately to timely modulate *BCL6* expression at transcriptional and post-transcriptional levels. BCL-6 prevents premature activation and differentiation of GC B-cells and provides an environment tolerant of the DNA breaks associated with Ig gene remodeling mechanisms involved in the production of high-affinity antibodies of different isotypes.[21] Important direct targets are *BCL2, TP53, IRF4,* and *BLIMP1*.[91]

Upon induction, BCL-6 binds its own promoter to negatively regulate its transcription. The importance of this mechanism is highlighted by the presence of genetic lesions that abrogate this autoregulatory circuit.[8] Chromosomal translocations affecting the band 3q27, where the *BCL6* gene is located, are one of the most common genetic abnormalities

in DLBCL (around 30% of patients).[101] Only half of the translocations involving *BCL6* affect an Ig locus; in other cases, the translocation partner is very diverse.[89]

1.4.2 Diagnosis

Awareness of DHL pathologic spectrum between BL, DLBCL and FL is important in directing ancillary testing to detect *IGH-BCL2* and *MYC* rearrangements; particularly in laboratories where conventional cytogenetics is not routinely performed or available.[87]

The majority of patients will present with advanced stage disease, involvement of extranodal locations, high serum lactate dehydrogenase level, and high-intermediate or high-risk IPI score. Among extranodal locations, a high proportion of patients will have involvement of the bone marrow at the time of diagnosis, and this may involve a circulating leukemic phase in the peripheral blood; a finding that is rarely seen in typical DLBCL. Central nervous system (CNS) involvement at diagnosis, or subsequently at relapse, is also increased in DHL relative to DLBCL patients.[103-105]

DHL are typically composed of a diffuse proliferation of medium- to large-sized transformed cells with few admixed small lymphocytes, but in rare cases, nuclei are relatively small and the chromatin is finely granular, resembling lymphoblastic lymphoma.[101] These lymphomas express B-cell markers such as CD19, CD20, CD22, and CD79 and are negative for one or more T-cell antigens, including CD2, CD3, CD4, CD7 or CD8. CD5 is positive in around 10% of the cases and CD10 in ~84-90%.[87,92,101,103] Cell proliferation (measured by Ki-67) is usually high, with a median typically reported in the 80% to 90% range, and often approaching 100%.[105,106]

The morphological and/or immunophenotypic parameters do not seem to effectively predict the detection of cytogenetic translocations involving *MYC, BCL2* and *BCL6*;[106] for example, 29-44% of DLBCL cases have overexpression of MYC and BCL-2 by IHC ('double-expresser' cases), but most of them do not harbor rearrangements.[88] A diagnosis of DHL is only determined following the results of a cytogenetic techniques (karyotyping) and/or fluorescent *in situ* hybridization (FISH).[86] Reproducibility is reported to be very high, with concordance of more than 98%.[85,96] For that reason, among all newly diagnosed lymphomas characterized morphologically and immunohistochemically as B-cell lymphoma unclassifiable with features intermediate between DLBCL and BL or HGBL, it would be prudent to perform FISH, given the high prevalence of double-hit cytogenetics within this cohort.[105]

1.4.3 Prognostic factors

DHL patients have a rapidly progressive disease with poor prognosis, even with high-intensity chemotherapy.[90] Median survivals are often in months, with most reported to be no more than ~1.5 years, and with OS at 3 or 5 years shorter than expected in DLBCL.[85]

Modern data suggest that a subset of DHL patients may have long-term survival,[107,108] demonstrating that some patients with DHL may indeed have a better outcome and that DHL does not predict the death sentence that was initially suggested.[105] There is a clear need to conduct prospective studies in patients with DHL, to determine their true prognosis.

Given the heterogeneity in outcome among patients with DHL, prognostic variables have been extensively evaluated. Patients with DHL often present with poor prognostic parameters, including elevated lactate dehydrogenase (LDH), BM and CNS involvement, and a high IPI score.[89,102] The IPI is predictive in DHL population, with patients with 0 to 1 risk factors having a very favorable overall prognosis of approximately 90%, compared with approximately 25% within IPI scores of 3 to 5. [107,109] Even so, it has not be taken into account that high or high-intermediate IPI is around 70% - 90% of the cases (clinical stage III/IV).[102,104]

1.4.4 Treatments

The mainstay of therapy for aggressive B-cell lymphoma is R-CHOP or R-CHOP-like regimens. Chemoimmunotherapy has improved the OS of DLBCL patients[110] but its failure to control the disease in DHL patients has motivated the development of new therapies that may overcome the adverse clinical impact of this genetic alteration.[84,105]

1.4.4.1 Current standard therapy (first-line treatment)

Despite the prognosis in DHL, R-CHOP chemotherapy remains the backbone of treatment given the good outcomes achieved in DLBCL patients as a whole. Optimizing therapy remains the key clinical challenge in DHL. The challenge is twofold: optimizing traditional chemoimmunotherapy regimens, and salvaging individuals with poor response to initial therapy.[110] A major problem with these regimens is that they are not well tolerated in older adults who comprise the majority of DHL patients.[88,111]

1.4.4.2 Second-line therapy

Patients who have primary R/R disease have extremely poor outcomes with no standard treatment recommendations.[112] SCT and allograft transplants for patients at high-risk of R/R disease have no effect on OS.[109] Furthermore, the utility of transplant is limited by poor tolerability in the elderly.[110]

1.4.4.3 Novel agents

As DHL is characterized by rearrangements of both *BCL2* and *MYC*, approaches targeting these specific pathways are particularly exciting areas of exploration.[110] Patients with R/R DHL should ideally be treated in the context of novel agents.[105]

MYC inhibition

Inhibition of MYC is likely pivotal to the development of truly novel approaches to the treatment of DHL. Although targeting transcription factors with direct inhibitors has proved technically challenging, progress is being made in the targeting of the regulation of *MYC* activity; there are several agents currently being developed including direct and indirect targeting, knockdown, protein/protein and DNA interaction inhibitors, and translation and expression regulation.[110,113]

Direct MYC inhibition can be achieved either by interference with its production or function. Antisense oligonucleotides like Resten-NG (AVI BioPharma)[114,115] have been used to promote degradation of target mRNA or preventing mRNA translation. However, none of these drugs were further developed. On the other hand, small molecule protein/protein interaction inhibitors such as inhibitors of MYC/MAX dimerization (10058-F4 and JY-3-094),[116,117] have been also studied. They have so far been limited by poor bioavailability, rapid metabolism, and inadequate target site penetration. Omomyc[118] is a MYC dominant negative that disrupt MYC/MAX interaction, sequester MYC away from DNA and occupy the E-box with transcriptionally inactive dimers.[119] Multiple studies in mouse models of cancer demonstrated Omomyc's therapeutic impact in different types of cancer.[120-122]

Targeting *MYC* itself has often proven very challenging. Because of that, many researchers have instead opted for an indirect approach, focusing on MYC transcriptional regulation or modulation of MYC stability and activity. The bromodomain and extra-terminal (BET) proteins inhibitors are a significant area of focus at the moment.[113]

Post-translational modifications of the histone proteins orchestrate chromatin organization and gene expression in normal and cancer cells.[123] BET proteins act as scaffolds for the recruitment of transcription factors and chromatin organizers required in transcription initiation and elongation.[124,125] The recent discovery of small molecules capable of blocking their lysine-binding pocket is the first paradigm of successful pharmacological inhibition of epigenetic readers.[123] Given the general role of BET proteins in transcriptional elongation, the discovery that inhibition of these proteins only affects the transcription of a small subset of genes was unexpected and suggested that inhibitors of bromodomains may specifically modulate the expression of some disease-promoting genes.[126]

The bromodomains and extra-terminal domain (BET) family proteins recognize acetylated chromatin through their bromodomains (BDs) and help in regulating gene expression. The BET family proteins include BRD2, BRD3, BRD4 and mBRDT.[125]

Inhibition of BRD4 (Bromodomain-containing protein 4) by the small molecule JQ1 has shown a downregulation of *MYC* transcription, followed by genome-wide downregulation of *MYC*-dependent target genes.[124,127] It has demonstrated preclinical activity in different cancers,

including DHL cell lines.[128] These exciting results from JQ1 encouraged the development of similar structure BET inhibitors.[123] CPI203 (Constellation Pharmaceuticals, Inc.)[129] is another BRD4 inhibitor structurally similar to JQ1 (Figure 5), but with an improved bioavailability profile in mice.[130] It has shown preclinical evidence of efficacy in lymphomas.[131,132]

CPI0610, a CPI203 analog, as well as other BET inhibitors are currently in phase I/II clinical trials: CPI0610 (ClinicalTrials. gov identifier: NCT01949883, NCT02157636, NCT02158858), GSK525762 (ClinicalTrials.gov identifier: NCT01943851, NCT01587703), and OTX015 (ClinicalTrials. gov identifier: NCT01713582).[110,123]

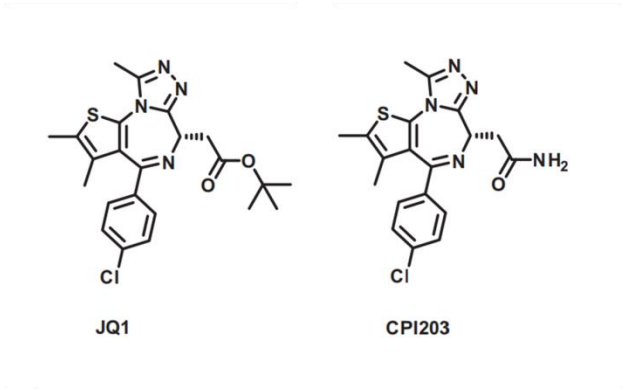

Figure 5 | **Structural view of JQ1 and CPI203.** (A) JQ1 chemical structure. (B) CPI203 chemical structure. Adapted from Hewitt MC et al., Bioorg Med Chem Lett, 2015.[133]

BCL-2 inhibition

Several inhibitors of the BCL-2 family are in development. The first extensively validated BCL-2 selective inhibitor was ABT-199 (venetoclax).[134] Venetoclax is approved in the USA for CLL patients with 17p deletion.[135] ABT- 199 has demonstrated preclinical efficacy in DHL cell lines, especially when used in combination with either traditional chemotherapy or other novel agents. In particular, ABT-199 enhances the antitumor effects of bortezomib, YM-155 (MCL1 inhibitor) and JQ1.[128] See chapter "2.4.4. Targeting anti-apoptotic BCL-2 family members" for more detailed information about venetoclax and other BCL-2 inhibitors.

Other novel therapeutic approaches

Small-molecule inhibitors of *BCL6* are also in development. One such compound binds to the corepressor-binding groove of the BCL-6 and is effective in killing BCL-6-positive DLBCL cell lines. It is likely that rational combinations of these agents with more effective chemotherapy platforms than CHOP, will offer the highest chance of altering the therapeutic outcome of DHLs.[95]

In a high number of GCB-DLBCL patients, the loss of expression of *PTEN* and consequent constitutive activation of the PI3K/AKT pathway leads to upregulation of MYC.[136] PI3K inhibition was selectively toxic to PTEN-deficient GCB-DLBCL models, suggesting that PI3K inhibitors could have activity in DHL. [88,95]

Other approaches currently being explored include optimized mAbs: anti-CD20, -CD22 and -CD40,[137-140] anti-PD1,[141] and vascular endothelial growth factor (VEGF) antibodies.[142]

More recently, modified autologous T-cells engineered to recognize CD19, also known as CAR-T cells, have shown promise in clinical trials of R/R DLBCL and may have good results in DHL.[83,143]

Finally, kinase inhibitors may also play a role in future attempts to better treat DHL. Drugs such as the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib[144] or the PI3Kδ inhibitor idelalisib[145] may play a role in new combination approaches. The benefits of ibrutinib after ASCT in DHL is currently being evaluated in a phase II clinical trial (ClinicalTrials.gov identifier: NCT02272686).

1.5 Mantle Cell Lymphoma

MCL is a rare B-cell lymphoma that represents 5–10% of all NHLs, with an incidence of 0.8 per 100,000 persons.[146] It occurs primarily among elderly individuals with a median age of approximately 60 years (range 29-85), and a high male-to-female ratio (around 2-7:1).[44] The clinical evolution is usually very aggressive, with poor responses to treatment and frequent relapses; few patients are cured with current therapies. However, there is a more indolent form of the disease that has a long survival.[44,147]

1.5.1 Origin and physiopathology of MCL

1.5.1.1 MCL subtypes

MCL classically has been recognized as an aggressive small B-cell lymphoma that developed in a linear fashion from naïve B-cells. Paradoxically, a subset of patients follow an indolent clinical evolution with a stable disease, even in the absence of chemotherapy, reflecting, in part, that MCL develops along 2 very different pathways.

Classical MCL is usually composed of IGHV-unmutated or minimally mutated B-cells that usually express SOX11 (SRY (Sex Determining Region Y)-Box 11), features genetic instability and typically involves lymph nodes and other extranodal sites. Acquisition of additional molecular/cytogenetic abnormalities can lead to even more aggressive blastoid or pleomorphic MCL. Leukemic non-nodal MCL develop from IGHV-mutated SOX11− B-cells, carrying epigenetic imprints of GC experienced B-cells. It usually involves peripheral blood (PB), BM, and often spleen. These cases feature genetic stability and are frequently clinically indolent; however, secondary abnormalities, often involving *TP53*, may occur and

lead to very aggressive disease. A third MCL subtype, ***in situ* mantle cell neoplasia**, is characterized by the presence of cyclin D1+ cells; most typically in the inner zones of the follicles. Although disseminated, they appear to have a low rate of progression (Figure 6).[2]

SOX11 is a transcription factor that has been reported to block terminal B-cell differentiation in aggressive MCL by regulating *PAX5* expression. There is also data demonstrating a role for *SOX11* as a driver of pro-angiogenic signals in MCL through the regulation of platelet-derived growth factor A (PDGFA) that contributes to a more aggressive phenotype.[148]

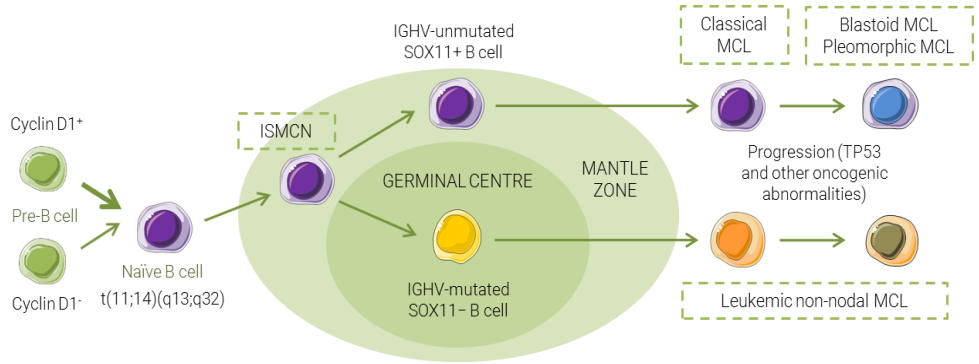

Figure 6 | **Hypothetical models of major subtypes of MCL.** Precursor B-cells may colonize the inner portion of the mantle zones, representing in situ mantle cell neoplasia (ISMCN). After the introduction of additional genetic and molecular abnormalities, ISMCN may progress, involving or not the transit through the GC, to classical MCL or leukemic non-nodal MCL respectively. More frequently classical MCL but also leukemic non-nodal MCL undergo additional molecular/cytogenetic abnormalities leading to clinical and sometimes morphological progression. Adapted from Swerdlow SH et al., Blood, 2016.[2]

1.5.1.2 Genetic alterations in MCL

Cyclin D1 overexpression

The t(11;14)(q13;q32) translocation leading to the constitutive overexpression of cyclin D1, is the genetic hallmark of MCL and is considered the primary genetic event. The role of cyclin D1 in promoting MCL lymphomagenesis is related to its function in cell cycle regulation, where participates in the control of the G1-S phase transition by binding to cyclin-dependent kinase 4 (CDK4) and CDK6 (Figure 7).[147,149]

Cyclin D1-negative MCL

A small subset (5%) of MCL is negative for cyclin D1 and the t(11;14), but have an expression profile and other features indistinguishable from conventional MCL. These cases have a high expression of cyclin D2 or cyclin D3, which highlight the relevance of the oncogenic deregulation of the G1 phase of the cell cycle in the pathogenesis of MCL.[149]

Secondary genetic events

CCND1, encoding cyclin D1, is a weak oncogene that requires the cooperation of other oncogenic events to transform lymphoid cells. MCL, especially classical MCL, represents one of the lymphoma subtypes with the highest genomic instability. Molecular studies have identified alterations in components of the cell-cycle regulation, DNA damage response, and cell survival pathways, but the profile of mutated genes contributing to the pathogenesis of MCL and cooperating with *CCND1*, is not well defined.[44,150]

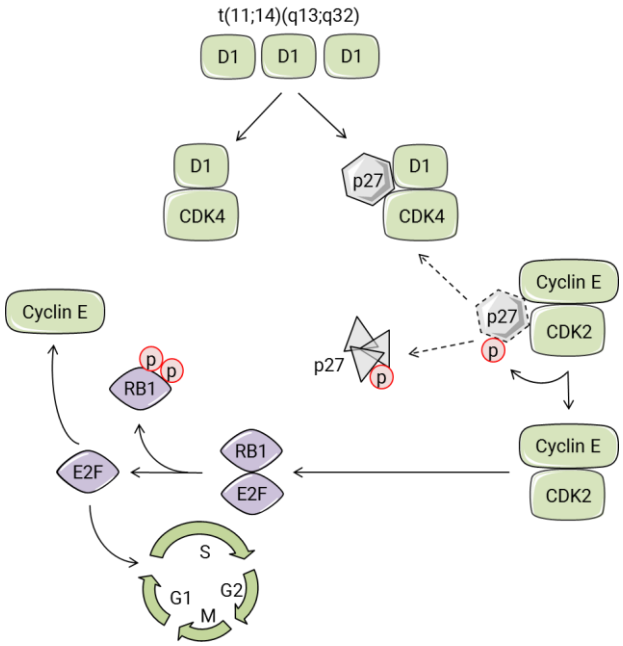

Figure 7 | **Cell cycle deregulation in MCL.** The t(11;14)(q13;q32) translocation results in the constitutive overexpression of cyclin D1. Cyclin D1 controls the G1-S-phase transition by binding to CDK4. Cyclin D1-CDK4 complexes phosphorylate and inactivate retinoblastoma 1 (RB1). The titration of p27 into cyclin D1-CDK4 complexes promote the increase of cyclin E-CDK2 active complexes that enhance p27 degradation and further phosphorylation of RB1, activating the transcription factor E2F and allowing the cell to progress into S phase. Adapted from Jares and Campo, Br J Haematol, 2008.[149]

The most common alterations further deregulating **cell cycle** in MCL include the deletion of the *CDKN2A* locus (9p21), implicated in the INK4a/CDK4/RB1 and ARF/MDM2/TP53 pathways. On the one hand, *CDKN2A* locus encodes for the CDK inhibitor INK4a (p16). It ensures the presence of high levels of cyclin D1/CDK4 activity. As an alternative to the loss of INK4a, the *BMI1* (polycomb ring finger) oncogene is amplified or highly expressed in some MCL cases. The direct inactivation of retinoblastoma 1 (*RB1*) could also occur in aggressive MCL variants. On the other hand, *CDKN2A* locus also encodes *ARF* (ADP-

Ribosylation Factor 1) that negatively regulates *MDM2* (murine double minute 2 homolog), which in turn promotes p53 degradation. These alterations, as well as *TP53* mutations (19-28% of MCL cases), lead to frequent deregulation of the p53 pathway, involved in cell cycle arrest, DNA repair and apoptosis.[147,151,152]

Table 2 | **Genetic alterations in MCL.**

Gene	Class/product
Cell cycle	
CCND1	Cyclin D1
CDK4	Cyclin-dependent kinase 4
MDM2	Murine double minute 2 homolog
RB1	Retinoblastoma 1
CDKN2A	INK4a and ARF
BMI1	BMI1 Proto-Oncogene, Polycomb Ring Finger
DNA damage / epigenetic modifiers	
TP53	Tumor protein p53
ATM	Ataxia-telangiectasia mutated
WHSC1 (NSD2)	Wolf-Hirschhorn syndrome candidate-1/ Nuclear Receptor Binding SET Domain Protein 2
KMT2D	Mixed-lineage leukemia protein 2
MEF2B	Myocyte-specific enhancer factor 2B
Cell survival	
BIRC3	Baculoviral IAP Repeat Containing 3
TRAF2	MAP3K14 negative regulator
MAP3K14	NF-kappa-B-inducing kinase (NIK)
IKBKB	Inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ)
TLR2	Member of the TLR family
PTEN	Phosphatase and tensin homolog
NOTCH1/2	NOTCH family members
SYK	Spleen Tyrosine Kinase
PKCβ II	Member of the protein kinase C family
Apoptosis	
BCL2	B-cell lymphoma 2
BCL2L11	Bcl-2-like protein 11 (BIM)

Recent genome-wide studies using next-generation sequencing have expanded the perspective of genes and pathways involved in the development of MCL. **DNA damage response pathway** dysfunction involves mutations of the DNA damage sensor *ATM*, which has been identified as the most common secondary alteration in MCL (42-55% of cases). Mutations in several chromatin modifiers such as *WHSC1* (10%), *KMT2D* (14%) and

MEF2B (3%) have also been detected, mostly in SOX11+ MCL. This differential distribution illustrates the relationship between genomic alterations and tumor heterogeneity. Additional pathways affected include **cell survival pathways**, which seems to contribute to the MCL oncogenesis. Constitutive activation of NF-κB, which regulates the expression of various genes involved in both survival and apoptotic signaling pathways, has been detected in MCL. *BIRC3* is the most commonly affected gene (6-10%) but there can be also mutations in *TLR2*, *TRAF2*, *MAP3K14* or *IKBKB*.

The PI3K/AKT/mTOR survival pathway is also activated in MCL; particularly in blastoid variants. It is associated with the loss of the PI3K phosphatase *PTEN* expression. Other alterations include activating mutations in *NOTCH1/2* (10% of cases), which are associated with an aggressive evolution of the tumor. Constitutive activation of the BCR signal transduction components *SYK* and *PKCβ II,* have been described in MCL. Within **apoptosis**, amplification of the anti-apoptotic *BCL2* and deletions of *BCL2L11*, member of the BH-3 only family with pro-apoptotic activity, have also been described in MCL (Table 2).[147,149,150,152,153]

1.5.2 Diagnosis

At diagnosis, 70% of patients or more have disseminated disease (stage III or IV),[44] with lymphadenopathy (75%), hepato-splenomegaly (35-60%), BM (>60%) and PB (13-77%) involvement. Other extranodal sites are frequently involved, including the gastrointestinal tract and Waldeyer's ring.[15,44]

Morphologically, four subtypes of MCL are recognized: the classic, the small cell, the blastoid and the pleomorphic variants. The last two subtypes have higher proliferation rates and are associated with inferior clinical outcome (Figure 8).[44,147]

The phenotype of MCL is relatively characteristic with high expression of the IgM/IgD surface Ig. By immunophenotyping, neoplastic cells are usually CD5+ and CD43+ and express the B-cell associated antigens: CD19, CD20, CD22, and CD79. They are usually negative for CD3, CD23, CD11c, CD10, and CD200. MCL cells are generally BCL-2 positive and BCL-6 negative.[44,149]

Demonstration of t(11;14)(q13;q32) by FISH or cyclin D1 overexpression by immunohistochemistry (ICH) is generally required to diagnose MCL, although a small number of cases are cyclin D1 negative. These cases have a high expression of cyclin D2 or cyclin D3; however, this is not helpful for diagnostics as these proteins are also overexpressed in other B-cell neoplasms. The nuclear SOX11 expression is a highly specific marker for both cyclin D1positive and negative MCL.[154]

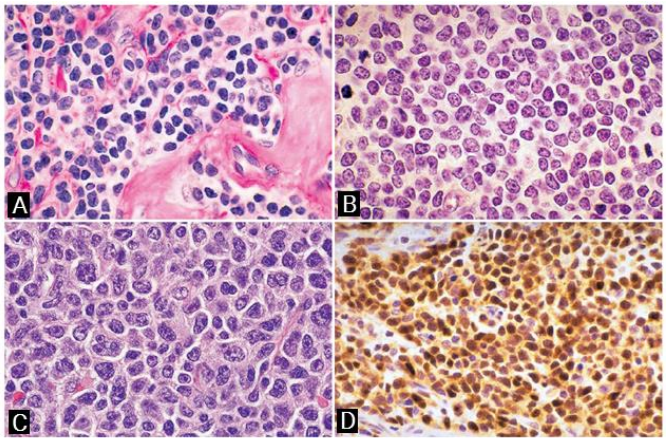

Figure 8. | **Mantle cell lymphoma.** (A) Classic variant. (B) Blastoid variant. (C) Pleomorphic variant. (D) Cyclin D1 immunostain. Adapted from Swerdlow et al., WHO Classification of Tumors, 2008.[15]

1.5.3 Prognostic factors

A specific MCL prognostic index, MIPI (MCL International Prognostic Index), classify MCL patients into low, intermediate, and high-risk groups, based on four independent prognostic factors: age, ECOG performance status,[43] LDH, and leukocyte count.[155,156] Other factors such as proliferation of the tumor, karyotypic complexity, genetic aberrations and DNA methylation are independent prognostic factors for MCL outcome.[147,157]

1.5.4 Treatments

1.5.4.1 Watch and wait strategy

Some newly diagnosed MCL patients can be diligently observed, deferring therapy to a later date. Asymptomatic, low tumor burden with non-nodal presentation and genetic stability are candidates for this strategy.[158] Delayed treatment in these patients does not adversely affect OS from time of treatment initiation.[159]

1.5.4.2 Current standard therapy (first-line treatment)

For patients in need of frontline therapy, the initial therapeutic decision is dictated by the age and the fitness of the patient. Although there is no accepted standard, there are 2 general approaches: HyperCVAD (hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone, alternating with cytarabine and methotrexate) alternating with high-dose immunochemotherapy, or HDC-ASCT.[152]

HyperCVAD usually combined with rituximab (R-HyperCVAD), leads to extremely high CR rates and durable responses.[160] The use of R-HyperCVAD alternating with high-dose methotrexate/cytarabine showed an ORR of 97%.[161] For patients that achieve remission, consolidation therapy is recommended.[162]

For older, less-fit patients there is no generally accepted front-line therapy. CHOP-based therapy, fludarabine and cyclophosphamide (FC), and bendamustine, have been the mainstay of therapy for them.[152]

Maintenance therapy is a well-established approach to postponing disease progression. Novel agents such as ibrutinib or lenalidomide are under investigation for their use as a maintenance therapy in MCL.[162]

1.5.4.3 Second-line therapy

ASCT was generally used in relapsed young patients but their use has better outcomes as first-line consolidation treatment.[163]

A growing number of novel biologically-targeted therapies are profoundly altering the landscape of MCL treatment options in both first-line and relapsed settings.[162]

There are currently four drugs licensed for use in MCL across the world: the proteasome inhibitor bortezomib (Velcade®), the mTOR inhibitor temsirolimus (Torisel®), the immunomodulatory agent lenalidomide (Revlimid®) and the BTK inhibitor ibrutinib (Imbruvica®). As single agents, the overall response rate (ORRs) for these drugs are 33%,[164] 22%,[165] 28%,[166] and 68%,[167] respectively (Table 3).

Table 3 | **Comparison of the drugs licensed for use in MCL.**

Treatment	Nº Patients	ORR	CR
Bortezomib[164]	155	33	8
Temsirolimus[165]	54	22	2
Lenalidomide[166]	134	28	8
Ibrutinib[167]	111	68	21

OOR, overall response rate; CR; complete response

With the possible exception of ibrutinib, it seems unlikely that these drugs will be used as single agents for the treatment of MCL outside of maintenance strategies, but they may have a role as part of combination therapy.[160,168] There are ongoing clinical trials with different combinations such as temsirolimus plus rituximab (OOR 60%; 19% CR), bortezomib with R-HyperCVAD (95% CR)[169] or lenalidomide plus rituximab (OOR 92%; 64% CR).[152,168,170]

1.5.4.4 Novel agents

Several novel agents using different target points have also been used with some reported efficacy in R/R MCL. Monotherapy novel agent studies are presented in Table 4. Among the most exciting recent advances in the management of B-cell malignancies, has been the development of CAR-T cells.[171] CDK4/6 inhibitors (e.g. abemaciclib,[163] palbociclib)[172]

are also an attractive therapeutic option given the role of cell-cycle deregulation in the pathogenesis of MCL. Anti-CD20 monoclonal antibodies (mAbs), such as ofatumumab[173] and obinutuzumab,[174] have single-agent activity in rituximab-treated patients and are good candidates to be used in combination with other therapies. Moreover, BH3 mimetic-type BCL inhibitors such as ABT-199 (venetoclax),[175,176] PI3Kδ inhibitors such as idelalisib,[177] HDACs inhibitors (e.g. abexinostat[178]), mTOR inhibitors (e.g. everolimus[179]) or other small molecules including some second-generation BTK inhibitors, are being developed and explored in MCL. Finally, the promising activity of anti-CD38 mAbs, such as daratumumab[180] in MM, has prompted the initiation of studies in other B-cell malignancies.[163,181]

Table 4 | **Novel agent studies in R/R MCL.**

Agent	Mechanism	Phase	Nº patients	%ORR (%CR)
Idelalisib	PI3Kδ inhibitor	I	40	40 (5)
Everolimus	mTOR inhibitor	II	58	8.6 (0)
Abexinostat	HDAC inhibitor	II	11	27.3 (0)
Venetoclax	BH3 mimetic	I/II	12	75 (0)
Abemaciclib	Cell cycle inhibitor (CDK4/6)	II	22	22 (0)

Adapted from Atilla E et al., Int J Hematol, 2017.[163]

1.6 Chronic Lymphocytic Leukemia

CLL is the most common leukemia in adults in the Western world, accounting for 25% of NHLs. CLL has an annual incidence of about 2-6 new cases per 100,000 people per year, increasing with age, reaching 12.8/100,000 at age 65. The median age at diagnosis is 65-70, with male predominance (1.5-2:1).[15,182]

There is a large variation in survival among individual patients, ranging from several months to a normal life expectancy.[15,182] A small fraction of CLL cases (around 2-15%) transform into a very aggressive form, known as Richter's syndrome (RS), which morphologically resembles DLBCL and is associated with a very poor clinical outcome.[183-185]

1.6.1 Origin and physiopathology of CLL

Most cases of CLL are preceded by Monoclonal B-cell Lymphocytosis (MBL), an indolent expansion of B lymphocytes characterized by less than $5x10^9$ B lymphocytes/L, but with a typical CLL immunophenotype. MBL is detectable in approximately 3% of healthy donors and will progress into overt malignancy only in a minor portion of cases.[186]

1.6.1.1 CLL subtypes

The IGHV mutational status defines subgroups of patients with CLL with distinct prognosis: those carrying unmutated *IGHV* genes (U-CLL) experienced an aggressive clinical course with clonal evolution and resistance to therapy, which translated into a shorter OS when compared with patients carrying mutated *IGHV* genes (M-CLL). U-CLL derived from antigen-experienced cells that acquire a memory phenotype in a T-, GC-independent fashion. Contrarily, M-CLL, with evidence of SHM, seems to derive from antigen-experienced post-germinal B-cell (Figure 9).[187,188] Both CLL subtypes display a highly restricted and biased repertory of IGHV genes (stereotyped BCR),[189] suggesting a derivation from progenitors that are reminiscent of antigen-experienced B-cells. These stereotyped BCR indicate that some BCR rearrangements have been positively selected by interactions with specific antigens, suggesting that this selection may be on the basis of disease development.[190]

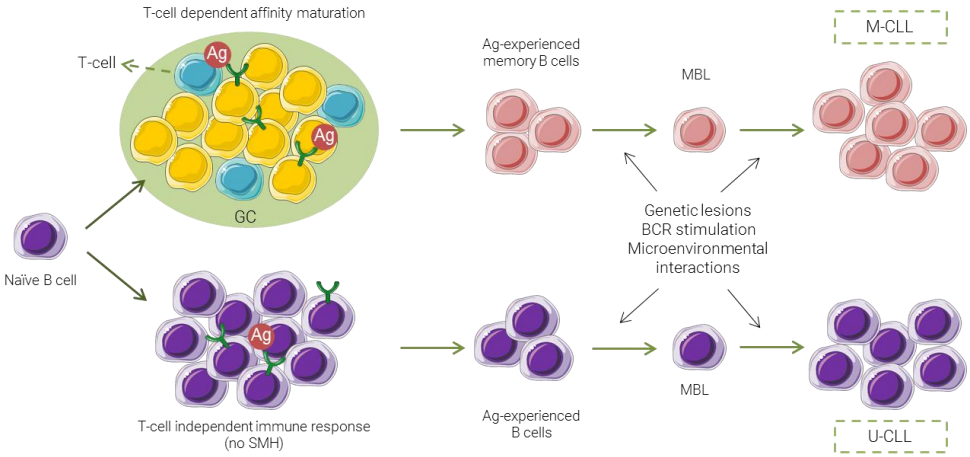

Figure 9 | **A model for the cellular origin of CLL.** The encounter of naive B-cells with antigen may proceed either through a T cell-dependent reaction occurring in the GC and leading to the generation of memory B-cells that have undergone somatic hypermutation of IGHV genes or in T cell-independent immune responses that may lead to the formation of antigen-experienced B-cells harboring unmutated IGHV genes. CLL originating from B-cells that have experienced somatic hypermutation carry mutated IGHV genes and are defined as M-CLL. Conversely, CLL originating from B-cells that have been involved in T cell-independent immune reactions harbor germline IGHV genes and are defined as U-CLL. MBL: monoclonal B-cell lymphocytosis. Adapted from Gaidano et al., J Clin Invest, 2012.[191]

1.6.1.2 Genetic alterations in CLL

Cytogenetic alterations in CLL

Although there is no CLL-specific genomic hallmark, more than 80% of CLL patients carry cytogenetic alterations at diagnosis. These genomic aberrations are important independent predictors of disease progression and survival. The most frequent alteration is the deletion 13q14 that occurs in 55% of CLL cases. This lesion may be an early event in the disease as it is often found as a single lesion and it is detectable at a similar frequency in MBL.[192] Other recurrent cytogenetic alterations include 12 trisomy (16% of cases), 11q22-23 deletion (18% of cases) and 17p13 deletion (6% of cases); table 5.[193]

Deletion of the 11q22-23 region, associated to ATM inactivation, has been shown to promote disease progression through its functional loss in regulation of cell cycle and DNA damage responses.[194] Deletion of 17p13 is associated with the inactivation of p53, thereby causing deregulation of apoptosis and cell cycle.[195]

Table 5 | **Recurrent cytogenetic alterations in CLL.**

Alteration	Frequency (%)	Biological effect
del13q14	50-60	Suppression of *MIR15A/MIR16A* that leads to alteration in NK-κB activity and overexpression of BCL-2[196,197]
Trisomy 12	15	Associated to NOTCH1 activation[198]
del11q22-q23	15	Inactivation of *ATM*, promoting deregulation of cell cycle and DNA damage[194]
del17p13	5-10	Inactivation of *p53* tumor supressor[195]

Other genetic alterations in CLL

Genome-wide analyses have enabled the characterization of recurrent genetic lesions present in the CLL genome, hence providing further insights into the disease pathogenesis and progression (Table 6).[199] According to these studies, the so-called "clonal" mutations are found in all or almost all tumor cells, and probably constitute founder alterations (e.g. trisomy 12, del13q14), whereas "subclonal" mutations are probably acquired over the course of the disease (e.g. *TP53, ATM, SF3B1*).[200]

These lesions affect genes implicated in different biological pathways including: NOTCH signaling (*NOTCH1* mutations; 10-20%),[201,202] mRNA processing (*SF3B1*; 5-15%),[203-205] inflammatory pathways (*MYD88*; 3-10%),[199,203] survival pathways (*BIRC3*; 4%),[206] cell cycle control (*TP53*; 10-15%),[203,207] telomere integrity (*POT1*; 3.5%)[208] and DNA damage (*ATM*; 11%).[207] Other lower frequency mutations identified are *XPO1, KLHL6*, and *CHD2*.[199,205,209]

Table 6 | **Recurrent genetic alterations in CLL.**

Gene	Class/product
TP53	Tumor protein p53
ATM	Ataxia-telangiectasia mutated
POT1	Protein essential for telomere function
BIRC3	Baculoviral IAP Repeat Containing 3
SF3B1	Splicing machinery cofactor
NOTCH1	NOTCH family member
MYD88	Adapter protein (activation of TLR pathway)

In contrast to the low number of highly recurrent gene mutations in CLL, genome-wide methylation studies have shown that CLL transformation is associated with a massive hypomethylation phenomenon frequently affecting the enhancer regions. Single genes with prognostic impact, such as the *ZAP70* (Zeta chain of T-cell receptor Associated Protein Kinase 70), have been identified within this epigenetic complexity.[210-212] The DNA methylation patterns have found to be different between U-CLL and M-CLL, similarly to the epigenetic imprints of their putative cells of origin.[212]

1.6.2 Diagnosis

In CLL patients, PB, BM, lymph nodes, liver, and spleen are typically infiltrated, and other extranodal sites may occasionally be involved. Most patients are asymptomatic, but some present with fatigue, autoimmune hemolytic anemia, splenomegaly, hepatomegaly, lymphadenopathy or extranodal infiltrates.[15] CLL is associated with a wide range of infectious, autoimmune, and malignant complications. These complications result in considerable morbidity and mortality that can be minimized by early detection and aggressive management.[213]

Morphologically, CLL cells are small mature-appearing lymphocytes with dense chromatin, a nucleus that virtually fills the cell with only a rim of visible cytoplasm and no (or occasionally small) nucleoli. In CLL, the presence of smudged cells on the blood smear is common and represents lymphocytes that were crushed in the process of making the slide. CLL cells also can appear as prolymphocytes, which are larger than typical CLL cells, have less-condensed nuclei and a single prominent nucleolus (Figure 10).[211,214]

According to the International Workshop on CLL (IWCLL) 2008, CLL diagnosis requires the evaluation of the blood count, blood smear, and the immune phenotype (by flow cytometry or immunohistochemical analyses) of the circulating lymphoid cells.[214] The lymphocyte count has to be of at least 5 x 10^9 B lymphocytes/L (5000/µL) in the peripheral blood for at least three months and the clonality should be confirmed by flow cytometry.[13,215]

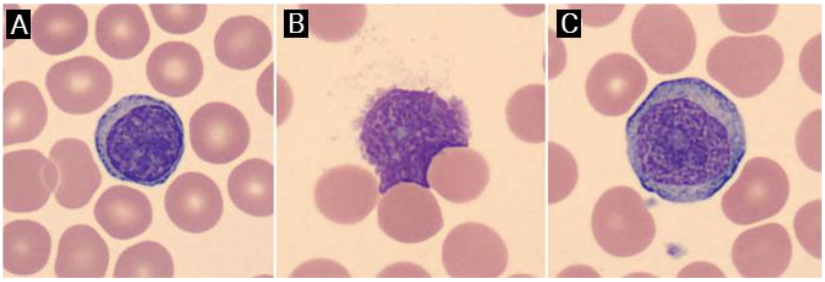

Figure 10 | **Chronic lymphocytic leukemia.** (A) Typical CLL B lymphocyte (B) Smudge cell (C) Prolymphocyte with prominent nucleolus Adapted from Kipps et al., Nat Rev Dis Primers, 2017.[211]

CLL B-cells typically express CD5, CD19, CD23, CD79a and CD43 and have low levels of CD20 and CD79b and lack of expression of CD10. CLL also express CD200, which helps to distinguish CLL from MCL.[15]

When such a population is detected in enlarged lymph nodes of patients without peripheral lymphocytes, the term small lymphocytic lymphoma (SLL) is used, indicating a clinical variant of the same histopathological and molecular entity. In reality, the heterogeneity within CLL or SLL is greater than the differences between CLL and SLL.[216]

1.6.3 Prognostic factors

Two clinical staging systems are widely used to divide patients with CLL into three broad prognostic groups. The Rai staging system,[217] more commonly used in the United States, and the Binet classification,[218] more commonly used in Europe. Both staging systems define late-stage, or high-risk, disease by the presence of pronounced anemia or thrombocytopenia.[211] These systems provide a simple inexpensive tool to identify those patients who are suitable for observation alone vs. those patients who require therapy.[217,218]

Genetic abnormalities, biochemical abnormalities, and patient characteristics are prognostic factors that add information to the classic clinical staging systems.[219,220]

IGHV mutation statues and molecular cytogenetics has remarkable impact on survival; hence, the distinction between M-CLL and U-CLL is clinically meaningful, while unmutated *IGHV* is a predictor of a bad prognosis.[191] Mutations in *TP53*, *ATM*, *NOTCH1, SF3B1*, and *BIRC3* are shown to have an adverse prognostic impact[219,221,222] while patients harboring del13q14 as the sole genetic lesion are included in a low-risk group of patients.[223-225]

High levels of CD49d (≥30%),[226,227] ZAP70 (≥20%),[228] and CD38 (≥30%),[229-231] as well as high levels of serum markers like β2-microglobulin, serum thymidine kinase, and soluble CD23[232] are associated with poor prognosis and a decreased response to chemotherapy.

Patients who are less than 65, with less comorbid conditions, show less frequent rates of severe infections related to the treatment.[223,233] An international prognostic index for CLL (CLL-IPI) has been created to integrate the major prognostic parameters. It discriminates low, intermediate, high and very high-risk prognostic subgroups.[234]

1.6.4 Treatments

1.6.4.1 Watch and wait strategy

In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0; Binet A; CLL-IPI low), should be monitored without therapy unless they have evidence of disease progression.[235] Whereas patients at intermediate (Rai I and II; Binet B; CLL-IPI intermediate) and high risk (Rai III and IV; Binet C; CLL-IPI high) usually benefit from the initiation of treatment, some of these patients can be monitored without therapy until they have evidence for progressive or symptomatic disease.[214,234,236]

1.6.4.2 Current standard therapy (first-line treatment)

The initial therapeutic decision is dictated by the illness stage and the fitness of the patient (Table 7).[237] The vast majority of CLL patients have an impaired physical condition (slow go) and may be offered a mild chemotherapy regimen containing chlorambucil for symptom control, but also dose-reduced fludarabine or bendamustine should be considered.[236] Chlorambucil in combination with an anti-CD20 antibody (obinutuzumab, rituximab) seems to lead to a higher number of CRs.[238] In the case of patients in good physical condition (go go), chemoimmunotherapy combination with rituximab plus fludarabine and cyclophosphamide (FCR) is the standard for treatment with an ORR of 95%.[239,240] Another possibility is the combination of bendamustine with rituximab (BR) with an ORR of 88%.[241]

The presence of del17p or mutated *TP53* carry a very dismal prognosis and there is no definitive data on the most effective first-line treatment. These patients can benefit from an alemtuzumab (anti-CD20)-containing regimen. Combinations of alemtuzumab with methylprednisolone (steroid) are amongst the most potent therapies, yielding ORR of 88% in previously untreated cases (all with *TP53* abnormalities), with 65% of cases achieving a CR.[242] More recently, novel kinase inhibitors have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in first-line for patients with 17p deletion (if chemotherapy is contraindicated). The approved agents include the BTK inhibitor ibrutinib and the PI3Kδ inhibitor idelalisib.[243]

Table 7 | **CLL first-line treatment.**

	Fitness	Del17p/ p53 mut	Therapy
Rai 0-II, Binet A-B, CL-IPI low-intermediate; inactive	Irrelevant	Irrelevant	Watch & wait
Rai III-V; Binet C; CLL-IPI high; active disease	Go go	No	FCR (BR above 65 years?)
		Yes	Ibrutinib, idelalisib + rituximab (allogeneic SCT)
	Slow go	No	Chlorambucil + obinutuzumab or + rituximab or + ofatumab
		Yes	Ibrutinib, alemtuzumab, HD rituximab or ofatumumab

F, fludarabine; C, cyclophosphamide; R, rituximab; B, bendamustine; SCT, stem cell transplantation; HD, high dose. Adapted from Hallek M et al., Am J Hematol, 2015.[235]

1.6.4.3 Second-line therapy

There is no standard treatment for R/R patients and the regimen choice is mainly based on first-line treatment and patient fitness (Table 8).

New therapies that have been recently approved for patients with R/R CLL include: the BTK inhibitor ibrutinib (ORR of 71%),[244] the PI3Kδ inhibitor idelalisib (plus rituximab; ORR of 81%),[245] the human anti-CD20 ofatumumab (CLL refractory to fludarabine and alemtuzumab; ORR 49%),[246] and the BCL-2 inhibitor venetoclax (for a patients harboring del17p who have received one prior therapy; ORR 85%).[247]

Table 8 | **CLL second-line treatment.**

	Fitness	Standard therapy	Alternatives (trials)
Refractory or progress within 2 years	Go go	Ibrutinib, FA, FCR, → Allogeneic SCT (?)	Lenalidomide, BR, (other kinase inhibitors, venetoclax)
	Slow go	Change therapy (include in trial)	Ibrutinib, idelalisib + rituximab, alemtuzumab for del17p, venetoclax, FCR-lite, BR, lenalidomide, ofatumumab, HD rituximab
Progress after 2 years	All	Repeat first-line therapy	

F, fludarabine; A, alemtuzumab; C, cyclophosphamide; R, rituximab; SCT, stem cell transplantation; B, bendamustine; HD, high dose. Adapted from Hallek M et al., Am J Hematol, 2015.[235]

1.6.4.4 Novel agents

Besides the recently approved agents, other novel exciting treatment approaches started being tested in the CLL field. These include second- and third-generation of BTK inhibitors, new PI3K inhibitors, checkpoint inhibitors, CAR-T cells, new mAbs and immunomodulatory drugs among others (Table 9).[211,243,248]

Table 9 | **Novel agents under development for the treatment of CLL.**

Agent	Phase	%ORR	Indication
BTK inhibitors			
cc-292[249]	I	53%	R/R CLL
ONO/GS-4059[250]	I	96%	R/R CLL
Acalabrutinib (ACP-196)[251]	I/II	95%	R/R CLL
PI3K inhibitors			
Duvelisib (IPI-145)[252]	I/II-III*	52-55% (phase I)	R/R CLL
TGR-1202[253]	I	94% PR	R/R CLL
Checkpoint inhibitors			
Pembrolizumab (MK-3475) (anti-PD-1)[254]	II	Ongoing (NCT02332980)	R/R CLL
mAbs			
Ublituximab (anti-CD20)[255]	I/II	50%	R/R CLL
Otlertuzumab (TRU-016) (anti-CD37)[256]	Ib#	54%	Untreated + R/R CLL
Immunomodulatory drugs			
Lenalidomide	II†	ongoing	Maintenance
CAR T-cells[257,258]	I	57%	R/R CLL

*Phase II and III trials of duvelisib alone or in combination with other agents are ongoing. ClinicalTrials.gov identifiers: NCT02711852, NCT02049515; # otlertuzumab + rituximab; † lenalidomide + rituximab as a maintenance therapy after Bendamustine + Rituximab chemoimmunotherapy (ClinicalTrials.gov identifier: NCT01754857).

1.7 Follicular lymphoma

FL is an indolent disease characterized by an abnormal proliferation of mature B-cells in the GC of lymphoid follicles.[15] FL has an annual incidence of 2-3 new cases per 100,000 people,[259] and it affects predominantly adults, with a median age in the 6th decade at diagnosis and a male:female ratio of 1:1.7. FL rarely occurs in individuals under age of 20 years; pediatric patients are predominantly males.[15]

It is generally considered incurable as the majority of patients undergoes multiple relapses and eventually develops resistance to standard therapies.[260] Furthermore, around 30-40%

of cases progresses towards histologic transformation to an aggressive malignancy, typically represented by DLBCL.[261,262]

1.7.1 Origin and physiopathology of FL

FL derives from the clonal expansion of multiple follicles containing apparently intact GCs with active SHM activity.[8,263] The observations that the neoplastic cells are organized in follicles, express GC surface markers and have a characteristic gene expression profile of GC B-cells, all suggest the GC B-cell origin of these tumors.[263-265]

The t(14;18)(q32;q21) translocation can be detected by FISH in 85% of all cases, and results in overexpression of anti-apoptotic BCL-2, considered a molecular hallmark of the disease.[15,266] It occurs due to a repair error during the V(D)J recombination process, at an early B-cell developmental stage in the BM.[267] Naive B-cells carrying the t(14;18) exit the BM and colonize secondary lymphoid tissue, where they undergo the GC reaction, but have a survival advantage due to their constitutive expression of BCL-2, which is not normally expressed in the GC.[268] BCL-2 may also rescue these cells from apoptosis due to weak BCR affinity.[269] Although BCL-2 provides a survival advantage that favors the acquisition of additional genetic aberrations during repeated GC transits,[270] BCL-2 overexpression is insufficient to induce lymphomagenesis as B-cells beating t(14;18) have been detected in the blood of healthy individuals, suggesting that these cells may selectively accumulate additional damage(s).[269,271]

Despite the loss of one Ig allele by the t(14;18) translocation, surface immunoglobulin (sIg) is retained. The variable region genes of this sIg carry sequence motifs for N-glycan additions that are introduced during SHM.[272,273] Oligomannose sugars are covalently bound within the antigen-binding region, suggesting a potentially important interaction of FL cells with mannose-binding lectins of the tumor microenvironment,[274] allowing FL cells to receive a constitutive antigen-independent stimulation through their BCR that activate survival and proliferation pathways.[275-277]

1.7.1.1 Genetic alterations in FL

Chromosomal alterations in FL

Apart from the t(14;18)(q32;q21) translocation, the most common chromosomal aberrations in FL include non-random losses of 6q (25-30%) and 1p36 (20-25%), as well as gains of 7 (~25%), X, 12q and 18q chromosomes.[278-281] These aberrations can constitute the most common distinct events arising secondary to t(14;18) in the early development of the FL.[266,282]

One of the most frequent secondary genetic abnormalities in FL are 6q deletions, which entails the loss of *TNFAIP3/A20*, a negative regulator of NF-κB signaling, as well as the receptor tyrosine kinase *EPHA7*, a potential tumor suppressors in FL.[281,283]

Other genetic alterations

GC-derived lymphomas are characterized by frequent mutations of histone-modifying genes.[22,266] In FL in particular, recurrent mutations have been reported in the histone methyltransferases *KMT2D*, that encodes for MLL2 protein (82-89%)[260,284] and *EZH2* (7.2-27%),[29,285] the histone acetylases *CREBBP* (32.6%),[22] *EP300* (8.7%),[22] and *MEF2B* (15.3%),[284] as well as the epigenetic regulator *ARID1A* (11%).[286] The high recurrence of these mutations illustrates that FL is likely a disease of the epigenome as well as the genome.[287] A negative regulator of NF-κB signaling, *TNFRSF14,* inactivation is found in 18-46% of FL cases. This inactivation can be due to 1p36 deletions or by somatic mutations, and is considered as a significant predictor of poor OS.[288,289] Other recurrent genetic aberrations include *TP53* mutations (<5% of cases) and *BCL6* translocation or deletion (39% of cases), both associated with the histologic transformation of FL. All these genetic alterations are summarized in table 10.

Table 10 | **Recurrent genetic alterations in FL.**

Gene	Class/product
BCL2	B-cell lymphoma 2
KMT2D	Mixed-lineage leukemia protein 2
IGHV/IGLV	N-glycosylation motifs
EPHA7	EPH Receptor A7
BCL6	B-cell lymphoma 6
TNFRSF14	TNF receptor superfamily member 14
CREBBP	CREB-binding protein
MEF2B	Myocyte-specific enhancer factor 2B
EP300	Histone acetyltransferase p300
EZH2	Enhancer of Zeste Homolog 2
ARID1A	AT-Rich Interaction Domain 1A
TNFAIP3/A20	Negative regulator of NF-κB signaling
TP53	Tumor protein p53

Adapted from Kridel R et al., J Clin Invest et al., 2012[266] and Kishimoto W et al., J Clin Exp Hematop, 2014[290]

1.7.2 Diagnosis

Patients with FL typically exhibit superficial lymph nodes of small to medium size, sometimes unnoticed or neglected by the patients for a prolonged period of time. Apart of the lymph nodes, FL also involves spleen, BM, PB, and Waldeyer ring. Involvement of non-hematopoietic extranodal sites may occur in a setting of widespread nodal disease.[15]

Most cases of FL have a predominantly follicular pattern with closely packed follicles that efface the nodal architecture. FL is typically composed of the two types of B-cells normally found in GC, centrocytes and centroblasts, and meshworks of follicular dendritic cells (FDCs) are present in follicular areas.[15]

Grading of lymph node biopsies is carried out according to the number of blasts/high-power field into grades 1, 2, 3A and 3B (Figure 11).[291] FL grade 3B, which resembles DLBCL in many features,[292,293] is considered an aggressive lymphoma, whereas grades 1, 2 and 3A should be treated as an indolent disease.[294] Mitotic counting following phosphohistone H3 immunohistochemistry correlates well with centroblast-based grading and offers a reliable quantification tool that can be recommended as an additional parameter for the precise sub-categorization of FL cases.[295]

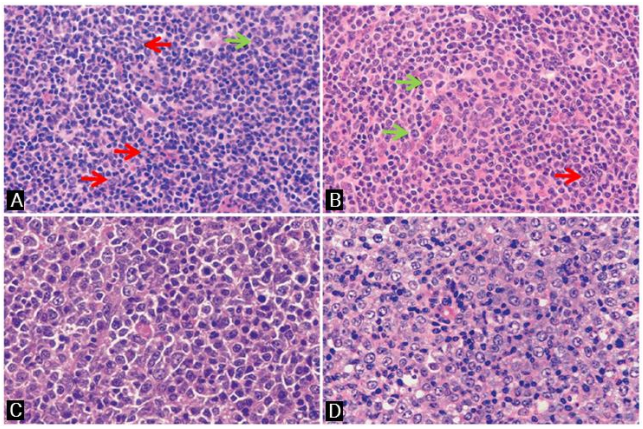

Figure 11 | **Follicular lymphoma grading.** (A) Grade 1-2 of 3. There is a monotonous population of small cells with irregular nuclei (centrocytes) with only rare large cells (centroblasts) with 1 or more basophilic nucleoli and a moderate amount of cytoplasm. Most of the large nuclei present in this field are those of FDCs (red arrows). (B) Grade 1-2 of 3. The majority of the cells are centrocytes, but more numerous centroblasts are present (green arrows). (C) Grade 3A. There are more than 15 centroblasts per high-power field, but centrocytes are still present. (D) Grade 3B. The majority of the cells are centroblasts. Adapted from Swerdlow et al., WHO Classification of Tumors, 2008.[15]

Since treatment largely depends on the stage of the disease, initial staging should be thorough. The staging is carried out according to the Ann Arbor classification system,[44] with mention of bulky disease (>7 cm) when appropriate.[291]

Diagnosis of FL is primarily based on a combination of physical examination, laboratory tests, serology, imaging, BM aspirate and biopsy, and toxicity evaluations.[291]

The immunophenotypic analysis of the cells can be used to confirm the diagnosis of FL. FL cells are usually sIg+ and express B-cell associated antigens, such as CD20, CD19,

CD22, and CD79a. Most cases are positive for the GC antigen CD10 and are negative for CD5 and CD43. Staining for FDC (CD21/CD23) may be necessary to distinguish between large follicles and diffuse areas.[15]

In addition, evaluation of *BCL2* gene rearrangement by FISH may assist in establishing a diagnosis of FL. *BCL2* translocation is neither necessary nor sufficient for diagnosis, as it is absent in 15% of FLs and present in about 10-15% of GCB-type DLBCLs.[20,266,296] Many grade 3B FL lack t(14;18)(q32;q21) and CD10 expression, but present *BCL6* translocation and increased p53 and MUM1/IRF4 expression, corresponding to a more aggressive phenotype.[292]

1.7.3 FL transformation

FL undergoes histologic transformation to a more aggressive malignancy, typically DLBCL, in around 30-40% of cases.[261,262] Transformed FL is a distinct disease that harbors unique combinations of oncogenic and tumor suppressor lesions compared to *de novo* DLBCL.[262]

The gold standard definition of higher-grade transformation is based on the histological demonstration of an increased proportion of large cells diffusely infiltrating the lymph nodes and effacement of the normal follicular architecture. It may be localized with persistence of a follicular pattern of proliferation in other lymph nodes.[259]

Discrete transformation-associated genetic alterations have been described involving genes implicated in cell cycle regulation, DNA damage response and proliferation (*MYC, CDKN2A, TP53, MYD88, CARD11, FOXO1*) and in immune evasion (*B2M*).[260,262,297-299] Together with this loss of genetic stability and deregulated proliferation, an increase in DNA methylation and a number of tumor microenvironment changes contribute to this progression.[262,300]

1.7.4 Prognostic factors

FL is a biologically heterogeneous disease, and the prognosis varies widely among individuals. It is important to determine factors associated with response to treatment and survival in order to guide treatment selection.[301]

In 2004, the Follicular Lymphoma International Prognostic Index (FLIPI) was developed. The index was based on age, stage, hemoglobin, the number of nodal site areas, and LDH.[302] Despite a widely accepted tool for risk assessment of FL, it was been built before the rituximab era and the initial cohort does not represent the present course of the disease. A new prognostic index, FLIPI-2, incorporates ages over 60, lymph node size larger than 6 cm, BM involvement, elevated β2-microglobulin and anemia as independent risk factors for progression-free survival (PFS).[303] More recently, a new clinic-genetic risk model (termed m7-FLIPI) has been established. It includes the mutation status of seven

genes (*EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP*, and *CARD11*), the FLIPI, and ECOG performance status (Table 4).[304]

1.7.5 Treatments

Although FL is considered incurable with standard chemotherapy, advances in treatment and our understanding of its biology have improved disease management and clinical outcomes. Treatment for FL varies among individuals depending on the symptomatology, the aggressiveness of the tumor, and general health (Figure 14).[305]

1.7.5.1 Watch and wait strategy

Asymptomatic, low-tumor-burden patients may be candidates for a strategy of watch and wait.[306] One randomized clinical trial compared single-agent rituximab to wait and watch and found that despite both groups of patients had the same OS, some benefits are associated with immediate rituximab therapy, such as improved PFS and a longer time to first chemotherapy, as well as a quality-of-life benefit in a subset of patients with particular difficulty adjusting to their diagnosis.[307]

When FL is localized to one area, it is important to distinguish between FL *In Situ* (FLIS) and Partial involvement FL (PFL). FLIS has a very low rate of progression to clinically significant FL, and conservative management (watch and wait) is advisable in cases where initial staging is negative for other sites of disease, whereas PFL patients are more likely to develop FL and local radiotherapy or rituximab monotherapy may be given.[291,305,308,309]

1.7.5.2 Current standard therapy (first-line treatment)

The majority of FL patients show advanced and symptomatic disease at diagnosis and, therefore, need therapy.

The addition of rituximab to conventional chemotherapy has improved outcomes in FL, including RR, PFS, event-free survival, and OS. The standard first-line treatment is rituximab in combination with chemotherapy,[291] being R-CHOP the most widely adopted regimen, with an ORR of 93%. Other combinations are R-CVP (rituximab plus cyclophosphamide, vincristine, and prednisone), with an ORR of 88%, R-FM (rituximab plus fludarabine and mitoxantrone) with an ORR of 91%.[310] BR (bendamustine plus rituximab) with an ORR of 93%, has gained widespread adoption as the preferred first-line treatment approach because of increased progression-free survival and fewer toxic effects than R-CHOP.[311]

A novel strategy, combining the immunomodulatory agent lenalidomide with rituximab, has an ORR of 98%. An international phase 3 study (NCT01476787) to compare this regimen with chemotherapy in patients with untreated FL is in progress.[312]

Rituximab maintenance improves PFS.[313,314] Another option for consolidation therapy is RadioImmunoTherapy, which consists in a combination of CD20 mAb with a radioactive isotope. The most widely used is ^{90}Y-ibritumomab tiuxetan (Zevalin®).[315,316]

1.7.5.3 Second-line therapy

Remission may last for several years, but the disease does return in most patients. For those patients with R/R FL, second-line therapies are often successful in providing another remission.

In early relapses (<12-24 months), a non-cross-resistant scheme should be preferred (bendamustine after CHOP or vice versa). Other options, including fludarabine-based, platinum salts-based or alkylating agents-based regimens, could also be useful. Rituximab monotherapy may be applied in symptomatic patients with low tumor burden.[291] Obinutuzumab plus bendamustine followed by obinutuzumab maintenance has improved outcome in rituximab-refractory patients.[317] Zevalin® also may represent an effective therapeutic approach, producing durable responses and prolonged OS (53% at 5 years).[318]

In later relapses, the PI3Kδ inhibitor idelalisib has been registered in double-refractory FL, based on a phase II study, where it has an ORR of 57% and a median PFS of 11 months.[319] Recent analyses suggest an increased mortality risk as a consequence of pulmonary morbidity, so appropriate prophylaxis is strongly recommended.[320,321] In selected younger patients with later relapses of high-risk profiles, autologous or allogeneic SCT may also be considered.[322]

In the case of transformed FL, there is no standard treatment. Usually, this entity is treated by analogy with the treatment of R/R DLBCL.[261] The advent of rituximab has improved the outcome of this group of patients.[323]

1.7.5.4 Novel agents

With the expanding knowledge of the pathogenesis of B-cell malignancies, in the last few years, several new therapies acting through a variety of mechanisms have shown promising results, including BTK inhibitors, BCL-2 inhibitors, PI3K inhibitors, EZH2 inhibitors, checkpoint inhibitors, and new mAbs among others.[324-326] Some of them are actually being studied on FL (Table 11).

Table 11 | **Novel agent studies in FL.**

Agent	Phase	%ORR	Indication
BTK inhibitors			
Ibrutinib[327]	I/Ib	90	R/R NHL
BCL-2 inhibitors			
Venetoclax[176]	I	38	R/R FL
PI3K inhibitors			
Copanlisib[328]	II	47	R/R FL
Duvelisib[329]	I	65-73	R/R FL
Duvelisib[330]	II	41	R/R NHL
EZH2 inhibitors			
Tazemetostat[331]	I	N/A	R/R FL
Checkpoint inhibitors			
Pidiluzumab + rituximab[332]	II	66	R/R FL
Nivolumab[333]	I	40	R/R FL
mAbs			
Epratuzumab + rituximab[334]	II	88	Untreated FL
Blinatumumab[335]	I	80	R/R FL
Ofatumumab + CHOP[336]	II	100	Untreated FL
Ofatumumab[337]	III	10	R/R FL
Obinutuzumab[338]	II	55	R/R NHL
immunomodulatory drugs			
lenalidomide[339]	II	53	R/R FL
CAR T-cells[340,341]	I	N/A	R/R FL

2. TARGETING SIGNALING PATHWAYS IN LYMPHOID NEOPLASMS

2.1 BCR signaling pathway

2.1.1 B-cell receptor (BCR) signaling

Despite accumulating mutations and translocations in the IGHV locus, malignant B-cells typically maintain expression of the BCR on the cell surface, suggesting that they may utilize the ability of the BCR to engage downstream proliferation and survival pathways.[7] B-cells require an intact BCR for survival and its depletion leads to rapid cell death.[342]

The BCR is composed of two Ig HC and two Ig LC forming the extracellular, antigen-binding part of the BCR. The BCR itself does not contain any signaling motifs but instead is linked to the CD79a-CD79b (Igα and Igβ, respectively) heterodimer, conforming the

cytoplasmatic tail of the BCR, which have immunoreceptor tyrosine-based activation motifs (ITAMs) and regulate BCR surface expression, internalization, and trafficking. Upon antigen encounter and BCR clustering, CD79a and CD79b are phosphorylated by an SRC family protein tyrosine kinase, most probably LYN, in their ITAMs. Dually phosphorylated ITAMS recruit and activates SYK.[7] In parallel, LYN phosphorylates tyrosine residues in the cytoplasmic tail of the BCR co-receptor CD19, which enables the activation of PI3K and VAV.[343] PI3K phosphorylates PIP2 to PIP3, which promotes the recruitment of several molecules, including SRC protein kinase BTK, to the cell membrane. BTK recruits and phosphorylates BLNK, which serves as a scaffold for various signaling molecules, including SYK, BTK and its crucial substrate phospholipase C- γ2 (PLCγ2). PLCγ2 mediates

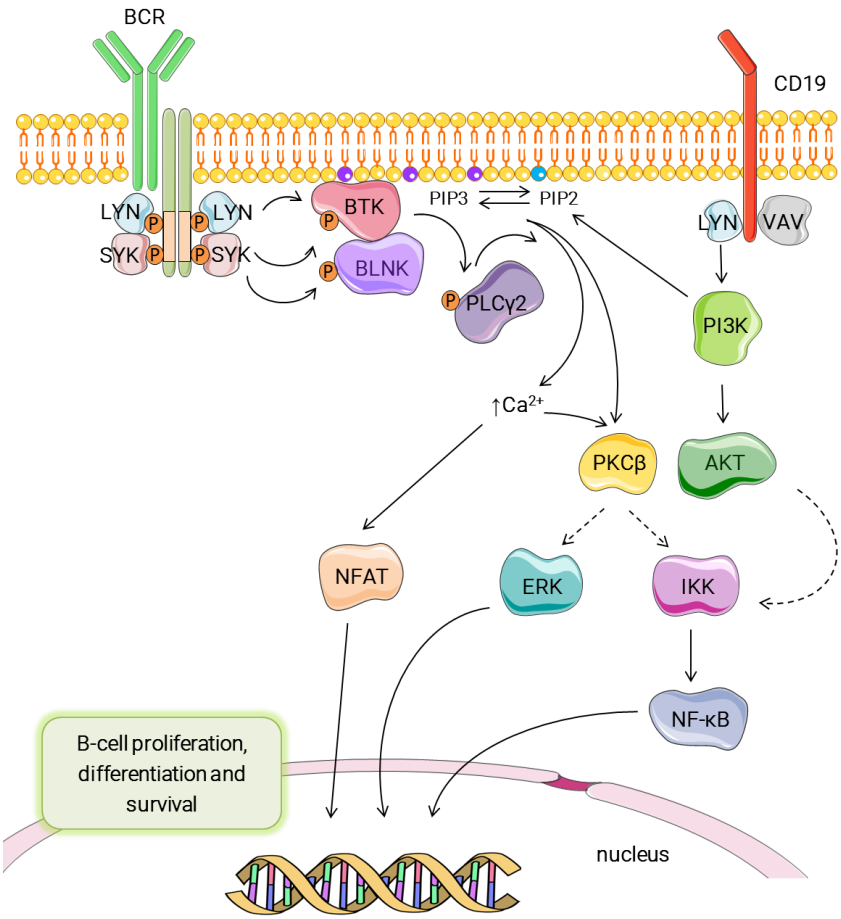

Figure 12 | **B-cell antigen receptor signal transduction.** After antigen ligation, LYN, SYK, and BTK are activated. B-cell adaptors such as BLNK fine-tune BCR signals by efficiently connecting the kinases with the effectors. Activation of PLCγ2 leads to the release of intracellular Ca^{2+} and activation of protein kinase C (PKC); both of which are crucial for the activation of MAPK, such as ERK and transcription factors, including NF-κB and NFAT. Adapted from Herrera and Jacobsen, Clin Cancer Res, 2014.[344]

activation of the transcription factors NFAT (nuclear factor of activated T-cells) and NF-κB, as well as of mitogen-activated protein kinases (MAPK) ERK1 or ERK2, which promote proliferation and survival of normal and malignant B-cells[345] (Figure 12). BTK activation can be regulated by the phosphatases PTEN and SHIP1, which dephosphorylate PIP3 and thereby inhibit BTK membrane association.[343,346]

There are two main models of BCR signaling: the tonic and the chronic signaling. The **tonic signaling** does not require antigen engagement, activates the PI3K pathway and it is essential for the survival of mature B cells.[347] The **chronic active signaling** is promoted by clustering of the BCR and results in the activation of multiple downstream pathways, including MAPK, PI3K, and NF-κB via the CBM complex, a signaling hub consisting of CARD11, BCL10, MALT1, and other proteins.[7]

2.1.1.1 Bruton's tyrosine kinase (BTK)

BTK belongs to the TEC kinase family and is expressed in most cells of the hematopoietic system, especially in B-cells, myeloid cells, and platelets, but not in T lymphocytes and plasma cells, in which the transcript levels are selectively downregulated.[348,349] BTK is a key component of BCR signaling that regulates B-cell proliferation and survival, having a crucial role in B-cell malignancies.[343]

BTK activation is initiated by cell membrane association and phosphorylation of Y551 in the kinase domain, either by an SRC family kinase or by spleen tyrosine kinase (SYK).[350] These events promote the catalytic activity of BTK and results in its autophosphorylation at position Y223.[343] Upon BCR stimulation, mature B-cells increase BTK levels.[351]

Apart from the role of BTK in BCR signaling, in B-cells, BTK participates in multiple pathways, including chemokine receptor and TLR signaling.

BTK and chemokine receptor signaling. BTK is involved in chemokine receptor pathways that are essential for B-cell trafficking, tissue homing and homeostasis. BTK is a key signaling molecule for the chemokine receptors CXCR4 and CXCR5. CXC-chemokine ligand 12 (CXCL12; also known as SDF1), which is highly expressed by stromal cells in the BM and in GC, induces BTK activation.[352]

BTK and TLR signaling. TLR signaling induces the downstream transcription factors NF-κB, activator protein 1 (AP1) and interferon regulatory factor 3 (IRF3), which, in B-cells, results in upregulation of activation markers, proliferation, antibody secretion, CSR and the production of pro-inflammatory cytokines. Upon activation, most TLRs recruit MYD88. BTK can directly interact with cytoplasmic Toll/IL-1 receptor (TIR) domains of most TLRs, as well as with downstream adaptors such as MYD88.[353,354]

Overall, BTK activity is crucial for the survival or proliferation of malignant B-cells, either in a B-cell-intrinsic manner or in the context of the tumor microenvironment.[343]

2.1.1.2 Spleen tyrosine kinase (SYK)

SYK (Spleen Tyrosine Kinase) belongs to the SYK family of cytoplasmic non-receptor tyrosine kinases and plays a critical role in transmitting signals from a variety of cell surface receptors, including the BCR, Fc receptors, T-cell receptor, and integrins, to downstream signaling events.[355,356] Although SYK is primarily expressed in hematopoietic tissues, SYK is also expressed in a variety of non-hematopoietic cells including epithelial and endothelial cells.[357] Mammals also express a SYK homolog, ZAP70, which is mostly restricted to T- and NK-lineage cells.[358]

Apart from its role in adaptive immune receptor signaling, SYK also mediates other biological functions including cellular adhesion, innate immune recognition, osteoclast maturation, platelet activation, and vascular development.[359,360]

2.1.1.3 Tyrosine-protein kinase LYN

The SRC family of protein tyrosine kinases (SFKs) regulates fundamental cellular processes, including cell growth, differentiation, cell shape, migration, and survival.[361] LYN is a member of the SRC family of intracellular membrane-associated tyrosine kinases. While LYN was originally identified in hematopoietic cells, it is expressed in many tissues such as prostate, colon, breast, and brain (neuronal/astrocytes).[362-366] It is involved in the transmission of signals from a number of receptors including BCR,[367] GM-CSF-receptor,[368] c-kit (stem cell factor receptor),[369] Fc,[370] as well as, integrins.[371] LYN has a dual role by both activating and inhibiting signaling pathways and, consequently, it is aptly described as a signaling modulator.[368] In the case of BCR signaling, LYN not only phosphorylates (and thereby activates) SYK, but it also activates phosphatases that in turn inhibit signal transduction through the BCR.[372]

It is clear that LYN has important functions in numerous hematopoietic cell types, from early stem/progenitors[369] through to multiple lineages of the lymphoid system (e.g. B-cell) and the myeloid system (e.g. macrophages, erythroid cells, platelets, mast cells, eosinophils). Interestingly, while LYN is not expressed in T-cells, it can have a significant impact upon T-cell function through modulating signaling in cells that interact with T-cells.[373]

2.1.2 BCR signaling in B-cell malignancies

Due to the key role of the BCR signaling in the development and maturation of normal B-cells, its deregulation is involved in various lymphoma subtypes, promoting B-cell growth and survival. Actually, in several B-cell malignancies, activating mutations in signal transduction components of the BCR pathway have been identified.[374]

Different ways of BCR stimulation have been identified in lymphoma. BCR self-reactivity with auto-antigens has been described in CLL and MCL, where cells use a restricted

repertory of IGHV genes, and in some cases express virtually identical BCRs; so-called "stereotyped BCRs".[189,375,376] In FL, the positive selection of N-glycosylation in the IGHV region is related with an antigen-independent stimulation of BCR, engaged by lectins,[272,273] suggesting an important interaction of FL cells with the tumor microenvironment.[274]

BCRs can be activated through extrinsic signaling but also through intrinsic signaling due to acquired mutations. Some of the aggressive lymphomas harbor genetic mutations that amplify external BCR signals (such as mutations in *CD79b*) or that provide autonomy from these external signals (such as mutations in *CARD11*) giving rise to chronic NF-κB activation.[79]

Of note, despite BCRs switching from IgM to IgG in the GC, many lymphomas derived from GC cells keep using IgM. This could be explained by the distinct signaling outputs of these two Ig subtypes. IgM-BCR signaling promotes the survival and proliferation of B-cells by activating many pathways, including NF-κB, whereas IgG-BCR signaling favors plasmacytic differentiation through the activation of ERK and MAPK pathways.[377,378]

2.1.2.1 BCR signaling in DLBCL

ABC subtype of DLBCL relies on the NF-κB pathway for survival, and has chronic active BCR signaling, depending on CARD11.[79,379] Mutations in members of BCR signaling (*CARD11, CD79A/B*) and the NF-κB pathway (*MYD88*) are classically found in this subtype.[380] BTK links BCR activity to NF-κB and is essential for the survival of ABC lines with chronic active BCR signaling.[79]

On the other hand, GCB-DLBCL utilizes the tonic, antigen-independent type of BCR signaling, which is transmitted via SYK and serves principally to activate the PI3K/AKT signaling pathway.[381]

2.1.2.2 BCR signaling in MCL

MCL is characterized by a highly distinctive IGHV gene repertory and with a biased BCR, suggesting a crucial role for antigenic selection in the pathogenesis of at least a subset of MCL.[375] A pro-survival role of BCR signaling is suggested by the observation of constitutive phosphorylation of different kinases of this pathway, including LYN, SYK, and PKCβ in a limited panel of patients.[382,383] Furthermore, MCL cells show constitutive activation of NF-κB and AKT, which might reflect BCR or TLR signaling.[384,385] In addition, BTK is strongly expressed in MCL,[386] and increased BTK autophosphorylation at Y223 was observed in unstimulated primary MCL cells.[387]

2.1.2.3 BCR signaling in CLL

BCR signaling plays an important pathogenic role in CLL.[388] CLL cells have elevated basal levels for the phosphorylated forms of BCR proximal kinases such as LYN, SYK [385] and PI3K.[389] Inhibition of both SYK[390] and PI3K[391] pathway prevents CLL cells from interacting with the microenvironment, and inhibition of LYN[392], SYK[390] and PI3K[391] all promote pro-apoptotic signals. BTK protein and mRNA are significantly overexpressed in CLL compared with normal B-cells. Although BTK is not always constitutively active in CLL cells, activation of these cells through BCR or CD40 ligands is accompanied by phosphorylation of BTK and effective activation of this pathway.[393] Cells with increased ZAP70 expression and carrying unmutated *IVGH* genes have generally enhanced BCR signaling.[393] Accordingly, U-CLL cells may be continuously stimulated *in vivo* by antigen, giving rise to a gene expression profile that is reminiscent of BCR signaling.[394] U-CLL BCRs are polyreactive and mostly recognizes autoantigens and other environmental antigens, while BCRs from M-CLL cells bind to a restricted set of more specific antigens.[395] In addition to the chronic active BCR signaling responses, CLL-BCRs induce antigen-independent autonomous signaling due to self-recognition of epitopes within the BCR. This cell-autonomous signaling may further potentiate the basal activity of CLL BCRs.[396]

2.1.2.4 BCR signaling in FL

As described before, FL cells may engage an unusual form of BCR signaling due to the frequent introduction of N-glycan motifs to the BCR during SHM. This BCR modification allows the interaction of FL BCR with mannose-binding lectins present on stromal cells in the tumor microenvironment, thereby crosslinking the BCR and initiating its signaling on FL cells.[275,276] Moreover, about 25% of FL BCRs were shown to exhibit some autoreactivity, despite ongoing SHM, contributing to their pathway activation.[397]

2.1.3 BCR inhibitors

Many lymphoma subtypes subvert BCR signaling to their malignant purpose, suggesting that pharmacological inhibition of this pathway holds promise in these cancers. Correct deployment of these inhibitors will require a careful understanding of the type of BCR signaling that is used in each lymphoma subtype. For example, chronic active BCR signaling in ABC DLBCL engages the SRC-family kinases SYK and BTK to activate downstream NF-κB and PI3K pro-survival pathways. In contrast, lymphomas that rely upon tonic BCR signaling, such as BL, depend upon SRC-family kinases and SYK to activate the PI3K pathway, but BTK and the NF-κB pathway are dispensable.[377]

Recent advances in the development of small molecule inhibitors of tyrosine kinases has resulted in great success in treating particular neoplasms and the therapeutic advantages

of these reagents is illustrated by the enormous success of imatinib mesylate (Gleevec, STI571)[398] and dasatinib for the treatment of chronic myeloid leukemia.[399,400]

2.1.3.1 BTK inhibitors

The fact that BTK is a crucial effector molecule for B-cell development and plays a major role in lymphomagenesis, makes this protein an interesting therapeutic target. However, we have seen that BTK is not only involved in BCR signaling, since BTK inhibition can also affect TLR signaling, B-cell adhesion and migration, and cells in the tumor microenvironment.[377]

Ibrutinib

Ibrutinib (Imbruvica®; PCI-32765; Pharmacyclics Inc. and Janssen Biotech, Inc.) is an orally active small molecule which binds irreversibly to the cysteine residue (C481) at the phosphorylation site of BTK, leading to irreversible inactivation and disruption of the signaling pathway from the BCR to the nucleus.[144] Apart of C481 in BTK, a small subset of tyrosine kinases in the human genome is thought to be susceptible to irreversible and durable inhibition by ibrutinib. These kinases include EGFR, HER2, HER4, ITK, BMX, JAK3, TEC, and BLK.[401] Treatment-specific side effects such as bleeding and atrial fibrillation may, at least partly, be related to off-target inhibition of non-BTK kinases.[402]

Ibrutinib has gained approval for the treatment of R/R patients with CLL, MCL, and Walderström macroglobulinemia (WM), and also for first-line therapy in patients with del17p CLL. Ibrutinib has also been studied in other lymphoid malignancies, such as R/R FL and DLBCL,[327,403] and is under evaluation in combination therapies.[402]

Ibrutinib is given orally once-per-day on a continuous schedule until progression or toxicity.[403] Ibrutinib is rapidly absorbed and eliminated after oral administration but BTK remains covalently bound to ibrutinib for at least 24 hours.[401] In clinical trials, it has been well tolerated and has demonstrated profound antitumor activity, inducing redistribution of malignant B-cells from the tissue compartments into the peripheral blood, along with resolution of enlarged lymph nodes and a surge in lymphocytosis.[401,404,405] Ibrutinib has direct effects on malignant B-cells. However, the effects of ibrutinib on the tumor microenvironment are also important as the molecular targets of ibrutinib are not restricted to B-cell compartment, but regulate key functions in other cellular elements such as NK, T-cells and macrophages.[406]

Resistance development is one of the factors that limit the use of ibrutinib. Few patients with CLL have had a relapse on ibrutinib,[407] but approximately one-third of patients with MCL do not respond to ibrutinib and many others eventually develop resistance to therapy.[408-410] Those who progress on ibrutinib tend to have poor outcomes with clinically aggressive disease and very short survival.[168,411]

In both MCL and CLL patients, a cysteine-to-serine missense mutation at BTK C481 (BTK^{C481S}) has been identified at relapse after durable response.[407,412,413] This mutation prevents irreversible binding of ibrutinib to BTK and results in a reduced degree of BCR signaling-inhibition. In addition to BTK^{C481S}, PLCγ2 mutations have been identified in patients with acquired resistance to ibrutinib. These PLCγ2 mutations are believed to be gain-of-function mutations that confer resistance to ibrutinib by allowing BCR-mediated activation that is independent of BTK.[407,412,414]

The absence of both BTK or PLCγ2 mutations in MCL and CLL patients with primary resistance or a transient response to ibrutini, suggest alternative mechanisms contributing to primary or rapid resistance to this drug. Recent studies have described alternative mechanisms involving sustained distal BCR signaling, specifically PI3K-AKT activation.[412,415] Furthermore, ibrutinib resistance in MCL may depend on downstream components of the BCR pathway such as ERK or AKT,[408] as well as on the alternative NF-κB signaling pathway, not mediated by BTK.[416]

Acalabrutinib

Acalabrutinib (ACP-196; AstraZeneca) is a novel irreversible second generation BTK inhibitor, more potent and selective than ibrutinib, with reduced off-target side effects.[251,417] In CLL mouse models, acalabrutinib significantly reduced tumor burden and increased survival.[367] In R/R CLL patients, including those with 17p deletion, acalabrutinib has shown promising safety and efficacy profiles (ORR of 95%).[251] At this time, a phase III study (NCT02477696) has commenced in which acalabrutinib is being compared with ibrutinib in high-risk patients with relapsed CLL. In addition, studies in treatment-naïve CLL are also being done,[417] as well as in patients with Richter syndrome (NCT02362035) and in MCL (NCT02213926).[418]

CC-292

Spebrutinib (CC-292; Celgene) is another covalent irreversible BTK inhibitor that binds BTK cysteine 481 with high specificity and effectively inhibits constitutive and induced BTK and PLCγ2 phosphorylation.[419] Recently, the results of a phase I study of CC-292 in patients with R/R CLL, B-NHL and WM were reported.[249]

CC-292 is well tolerated as a daily oral monotherapy. Administrated as single-agent, it achieved high BTK receptor occupancy, and resulted in dose-dependent responses in R/R CLL patients, including those with high-risk cytogenetic features (ORR of 53% in patients receiving twice-daily dosing).[249] However, its clinical activity (in particular, durability of response) was inferior to that of ibrutinib or acalabrutinib.[251,405]

ONO/GS-4059

Tirabrutinib (ONO/GS-4059; ONO and Gilead) is a potent and selective oral BTK inhibitor. As monotherapy, ONO/GS-4059 showed response in CLL (ORR of 92%), MCL (ORR of 92%) and in DLBCL (ORR of 35%), with no significant toxicities.[250] A long-term extension study (NCT02457559) did not reveal new safety or toxicity concerns.[420] Additionally, a clinical trial is currently underway to evaluate the combination of PI3Kδ inhibitor idelalisib and ONO/GS-4059 in R/R B-cell malignancies (NCT02457598).[421]

2.1.3.2 SYK inhibitors

SYK is recognized as a critical element in the BCR signaling pathway and it is also a key component in signal transduction from other immune receptors, such as Fc receptors and adhesion receptors. Several SYK inhibitors including fostamatinib (R788), entospletinib (GS-9973), cerdulatinib (PRT062070), and TAK-659 are being assessed in clinical trials.[422]

Fostamatinib

Fostamatinib (R788; Rigel Pharmaceuticals) is the first oral SYK inhibitor that can selectively abrogate the BCR signaling pathway and has potent anti-inflammatory effects.[423] In a murine model of CLL, fostamatinib was found to induce an early and transient mobilization of both normal and malignant B-cells, but selectively inhibited the growth of the malignant B-cell population.[424] Fostamatinib has been tested in a phase I/II study in patients with R/R B-cell lymphomas, with an ORR of 3-22% for DLBCL, 10% for FL, 55% for CLL, and 11% for MCL.[425,426]

2.2 PI3K/AKT signaling pathway

Sometimes considered as part of the BCR pathway, the PI3K signaling pathway is involved in a wide variety of essential cellular processes, such as proliferation, growth, apoptosis and cytoskeletal rearrangement. Elevated PI3K signaling can contribute to tumorigenesis and is a hallmark of human cancer.[427,428]

Human cells express three classes of PI3K enzymes. The most important in cancer is the class I, which involves p85 regulatory subunit and four catalytic isoforms (p110α, β, δ, and γ). The p110α and p110β proteins are expressed ubiquitously, whereas expression of p110δ and p110γ is enriched in immune cells.[427]

Activation of class I PI3Ks occurs through multiple upstream pathways such as receptor-coupled tyrosine kinase activities and BCR stimulation. PIP3 is rapidly metabolized by lipid phosphatases, including the tumor suppressor PTEN. Once activated, PI3K phosphorylates PIP2 to PIP3 which acts as a second messenger to recruit cytoplasmic proteins to the plasma membrane or endomembrane.[427]

One of the PI3K effectors is AKT/PKB. PDK1 (Pyruvate Dehydrogenase Kinase, isozyme 1) is recruited by PIP3 and phosphorylates and activates AKT. AKT, in turn, phosphorylates many substrates involved in cell proliferation, metabolism, survival, and motility, such as MDM2, BAD, FOXO1 or GSK3β.[428,429] AKT can exert a positive effect on NF-κB function by phosphorylation of IκB kinase (IKK); a kinase that induces degradation of the NF-κB inhibitor IκB3 (Figure 13).[428]

Below AKT, mTOR (mechanistic Target of Rapamycin) is another PI3K effector. This serine-threonine kinase forms two cellular complexes known as mTORC1 and mTORC2. mTORC1 phosphorylates numerous substrates that promote anabolic metabolism to support cell growth and proliferation, and mTORC2 phosphorylates AKT and is also involved in regulation of the cytoskeleton.[427,430]

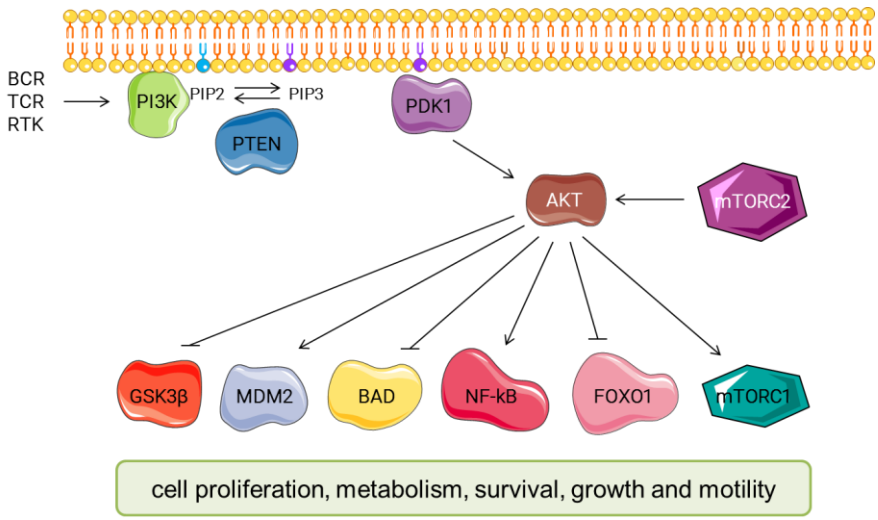

Figure 13 | **PI3K/AKT signaling pathway.** Upon pathway activation, PI3K catalyzes the production of PIP3, which serves as a second messenger to activate AKT. AKT mediates the activation (i.e. MDM2, NF-κB) and inhibition (i.e. GSK3β, BAD, FOXO1) of several targets, resulting in cell proliferation, metabolism, survival, growth, and motility. Moreover, mTORC1 is indirectly activated by AKT and mTORC2 directly phosphorylates AKT increasing its activity. BCR: B-cell receptor; TCR: T-cell receptor; RTK: receptor tyrosine kinase. Adapted from Vivanco et al., Nat Rev Cancer, 2002.[428]

2.2.1 PI3K inhibitors

The recognition that PI3K signaling is aberrantly activated in the majority of human cancers, spurred expectations that PI3K pathway inhibitors would spawn a major paradigm shift in cancer therapy.

There are two categories of PI3K inhibitors: pan-PI3K inhibitors and isoform-selective PI3K inhibitors. The pan inhibitors usually have more off-target effects and toxicity problems.

Also, compensatory mechanisms and intrinsic or acquired resistance make it difficult to use.[427] Despite the complications, some pan-PI3K inhibitors like buparlisib (NVP-BKM120)[431,432] are being investigated for the treatment of lymphoid neoplasms.

Isoform-selective PI3K inhibitors that preferentially inhibit the activity of one or more PI3K isoforms might circumvent the intrinsic toxicity associated with pan-PI3K inhibition and might be more permissive for exploration. Idelalisib (GS-1101, CAL-101) is a PI3Kδ specific inhibitor approved for the treatment of R/R CLL and FL. Clinical trials for the evaluation of idelalisib as a first-line therapy revealed high toxicity rates in naïve patients. At present, attempts are being made to define the optimal combinations and populations.[433]

2.3 NF-κB signaling pathway

The nuclear factor κB or NF-κB transcription factor family that consists of five molecules: p50/NF-κB1, p52/NF-κB2, RelA (p65), c-Rel, and RelB. p50 and 52 are generated through the processing of their respective precursors, p105 and p100.[434] Upon activation, NF-κB controls the expression of a wide range of genes involving several cellular functions including inflammation, apoptosis, cell survival, proliferation, angiogenesis, and innate and acquired immunity.[435] In B cells, many receptors, including BCRs, activate the NF-κB pathway and is shown to be constitutively active in some B-cell malignancies.[436] The NF-κB pathway is activated through either the canonical (also called "classical") or non-canonical (also called "alternative") pathway.

The canonical pathway is activated by a large series of stimuli, including the TNF receptors' family, toll-like receptors such as TLR4, the antigen receptors BCR and TCR, lymphocyte co-receptors such as CD40, CD30, or receptor activator of NF-κB (RANK). Upon activation, the IκB-kinase (IKK) complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also named NF-κB essential modulator or NEMO), phosphorylates and causes the degradation of the inhibitor of NF-κB α (IκBα), releasing RelA/p50 complex, which can translocate into the nucleus and starts the transcription of target genes. The non-canonical pathway is dependent on the activation of the RelB subunit associated with p50 or p52. Differing to the canonical one, this pathway is activated by a more restricted number of receptors that belong to the TNF receptor superfamily, including BAFF-R, CD40, CD30, and LTβ-R, through the NF-κB-inducing kinase (NIK), which activates IKKα and, in turn, causes the degradation of p100 to p52. Upon p100 degradation, p52 preferentially dimerizes with RelB and translocates into the nucleus, where it begins the transcription of target genes (Figure 14).[434,437,438]

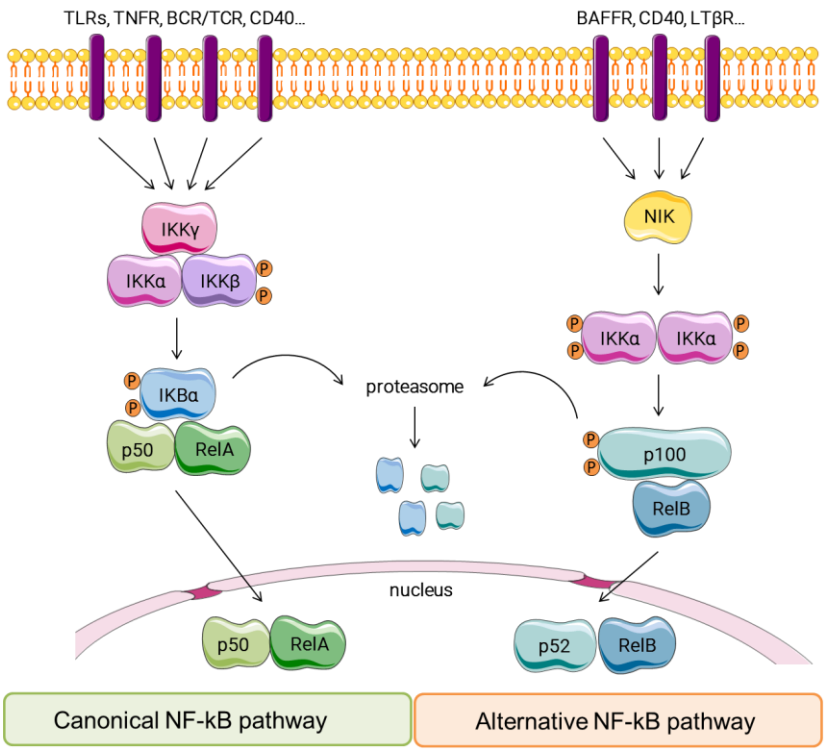

Figure 14 | **Canonical and alternative NF-κB signaling pathways.** Canonical pathway is triggered by numerous signals, including those mediated by innate and adaptive immune receptors. It relies on inducible degradation of IκBs, particularly IκBα, leading to nuclear translocation of various NF-κB complexes, predominantly the p50/RelA dimer. Non-canonical NF-κB pathway relies on phosphorylation-induced p100 processing, which is triggered by signals from a subset of TNFR members. This pathway is dependent on NIK and IKKα and mediates the activation of RelB/p52 complexes. Adapted from Jost and Ruland, Blood, 2007.[437]

Oncogenic mutations that result in NF-κB activation are recurrent in lymphoid malignancies, but most of these mutations affect upstream components of NF-κB signaling pathways, rather than NF-κB family members themselves.[69,436,439,440]

2.3.1 NF-κB inhibitors

Given their relevance in cancer pathogenesis and progression, NF-κB and its signaling components are considered a good therapeutic target for the treatment of these malignancies. To this purpose, numerous small inhibitory peptides have been designed to target specific patterns of the molecules most relevant to NF-κB activation, i.e. IKKs kinases, the proteasome 26S, or, more recently, the NF-κB subunits, and block their activity.[435]

Bortezomib (Velcade®; Millenium Pharmaceuticals, Inc) is a reversible 26S proteasome inhibitor approved for the treatment of MM. Although bortezomib affects other signaling

pathways, its efficacy may in part be due to inhibition of NF-κB activity.[441] Bortezomib is being tested in clinical trials for R/R B-NHL.[442]

Lesions in the alternative NF-κB pathway conferred dependence on the protein kinase NIK. In fact, NIK is a new therapeutic target for lymphomas that are refractory to BCR pathway inhibitors.[416] For example, IAP antagonists can induce NIK stabilization and lead to strong induction of NF-κB signaling.[443]

2.4 Apoptotic signaling pathways

Apoptosis is an essential programmed cell death pathway used by multicellular organisms to dispose of unwanted cells in a diversity of settings. It can be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological. Apoptosis seems to be involved in cell turnover in many healthy adult tissues and is responsible for the focal elimination of cells during normal embryonic development. Deregulated apoptosis has a major role in tumorigenesis. It occurs spontaneously in untreated malignant neoplasms, and participates in at least some types of therapeutically induced tumor regression.[444]

The defining morphological characteristics of apoptosis include cell shrinkage, nuclear fragmentation, chromatin condensation and membrane blebbing; all of which are due to the proteolytic activity of the caspase proteases.[444] In vertebrate cells, apoptosis typically proceeds through two signaling cascades termed the intrinsic and extrinsic pathways, both of which converge on the activation of the major effector caspases: caspase-3, -6 and -7 (Figure 15).[97,99,445].

The **intrinsic or mitochondrial pathway** is activated by various developmental cues or cytotoxic insults, such as viral infection, DNA damage, and growth-factor deprivation.[99] The mitochondrial outer membrane permeabilization (MOMP), which leads to the release of *pro-apop*totic proteins from the mitochondrial intermembrane space (IMS), is the crucial event driving initiator caspase activation and apoptosis. Following its release from mitochondria, cytochrome c binds apoptotic protease-activating factor 1 (APAF1), inducing its conformational change and oligomerization, leading to the formation of a caspase activation platform termed the apoptosome. The apoptosome recruits, dimerizes and activates caspase-9, which, in turn, cleaves and activates effector caspases. Mitochondrial release of second mitochondria-derived activator of caspase (SMAC; also known as DIABLO) and OMI (also known as HTRA2) blocks X-linked inhibitor of apoptosis protein (XIAP)-mediated inhibition of caspase activity.[97,446]

Caspases are a family of proteases that have an essential Cys residue in their active site and a requeriment for an Asp residue in the substrate cleavage site.

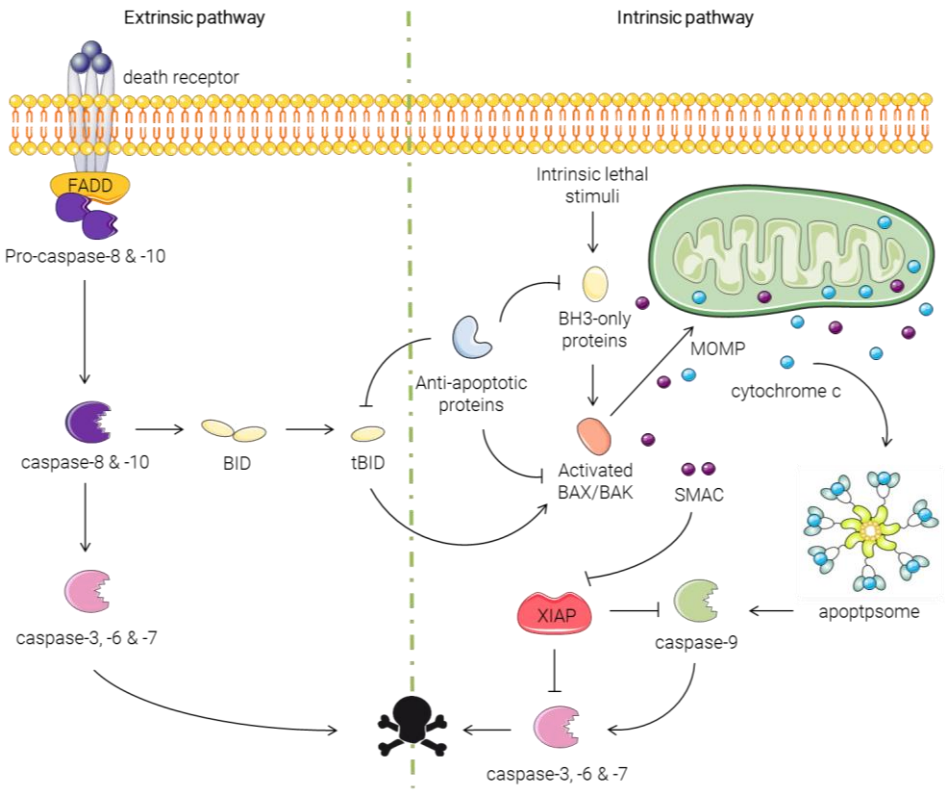

Figure 15 | **Apoptotic pathways.** Caspase activation by the **extrinsic pathway** involves the binding of extracellular death ligands to transmembrane death receptors tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) and FAS. It induces the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD), which in turn recruit and aggregate several molecules of caspase-8 and -10. Active caspase-8 and -10 cleave and activate the effector caspase-3 and -7, leading to apoptosis. In some situations, extrinsic death signals can crosstalk with the intrinsic pathway through caspase-8-mediated proteolysis of the BH3-only protein BID (BH3-interacting domain death agonist). Truncated BID (tBID) can promote mitochondrial cytochrome c release and assembly of the apoptosome. The **intrinsic (or mitochondrial) pathway** of apoptosis requires mitochondrial outer membrane permeabilization (MOMP). Intrinsic lethal stimuli, such as cell stress or DNA damage, typically activate one or more members of the BH3-only protein family, leading to BAX and BAK activity that triggers MOMP. Following MOMP, intermembrane space proteins, such as SMAC and cytochrome c, are released into the cytosol. On release from mitochondria, cytochrome c can seed apoptosome assembly, which activates caspase-9. Active caspase-9 in turn, activates caspase-3 and caspase-7, leading to apoptosis. Adapted from Ichim and Tait, Nat Rev Cancer, 2016.[446]

MOMP is a highly regulated process, primarily controlled through interactions between pro- and anti-apoptotic members of the B cell lymphoma 2 (BCL-2) family.[445] This pathway predominantly leads to the activation of caspase-9[447] but, at least in certain cell types, the intrinsic pathway can proceed in the absence of caspase-9 or its activator, APAF1.[448]

The **extrinsic or death-receptor pathway** is triggered by ligation of so-called death receptors (members of the TNF receptor family, such as Fas or TNF receptor-1 (TNFR1)) that contain an intracellular death domain, which can recruit and activate caspase-8 through the adaptor protein Fas-associated death domain (FADD; also known as MORT1) at the cell surface. Active caspase-8 cleaves and activates the effector caspases. In some cells, the extrinsic pathway can intersect the intrinsic pathway through caspase-8-mediated cleavage of BCL-2 homology 3 (BH3)-interacting domain death agonist (BID).[449] The C-terminal truncated form of BID (tBID) translocates to mitochondria and promotes MOMP and further caspase activation through the intrinsic pathway.[99,445]

2.4.1 Impairment of apoptosis in lymphoid malignancies

Deregulation of apoptosis is a characteristic feature of many cancers, confering them a survival advantage over normal cells.[450] That gives time for accumulation of genetic alterations that deregulate cell proliferation, interfere with differentiation, promote angiogenesis, and increase invasiveness during tumor progression.[451]

There are varieties of molecular mechanisms that tumor cells use to suppress apoptosis, which usually involves modulation of BCL-2 proteins. The prototypic member of the family, BCL-2, was first identified in B-cell lymphoma.[452] Since then, pro-survival BCL-2 family proteins have been implicated in a variety of cancers, including CLL, acute lymphoblastic leukemia (ALL), MCL and DLBCL.[98] Similarly, MCL-1 expression is associated with MM and chronic myeloid leukemia (CML) among others. High levels of expression of pro-survival proteins are often associated with increased chemoresistance and are therefore attractive targets for cancer therapy.[453]

2.4.2 The BCL-2 protein family

B-cell lymphoma-2 (BCL-2)-family proteins have a crucial role in the regulation of apoptosis through their ability to regulate mitochondrial cytochrome c release. This family is divided into three groups based on their BH domain organization.[445] The **anti-apoptotic** or pro-survival BCL-2 proteins, which contain four BH domains (BH1-BH4) and suppress cell death by binding and inhibiting the pro-apoptotic BCL-2 proteins; the **pro-apoptotic** proteins, which present BH1-BH3 and directly promote MOMP; and the **BH-3 only proteins**, which, except BID, only contain one highly conserved BH3 domain and are very heterogeneous (Figure 16).[454]

In addition to regulation of mitochondrial apoptosis, proteins of the BCL-2 family play important roles in regulating other cellular pathways such as autophagy, endoplasmic reticulum (ER) stress response, intracellular calcium dynamics, cell cycle progression, mitochondrial dynamics, and energy metabolism.[455]

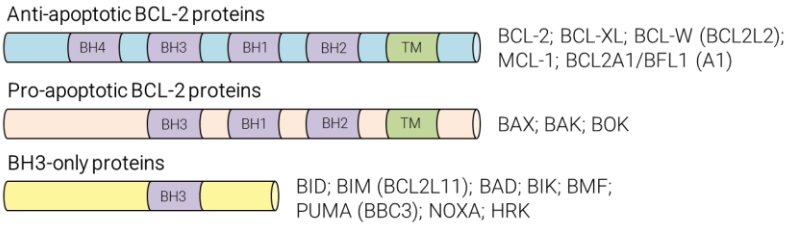

Figure 16 | **The BCL-2 family.** The BCL-2 family comprises three subfamilies that contain between one and four BCL-2 homology (BH) domains. The anti-apoptotic subfamily comprises proteins that contain four BH domains. Most members of this subfamily also contain transmembrane domains (TM) and are therefore typically associated with membranes. The pro-apoptotic subfamily lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes. The BH3-only subfamily is a structurally diverse group of proteins that only display homology within the small BH3 motif. Adapted from Tait and Green, Nat Rev Mol Cell Biol, 2010.[445]

2.4.2.1 Anti-apoptotic BCL-2 proteins

BCL-2 and its close relatives (BCL-XL, MCL-1, BFL-1, and BCL-W) contain four BH and are generally integrated within the outer mitochondrial membrane (OMM), but may also be in the cytosol, the nuclear envelope or in the ER membrane.[99,456] The anti-apoptotic BCL-2 proteins block apoptosis by preventing BH3-only protein-induced oligomerization of the pro-apoptotic BCL-2-family members BAX and/or BAK in OMM, which would otherwise lead to the efflux of cytochrome c and other mitochondrial intermembrane space proteins.

The anti-apoptotic BCL-2 proteins differentially bind to the BH3-only proteins. Some BH3-only proteins (BIM, PUMA, and BID) interact with essentially all anti-apoptotic family members, whereas other members (for example, NOXA), display differential affinity towards various pro-survival proteins (Figure 17).[97,453]

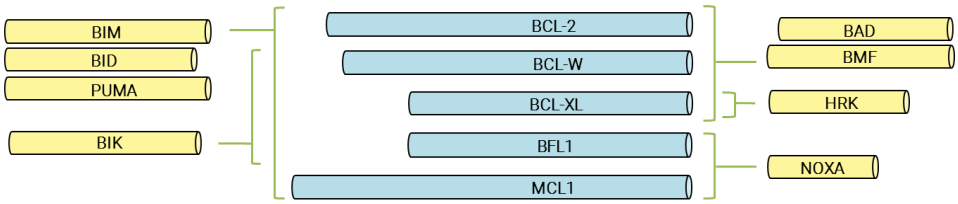

Figure 17 | **BH3-only protein binding specificity for BCL-2 homologs.** BIM and PUMA bind to all five anti-apoptotic BCL-2 family members. By contrast, NOXA only binds to MCL1 and A1, and BAD binds selectively to BCL-W, BCL-2, and BCL-XL. BID binds avidly to BCL-XL, BCL-W, MCL-1, and BFL-1, but only weakly to BCL-2. The HRK BH3-only family member has remarkable specificity for BCL-XL. These binding specificities recapitulate the ability of these proteins to activate apoptosis. For example, BIM, BID or PUMA alone can induce apoptosis, whereas a combination of NOXA and BAD is required. Adapted from Youle and Strasser, Nat Rev Mol Cell Biol, 2008[99] and Opferman JT, FEBS J, 2019.[457]

2.4.2.2 Pro-apoptotic BCL-2 proteins

BAX and BAK are members of the BCL-2 family and core regulators of the intrinsic pathway of apoptosis. Upon apoptotic stimuli, they are activated and homo-oligomerize into pores within the OMM to promote its permeabilization (mitochondrial outer membrane permeabilization, MOMP), which is considered a key step in apoptosis. There is a potential third effector molecule, BOK, with high homology to BAX and BAK, but with a more mysterious role.[454]

Under normal conditions, BAX is largely cytosolic via constant retro-translocation from mitochondria to the cytosol mediated by BCL-XL.[455,458] In contrast, BAK is mainly mitochondrial with its transmembrane domain spanning the OMM and presents only a small cytosolic fraction due to retrotranslocation.[459] On the other hand, BOK is predominantly found in the Golgi and ER membranes,[460] and it appears to be constitutively active and controlled at the level of protein stability by components of the ER-associated degradation pathway.[461]

BAX and BAK are controlled by pro-survival family members. Although all the indicated pro-survival members can restrain BAX, BAK is constrained primarily by BCL-XL, MCL-1, and BFL-1.[462]

2.4.2.3 BH3-only proteins

The mammalian BH3-only protein family currently comprises eight members (BID, BAD, BIM, BIK, BMF, NOXA, PUMA and HRK) that differ in their expression patterns and mode of activation. The BH3-only proteins are considered to be essential initiators of the mitochondrial apoptotic pathway and are up-regulated by many forms of stress.[453] They are functionally categorized as direct activators and depressors or sensitizers on the basis of whether they directly trigger the effectors or antagonize anti-apoptotic BCL-2 proteins, respectively.[456,463]

In some reviews, BCL-2 interacting protein 3 or BCL-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)[445] is included in the BH3-only protein group, based on limited sequence homology to the BH3 domain, but its ability to bind to and regulate pro-survival BCL-2 proteins or pro-apoptotic BAX and BAK has not been established.[464]

2.4.3 Activation of MOMP

MOMP is commonly seen as the point-of-no-return in the intrinsic apoptotic pathway; it unleashes the activation of apoptotic caspases that are responsible for the degradation of many vital cellular substrates, and consequently the morphological changes observed during apoptosis.[460] Moreover, other proteases besides caspases are activated during cell death, such as calpains, serine proteases (granzymes), and lysosomal proteases.[465]

There are two models of activation of MOMP that may exist in a cell type- or stimulus-dependent manner. In the **direct activator model**, BAX and BAK are activated following interaction with a subset of BH3-only proteins known as direct activators, and anti-apoptotic BCL-2 proteins prevent MOMP either by sequestering the activating BH3-only proteins or by directly inhibiting activated BAX and BAK. A second subset of BH3-only proteins, termed sensitizers, cannot directly activate BAX and BAK but neutralize anti-apoptotic BCL-2 proteins, displacing activators from the BCL-2 complex. The **indirect activator model** asserts that BAX and BAK are bound in a constitutively active state by anti-apoptotic BCL-2 proteins, and that competitive interactions of BH3-only proteins with anti-apoptotic BCL-2 family members is sufficient to displace and release activated BAX and BAK. [445,453,463] Moreover, a **unified model** has been described.[466] It assumes that the anti-apoptotic proteins prevent MOMP either by sequestering the activating BH3-only proteins or by directly inhibiting activated BAX and BAK (Figure 18).[454,455] Finally, the recent 'interconnected hierarchical' model established a sequence of events for the execution of apoptosis.[467]

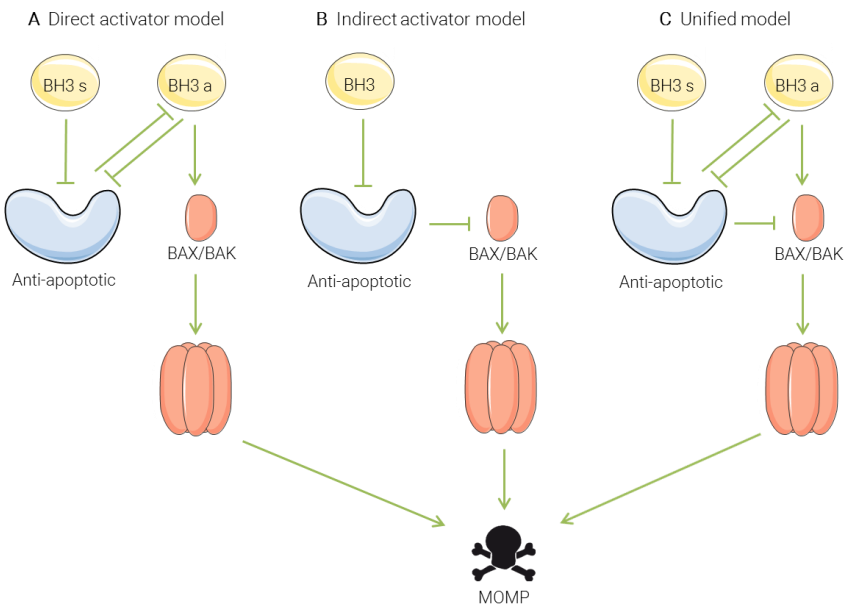

Figure 18 | **Regulation of MOMP by the BCL-2 family.** A graphical scheme of the direct and indirect activation model. (A) The direct activation model requires a separate activation step for BAK and BAX by some BH3-only proteins (activators). (B) The indirect activation model assumes BAK and BAX to be constitutively active, which do not require separate activation. BH3-only proteins liberate BAK and BAX by interacting with anti-apoptotic proteins. (C) In the unified model anti-apoptotic proteins may inhibit directly BAX/BAK or BH3-only activators. BH3a: activators; BH3s: sensitizers.

2.4.4 Targeting anti-apoptotic BCL-2 family members

The network of protein-protein interactions among the BCL-2 protein family plays a critical role in regulating cellular commitment to mitochondrial apoptosis. Anti-apoptotic BCL-2 proteins are considered promising targets for drug discovery and exciting clinical progress has stimulated intense investigations in the broader family.[468] Attempts to overcome the cytoprotective effects of anti-apoptotic BCL-2 proteins in cancer include three strategies: shutting off gene transcription, inducing mRNA degradation with antisense oligonucleotides and directly inhibiting the proteins.[450,451] The latter has emerged as the most successful approach. Over the past 30 years, research on the BCL-2-regulated apoptotic pathway has led to the development of small-molecule compounds, known as 'BH3-mimetics', that bind to pro-survival BCL-2 proteins to directly activate apoptosis of malignant cells.[464] Their design is based on how the BH3-domain of BH3-only molecules fits into the hydrophobic cleft of the anti-apoptotic molecule. BH3-mimetics are typically designed to competitively bind to the BH3-binding groove of anti-apoptotic molecules to displace pro-apoptotic molecules.[457] However, it must be remembered that the BCL-2 family proteins are important to a number of physiological functions that are beyond the regulation of apoptosis.[469]

2.4.4.1 ABT family of BH3-mimetics

ABT-737 (AbbVie; figure 19a), which binds to and inhibits BCL-2, BCL-XL, and BCL-W, was the 'first-in-class' BH3-mimetic. *In vitro,* ABT-737 exhibited cytotoxicity in lymphoma and small-cell lung carcinoma cell lines, as well as primary patient-derived cells. In animal models, ABT-737 improved survival, caused regression of established tumors, and produced cures in a high percentage of the mice.[470] It also displayed synergistic cytotoxicity with chemotherapeutics and radiation. However, ABT-737 was not orally bioavailable, which limited chronic single-agent therapy and flexibility to dose in combination regimens. Consequently, ABT-263 (navitoclax; AbbVie; figure 19b), an orally available analog was developed.[471] It displayed a similar binding selectivity for pro-survival BCL-2 proteins *in vitro* as ABT-737, and is currently in clinical trials in solid tumors[472] and B-cell malignancies.[473,474] Although chronic dosing of ABT-263 was well tolerated, it induced a thrombocytopenia that was dose-related and maintained throughout the period of drug treatment; probably due to BCL-XL inhibition.[473,475,476] Subsequently, ABT-199 (venetoclax; Venclexta®; AbbVie+Roche; figure 19c), a first-in-class BCL-2 selective inhibitor was developed. Venetoclax shows potent cytotoxicity *in vitro* and antitumor efficacy *in vivo* in various lymphoid malignancies such as CLL,[477] AML,[478] DLBCL,[479] or DH[128]. The lack of BCL-XL inhibition with ABT-199 allow for higher circulating concentrations of the drug without dose-limiting thrombocytopenia.[134] Venetoclax entered the clinic in 2011. It has proved highly efficacious and tolerable in a Phase I trial (79% ORR)[480] and has rapidly progressed into Phase II and III clinical trials as a single agent for

the treatment of patients with R/R lymphoid malignancies (particularly CLL; ORR 79.4%).[247] Venetoclax is generally well tolerated, with the significant adverse effect being tumor lysis syndrome (TLS),[481] for which there are formal management recommendations, such as a dose escalation scheme.[482]

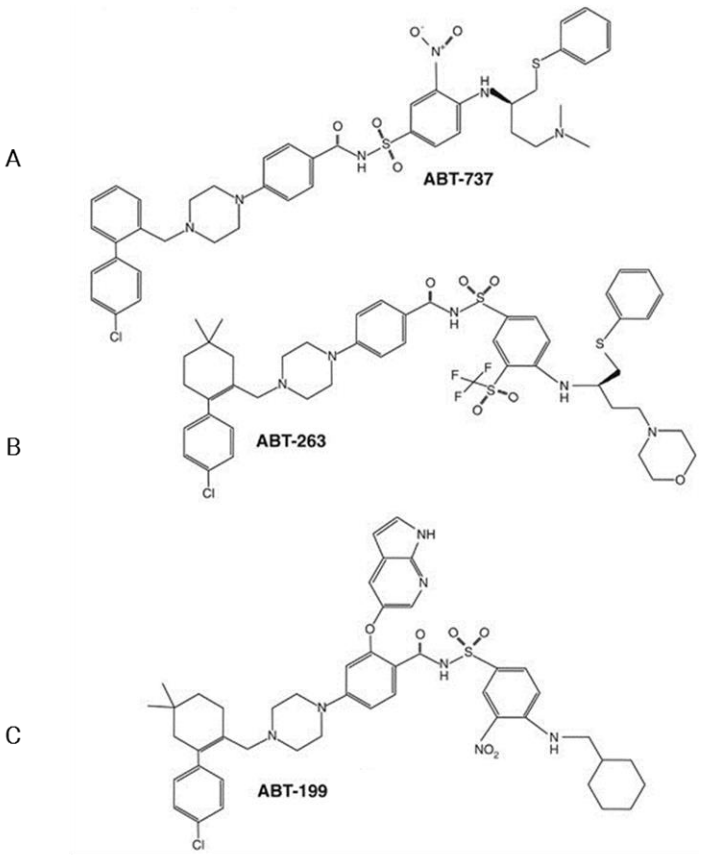

Figure 19 | **Structural view of BH3 mimetics.** (A) ABT-737 is a small-molecule BH3 mimetic that binds to the hydrophobic BH3-binding groove of BCL-XL, BCL-2, and BCL-W. (B) ABT-263 (navitoclax) is structurally related to ABT-737. It represents an orally bioavailable small-molecule BH3 mimetic which efficiently antagonizes BCL-XL, BCL-2, and BCL-W. (C) ABT-199 is a high-affinity BCL-2-selective small-molecule BH3 mimetic. Adapted from Brinkmann K and Kashkar H, Cell Death Dis, 2014.[483]

Venetoclax was FDA-approved for use as monotherapy in patients with R/R CLL with 17p deletion.[135] Ongoing single agent (Table 12) and combination studies (for example with anti-CD20 mAbs; 86% ORR)[484] are likely to lead to broader approvals for this drug in the near future.[485] More than 50 phase I-III clinical trials of venetoclax in AML, CLL, DLBCL, MCL, MM, and NHL are underway.[469,486]

Table 12 | **Single agent clinical trials of venetoclax.**

Phase	Indication	N enrolled	NCT number
I	NHL, MM, CLL, SLL, AML	36	NCT02265731
	Advanced NHL	12	NCT01969695
	R/R NHL	135	NCT03236857
	R/R NHL; R/R CLL	211	NCT01328626
II	R/R CLL del17p	70	NCT02966756
	FL	56	NCT03113422
	R/R CLL	120	NCT02141282
	R/R CLL del17p	150	NCT01889786
III	R/R CLL	250	NCT02756611
	R/R CLL	200	NCT02980731

NHL, Non-Hodgkin's lymphoma; MM, multiple myeloma; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; AML, acute myeloid leukemia; FL, follicular lymphoma.

2.4.4.2 BH3-mimetics acquired resistance

BH3-mimetics such as ABT-737 and its analogs fail to maintain a significant antitumor activity, and acquired resistance arises inevitably following its continuous administration to lymphoma cells.[100,487] In many tumors, overexpression of pro-survival BCL-2 proteins contribute to apoptosis resistance[99,456] and is associated with resistance to chemotherapy.[488] This is the case of ABT-737, where an upregulation of MCL-1[100,489,490] and BFL-1[100] has been reported in resistant cells. The sequestration of the BH3-only BIM by MCL-1 and BFL-1 seems to have an essential role in maintaining cell survival in ABT-737 and ABT-199 treated cells (Figure 20).[100,491] NOXA is reported to act synergistically with ABT-737 to induce cell death in cells expressing MCL-1 or BFL-1.[492] Accordingly, NOXA-inducing anti-cancer drug, bortezomib exhibits synergistic pro-apoptotic effect in

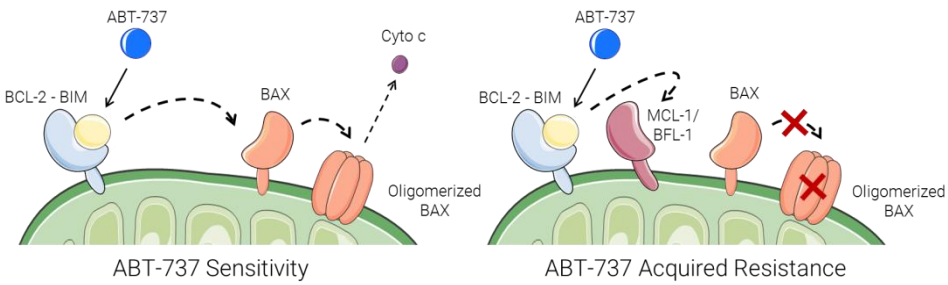

Figure 20 | **Proposed mechanism of ABT-737 acquired resistance.** In responding cells, BIM is displaced from BCL-2, inducing BAX activation and apoptosis. In resistance cells, BIM is still displaced but is captured by MCL-1 or BFL-1, preventing BAX activation and maintaining survival. Adapted from Yecies D, et al., Blood, 2015.[100]

combination with ABT-737 (Figure 21).[492] In the same way, obatoclax, through its ability to bind MCL-1, can overcome resistance to apoptosis mediated specifically by MCL-1.[493]

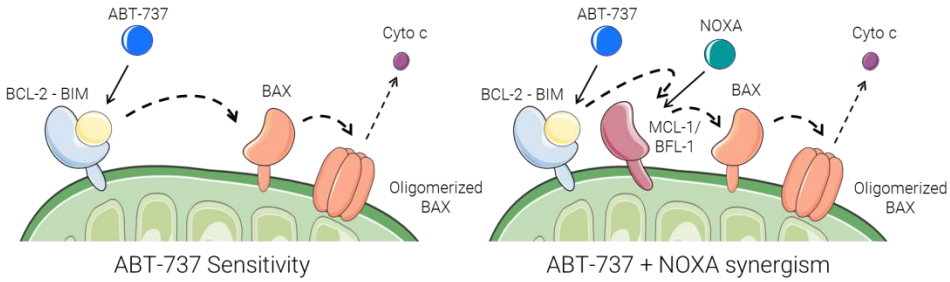

Figure 21 | **Proposed mechanism of ABT-737 and NOXA synergism.** NOXA acts synergistically with ABT-737, displacing BIM from BCL-2 and MCL-1/BFL-1.

Kinase inhibitors also have shown to overcome venetoclax resistance by altering microenvironmental signaling pathways.[494] Many signal transduction pathways control the transcription, translation, post-translational modification or degradation of BCL-2 family members.[495] Modification of these pathways with kinase inhibitors can prevent the upregulation of anti-apoptotic proteins.[496]

Some studies have revealed a synergistic antitumoral effect between BET inhibitors such as JQ1 and BH3-mimetics like ABT-263.[497] While MYC downregulation was initially proposed as a key mechanistic property of BET inhibitors, additional anti-tumor activities are important, including the modulation of the expression of pro-apoptotic (BIM) and anti-apoptotic (BCL-2; BCL-XL) BCL-2 family members to directly engage the mitochondrial apoptotic pathway.[498] It will be of special interest in the treatment of DHL.

AIMS

GENERAL AIMS

The main goal of this dissertation is to explore new therapeutic approaches for chemo-refractory B-NHL. In the last decade, a number of targeted therapies have been introduced in the clinical settings for the treatment of lymphoid malignancies. Venetoclax and ibrutinib are two of these targeted drugs. Despite their high response rates, intrinsic and acquired resistances to these agents limit their use, and challenge the search for new therapies or combinations able to overcome resistance, and to improve patients' outcome.

We have focused our investigation in two main hypotheses:

- The deregulation of some BCL-2 family members can be related with the acquired resistance to venetoclax in DHL, and may represent a promising therapeutic target to overcome this phenomenon.
- The inhibition of more than one BCR kinase, such as BTK, SYK and LYN, can be useful in B-NHL cases that are resistant to the sole inhibition of BTK.

Following these lines of investigation, the first aim of this thesis is to analyze the mechanisms involved in the acquired resistance to venetoclax in DHL and explore pharmacological ways to counteract it. On the other hand, the second aim of this thesis is to validate the antitumoral activity of pleiotropic BCR kinase targeting.

SPECIFIC AIMS

1.- To investigate the mechanisms underlying the acquired resistance to venetoclax in DHL cells and to explore therapeutic strategies to overcome this phenomenon.

1.1. To evaluate the antitumoral activity of the BH3-mimetic venetoclax in *in vitro* models of DLBCL and DHL and determine the molecular basis of ABT-199 resistance in DHL.

1.2. To analyze the effect of the BET inhibitor CPI203 treatment in DHL and to examine the molecular mechanism underlying its activity.

1.3. To study the antitumoral activity of the combination of ABT-199 and CPI203, both *in vitro* and *in vivo*.

2.- To evaluate the antitumor profile of a novel pleiotropic BCR kinase inhibitor.

2.1. To determine the antitumoral activity of IQS019 in *in vitro* and *in vivo* models of CLL, MCL, FL, and DLBCL.

2.2. To compare the efficacy of the co-inhibition of LYN, SYK, and BTK over the inhibition of BTK alone by ibrutinib and to evaluate its benefit in ibrutinib-resistant cases.

RESULTS

FIRST PAPER

The BET bromodomain inhibitor CPI203 overcomes resistance to ABT-199 (venetoclax) by downregulation of BFL-1/A1 in *in vitro* and *in vivo* models of *MYC+/BCL2+* double hit lymphoma

Esteve-Arenys A,[1;2;7] Valero JG,[1;2;7] Chamorro-Jorganes A,[1;2] Gonzalez D,[1;2] Rodriguez V,[2] Dlouhy I,[3] Salaverria I,[2] Campo E,[2;4] Colomer D,[2;4] Martinez A,[4] Rymkiewicz G,[5] Pérez-Galán P,[2] Lopez-Guillermo A,[2;3;8] and Roué G.[1;2;6;8]

1 Aggressive B-cell Lymphoma Study Group and 2 Division of Hematology and Oncology, Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), CIBERONC, Barcelona, Spain; 3 Department of Hematology, and 4 Hematopathology Unit, Department of Pathology, Hospital Clinic, Barcelona, Spain; 5 Department of Pathology, The Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. 6 Laboratory of Experimental Hematology, Department of Hematology, Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, Barcelona, Spain.

7 Both authors contributed equally to this work.

8 AL-G and GRo share the senior authorship of this work.

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7 Both authors contributed equally to this work.

8 AL-G and GRo share the senior authorship of this work.

Running Title: BCL-2 and BRD4 dual targeting in DHL

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Corresponding author: Gaël Roué, PhD
IDIBAPS, Division of Hematology and Oncology
Centre Esther Koplowitz
Roselló 153,
Barcelona 08036, Spain.
Phone: +34-932275400 ext 4525
Fax: +34-933129407
E-mail: groue@clinic.ub.es

ABSTRACT

High-grade B-cell lymphoma (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements, mostly known as double-hit lymphoma (DHL), is a rare entity characterized by morphologic and molecular features between Burkitt lymphoma and the clinically-manageable diffuse large B-cell lymphoma (DLBCL). DHL patients usually undergo a rapidly progressing clinical course associated with resistance to standard chemo-immunotherapy. As a consequence, the prognosis of this entity is particularly poor with a median overall survival inferior to one year. ABT-199 (venetoclax) is a potent and selective small-molecule antagonist of BCL-2 recently approved for the treatment of a specific subtype of lymphoid neoplasm. In this study, we demonstrate that single-agent ABT-199 efficiently displaces BAX from BCL-2 complexes but fails to maintain a significant antitumor activity over time in most *MYC+/BCL2+* DHL cell lines and primary cultures, as well as in a xenograft mouse model of the disease. We further identify the accumulation of the BCL2-like protein BFL-1 to be a major mechanism involved in acquired resistance to ABT-199. Noteworthy, this phenomenon can be counteracted by the BET bromodomain inhibitor CPI203, since gene expression profiling identifies *BCL2A1*, the BFL-1 coding gene, as one of the top apoptosis-related gene modulated by this compound. Upon CPI203 treatment, simultaneous downregulation of MYC and BFL-1 further overcomes resistance to ABT-199 both *in vitro* and *in vivo*, engaging synergistic caspase-mediated apoptosis in DHL cultures and tumor xenografts. Together, these findings highlight the relevance of BFL-1 in DH lymphoma-associated drug resistance and support the combined use of a BCL-2 antagonist and a BET inhibitor as a promising therapeutic strategy for patients with aggressive DHL.

Keywords: BCL-2 proteins, MYC, drug resistance, B-NHL, mouse model

INTRODUCTION

Double hit lymphoma (DHL) is a rare subtype non-Hodgkin B-cell lymphoma (NHL), characterized by recurrent chromosomal translocations affecting *MYC* and *BCL2* or *BCL6* oncogenes.[1,2] In most cases, concomitant t(14;18)/IGH-*BCL2* and t(8;14)/IGH-*MYC* or variants, leads to the overexpression of BCL-2 and MYC, respectively, conferring to the tumor increased proliferation with improper control of apoptosis, and to the patients an extremely poor prognosis.[3,1,4] In some extremely rare cases, DHL is defined by three recurrent chromosomal translocations affecting *MYC* and *BCL2* loci, in combination with *BCL6/3q27* chromosomal translocation.[5,6] Although these alterations can be found in distinct NHL subtypes such as follicular lymphoma (FL), acute lymphoblastic leukemia and B cell lymphoma unclassifiable, they mainly arise from diffuse large B-cell lymphoma (DLBCL).[7] Covering almost 40% of newly diagnosed lymphomas, DLBCL is the main adult lymphoma and represents an heterogeneous group of tumors with distinct subtypes that differ in genetic abnormalities, clinical outcome, response to treatment and prognosis. Gene

expression profiling has defined three distinct molecular cell-of-origin subtypes of DLBCL: germinal center B-cell (GCB), activated B-cell (ABC) and primary mediastinal B-cell lymphoma (PMBL).[8] DLBCL is biologically aggressive but can be cured in > 50 % of cases, even in advanced stages, thanks to the introduction in the clinical of the immuno-chemotherapy R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone) or other similar regimens. Although the 2-year overall survival (OS) of DLBCL patients could reach 70 % with to these strategies,[9] these latest are usually insufficient in DHL, where the median OS is closed to 12 months.[10,11] Therefore, new rationally-based therapeutic approaches are urgently needed for this last subgroup of patients.

In this context, approaches targeting BCL-2 and MYC expression and/or activity are particularly promising for the management of DHL patients. *MYC* is a powerful transcriptional amplifier involved in the regulation of genes implicated in numerous physiological functions, including cell proliferation and growth, DNA replication, metabolism and energy modulation among others.[12] Several strategies that could modulate indirectly *MYC* transcription are being developed. One of the most promising approaches consists in the targeting of BRD4, a member of the bromodomain and extraterminal (BET) chromatin adaptor family. This family of chromatin adaptors, that also includes BRD2 and BRD3, is functionally linked to pathways important for cellular viability and cancer signaling. In particular, BRD4 has a direct role in the transcriptional regulation of different genes related to cell division and cell growth regulation.[13,14,15] Therefore, its deregulation can influence cancer cell biological activity and the inhibition of BRD4 could effectively disrupt tumor growth.[16] Different BET inhibitors (BETis) have recently emerged as promising molecules for the treatment of hematological malignancies.[17] Among them, the BETi (+)-JQ1 selectively antagonizes BRD4 by competitively binding to the acetyl-lysine recognition pocket of BET bromodomains from chromatin.[18,19] This displacement of BRD4 leads to a drastic transcriptional downregulation of MYC in a dose- and time-dependent manner. However, although gene expression changes observed after BET bromodomain inhibition are mainly dominated by the *MYC* transcriptome, BETis influence the expression of a more extensive assortment of nearly 3000 genes.[20] CPI203 is an analog of (+)-JQ1 with superior bioavailability after either oral or intraperitoneal administration.[13] The antitumoral effects of this compound are comparable and even higher in some cases than (+)-JQ1, both *in vitro* and *in vivo* settings.[13,21] Previous studies in multiple myeloma and mantle cell lymphoma highlighted the capacity of CPI203 to inhibit cancer cell growth, including in cases with acquired or intrinsic resistance to conventional chemotherapeutics. In these models, the compound was able to re-sensitize tumor cells to standard treatments, by promoting a synergistic MYC-related antiproliferative and pro-apoptotic response.[22,23]

On the other hand, BCL-2 protein promotes the survival of cells upon major stressors, as well as the accumulation of cancer cells by opposing to cell death. It is also associated to resistance to chemotherapy, and its overexpression correlates with a poor prognosis in DLBCL patients.[3] This protein is the main member of the anti-apoptotic subgroup of the BCL-2 family of proteins, which also includes BCL-XL, BCL-W, MCL-1 and BFL-1/A1. Members of the BH3-only protein subgroup of this family (BIM, cleaved BID, PUMA, NOXA) bind to the pro-survival proteins, leading to the

release of BAX and BAK from their anti-apoptotic counterparts. This step is followed by the permeabilization of the mitochondrial outer membrane and the subsequent cytosolic release of cytochrome c and activation of caspases.[24] In the last decade, this knowledge has stimulated efforts to develop a number of small molecules inhibitors of BCL-2, the so-called "BH3 mimetics", able to trigger apoptosis in cells with high levels of BCL-2. ABT-737 and the orally available analog, ABT-263, were among the first molecules to show antitumor activity against chronic lymphocytic leukemia (CLL), a hematologic malignancy that expresses particularly high levels of BCL-2.[25] As ABT-263 was shown to trigger thrombocytopenia due to the inhibition of BCL-XL, a factor required for platelet survival,[3,26] ABT-199, a potent and selective small-molecule antagonist of BCL-2 capable of displacing pro-apoptotic BAX from BCL-2 complexes, was developed and rapidly showed unprecedented clinical activity with acceptable toxicity.[27] Indeed, although it displayed remarkable, although variable, single-agent activity in different NHL subtypes,[28] ABT-199 was particularly active in patients with CLL,[29] including patients with unfavorable prognosis harboring 17p/*TP53* deletion.[30] These studies led to its recent approval by the FDA for this entity. However, this drug fails to maintain a significant antitumor activity and acquired resistance arises inevitably following its continuous administration to lymphoma cells.[31] A previous work from Letai's lab has demonstrated that in B-cell lymphoma cell lines the major mechanism underlying acquired resistance to the first generation inhibitor ABT-737, was related to the accumulation of other BCL-2-like proteins with potential compensatory functions, *i.e.* MCL-1 and/or BFL-1.[32] Further studies are still needed to ascertain whether such mechanisms could be involved in ABT-199 activity loss in these models.

In the present work, we have evaluated the combinatorial activity of CPI203 and ABT-199 in *in vitro* and *in vivo* models of MYC+/BCL2+ double hit lymphoma, and identified the regulation of the BCL-2-like protein BFL-1 as the main molecular mechanism underlying the cooperation between these two drugs.

RESULTS

Decreased sensitivity to ABT-199 correlates with up-regulation of anti-apoptotic BFL-1 in DHL

To evaluate the sensitivity of DHL cells to ABT-199, a panel of 6 DHL and 3 representative GCB-DLBCL cell lines was exposed to doses of ABT-199 ranging from 1 to 100 nM and cell viability was measured by MTT assay at 24 and 96 hours. Figure 1a shows that the viability decreased in a dose-dependent manner in all the samples tested and in the two set of cell lines. At 24 hours, no difference in term of drug sensitivity could be observed between DHL and standard GCB-DLBCL cells, as at the 100 nM dose the cytotoxic effect of the compound reached 41.2 % (range: 11.6 – 73.0 %) and 46.3 % (range: 27.2 – 77.6 %) in these two subgroups, respectively. However, while ABT-199 antitumoral effect was increased in GCB-DLBCL cells after 96 hours of treatment (mean cytotoxic effect at the 100 nM dose : 78.1 %, range: 71.3 – 85.6 %), in the same period of time DHL cells recovered part of their proliferative properties despite the presence of ABT-199, as the mean cell viability progressed up to 77.5 % (Figure 1a). Of note, DHL cells showed similar capacity to recover their viability at the different doses of ABT-199 tested (Supplemental Figure S1a). This

process was not related to a defective capacity of ABT-199 to displace BAX from BCL-2 in DHL cells, as BCL-2 immunoprecipitation assay showed similar degrees of BAX level decrease in BCL-2 complexes and/or BAX accumulation out of these complexes, in DHL and GCB-DLBCL cell treated with the inhibitor (Figure 1b). In order to assess if the acquired resistance phenotype in DHL cells could be related to basal BCL-2 and/or BCL-2-like protein levels, we analyzed the expression of BCL-2, BFL-1, MCL-1 and BCL-XL by Western blot, in the same 9 cell lines as before (Supplemental Figure S1b), and plotted the relative protein expression of each protein against ABT-199 antitumoral effect. As shown in figure 1c, no significant correlation could be found between the basal expression of these proteins and DHL sensitivity to ABT-199. We then investigated the dose- and time-dependent modulation of these four proteins in the 3 representative DHL cell lines, OCI-LY8, SUDHL-4 and Toledo, and in the representative GCB-DLBCL cell line SUDHL-16 cells exposed to 1, 10 or 100 nM ABT-199. We observed that the upregulation of BFL-1, but not other BCL-2-like protein, significantly correlated with the cell proliferation recovery observed in DHL cells between 24h and 96h, when exposed to the different doses of ABT-199 (Figure 1d and Supplemental Figures S1d and S1e). Of interest, this upregulation of BFL-1 was maintained up to 14 days of exposure to ABT-199 in DHL cells (data not shown).

To confirm a protective role of BFL-1 toward ABT-199 antitumoral effect in B-cell lymphoma, we further transduced the ABT-199 high sensitive cells SUDHL-16 with lentiviral particles containing either a GFP+ control or a GFP/BFL1+ vector (see details in Materials and Methods section). After several rounds of selection, the GFP/BFL1+ cells presented a 67 fold increase in *BCL2A1* transcript levels when compared to control GFP+ cells (Figure 1e, *left panel*). The associated overexpression of BFL-1 did not affect the levels of the other antiapoptotic BCL-2 family members or of the *MYC* oncogene (Figure 1E, *central panel*), but it conferred to these cells a significant protection against ABT-199. Indeed, at the highest dose tested (100 nM), the compound induced a 9 % cell proliferation blockade at 96 hours in these cells, contrasting with the 99 % antitumoral effect observed in the control GFP+ SUDHL-16 cell line (Figure 1e, *right panel*). Most important, while the effect of ABT-199 was largely increased over the time in the control cell line, GFP+/BFL-1+ SUDHL-16 cells experimented an almost complete recovery of viability between 24 hours and 96 hours of treatment (Figure 1e, *right panel*), as illustrated in Supplemental Figure S1f, thus resembling the acquired resistance phenotype observed in DHL cells. Altogether, these results strongly suggest that the loose of ABT-199 efficacy observed in DHL cells after prolonged exposure to the drug, involved to the upregulation and/or stabilization of BFL-1 protein.

Regulation of an apoptosis gene signature by the BET bromodomain inhibitor CPI203 in DHL cells

According to the impact of posttranslational modifications in the stability and the role of BFL-1 in lymphomagenesis,[33] different strategies have been proposed that can target the expression of this BCL-2 similar by means of kinase-mediated protein destabilization.[34] However, recent works have highlighted as a most efficient strategy the transcriptional modulation of *BCL2A1* gene by pharmacological inhibition of the BET family member, BRD4.[35] We thus assessed the sensitivity of 6 DHL cell lines and 4 primary patient cultures (see characteristics in Supplemental Table 1) to

the BET inhibitor CPI203, using pre-established conditions.[22] As shown in figure 2a and Table 1, CPI203 exerted a dose- and time-dependent cytostatic effect in the nanomolar range in all the 6 DHL cell lines analyzed, independently of their sensitivity to ABT-199. The mean antitumoral effect of CPI203 at the 500 nM dose was 30.3 % (range: 23.2 – 45.7 %) at 24 hours, and 52.2 % (range: 33.8 - 61.2 %) at 48 hours. Accordingly, we were able to detect a 7.5 % relative increase in cell cycle blockade at the G1 phase in 4 DHL primary cultures exposed for 48 hours to a 100 nM dose of the compound (Figure 2b). To characterize the main factors involved in DHL response to CPI203, we then performed a gene expression profiling (GEP) analysis with the three representative cell lines OCI-LY8, SUDHL-4 and SUDHL-6 and two primary cases (PT#1 and PT#2), either untreated or treated for 6 hours with 100 nM CPI203. Gene sets with a false discovery rate (FDR) below 0.05 and a normalized enrichment score (NES) greater than 1.5 were considered significant. In agreement with previous reports carried out in B-cell lymphoma,[36,22] gene set enrichment (GSEA) analysis yielded an enrichment of genes up-regulated by MYC or involved in BLIMP-1-dependent plasmacytic differentiation, as well as higher NF-κB and interleukin-2-related gene signatures, in control cells, when compared to CPI203-treated samples (Supplemental Table 2 and Supplemental Figure S2). Noteworthy, we also observed that CPI203 was able to modulate an apoptosis-related gene set in DHL cells (Supplemental Table 2). A deeper analysis of this gene set pointed out a simultaneous modulation of several *BCL2* family genes after exposure to the BET inhibitor, being the downregulation of *BCL2A1* and the upregulation of *BCL2L11*, codifying respectively for anti-apoptotic BFL-1 and pro-apoptotic BIM proteins, among the main events observed in CPI203-treated cells (Figure 2c). Quantification of the corresponding transcripts by quantitative RT-PCR in these 5 DHL samples revealed that, upon CPI203 treatment, *BCL2A1* and *BCL2L11* underwent respectively a 27% decrease and a 37% upregulation, while *MYC* mRNA levels, used here as a hallmark of BRD4 activity, were decreased by 41% (Figure 2d). Western blot analysis confirmed that, together with MYC, BFL-1 and BIM, but not BCL-2 protein levels were significantly altered in CPI203-treated cells, and in a time- and dose-dependent fashion. Indeed, the downregulation of BFL-1 reached 70 %, while the upregulation rate for BIM was closed to 60 %, after a 48 hour treatment of the cells with 500 nM CPI203 (Figure 2e). As a consequence to the increased intracellular pool of BIM, and in the absence of BFL-1, the pro-apoptotic protein was displaced from BFL-1 to BCL-2 complexes in CPI203-treated cells, suggesting that the BET inhibitor could represent a potent strategy to alter the balance of pro-apoptotic and anti-apoptotic BCL-2 family members, thereby allowing to a kind of "priming" of DHL cells for death (Figure 2f).[37]

CPI203 restores ABT-199 apoptogenicity in DHL cultures

According to the above results, we investigated whether the redistribution of BIM-BCL-2 complexes by mean of CPI203 could represent an effective strategy to sensitize DHL cells to ABT-199. For this aim, the 6 DHL cell lines were treated for 24-48 hours with 100-500 nM CPI203 and/or 10-50 nM ABT-199, and cytotoxicity was determined by MTT assay. A strong synergistic effect was observed between the two compounds at all the doses tested and in every cell lines, as attested by combination index (CI) values below 0.8 (Figure 3a). The simultaneous detection of

reactive oxygen species (ROS) and mitochondrial depolarization by flow cytometry further confirmed that the cooperation between the two compounds was mainly mediated by an increased apoptotic processing, although most of this effect was related to ABT-199 activity (Figure 3b). Similarly, while CPI203 alone was almost ineffective as an apoptosis inducer in DHL primary samples, a 50 nM dose of ABT-199 could trigger cell death in almost half of them (Figure 3c). Most interestingly, when compared to control group, the drug combination achieved an 88 % relative increase in hypodiploid sub-G1 cell population in these samples (Figure 3c).

At the molecular level, we confirmed by Western blot assay and immunofluorescence analysis that cell exposure to CPI203 efficiently counteracted both basal expression and ABT-199-mediated upregulation of BFL-1 in the representative cell line OCI-LY8 (Figure 3d) and in two representative primary cultures (Figure 3e), respectively. In accordance with the flow cytometry data in OCI-LY8 cells, the detection of the processed form of the effector caspase-3 as well as the degradation of its substrate PARP, confirmed the activation of apoptosis in DHL cells treated with both agents (Figure 3e). As expected, in cells treated with the combination, the increased apoptotic signaling was associated with an improved dissociation of BAX from BCL-2 complexes (Figure 3f). Thus, these results demonstrate that simultaneous targeting of BCL-2 and BFL-1/BIM by ABT-199 and BET inhibition efficiently activates the cell death program in DHL cells resistant to ABT-199 single agent.

In vivo antitumoral activity of CPI203/ABT-199 combination involves BFL-1/A1 downregulation and apoptosis restoration

In order to validate the synergistic effect of CPI203/ABT-199 combination *in vivo*, OCI-LY8 tumor-bearing mice were randomly assigned into four treatment groups (n=4–5 mice per group) to receive either CPI203 2.5 mg/kg BID, ABT-199 20 mg/kg weekly, the combination of both agents or the equivalent volume of vehicle. Tumor growth was recorded for two weeks as detailed in Materials and Methods. In the ABT-199 treatment group, we observed that the drug allowed to a transient control of tumor outgrowth during the first week of treatment, that was not maintained during the second half of the assay, as the final tumor volumes were superior (+ 18 %) to those of the vehicle group (Figure 4a). In contrast, CPI203-treated mice showed a 39.4 % reduction in tumor size when compared to the control group (p = 0.016). This effect was remarkably enhanced in the combo group, that reached an overall reduction of 80.4 % in tumor burden when compared to the control group (p = 0.0003, Figure 4a). Accordingly, while the glucose uptake underwent a two-fold increase in ABT-199-treated *vs* control tumors, this value decreased down to 86.4 % and 29.9 % in the CPI203 and combination groups, respectively (Figure 4b), thus arguing in favor of a synergistic inhibition of tumor metabolism in combo-receiving animals. Immunohistochemical staining of consecutive sections from representative tumors further demonstrated a synergistic decrease in mitotic index and induction of apoptosis by the CPI203-ABT199 combination, as revealed by phospho-histoneH3 and activated caspase-3 staining, respectively, together with a global downregulation of MYC, BFL-1 and BCL-2 (Figure 4c). Interestingly, the increased levels of BFL-1 mRNA (Figure 4d) and protein (Figures 4c and 4e) detected in ABT-199-treated tumors were in agreement with our *in vitro* observation, and

confirmed a potential role of this protein in acquired resistance to the BCL-2 antagonist. Moreover, in accordance with our *in vitro* data, CPI203 therapy allowed to neutralize both basal and ABT-199-related transcription of *BCL2A1* gene, leading to consequent dowregulation of BFL-1 protein *in vivo* (Figures 4d and 4e). Thus, these results confirmed our *in vitro* data, showing that the combination of the BET inhibitor with the BCL-2 antagonist augments the antitumor properties of this latest, as a result of the abrogation of BFL-1 expression and consequent blockade of tumor growth.

DISCUSSION

DHL is an aggressive disease characterized by frequent failures of standard chemotherapeutic regimens. Among the multiple novel targeted agents currently in clinical development and that may offer hope for better outcomes in those patients, are the specific inhibitors of the key oncogenes, BCL-2 and MYC. First studies comparing the first generation dual BCL-XL/BCL-2 antagonist, ABT-737, with the novel BCL-2 specific drug, ABT-199, have shown this latest to be more effective than its predecessor in DHL cell lines and human primary DHL tumor samples.[38] These first results suggested that DHL cells were mostly dependent on BCL-2, rather than other anti-apoptotic member of this family, for their survival. However, similar studies performed in aggressive B-cell lymphoma and leukemic mature B cells, that included prolonged exposure to these drugs, highlighted a role for high MCL-1 and BCL-XL levels in cell resistance to ABT-199,[39,40] as well as a negative impact of *BCL2A1* and/or *MCL1* upregulation on ABT-737 responsiveness.[32] Here, by analyzing the time- and dose-dependent modulation of the main BCL-2-like proteins, we identified BFL-1 upregulation as the molecular mechanism preferentially associated to the loss of ABT-199 response in DHL cell lines and primary samples. By employing a standard GCB-DLBCL cell line modified to specifically overexpress BFL-1, we further confirmed a crucial role of this protein in the acquisition of resistance to ABT-199 in B-cell lymphoma.

In the clinical setting, the first results of the phase I trials evaluating BH3 mimetics have demonstrated high response rates and good toxicity profiles in different subtypes of refractory/relapsed cancers, but the determination of optimal doses pointed out the need for combination-based phase II studies.[28] Among the main partner dugs currently under clinical evaluation with ABT-199 in cancer patients, are the proteasome inhibitors bortezomib and carfilzomib, the B-cell receptor kinase inhibitor ibrutinib, the anti-CD20 antibody rituximab and the epigenetic drug azacitidine.[39] Regarding this last class of agents, and unlike older epigenetic therapies, the preclinical evaluation of BET inhibitors has offered numerous mechanistic insights of their antitumor activity, facilitating their optimal therapeutic targeting in different cancer models. In this regard, a number of preclinical studies have demonstrated the synergistic effect of (+)-JQ1 (the precursor of CPI203) with conventional therapies, in different hematological malignancies,[36,22,23] as well as with ABT-199.[38] In agreement with this last work reporting a promising drug-drug interaction profile of ABT-199 with (+)-JQ1, we demonstrate here the capacity of CPI203 to overcome resistance to the BCL-2 antagonist in DHL cell lines, primary

cultures, and mouse xenografts, thereby achieving a remarkable antitumor activity either as single agent or when employed in combination.

Of special interest, although this strategy was initially bound to interfere, among others, with MYC transcriptome and NF-κB signaling,[36,20] we did not observe any significant alterations of these pathways in DHL cell with refractoriness to ABT-199 (data not shown). Conversely, thanks to the capacity of BETis to interfere with apoptosis-related genes,[35] we show that the blockade of BFL-1 transcription, rather than MCL-1 or BCL-2, was involved in CPI203 sensitizer effect to ABT-199 *in vitro* and *in vivo*. Thus, we introduce here a clear rational explaining the high synergistic interaction between the BCL-2 antagonist and the BET inhibitor in DHL cells and DHL-tumor bearing mice.

Binding of released BIM to BCL-2-like counterparts is a known mechanism of intrinsic resistance to ABT-199.[41] In this sense, the capacity of CPI203 to increase the disbalance between intracellular pools of BIM and BFL-1 may be crucial for the capacity of the BET inhibitor to overcome ABT-199 resistance. Our immunoprecipitation analysis suggest that the events downstream transcriptional normalization by CPI203 include the redistribution of BIM from BFL-1-dependent to BCL-2-dependent complexes, and the consequent triggering of apoptotic signaling in cells exposed to ABT-199 (summarized in Figure 5). Supporting a crucial role of BIM in the response to ABT-199/CPI203 combination, the deletion of this BH3-only gene in a transgenic mouse model of MYC+/BCL2+ lymphoma accelerated the progression of the disease under ABT-199 treatment.[42]

Taken together, our results demonstrate that the combination of a BET inhibitor with a BCL-2-specific BH3-mimetic is effective and can produce synergistic antitumor activity in *in vitro* and *in vivo* models of DH lymphoma. This rationally-based strategy could constitute a promising therapeutic alternative for aggressive DHL cases, thus warranting its clinical evaluation in those patients with a very unfavorable prognosis.

MATERIALS AND METHODS

Cell lines and patients samples

Nine DHL and GCB-DLBCL cell lines were used in this study (Table 1). The DOHH-2, SUDHL-4, SUDHL-6, SUDHL-16 cell lines were acquired at DSMZ (Braunschweig, Germany). WSU-DLCL2 was obtained from ATCC cell bank (LGC Standards, Teddington, UK). OCI-LY8, Toledo, Karpas-422 and Pfeiffer cell lines were kindly provided by Dr M. Raffeld (NCI, Bethesda, MD, USA), Dr MA Piris (Fundación Jiménez Díaz, Madrid, Spain), Dr JI Martin-Subero (Department of Basic Clinical Practice, University of Barcelona, Barcelona, Spain) and Dr JA Martinez-Climent (Division of Hemato-Oncology, Center for Applied Medical Research, Pamplona, Spain), respectively. Short tandem repeat (STR) profiling was used for the authentication of each cell line upon reception, using the AmpFlSTR identifier kit (Thermo Fisher, Waltham, MA, USA), and based on available STR profiles. Cells were cultured at a density of 4-6 x 10^5 cells/ml in a humidified atmosphere containing 5% CO_2, in RPMI 1640 (IMDM for OCI-LY8) supplemented with 10% to 20% heat-

inactivated fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml penicillin-streptomycin (Thermo Fisher). Mycoplasm infection was tested by PCR.

Primary tumor cells from 4 DHL patients carrying *MYC*, *BCL2* and/or *BCL6* chromosomal rearrangements were used (see supplemental Table S1 for biological and clinical characteristics). The ethical approvals including the informed consent of the patients were granted following the guidelines of the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology. Tumor cells were isolated, cryopreserved and conserved as previously described.[43] The percentage of CD20+ tumor B-cells was evaluated by flow cytometry. In order to prevent *ex vivo* apoptosis, primary cells were co-cultured with the mesenchymal bone marrow-derived StromaNKTert cell line (Riken BioResource Center, Ibaraki-ken, Japan)[45].

Drugs

CPI203 was kindly provided by Constellation Pharmaceuticals (Cambridge, MA, USA) and ABT-199 was purchased at Selleck Chemicals (Munich, Germany). Both drugs were used at previously described effective doses: 50-1000 nM for CPI203 and 1-100 nM for ABT-199.

Proliferation, apoptosis assays and cell cycle analysis

Cell lines were exposed to CPI203 and/or ABT-199 for the indicated times and doses. Measurement of cell proliferation was performed in triplicate by the MTT assay, based on the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide by proliferating cells. Untreated control cells were used as references. Calcusyn software version 2.0 (Biosoft, Ferguson, MO, USA) was used to calculate the combination indexes (CIs). CIs lower than 0.8 indicated a substantially synergistic interaction between the two drugs. Apoptosis was determined by cytofluorimetric detection of mitochondrial transmembrane potential ($\Delta\Psi m$) loss and reactive oxygen species (ROS) production on an Attune acoustic focusing cytometer (Thermo Fischer) after simultaneous staining of the cells with DiOC(6)3 and dihydroethidine (DHE) (Thermo Fisher), respectively.

Primary samples (5 x 10^5 cells/sample) were cultured for 48h in the presence of CPI203 ± ABT-199, at the indicated doses. Afterward, apoptotic hypodiploid cells were evaluated by cell cycle analysis. For this aim, cells were fixed with 70% ethanol overnight, stained with propidium iodide (Sigma-Aldrich, Saint-Louis, MO, USA) and analyzed through a FACScalibur flow cytometer using Modfit v2.0 software (Becton-Dickinson, San Jose, CA, USA).

Immunoprecipitation and Western blot

A monoclonal anti-BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a polyclonal anti-BIM (Cell Signaling Technology, Danvers, MA, USA) antibodies were used to perform BCL-2 and BIM immunoprecipitation, as previously described.[45] For Western blot analysis, 3-5 x 10^6 cells were lysed on ice for 30 min in RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors. Protein concentration was determied by Lowry protein assay (Bio-Rad, CA, USA). Protein lysates (30-50 µg) were separated on 12% acrylamide/bisacrylamide gels and transfered onto polyvinylidene difluoride membranes (Immobilon-P, Merck-Millipore, Darmstadt,

Germany). Membranes were incubated with primary antibodies (anti-MYC, anti-BIM, anti-activated caspase-3 (Cell Signaling Technology), anti-MCL-1, anti-BCL-X_L, anti-BCL-2, anti-BAX (Santa Cruz), anti-BFL-1 (Merck-Millipore), and anti-PARP (Roche, Basel, Switzerland) followed by the appropriate secondary antibody (anti-rabbit, Cell Signaling Technology; anti-mouse, Sigma-Aldrich). Anti-tubulin (Sigma Aldrich) was used as a loading control. Chemiluminescence detection was done using the ECL system (Pierce) and visualized on a mini-LAS4000 device using Image Gauge software (Fujifilm, Valhalla, NY, USA) was used for relative quantification of protein levels.

Generation of BFL-1-overexpressing cells

To generate *BCL2A1*-coding retroviral particles, 3 × 10^6 Phoenix™ Ampho Cells cells were transfected with 15 µg of pRetroX-IRES-ZsGreen1 or pRetroX-BFL1-IRES-ZsGreen1 plasmid in the presence of X-tremeGENE™ HP DNA Transfection Reagent (Roche) according to manufacturer's protocol. The viral supernatant was collected 48 hour post-transfection, passed through a 0.45-µm filter and used to infect 10^6 SUDHL-16 cells in the presence of 4 µg/ml polybrene. Cells were spun in 24-well plates at 2 200 rpm for 2h at 32º. Transduced cells were sorted based on GFP expression by using a FACSAria cell sorter (Becton-Dickinson).

RNA isolation and quantitative real-time PCR

For total RNA isolation, 3-5 x 10^6 cells were homogenized with TRIzol reagent (Thermo Fisher), following the manufacturer's protocol. cDNA was synthesized from total RNA using PrimeScript™ RT Master Mix (Takara, Saint-Germain-en-Laye, France) and quantitative real-time PCR (qPCR) was then performed on the StepOne Real-Time PCR System (Thermo Fisher). Premix Ex Taq (Probe qPCR) Master Mix (Takara) and pre-designed TaqMan® Probes (Thermo Fisher) were used. The comparative cycle threshold method (ΔΔCt) was used to quantify the relative expression of each gene. β-actin was used as an endogenous control. qPCR were performed in duplicate.

Gene expression and gene set enrichment (GSEA) analysis

OCI-LY8, SUDHL-6 and SUDHL-4 cell lines (5 x 10^6 cells), as well as primary DHL cultures from PT#1 and PT#2 (3 x 10^6 cells) were cultured for 6 hours in the presence or the absence of CPI203 (100 nM). Total RNA was then extracted as above and cRNA was hybridized on the HG-U219 GeneChip (Affymetrix, Santa Clara, CA, USA). A Gene Titan instrument and a GeneChip Command Console Software were used for the scanning of the results. Raw data were normalized using Expression Console Software v1.1 (Affymetrix). Gene signatures were determined with GSEA version 2.0 (Broad Institute, Cambridge, MA USA) using the hallmark gene sets, the C2 curated gene sets, the C3 motif gene sets, the C5 gene ontology gene sets, the C6 oncogenic signatures (Molecular Signature Database v2.5), and custom gene sets (www.lymphochip.com). A two-class analysis with 1000 permutations of gene sets and a weighted metric was used. Expression heatmap was generated using Morpheus software (Broad Institute). Data have been deposited at

the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (GSE98905).

Immunofluorescence

DHL primary tumor cells (2-3 x 10^5) from PT#1-2 were co-cultured and treated as indicated. After 48 hours of treatment, cells were seed on poly-L-lysine–coated glass coverslips and fixed with 4% paraformaldehyde. After one wash, cells were permeabilized with 0.1% saponin containing 10% FBS, and were incubated with primary (anti–BFL-1, Merck-Millipore) and secondary (anti–rabbit immunoglobulin G–FITC; Sigma-Aldrich) antibodies. Coverslips were mounted on glass slides with Fluoroshield-DAPI mounting medium (Sigma-Aldrich) and fluorescence was visualized on a Nikon H5505 microscope (Nikon, Amsterdam, Netherlands). Pictures were acquired using the Isis Imaging System v5.3 software (MetaSystems GmbH, Heidelberg, Germany).

Xenograft mouse model and tumor phenotyping

OCI-LY8 cells 10 x 10^6 per mouse)were subcutaneously injected in CB17-SCID mice (Janvier labs, Le Genest-Saint-Isle, France) and tumor growth was evaluated every 3 days. Animal vital parameters were monitored as previously described [22] and all procedures were carried out following protocols approved by the Animal Ethics Committee of the University of Barcelona (agreement #154/16). When the tumors reached an average volume of 100 mm^3, mice were randomized into cohorts of 4-5 animals each and treatments were administered for two weeks. CPI203 was used at a 2.5 mg/kg intraperitoneal dose, twice a day, and/or ABT-199 at a 20 mg/kg oral dose, once a week. Animals of the control group were dosed with equal volume of vehicle. Animals were euthanized according to institutional guidelines and tumor samples were excised. Tumors were snap-frozen in OCT medium (Sakura Tissue Tek, Alphen aan den Rijn, Netherlands) or fixed in PFA and embedded in paraffin. Immunohistochemical staining was performed as previously described.[22] The primary antibodies used were phospho-histone H3 (Epitomics, Burlingame, CA, USA), cleaved-caspase-3 and MYC (Cell Signaling Technology), BCL-2 (Dako, Glostrup, Denmark), and BFL-1 (Merck-Millipore). Images were acquired using an Olympus DP70 microscopewith the Cell B Basic Imaging Software (Olympus, Hamburg, Germany). One representative tumor of each group was used for the quantification of *MYC*, *BCL2* and *BFL1* mRNA expression. qPCR analysis were performed as above. For MYC, BCL-2 and BFL-1 protein levels analysis, whole protein extracts were separated by SDS-PAGE and analyzed as previously published.[46]

Statistical analysis

Data are represented as mean ± SD or SEM of 3 independent experiments. GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform all statistical analyses, using Student's t-test or nonparametric Mann-Whitney test/ANOVA for comparisons between two groups of samples. Spearman test was used to evaluate correlation coefficients. Results were considered statistically significant when $p < 0.05$ (* $p< 0.05$; ** $p < 0.01$; *** $p < 0.001$).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTION

AE-A and JGV designed study, performed experiments, analyzed data and co-wrote the manuscript. AC-J, DG and ID were in charge of and/or provided support to cell line transfection, western blot assay, flow cytometry analysis, and interpretation of the data. VR provided support in animal studies. IS supervised cell line authentication. EC and DC reviewed the manuscript. AM helped in designing immunohistochemical assays. GRy provided essential primary samples. PP-G helped in interpreting the results and reviewed the manuscript. AL-G co-designed the study and reviewed the manuscript. GRo conceived and designed the study, analyzed data, interpreted the results and wrote the manuscript.

SUPPLEMENTARY INFORMATION

Supplementary Information accompanies the paper on the *Oncogene* website (http://www.nature.com/onc)

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Table 1 –Drug sensitivity of DHL and GCB-DLBCL cell lines

Cell Line	Subtype	t(8;14) or variants	t(14;18)	t(3q27;v)	Inhibition of cell proliferation (%) by ABT-199 (100nM)		Inhibition of cell proliferation (%) by CPI203 24h		CI 24h
		MYC	BCL2	BCL6	24h	96 h	100 nM	500 nM	(50nM ABT-199 + 100nM CPI203)
WSU-DLCL2	DHL	+ (non-IG)	+	+	23.3	10.8	27.9	38.3	0.1
OCI-LY8	DHL	+ (IG)	+	+	66.9	30.3	21.7	22.8	0.3
SUDHL-4	DHL	+ (non-IG)	+	+	11.6	0.0	19.7	27.6	> 0.8
DOHH2	DHL	+ (IG)	+	-	16.8	0.0	19.8	45.7	0.2
SUDHL-6	DHL	+ (non-IG)	+	-	47.3	30.3	18.9	23.2	0.8
TOLEDO	DHL	+ (non-IG)	+	-	73.1	59.0	23.8	24.3	0.3
PFEIFFER	GCB-DLBCL	-	+	-	34.1	71.3	ND	ND	ND
KARPAS-442	GCB-DLBCL	-	+	-	28.5	77.5	ND	ND	ND
SUDHL-16	GCB-DLBCL	-	+	-	77.6	85.6	ND	ND	ND

ND: not determined; v: variable

FIGURE LEGENDS

Figure 1. Prolonged exposure to ABT-199 triggers BFL1 overexpression and consequent desensitization of DHL cell lines to the BCL-2 antagonist. **(a)** Antitumoral activity of ABT-199 was evaluated by MTT assay at 24 h and 96 h in a panel of n=6 DHL and n=3 GCB-DLBCL cell lines, using untreated cells as reference. **(b)** Representative DHL and GCB-DLBCL cell lines were treated with 100 nM ABT-199 for 3 hours and BCL-2 immunoprecipitation was performed as described in "Materials and Methods", analyzing BAX and BIM protein contents in BCL-2–bound (B) and –unbound (UB) fractions by Western blot. **(c)** Correlations between ABT-199 cytotoxicity in DHL cell lines and anti-apoptotic BCL-2-like protein levels assessed by Western blot. α-tubulin was used as a loading control. Cytotoxic effect was determined after a 24 hour incubation with 100 nM ABT-199 and referred to the corresponding untreated control. **(d)** *Left panels*: time- and dose-dependent modulation of anti-apoptotic BCL-2-like proteins in DHL and GCB-DLBCL cells upon ABT-199 treatment. Representative blots from OCI-LY8 and SUDHL-16 cell lines are shown. BCL-2, MCL-1, BCL-XL and BFL-1 protein level changes were plotted against the mean relative cell proliferation recovery observed between 24h and 96h in the two cell lines (*right panels*). **(e)** Comparative analysis of BFL1 mRNA levels (*left panel*) and protein expression (*central panel*) in control GFP+ versus GFP+/BFL-1+ SUDHL-16 cell lines by qPCR and Western blot analysis, respectively. Right panel: sensitivity of GFP+ and GFP+/BFL-1+ SUDHL-16 cells to ABT-199 was evaluated by MTT at different time points using untreated cells as reference.

Figure 2. Modulation of BIM and BFL-1 expression by the BET inhibitor CPI203 in DHL cells. Antitumoral activity of CPI203 was evaluated at the indicated times by MTT assay and cytofluorimetric quantification of G0/G1 cell cycle fraction in, respectively, n=6 DHL cell lines **(a)** and n=4 DHL primary samples co-cultured on a mesenchymal stromal cell monolayer **(b)**. **(c)** Expression heatmap of BCL-2 family genes in CPI203-treated DHL cell lines and primary samples. Values are referred to their corresponding untreated controls. Mean gene modulation of the 5 samples is shown in the right column. **(d)** Relative changes in *MYC*, *BCL2A1* and *BCL2L11* transcript levels after CPI203 treatment was evaluated by quantitative RT-PCR in the 2 primary samples and the 3 DHL cell lines used in *(c)*, using β-actin as an endogenous control gene. Values

are referred to control untreated cells. **(e)** Time- and dose-dependent modulation of MYC and BCL-2 family proteins in a representative DHL cell line upon exposure to CPI203. Protein levels were quantified as above using α-tubulin as a loading control, and referred at each time point to the corresponding untreated cells. **(f)** OCI-LY8 cells were treated with 100 nM CPI203 for 24 hours and BIM immunoprecipitation was performed as described in "Materials and Methods", analyzing BCL-2 and BFL-1 protein contents in BIM–bound and –unbound fractions by Western blot.

Figure 3. CPI203 and ABT-199 combination elicits a synergistic apoptotic cell death in DHL cell lines and primary tumor samples. (a) A set of 6 DHL cell lines were treated for 24-48 hours with 100-500 nM CPI203 and/or 10-50 nM ABT-199. Cytotoxicity was determined by the MTT assay, and the combination indexes (CIs) were calculated by using the Calcusyn software version 2.0 (Biosoft). Shown are the mean values obtained from the 6 cell lines exposed to 100 nM CPI203 ± 50 nM ABT-199. **(b)** OCI-LY8 cells were treated as above with CPI203 and/or ABT-199 and mitochondrial transmembrane potential (ΔΨm) loss and reactive oxygen species (ROS) production were evaluated by cytofluorimetric analysis of $DiOC(6)_3$ and dihydroethidine labeling, respectively. **(c)** DHL primary cells were cultured as previously in the presence or absence of 100 nM CPI203 + 50 nM ABT-199 and apoptotic fraction was evaluated by cytofluorimetric determination of hypodiploid sub-G1 cell population. **(d)** Levels of BCL-2, BFL-1, BIM, MYC and processing of caspase-3 and poly(ADP-ribose) polymerase (PARP) were determined by Western blot in OCI-LY8 cells treated as above. **(e)** Microphotographs of BFL-1 (green) and nuclei (blue) labeling in 2 representative DHL primary cultures in the absence (CNT) or the presence of CPI203 ± ABT-199. **(f)** OCI-LY8 cells were treated as previously and BCL-2-bound and -unbound fractions were analyzed by immunoprecipitation (IP) and Western blot for the presence of BAX and BIM.

Figure 4. CPI203 plus ABT-199 synergistically inhibits the growth of DHL tumors. (a) 10×10^6 OCI-LY8 cells were subcutaneously inoculated into the right flank of SCID mice. Tumor-bearing animals (n=4–5 mice per group) received intraperitoneal injection of 2.5 mg/kg CPI203 (BID) and/or oral administration of 20 mg/kg ABT-199 (once weekly), or an equal volume of vehicle, 5 days a week, for 2 weeks. Tumor volumes were measured each 2-3 days with external calipers. **(b)** *Left panel*, intratumoral glucose uptake was evaluated in representative mice injected intravenously with an IR800-labeled 2-deoxy glucose probe 72 h prior killing, and visualized with an Odyssey infra-red scanner (Li-Cor). *Right panel*, relative fluorescence quantification by means of the Image Studio software (Li-Cor) shows markedly reduced glucose uptake in tumor masses from mice receiving CPI203/ABT-199 combination, when compared with either vehicle- or ABT-199-treated animals. **(c)** Immunohistochemical staining of consecutive sections from representative tumors, showing the synergistic decrease in mitotic index and induction of apoptosis by the CPI203-ABT199 combination, as revealed by p-histone H3 and activated caspase-3 staining, respectively, together with the global downregulation of MYC, BFL-1, and (in lower extents) BCL-2 (magnification × 200). **(d)** *BCL2, MYC and BCL2A1* transcripts were quantified by RQ-PCR in 3 representative specimens per tumor group. **(e)** BCL-2, MYC and BFL-1 protein levels were evaluated by Western blot analysis in 4 representative tumor samples.

Figure 5. Possible mechanism of DHL cell priming by CPI203 and sensitization to ABT-199. In ABT-199 sensitive cells, the BH3 mimetic displaces BIM from BCL-2, allowing to the de-repression and/or direct activation of BAX, leading to subsequent mitochondrial depolarization and activation of caspases. In DHL cells, although ABT-199 achieves the dissociation of BCL-2-BIM complexes, overexpressed BFL-1 binds to and inactivates BIM, avoiding BAX activation and preserving cell survival. CPI203 primes cells to death by decreasing BFL-1 and increasing BIM protein levels, allowing its combination with ABT-199 to tip the balance between pro- and anti-apoptotic signaling toward induction of cell death.

Fig.1

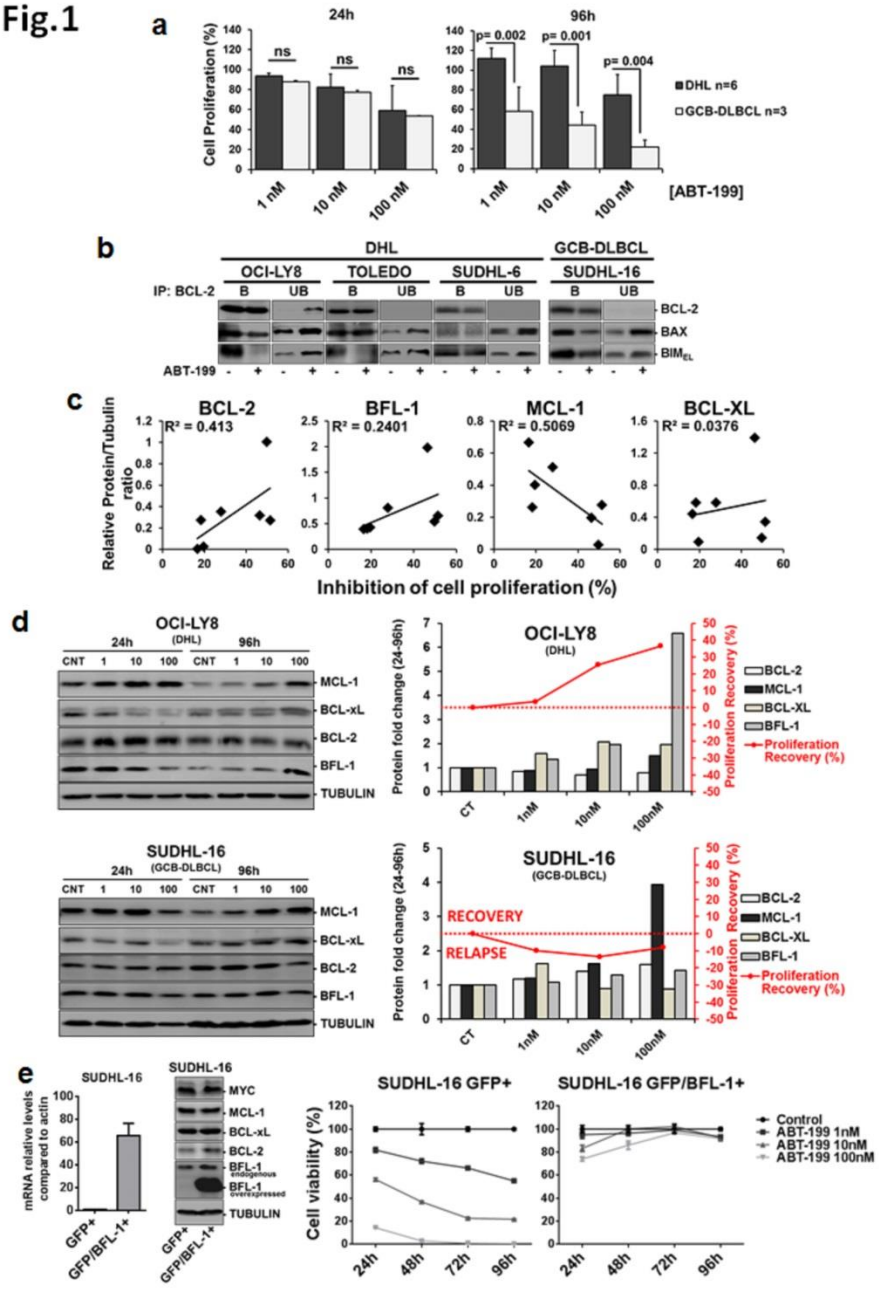

a
24h
96h
Cell Proliferation (%)
ns
p= 0.002
p= 0.001
p= 0.004
DHL n=6
GCB-DLBCL n=3
1 nM
10 nM
100 nM
[ABT-199]
b
DHL
GCB-DLBCL
OCI-LY8
TOLEDO
SUDHL-6
SUDHL-16
IP: BCL-2
B
UB
BCL-2
BAX
BIM EL
ABT-199
c
BCL-2
BFL-1
MCL-1
BCL-XL
R² = 0.413
R² = 0.2401
R² = 0.5069
R² = 0.0376
Relative Protein/Tubulin ratio
Inhibition of cell proliferation (%)
d
OCI-LY8
(DHL)
24h
96h
CNT
MCL-1
BCL-xL
BCL-2
BFL-1
TUBULIN
Protein fold change (24-96h)
Proliferation Recovery (%)
Proliferation Recovery
SUDHL-16
(GCB-DLBCL)
RECOVERY
RELAPSE
CT
1nM
10nM
100nM
e
SUDHL-16
mRNA relative levels compared to actin
GFP+
GFP/BFL-1+
MYC
BFL-1 endogenous
BFL-1 overexpressed
SUDHL-16 GFP+
SUDHL-16 GFP/BFL-1+
Cell viability (%)
24h
48h
72h
96h
Control
ABT-199 1nM
ABT-199 10nM
ABT-199 100nM

Fig.2

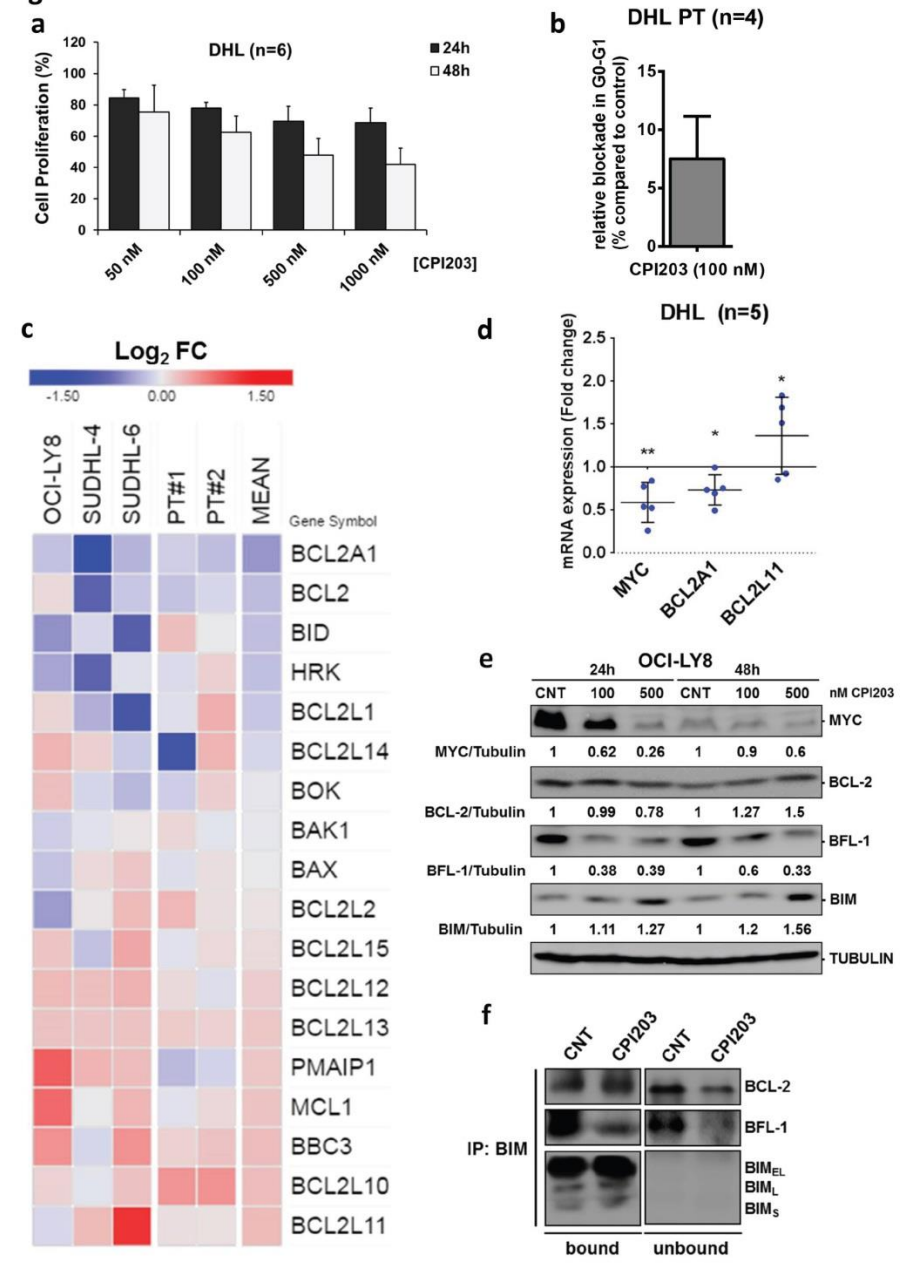

a
DHL (n=6)
24h
48h
Cell Proliferation (%)
120
100
80
60
40
20
0
50 nM
100 nM
500 nM
1000 nM
[CPI203]
b
DHL PT (n=4)
relative blockade in G0-G1 (% compared to control)
15
10
5
0
CPI203 (100 nM)
c
Log2 FC
-1.50
0.00
1.50
OCI-LY8
SUDHL-4
SUDHL-6
PT#1
PT#2
MEAN
Gene Symbol
BCL2A1
BCL2
BID
HRK
BCL2L1
BCL2L14
BOK
BAK1
BAX
BCL2L2
BCL2L15
BCL2L12
BCL2L13
PMAIP1
MCL1
BBC3
BCL2L10
BCL2L11
d
DHL (n=5)
mRNA expression (Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
MYC
BCL2A1
BCL2L11
e
OCI-LY8
24h
48h
CNT
100
500
CNT
100
500
nM CPI203
MYC
MYC/Tubulin
1
0.62
0.26
1
0.9
0.6
BCL-2
BCL-2/Tubulin
1
0.99
0.78
1
1.27
1.5
BFL-1
BFL-1/Tubulin
1
0.38
0.39
1
0.6
0.33
BIM
BIM/Tubulin
1
1.11
1.27
1
1.2
1.56
TUBULIN
f
CNT
CPI203
CNT
CPI203
IP: BIM
BCL-2
BFL-1
BIMEL
BIML
BIMS
bound
unbound

Fig.3

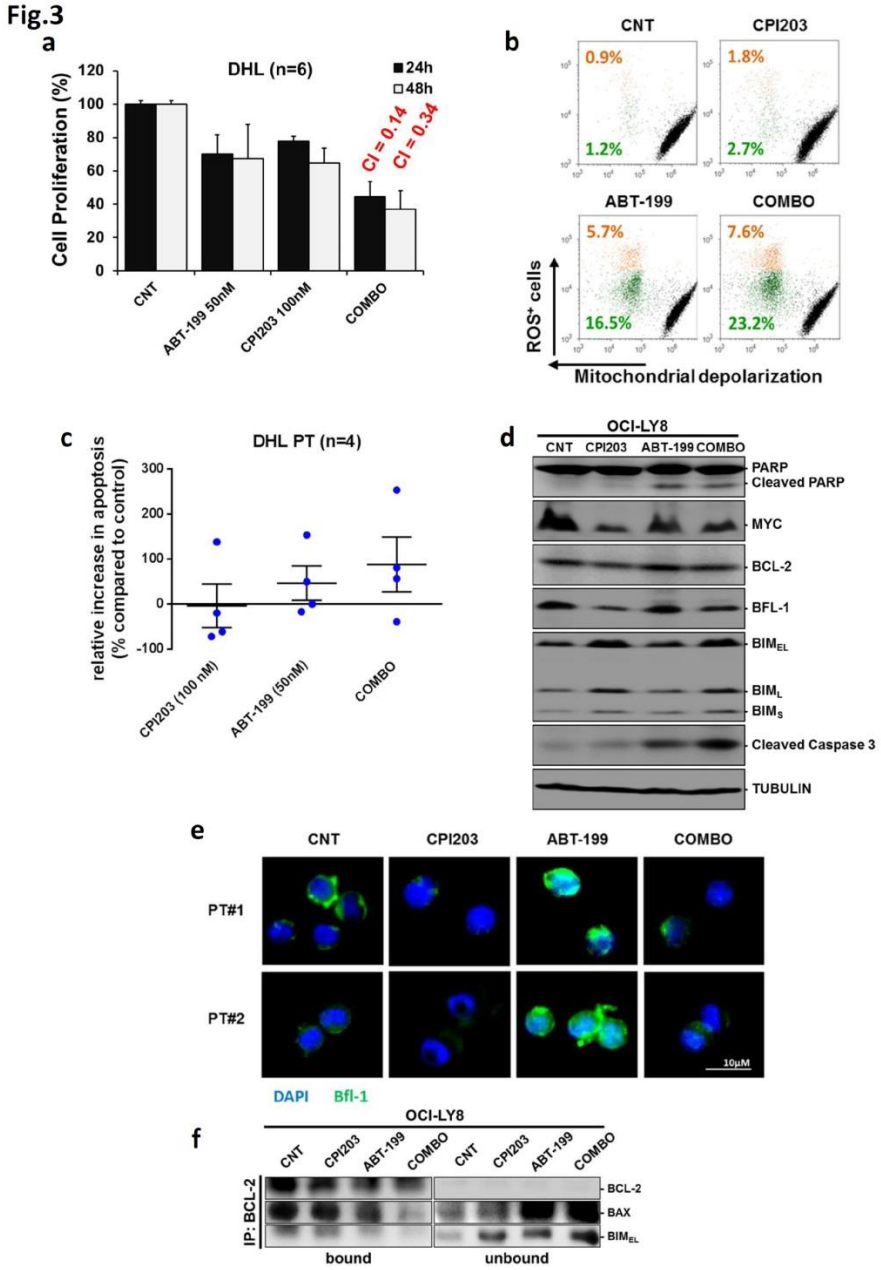
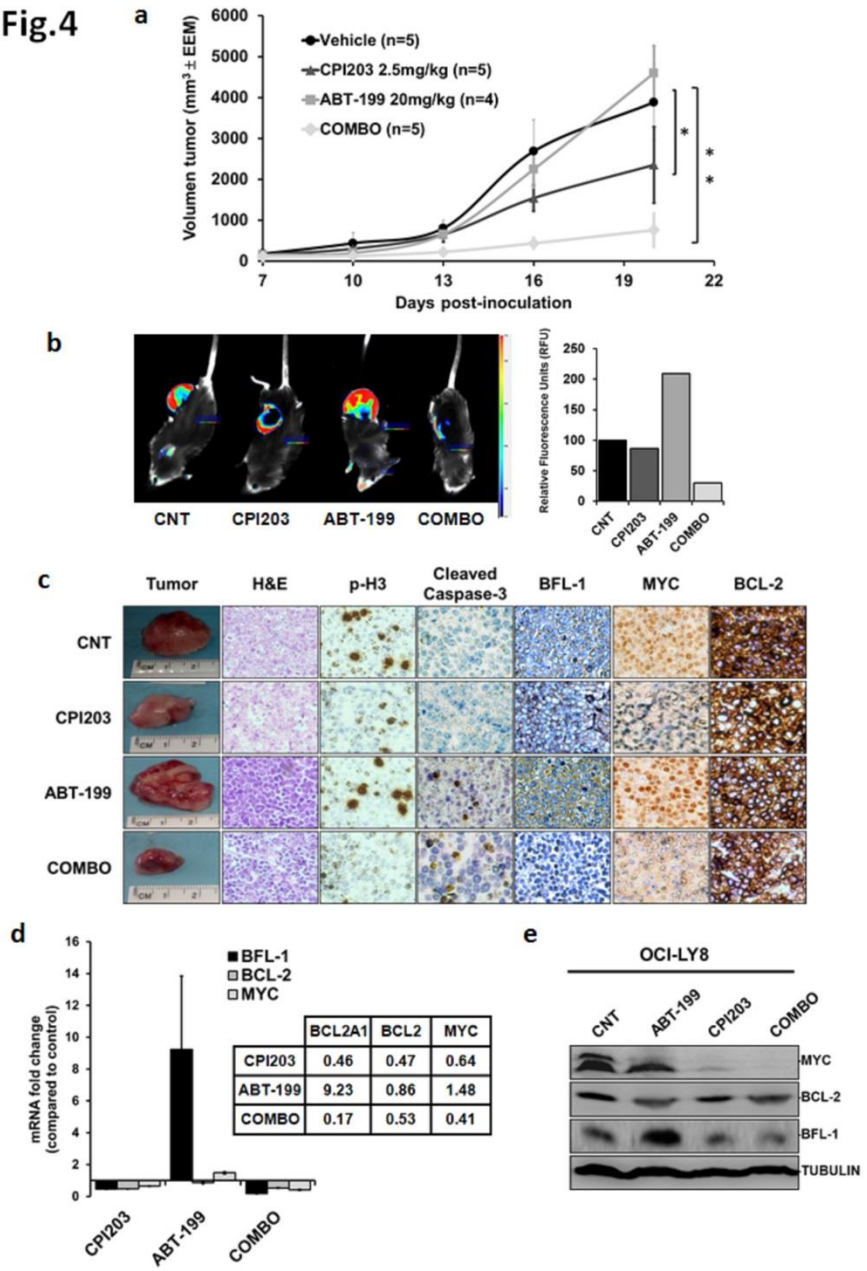
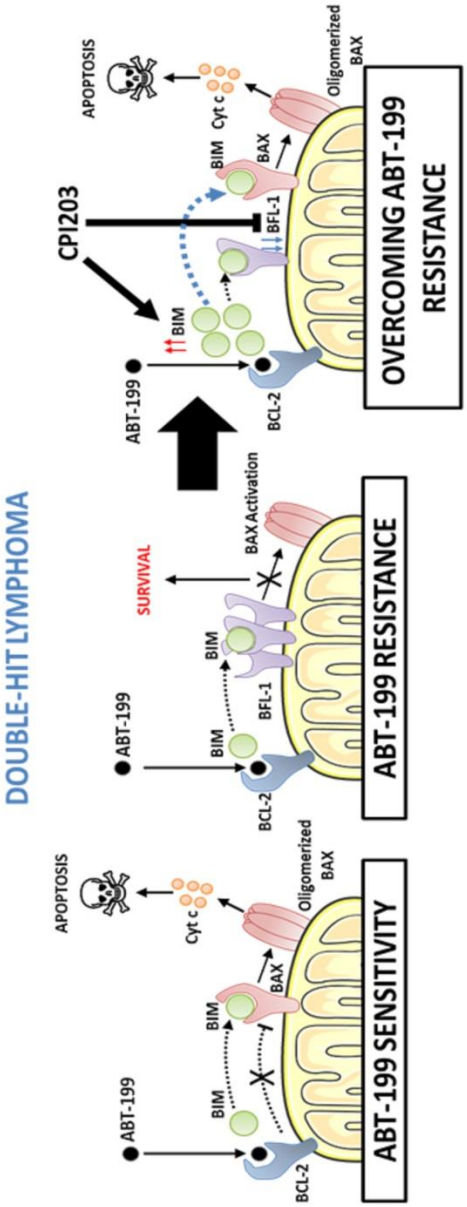

a
DHL (n=6)
24h
48h
Cell Proliferation (%)
120
100
80
60
40
20
0
CI = 0.14
CI = 0.34
CNT
ABT-199 50nM
CPI203 100nM
COMBO
b
CNT
CPI203
0.9%
1.8%
1.2%
2.7%
ABT-199
COMBO
5.7%
7.6%
16.5%
23.2%
ROS+ cells
Mitochondrial depolarization
c
DHL PT (n=4)
relative increase in apoptosis
(% compared to control)
300
200
100
0
-100
CPI203 (100 nM)
ABT-199 (50nM)
COMBO
d
OCI-LY8
CNT
CPI203
ABT-199
COMBO
PARP
Cleaved PARP
MYC
BCL-2
BFL-1
BIM$_{EL}$
BIM$_L$
BIM$_S$
Cleaved Caspase 3
TUBULIN
e
CNT
CPI203
ABT-199
COMBO
PT#1
PT#2
10μM
DAPI
Bfl-1
f
OCI-LY8
CNT
CPI203
ABT-199
COMBO
CNT
CPI203
ABT-199
COMBO
IP: BCL-2
BCL-2
BAX
BIM$_{EL}$
bound
unbound

Fig.4



	BCL2A1	BCL2	MYC
CPI203	0.46	0.47	0.64
ABT-199	9.23	0.86	1.48
COMBO	0.17	0.53	0.41

Fig.5


DOUBLE-HIT LYMPHOMA
ABT-199 SENSITIVITY
ABT-199
BIM
BCL-2
BAX
Oligomerized BAX
Cyt c
APOPTOSIS
ABT-199 RESISTANCE
SURVIVAL
BAX Activation
BFL-1
OVERCOMING ABT-199 RESISTANCE
CPI203

SUPPLEMENTAL TABLES

Supplemental Table S1.- Characteristics of DHL patients

Patients	Gender [1]	Age at diagnosis (years)	Cell Source [2]	Tumoral cells (%) [3]	Cytogenetic alterations	Treatment	BM involvement
PT#1	M	63	LN	34	BCL2+ (3 copies) BCL6+ (4 copies) MYC+ (3 copies)	R-CHOP	no
PT#2	F	57	LN	79	BCL2+ (3 copies) BCL6+ (3 copies) MYC+ (3 copies)	DA-EPOCH-R	yes (15%)
PT#3	M	59	soft tissue/ bulky	92	BCL2+ (3 copies) BCL6+ (3 copies) MYC+ (rear)	DA-EPOCH-R	no
PT#4	M	33	soft stomach	87	BCL2- BCL6+ (4 copies)/ MYC+ (rear)	R-CHOP	no

[1] F: female; M: male
[2] LN: lymph node
[3] CD20+ tumor cells determined by flow cytometry

Supplemental Table S2.- Modulation of gene expression by CPI203 in DHL cultures

DOWNREGULATED GENE SETS	SIZE	NES	FDR q-val
Hallmark gene sets			
MYC TARGETS V2	58	2.94	<0.0001
MYC TARGETS V1	197	2.28	<0.0001
INTERFERON ALPHA RESPONSE	97	1.95	<0.0001
INTERFERON GAMMA RESPONSE	199	1.89	<0.0001
IL2 STAT5 SIGNALING	198	1.61	0.008
Motif gene sets (C2)			
BROWNE INTERFERON RESPONSIVE GENES	67	2.29	<0.0001
BILD MYC ONCOGENIC SIGNATURE	190	2.27	<0.0001
JAIN NFKB SIGNALING	75	2.00	0.005
Motif gene sets (C3)			
MYCMAX 01	247	1.86	0.043
NFKB Q6	244	1.77	0.044
Motif gene sets (C5)			
GENE ONTOLOGY RIBOSOME BIOGENESIS	292	2.66	<0.0001
GENE ONTOLOGY PRERIBOSOME	56	2.65	<0.0001
GENE ONTOLOGY RRNA METABOLIC PROCESS	243	2.58	<0.0001
GENE ONTOLOGY B CELL ACTIVATION	130	2.25	<0.0001
Motif gene sets (C6)			
MYC_UP.V1_UP	167	2.82	<0.0001
IL2 UP.V1_UP	178	1.82	0.008
MTOR UP.N4.V1.UP	195	1.71	0.015
MTOR UP.V1.UP	167	1.70	0.014
Custom gene sets[1]			
BLIMP B-CELL REPRESSED	64	2.97	<0.0001
BLIMP1 TARGETS	145	2.56	<0.0001
GENES UPREGULATED BY MYC	27	2.51	<0.0001
C-MYC TARGETS	44	1.84	0.006
NFKB PATHWAY	237	1.83	0.006
UPREGULATED GENE SETS	**SIZE**	**NES**	**FDR q-val**
Hallmark gene sets			
P53 PATHWAY	198	-1.74	0.013
APOPTOSIS	160	-1.60	0.016
Motif gene sets (C2)			
WU_APOPTOSIS_BY_CDKN1A_VIA_TP53	54	-1.87	0.027
CONCANNON_APOPTOSIS_BY_EPOXOMICIN_UP	234	-1.66	0.099
Motif gene sets (C6)			
MYC UP.V1 DN	149	-1.63	0.024

GSEA was used to test for significant enrichment of defined gene signatures.
NES indicates Normalized Enriched Score; FDR, False Discovery Rate.
Thresholds: FDR <0.10 and NES>1.5
[1]Downloaded from http://lymphochip.nih.gov/signaturedb/index.html

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. (a) Relative proliferation recovery between 24h and 96h of treatment by ABT-199 in DHL and GCB-DLBCL (GCB) cell lines, as determined by MTT assay, and referred to control untreated cells. (b) Western blot analysis of basal BCL-2 and BCL-2-like protein expression in DHL and GCB-DLBCL cell lines. (c) Upper panels: time- and dose-dependent modulation of anti-apoptotic BCL-2 proteins in SUDHL-6 and Toledo cells upon ABT-199 treatment. BCL-2, MCL-1, BCL-XL and BFL-1 protein levels were plotted against the mean relative cell proliferation recovery observed between 24h and 96h (lower panels). (d) Microphotographs of GFP signals in control and BFL1+ SUDHL-16 cell lines upon a 4 day treatment with 1, 10, or 100 nM ABT-199.

Figure S2. Representative gene sets, either downregulated (a) or upregulated (b) by CPI203 treatment in DHL cells and primary samples

Fig. S1

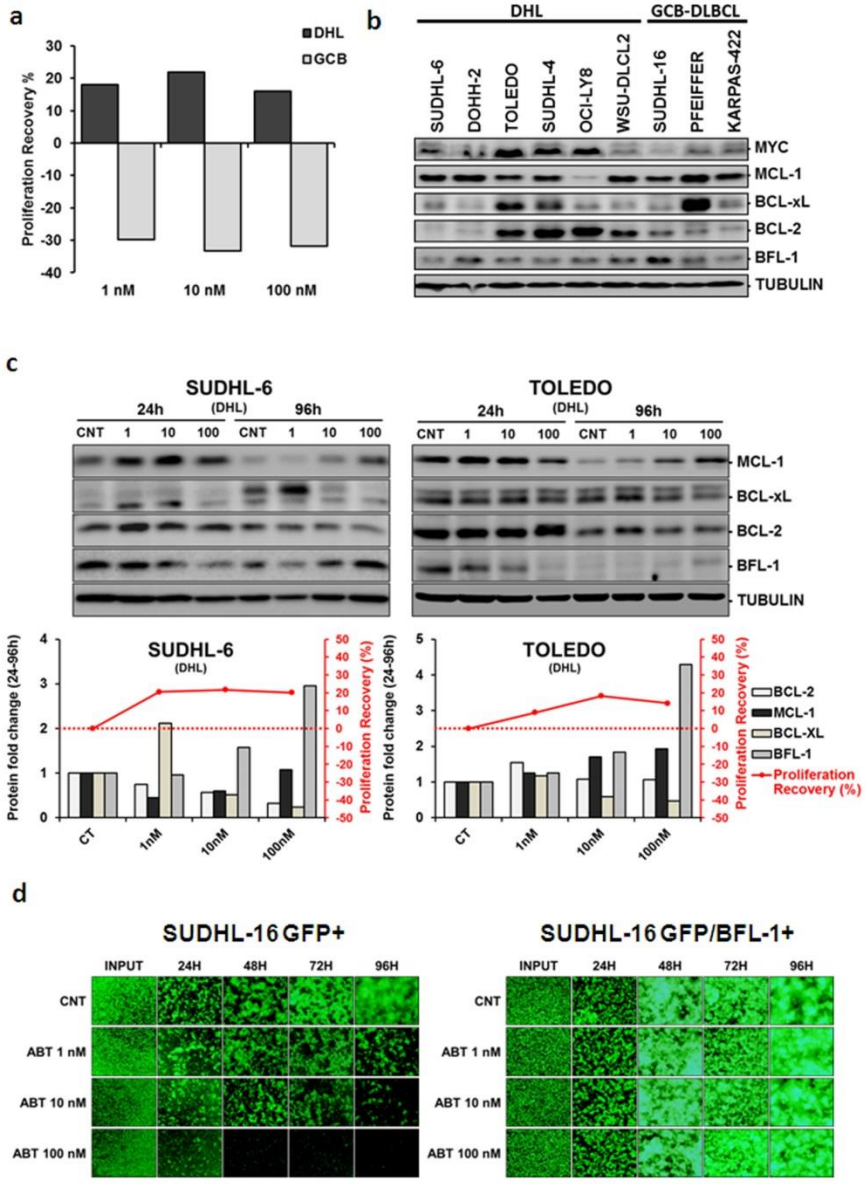
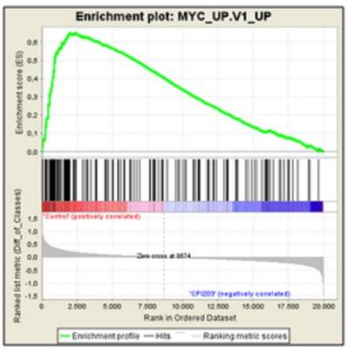

a
DHL
GCB
Proliferation Recovery %
30
20
10
0
-10
-20
-30
-40
1 nM
10 nM
100 nM
b
DHL
GCB-DLBCL
SUDHL-6
DOHH-2
TOLEDO
SUDHL-4
OCI-LY8
WSU-DLCL2
SUDHL-16
PFEIFFER
KARPAS-422
MYC
MCL-1
BCL-xL
BCL-2
BFL-1
TUBULIN
c
SUDHL-6
(DHL)
24h
96h
CNT 1 10 100 CNT 1 10 100
TOLEDO
(DHL)
24h
96h
CNT 1 10 100 CNT 1 10 100
MCL-1
BCL-xL
BCL-2
BFL-1
TUBULIN
SUDHL-6
(DHL)
Protein fold change (24-96h)
Proliferation Recovery (%)
CT
1nM
10nM
100nM
TOLEDO
(DHL)
Protein fold change (24-96h)
Proliferation Recovery (%)
CT
1nM
10nM
100nM
BCL-2
MCL-1
BCL-XL
BFL-1
Proliferation Recovery (%)
d
SUDHL-16 GFP+
INPUT
24H
48H
72H
96H
CNT
ABT 1 nM
ABT 10 nM
ABT 100 nM
SUDHL-16 GFP/BFL-1+
INPUT
24H
48H
72H
96H
CNT
ABT 1 nM
ABT 10 nM
ABT 100 nM

Fig. S2

a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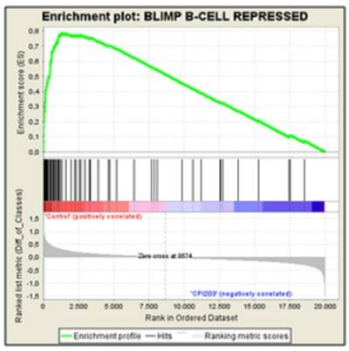
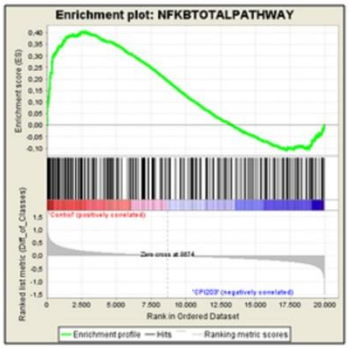
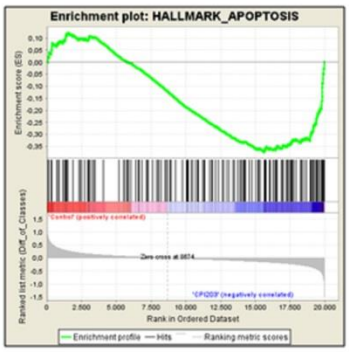
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


Enrichment plot: BLIMP B-CELL REPRESSED
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


Enrichment plot: NFKBTOTALPATHWAY
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

b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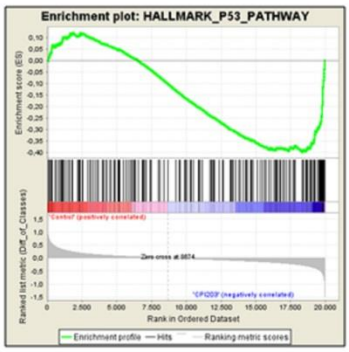
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


Enrichment plot: HALLMARK_P53_PATHWAY
Enrichment score (ES)
Ranked list metric (Diff_of_Classes)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

SECOND PAPER

Activity of the novel BCR kinase inhibitor IQS019 in preclinical models of B-cell non-Hodgkin lymphoma

P. Balsas[1]*, A. Esteve-Arenys[1]*, J. Roldán[1], L. Jiménez[1], V. Rodríguez[1], J. G. Valero[1], A. Chamorro-Jorganes[1], R. Puig de la Bellacasa[2], J. Teixidó[2], A. Matas-Céspedes[1], A. Moros[1], A. Martínez[3], E. Campo[1,3], A. Sáez-Borderías[4], J. I. Borrell[2], P. Pérez-Galán[1], D. Colomer[1,3] and G. Roué[1]

1 Division of Hematology and Oncology, Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Barcelona, Spain

2 Grup d'Enginyeria Molecular, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain

3 Department of Pathology, Hematopathology Unit, Hospital Clinic, Barcelona, Spain

4 Pangaea Biotech S.L., Quiron Dexeus University Hospital, Barcelona, Spain

5 Division of Hematology and Oncology, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

* These authors contributed equally to this work

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DOI 10.1186/s13045-017-0447-6

Journal of Hematology & Oncology

RESEARCH Open Access

Activity of the novel BCR kinase inhibitor IQS019 in preclinical models of B-cell non-Hodgkin lymphoma

P. Balsas[1†], A. Esteve-Arenys[1†], J. Roldán[1], L. Jiménez[1], V. Rodríguez[1], J. G. Valero[1], A. Chamorro-Jorganes[1], R. Puig de la Bellacasa[2], J. Teixidó[2], A. Matas-Céspedes[1], A. Moros[1], A. Martínez[3], E. Campo[1,3], A. Sáez-Borderías[4], J. I. Borrell[2], P. Pérez-Galán[1], D. Colomer[1,3] and G. Roué[1*]

Abstract

Background: Pharmacological inhibition of B cell receptor (BCR) signaling has recently emerged as an effective approach in a wide range of B lymphoid neoplasms. However, despite promising clinical activity of the first Bruton's kinase (Btk) and spleen tyrosine kinase (Syk) inhibitors, a small fraction of patients tend to develop progressive disease after initial response to these agents.

Methods: We evaluated the antitumor activity of IQS019, a new BCR kinase inhibitor with increased affinity for Btk, Syk, and Lck/Yes novel tyrosine kinase (Lyn), in a set of 34 B lymphoid cell lines and primary cultures, including samples with acquired resistance to the first-in-class Btk inhibitor ibrutinib. Safety and efficacy of the compound were then evaluated in two xenograft mouse models of B cell lymphoma.

Results: IQS019 simultaneously engaged a rapid and dose-dependent de-phosphorylation of both constitutive and IgM-activated Syk, Lyn, and Btk, leading to impaired cell proliferation, reduced CXCL12-dependent cell migration, and induction of caspase-dependent apoptosis. Accordingly, B cell lymphoma-bearing mice receiving IQS019 presented a reduced tumor outgrowth characterized by a decreased mitotic index and a lower infiltration of malignant cells in the spleen, in tight correlation with downregulation of phospho-Syk, phospho-Lyn, and phospho-Btk. More interestingly, IQS019 showed improved efficacy in vitro and in vivo when compared to the first-in-class Btk inhibitor ibrutinib, and was active in cells with acquired resistance to this latest.

Conclusions: These results define IQS019 as a potential drug candidate for a variety of B lymphoid neoplasms, including cases with acquired resistance to current BCR-targeting therapies.

Keywords: B-NHL, Btk, Lyn, Syk, Cell migration, Mouse model

Background

The B cell receptor (BCR) regulates multiple cellular processes which are critical for maintenance and survival of B cells, including proliferation, differentiation, and cell migration [1]. Antigen engagement to BCR extracellular domain leads to phosphorylation and activation of immunoreceptor tyrosine-based activation motifs located in the cytoplasmic portion and other proteins downstream the receptor. Within BCR signalosome, the Lck/Yes novel tyrosine kinase (Lyn) recruits and phosphorylates the spleen tyrosine kinase (Syk), triggering a proliferation and survival cascade signaling that involves the phosphorylation and activation of Brutons' tyrosine kinase (Btk), which subsequently phosphorylates phospholipase Cγ2 (PLCγ2), leading to calcium mobilization and activation of several downstream pathways, including MAP kinases, Akt and NF-κB [2]. In addition to tonic, ligand-mediated BCR signaling, chronic BCR activation can occur in the absence of antigen engagement [3], leading to aberrant, constitutive BCR activation in several B cell non-Hodgkin

* Correspondence: groue@clinic.ub.es
†Equal contributors
[1]Division of Hematology and Oncology, Institut d'Investigacions Biomèdiques August Pi iSunyer (IDIBAPS), Barcelona, Spain
Full list of author information is available at the end of the article

lymphoma (B-NHL) subtypes, including diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), follicular lymphoma (FL), and chronic lymphocytic leukemia (CLL) [4–7]. In these entities, BCR signaling represents an important pro-survival stimulus that may be stronger than in normal B cells, supporting the recent emergence of several BCR-targeting therapies [7]. But despite the promising results obtained with the first kinase inhibitors, such as fostamatinib and ibrutinib, specific for the Src-family kinases Syk and Btk [8], the design of new compounds is warranted to improve treatment efficacy and to by-pass the resistance appearing in primarily responsive patients [9–11]. In this context, we recently described the synthesis of a new family of 4-aminopyrido[2,3-*d*]pyrimidines with kinase inhibitory property and antitumoral activity in B lymphoid cells. Compound 19 (thereafter referred as IQS019) was identified as the most effective and specific molecule, with growth inhibitory 50 (GI_{50}) doses in the low micromolar range. Docking studies and biochemical assays further showed that the compound inhibited the active site of the BCR kinases Syk, Lyn, and Btk with higher efficacy than the reference kinase inhibitors [12, 13]. Here, using an extended panel of B-NHL cell lines and primary samples, we describe the full mechanism of action of this compound and report its remarkable antitumoral activity in vitro and in distinct B-NHL xenotransplant mouse models.

Methods

Cell lines and patients samples

Twenty-one cell lines from the different subtypes of B lymphoid neoplasm were used in this study (Table 1 and Additional file 1 Methods). All cell lines were routinely culture at 37 °C in a humidified atmosphere with 5% carbon dioxide in RPMI-1640, DMEM, or IMDM culture medium supplemented with 10–20% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 50 μg/ml penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Primary tumor cells from 13 CLL patients (Additional file 1: Table S2) were used. Tumor cells were isolated, cryopreserved, and conserved within the Hematopathology collection of our institution (Hospital Clínic-IDIBAPS Biobank R121001-094), as previously described [14].

Table 1 Sensitivity of B lymphoid cell lines to IQS019

Cell lines	B lymphoid subtypes	*TP53* status[a]	IQS019 cytotoxic effect (referred to untreated cells)	
			1 μM, 48 h	5 μM, 48 h
DOHH-2	FL	wt	26%	100%
WSU-NHL	FL	del/mut	6%	80%
WSU-FSCCL	FL	wt	26%	77%
SC-1	FL	del/mut	4%	43%
JEKO-1	MCL	del/mut	21%	75%
MAVER-1	MCL	del/mut	15%	70%
UPN-1	MCL	del/mut	14%	66%
HBL-2	MCL	del/mut	12%	64%
MINO	MCL	del/mut	19%	64%
GRANTA-519	MCL	wt	26%	63%
Z-138	MCL	wt	18%	62%
JVM-2	MCL	wt	16%	58%
REC-1	MCL	wt	12%	48%
MEC-2	CLL	del/mut	12%	51%
JVM-13	CLL	wt	7%	46%
MEC-1	CLL	wt	9%	33%
SUDHL-16	GCB-DLBCL	del/mut	15%	47%
OCI-LY8	GCB-DLBCL	del/mut	2%	29%
SUDHL-8	GCB-DLBCL	del/mut	15%	32%
OCI-LY10	ABC-DLBCL	wt	3%	47%
U-2932	ABC-DLBCL	del/mut	15%	51%

[a]17p13 deletion was assessed by fluorescence in situ hybridization and *TP53* mutational status was analyzed by direct sequencing
Abbreviations: *FL* follicular lymphoma *MCL* mantle cell lymphoma, *CLL* chronic lymphocytic leukemia, *DLBCL* diffuse large B cell lymphoma

Kinase inhibition profiling

The kinase inhibition profile of IQS019 (0.1 and 10 μM) was evaluated at Proqinase (Freiburg, Germany) using a Kinase 400-Profiler Panel, according to previously described procedures [13]. The residual activity (in %) for each compound well was calculated by using the following formula: Residual activity (%) = 100 x [(signal of compound−low control)/(high control−low control)].

Cell-based tyrosine kinase assay

In vitro inhibitory activity of IQS019 against BCR-related kinase was determined by Advanced Cell Dynamics (San Diego, CA, USA). Briefly, the Ba/F3 murine B lymphoid cell line was transfected with either a control vector or a vector containing the kinase domain of Btk, Syk, or Lyn, rending each cell line dependent upon activity of the recombinant kinase for survival. Cells were treated for 48 h with the indicated doses of IQS019 and cell viability was monitored via ATP concentration using CellTiter-Glo assay (Promega, Madison, WI, USA). IC_{50} values were determined using the GraphPad Prism software version 5.04 (San Diego, CA, USA)

Cell proliferation assay

Cells (4–6 x 10^5 cells/ml) were treated for the indicated times with IQS019 or ibrutinib (Selleck Chemicals, Munich, Germany) at doses ranging from 0.1 to 20 μM, and cell proliferation was determined by a modification of the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction method.

BCR stimulation and phospho-kinase detection

Cell lines (3–5 x 10^6 cells) and primary CLL samples (8–10 x 10^6 cells) were pretreated with 1 or 2.5 μM IQS019 for 90 min in FBS-free RPMI medium. Once starved, cells were incubated at 37 °C with 10 μg/ml of either anti-IgM (UPN-1, JVM-13, OCI-LY10 and primary CLL cells) or anti-IgG (DOHH-2) antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Based on preliminary experiments showing a cell type-dependent variation in the optimal duration of the stimulation, cells were exposed to their respective anti-Ig for 2 min (UPN-1 and OCI-LY10 cells), 30 min (DOHH-2 and JVM-13 cells), and 15 min (CLL primary cells). Detection of phospho-Syk, phospho-lyn and phospho-Btk was carried out by western blot and flow cytometry, respectively, as detailed in Additional file 1 Methods.

CXCL12-mediated chemotaxis

Cell lines and CLL primary cells were exposed as indicated to IQS019, with or without BCR ligation, and CXCL12-induced migration was evaluated using 24-well chemotaxis chambers containing 8 μm (cell lines) or 5 μm (primary cells) pore size inserts (Corning Life Science, Tewksbury, MA, USA), as previously described [15]. To quantify CXCR4-dependent F-actin polymerization, cells (300.000–500.000) treated as above were fixed on poly-L-lysine–coated glass coverslips with 4% paraformaldehyde, washed in PBS, permeabilized for 10 min with a solution containing 0.1% saponin (in PBS), followed by a 30 min incubation with 50 μg/ml phalloidin-TRITC (Sigma-Aldrich). Then, coverslips were washed three times with saponin 0.03%, mounted on glass slides with DAPI-containing Fluoroshield mounting medium (Sigma-Aldrich), and visualized on a Nikon H5505 microscope by means of a 60X NA oil objective (Nikon, Amsterdam, Netherlands) with the use of Isis Imaging System v5.3 software (MetaSystems GmbH, Heidelberg, Germany).

Xenograft mouse models and immunohistochemical studies

For MCL xenotransplant model, CB17-SCID female mice (Janvier Labs, Le Genest-Saint-Isle, France) were inoculated subcutaneously with UPN-1 cells as previously described [14].Tumor-bearing mice were randomly assigned into equivalent cohorts and received a daily dose of 2 mg/kg, 10 mg/kg (i.p.), or 25 mg/kg (p.o.) IQS019-2$MeSO_3H$ or ibrutinib, or equal volume of vehicle, for 15 days, in a five/two (on/off) schedule. Animals were sacrificed and tumor samples were processed and stained for phospho-Histone H3 and cleaved caspase-3 as previously described [14]. Detection of phospho-Syk, phospho-Lyn and phospho-Btk was carried out from OCT tumor section as explained in Additional file 1 Methods. For systemic FL model, 12 SCID mice were intravenously inoculated via tail vein with 1.5 x 10^7 DOHH-2 cells per mouse. One week later, animals were randomly assigned into two equivalent cohorts and treated intraperitoneally with 2 mg/kg IQS019-2$MeSO_3H$ or vehicle, as before. Mice were then sacrificed and immunodetection of phospho-BCR kinases was performed as detailed in Additional file 1 Methods.

Statistical analysis

Unless otherwise specified, the data are depicted as the mean ± SD of three independent experiments. Unpaired and paired T-tests were used to obtain the statistical analysis using Graph Pad Prism software 4.0. Results were considered statistically significant when $p < 0.05$ (*, **$p < 0.01$, ***$p < 0.001$).

Results

Antitumor effect of the 4-aminopyrido[2,3-*d*]pyrimidine IQS019 in B lymphoid cell lines and primary samples

To assess the selectivity of the kinase inhibitor IQS019 (Fig. 1a and ref [13]), we first evaluated its inhibitory property against a panel of 400 kinases, including 70 disease-relevant protein kinase mutants and 13 lipid kinases, covering about 60% of the human kinome. We

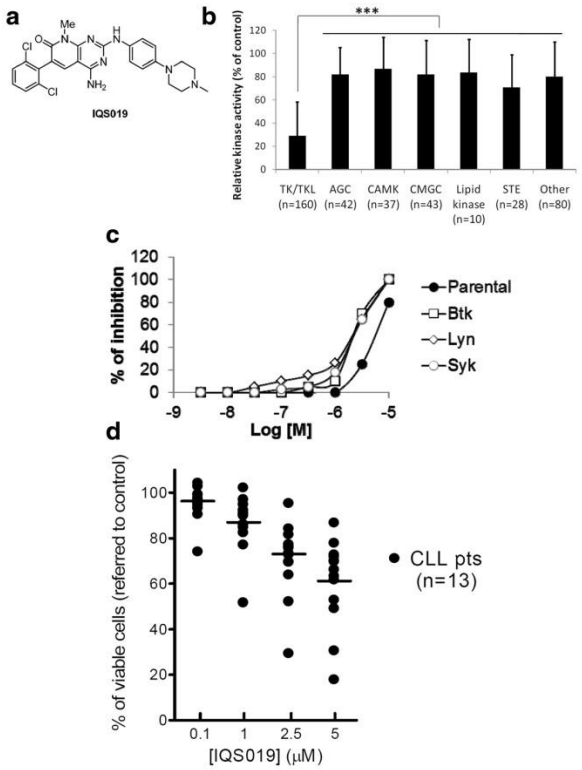

Fig. 1 IQS019 simultaneously targets Syk, Lyn and Btk tyrosine kinases and blocks cell proliferation in distinct subtypes of B lymphoid neoplasms. **a** Chemical structure of IQS019. **b** Selectivity kinase inhibition profile of IQS019 against tyrosine kinase (TK) and tyrosine kinase-like (TKL), when compared to the other subfamilies of protein kinases (*** $p < 0.001$). **c** Effect of IQS019 on the proliferation of control (parental), Syk-, Lyn-, and Btk-overexpressing Ba/F3 cells. The different cell lines were treated with increasing dose of IQS019 and proliferation blockade was assessed by MTT assay, using untreated cells as a reference. Measured data represent the activities of duplicate determinations. **d** Effect of IQS019 on the proliferation of CLL primary cultures. Cells (2 x 10^5) were incubated with increasing concentrations of IQS019 for 24 hours and cell viability was determined by the MTT reduction assay. Results are the mean values ± SD of four replicates, and are referred to control, untreated cells

found the compound to be preferentially active against tyrosine kinase (TK) and tyrosine kinase-like (TKL) families, reaching a mean residual kinase activity of 28% at a 10 μM dose, while this activity remained above 70% in all the other kinase subgroups (Fig. 1b, *** $p < 0.001$ and Additional file 1: Figure S1). Of special interest, in a set of 17 TK/TKLs, the compound was able to inhibit at least 20% of the kinase activity at the lowest dose (0.1 μM) and to achieve an almost complete kinase inactivation at the 10 μM concentration. These kinases corresponded to leucocyte-, BCR-, or T-cell receptor (TCR)-related kinases (Lyn, Blk, Lck, Src, Frk, Csk, Hck, Fyn, Btk, Syk), the member of the Tec family of non-receptor tyrosine kinases, Bmx, and other receptor tyrosine kinases with lower relevance in B-NHL, such as Ddr2, Egfr, EphA, Erbb, Fgr and Braf (Additional file 1: Table S1). Among these potential targets, a radiometric kinase activity study further showed that IQS019 had an IC_{50} in the low micromolar range for the BCR kinases Lyn (0.15 μM), Syk (1.6 μM) and Btk (2.1 μM), corresponding to those kinases able to bind the compound in their active site [13]. Accordingly, ectopic expression of each individual kinase in B lymphoid cells rendered them dependent of these kinases for their survival and increased cell sensitivity to IQS019. Indeed, while the calculated IC_{50} of the compound was 5.4 μM in parental Ba/F3 cells, this value decreased to 2.2, 1.4, and 2.2 μM in Btk-, Lyn-, or Syk-overexpressing cells, respectively

(Fig. 1c). Thus, these results confirm that IQS019 is a potent tyrosine kinase inhibitor, with the unique ability to bind and to simultaneously inhibit the three BCR kinases Lyn, Syk, and Btk.

We further assessed the activity of the compound in vitro using a panel of 21 B-NHL cell lines representative of the CLL, MCL, FL and DLBCL subtypes (Table 1). We show that a 5 μM dose of the compound decreased cell proliferation in all the cell lines (range: 29–100%), being MCL and FL cells significantly more sensitive to the compound (mean cytotoxicity at 48 h: 67.2 ± 15%) than CLL cells and DLBCL cells of either activated B-cell (ABC) or germinal centre B-cell (GCB) subtype (mean cytotoxicity at 48 h: 42 ± 9%) ($p = 0.0002$). Based on these results, a set of 13 CLL primary cultures were exposed for 24 h to increasing doses of IQS019 and cell viability was measured by MTT assay. Although a high variability was observed among cases, the viability decreased in a dose-dependent manner in all the samples treated with the compound (Fig. 1d). The calculated IC_{50} was 6.1 μM in this set of samples, corresponding to the upper range of the values found in the cell lines. Similar responses were observed in FL and MCL primary cultures (data not shown). Of note, no association could be established between sensitivity to IQS019 and common cytogenetic alterations, *TP53* mutation and/or deletion, or *IGHV* mutational status (Table 1, Additional file 1: Table S2 and Figure S2a). Of interest, a 24 h treatment with a 5 μM dose of the compound induced about 35% apoptosis increase in the representative cell lines UPN-1 and DOHH-2 (Additional file 1: Figure S2b). In CLL and primary cultures ($n = 6$) the average cell death induction reached 26% (range: 9.5–51.5%), as shown in the representative cases, CLL n.2 and CLL n.10 (Additional file 1: Figure S2b and data not shown). This phenomenon was completely abrogated in the presence of the pan-caspase inhibitor Q-VD-OPh. In parallel, the analysis of phospho-histone H3 levels as a surrogate of mitotic progression indicated a notable decrease of this marker in five out of six primary CLL cases treated with the compound (Additional file 1: Figure S2c). Thus, altogether these results demonstrate that IQS019 antitumor activity in B lymphoid cells involved both a blockade in cell proliferation and the induction of a caspase-dependent cell death.

IQS019 antagonizes constitutive and antigen-mediated BCR signaling

Based on the above results, we analyzed the effect of IQS019 on the phosphorylation status of Syk, Lyn and Btk in four cell lines representative of MCL (UPN-1), FL (DOHH-2), CLL (JVM-13), and DLBCL (OCI-LY10) subtypes. Cells were incubated for 6 h with increasing concentrations of IQS019 and phosphorylation levels of Syk and Lyn at their respective Tyr352 and Tyr396 residues, were evaluated by Western blot, while Btk phosphorylation at Tyr223 residue was analyzed by flow cytometry. As observed in Fig. 2a, IQS019 treatment led to a dose-dependent dephosphorylation of Syk and Lyn in the four cell lines tested. Consistent with the cytotoxicity of the compound (Table 1), a complete dephosphorylation of the two kinases was observed in UPN-1 and DOHH-2, while a slight, persistent phosphorylation of both Syk and Lyn was detected in OCI-LY10 and JVM-13 cells (Fig. 2a). Regarding Btk phosphorylation, flow cytometry analysis showed a 30% (UPN-1 and OCI-LY10) and a 60% (DOHH-2 and JVM-13) decrease in the relative mean fluorescence intensity ratio (r) of phospho-Btk signal in cells exposed to a 5 μM dose of the compound (Fig. 2b).

In a second step, the four previous cell lines and two representative primary CLL cases were BCR-stimulated with their corresponding anti-Ig in the presence of increasing concentrations of IQS019, and phospho-Syk, phospho-Lyn and phospho-Btk levels were analyzed as above. As shown in Fig. 3a, BCR ligation induced an increase in the phosphorylation levels of Syk and Lyn in all the samples tested, that was hampered by IQS019 in a dose-dependent manner. Remarkably, a dose of IQS019 as low as 1 μM was sufficient to completely counteract the anti-IgM-mediated activation of Syk and Lyn in the highly IgM-responsive (unmutated *IGHV*) CLL sample showing the greatest efficacy of the stimulation (CLL#10, Fig. 3a). Similarly, IQS019 efficiently counteracted Ig-induced Btk phosphorylation in cell lines, as shown by a 30 to 70% reduction in relative phospho-Btk levels (Fig. 3b). In CLL primary cells, for all but 1 cases out of the 6 examined, IQS019 achieved a 30% reduction in phospho-Btk levels ($p = 0.0005$), as shown in the representative CLL no.10 (Fig. 3b and data not shown). Of note, in the representative cell line UPN-1, Syk-dependent phosphorylation of Btk at Tyr551 was negligible upon BCR triggering and remained unaffected in the presence of IQS019 (data not shown), suggesting that IQS019-mediated inhibition of Btk requires a direct interaction of the compound with the kinase, rather than an indirect, Syk-mediated signal transduction. Altogether, these results suggest than IQS019 counteracts both constitutive and antigen-induced BCR signaling in B lymphoid cell lines and primary cells.

IQS019 inhibits CXCL12-mediated migration of malignant B cells

Migration of neoplastic B cells has been shown to be heavily affected upon exposure to drugs targeting the BCR-associated kinases, as these latest tightly regulate the re-organization of the cytoskeleton required for cell chemotaxis [15]. Thus, we evaluated the effect of

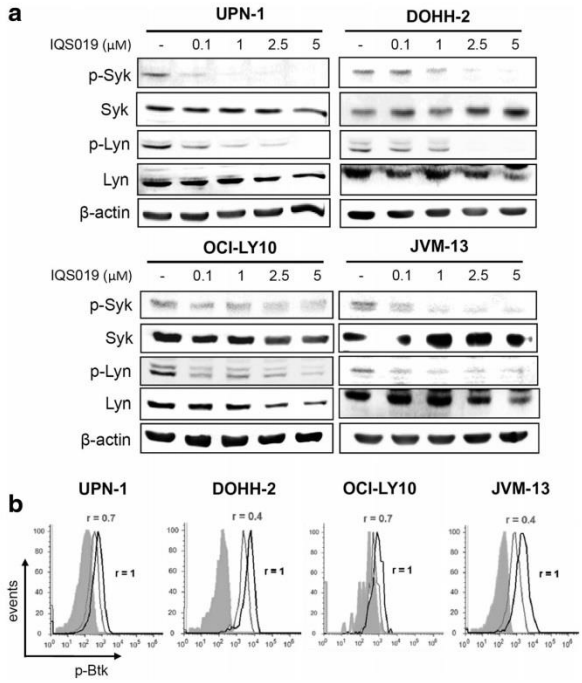

Fig. 2 IQS019 impairs constitutive phosphorylation of Syk, Lyn and Btk tyrosine kinases in malignant B cells. **a** UPN-1, DOHH-2, OCI-LY10, and JVM-13 cells (6 x 10^6) were treated with increasing concentrations of IQS019 for 6 h, followed by Western Blot detection of phospho(p)-Syk, and p-Lyn, using β- actin as a loading control. Shown is a representative experiment from two replicates. **b** Flow cytometry analysis of p-Btk levels in cell lines treated as before. A PE-labeled mouse IgG1 κ was used as an isotype control (*grey* filled histogram). Indicated are the relative median fluorescence intensity (*r*) values observed after treatment with 5 μM IQS019 (*grey curves*), and referred to control untreated cells (*black curves*)

IQS019 on the migratory capacity of malignant B cells, using a CXCL12-dependent chemotaxis assay with 3 cell lines harboring detectable levels of CXCR4 (Additional file 1: Figure S3) and in a set of seven CLL primary samples, either untreated or pre-treated with IQS019 or with the standard CXCR4 antagonist AMD3100. The migration induced by recombinant CXCL12 in MCL, FL and DLBCL cell lines was significantly inhibited by the compound at all the doses tested (Fig. 4a). The statistical significance of this effect was higher at the 2.5 μM than at the 1 μM dose in DOHH-2 and OCI-LY10 cells, while an almost complete inhibition of cell migration was achieved in UPN-1 cells at the lowest dose. In the case of CLL primary cells, since the stimulation of BCR has been shown to facilitate CXCL12-mediated migration [15], we evaluated the activity of IQS019 after BCR crosslinking. As shown in Fig. 4b, c, IQS019 significantly overcame IgM-activated, CXCL12-dependent chemotaxis in all the primary samples tested, either at the 1 μM dose (mean inhibition: 51.5%; range: 23.9–85.5%; $p = 0.0013$) or at the 2.5 μM dose (mean inhibition: 82.9%; range: 63.4–97.6%; $p < 0.0001$), when compared to untreated control cells. Accordingly, the mean fraction of cells with detectable F-actin polymerization shifted from 13.8% in control cells to 75.1% after CXCL12 stimulation, and was lowered down to 25.9% in the presence of IQS019 (Fig. 4d, *** $p = 0.0003$). Of special interest, when comparing with AMD3100, IQS019 showed similar, or even superior anti-migratory activity (Fig. 4a, b and c). These results indicate that IQS019-mediated inhibition of BCR upstream kinases may interfere with B cell chemotaxis and tumor cell dissemination.

IQS019 is safe and impairs tumor outgrowth and malignant B cell homing to spleen in vivo

In order to validate the activity of IQS019 in vivo, we first synthesized the salt form of the compound, thereafter labeled as IQS019-2MeSO3H, and evaluated its

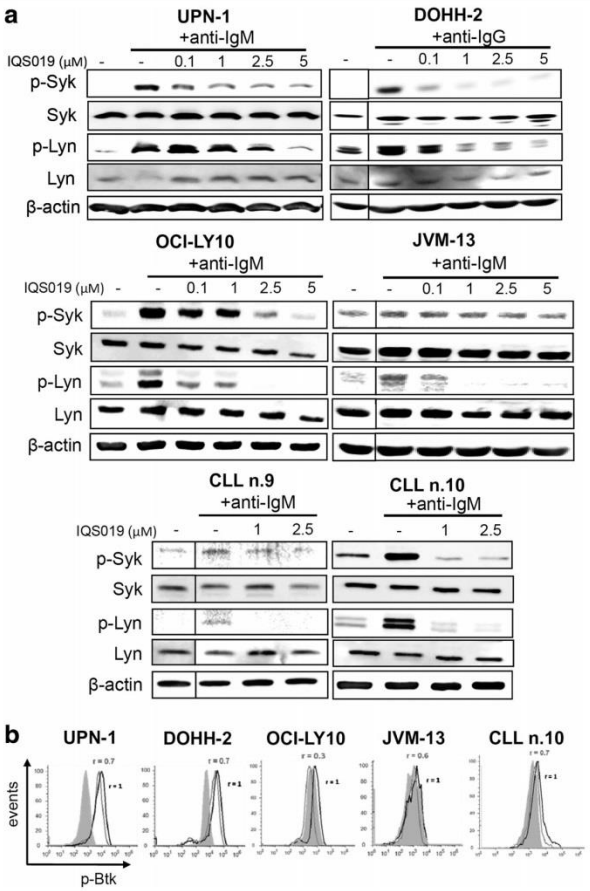

Fig. 3 IQS019 overcomes anti-Ig-mediated BCR activation in B lymphoid cell lines and primary samples. Representative cell lines and primary cultures were pre-treated for 1.5 h with increasing concentrations of IQS019, followed by anti-Ig-mediated BCR ligation, as described in methods section. **a** Expression levels of p-Syk and p-Lyn were analyzed by Western Blot, as previously, using β-actin as a loading control. Shown is a representative experiment from two replicates. **b** p-Btk levels were analyzed as previously by flow cytometry in B lymphoid cell lines and primary cultures, pre-stimulated with their respective anti-Ig and either untreated (*black curves*) or exposed to 5 μM IQS019 (*grey curves*). Shown is a representative experiment from a CLL patient out of a series of six cases

single-dose toxicity over 14 days after intravenous administration in healthy immunodeficient (SCID) mice (details in Additional file 1 Methods). As the maximum tolerated dose was not reached, 2 and 10 mg/kg doses were selected for further in vivo experiments. We then developed two different, complementary xenotransplant animal models of the two entities showing increased sensitivity to the compound in vitro, i.e., MCL and FL. Heterotopic MCL tumors were generated in SCID mice subcutaneously inoculated with UPN-1 cells, while a systemic (i.e., characterized by homing of tumor B cells from peripheral blood to spleen) FL tumor model was obtained by intravenous injection of DOHH-2 cells in SCID mice. As shown in Fig. 5a, after two weeks of treatment, mice bearing MCL tumors and dosed with IQS019-2MeSO$_3$H showed a 63% reduction in tumor volume, when compared to the vehicle group ($^*p < 0.05$). There was not subsequent improvement of the anti-tumor activity of the compound between the 2 mg/kg and the 10 mg/kg dosing, suggesting that optimal

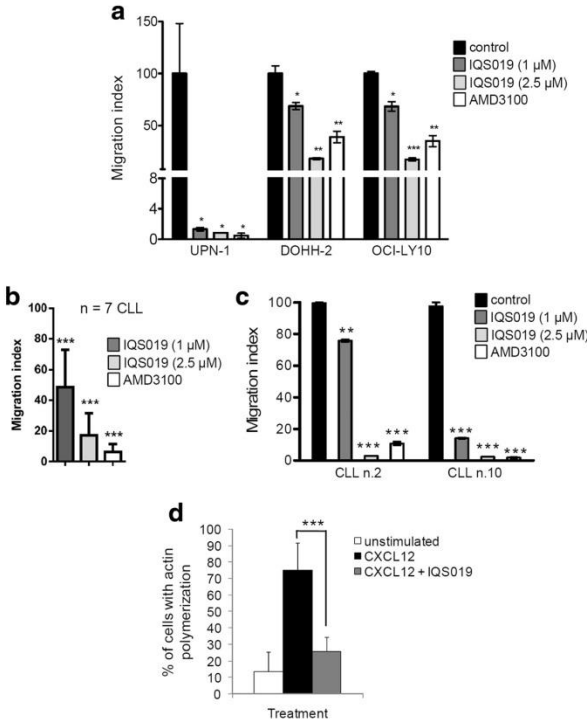

Fig. 4 IQS019 interferes with malignant B cell chemotaxis. Representative, CXCR4-expressing B lymphoid cell lines (**a**) and a set of $n = 7$ CLL primary samples (**b**) were exposed for 1.5 h to 1 or 2.5 μM IQS019, with or without Ig-mediated BCR stimulation, followed by cytofluorimetric recounting of cells migrated towards recombinant CXCL12 in a 4-h transwell assay. Treatment with the CXCR4 antagonist AMD3100 (40 μM) was used as a control of chemotaxis blockade. Expressed are the ratios between CXCL12-dependent and CXCL12-unspecific migration. **c** Cell migration profiles from two representative CLL cases are shown. **d** A set of six CLL cultures were treated as above with CXCL12 +/- IQS019 and stained with phalloidin-TRITC. Cells with high levels of polymerized F-actin were recounted for each condition by two independent reviewers. Shown are the mean results obtained from the six cases. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

activity was reached at the lowest dose. Consistently, tumor metabolism was similarly decreased in both treatment groups, as glucose uptake fell to 50–52% in tumors from all IQS019-2MeSO$_3$H-exposed animals, irrespective of the dose (Fig. 5b). This effect was closely related to the inhibition of the three BCR-related kinases Syk, Lyn, and Btk, as shown by a complete reduction of their phosphorylated forms in the drug-treated specimens, when compared to the control group (Fig. 5c). Immunohistochemical analysis of representative tumor sections further revealed that IQS019 therapy efficiently reduced the mitotic index and induced apoptosis in UPN-1-derived tumors, as shown by a decreased labeling of phospho-histone H3 and an intracellular increase in the activated form of caspase-3 (Fig. 5c).

In the systemic DOHH-2 mouse model, mice dosing was initiated at day 7 post-inoculation, with a 2 mg/kg IQS019-2MeSO$_3$H regimen, daily, for 15 days. Once inoculated, FL cells rapidly migrate to the spleen [16]. Therefore, at the end of the procedure, entire spleens were processed, and the presence of malignant B cells was evaluated by labeling with anti-human CD45 antibody and tumor cell recounting on a flow cytometer. IQS019-2MeSO$_3$H treatment induced a 52% reduction in tumor cell infiltration into the spleen, when compared to vehicle group (Fig. 5d, * $p = 0.01$). Accordingly, the r fluorescence values of phospho-Syk, phospho-Btk, and phospho-Lyn, decreased by 83, 57, and 33% in tumors B cells purified from IQS019-treated animals (Fig. 5e). Altogether, these results demonstrate that IQS019 is safe and exhibits in vivo efficacy against MCL and FL tumor burden, involving the inhibition of BCR signaling and the blockade of tumor cell homing to lymphoid compartment.

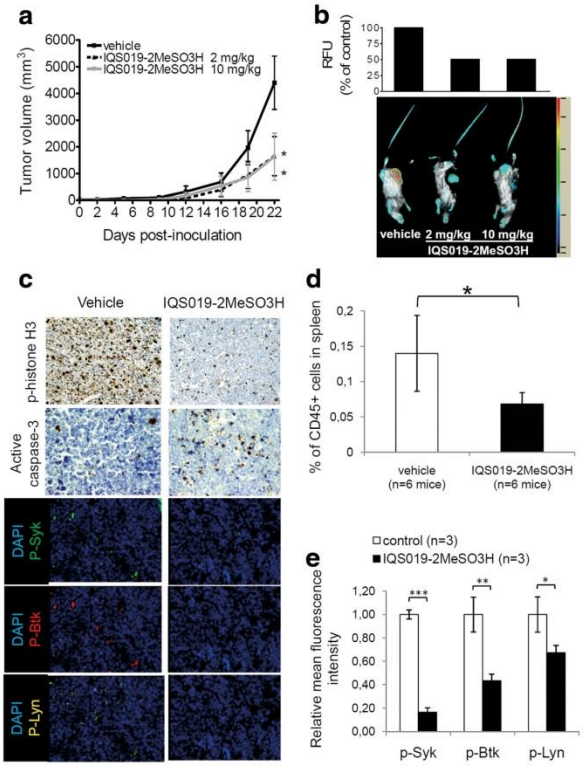

Fig. 5 IQS019 impairs tumor growth and homing of B lymphoid cells to spleen *in vivo*. **a** SCID mice were inoculated with UPN-1 cells and began treatment at day 10 post-inoculation with 2 or 10 mg/kg IQS019-2MeSO$_3$H or equal volume of vehicle. IQS019-2MeSO$_3$H was administrated 5 days a week for 2 weeks. **b** *Lower panel*, intratumoral glucose uptake was evaluated in representative mice injected intravenously with an IR800-labeled 2-deoxy glucose probe 24 h prior sacrifice, and visualized with an Odyssey infra-red scanner (Li-Cor). *Upper panel*, relative fluorescence quantification by means of the Image Studio software shows markedly reduced glucose uptake in tumor masses from mice receiving IQS019-2MeSO$_3$H, when compared to vehicle-treated animals. **c** Immunostaining of consecutive sections from representative UPN-1-derived tumors, showing the decrease of proliferation and the induction of apoptosis accompanying the downregulation of p-Syk, p-Lyn and p-Btk upon IQS019-2MeSO$_3$H treatment (magnification 200X). **d** Mice were injected intravenously with DOHH-2 cells and, after one week, received a 2 mg/kg dose of IQS019-2MeSO$_3$H or vehicle, daily, for up to 14 days. Mice were then sacrificed and human (CD45+) malignant B cells were recounted from spleen as described in methods section (statistical significance: * $p < 0.05$). **e** In each treatment group, CD45+/CD20+ human B cells isolated from $n = 3$ representative animals, were labeled with anti-p-Syk, anti-p-Lyn, or anti-p-Btk antibody, and fluorescence was recorded on a cytometer. Shown are the relative *r* values among control and vehicle group, calculated for each phospho-kinase

IQS019 shows superior anti-tumor activity than ibrutinib in vitro and in vivo

We previously reported that IQS019 presented an increased anti-proliferative activity in vitro when compared to ibrutinib, in a single MCL cell line and at a single time point [13]. To confirm this preliminary experiment, we compared by MTT assay the anti-proliferative effect of IQS019 and ibrutinib at 24, 48, and 72 h, using doses ranging from 0.5 to 10 μM, in a panel of eight cell lines that included the ibrutinib-sensitive MINO, REC-1, UPN-1, DOHH-2, and WSU-NHL and the ibrutinib-resistant Z-138, GRANTA-519, and JVM-2 cells. Figure 6a shows that the mean IC_{50} of ibrutinib remained significantly high (i.e., > 10 μM) in this set of cell lines, even after a 72-h drug exposure, mainly due to the high values observed in the resistant cell lines (152.4 μM for Z-138, 22.1 μM for GRANTA-519 and 77.4 μM for JVM-2). In contrast, IQS09-mediated proliferation blockade was almost completely reached in all the cell lines after only 24 hours, with a

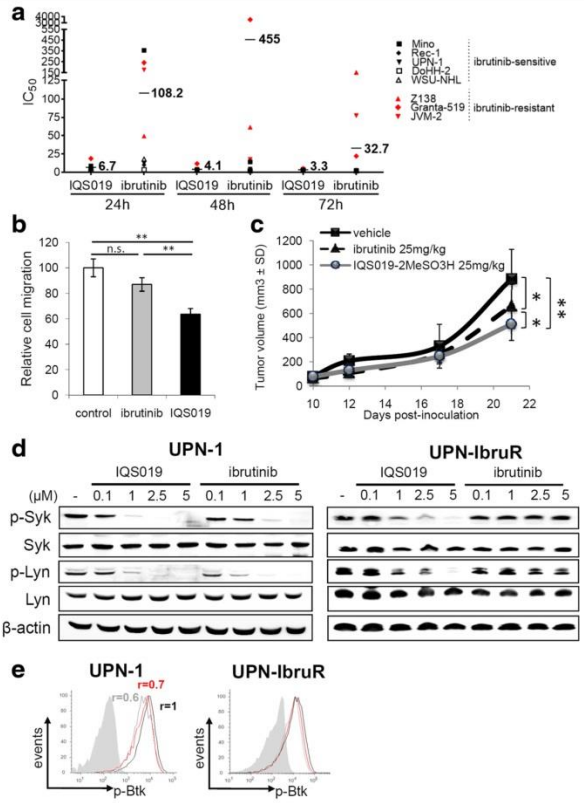

Fig. 6 Improved in vitro and in vivo activity of IQS019 *vs* the Btk inhibitor ibrutinib. **a** A panel of eight B-NHL cell lines was incubated with increasing concentrations of IQS019 or ibrutinib for 24, 48, and 72 h and IC_{50} was calculated as previously. **b** IQS019 has improved anti-migratory activity. UPN-1 cells were exposed to 5 μM IQS019 or ibrutinib for 90 min followed by evaluation of CXCL12-dependent cell chemotaxis, as described in methods section. Statistical significance: ** $p < 0.01$. **c** Orally administered IQS019 impairs tumor outgrowth more efficiently than ibrutinib. SCID mice were inoculated with UPN-1 cells subcutaneously and, at day 10 post-inoculation, started to be dosed p.o., 5 days a week, for two weeks, with 25 mg/kg IQS019-2MeSO3H, 25 mg/kg ibrutinib or equal volume of vehicle ($n = 10$ animals per group). Tumor volumes were recorded as above. Statistical significance: * $p < 0.05$, ** $p < 0.01$. **d** Western blot analysis of phospho-Lyk, phospho-Syk and (**e**) cytofluorimetric analysis of phospho-Btk in UPN-1 and UPN-IbruR cells treated for 6 h with indicated doses of IQS019 or ibrutinib. For phospho-Btk analysis, cells were exposed to a single dose of each agent (5 μM) 90 min prior to IgM stimulation and cell labeling. Indicated are the *r* values observed after treatment with IQS019 (*grey curves*) or ibrutinib (*red curves*), and referred to control IgM-stimulated cells (*black curves*). Isotype controls are represented by *grey* filled histograms. Shown are representative experiments from two replicates

mean IC_{50} of 6.7 μM (range: 2.7–8.7 μM), which decreased down to 4.1 μM (range: 2.2–11.7 μM) and 3.3 μM (range: 2.2-5.1 μM) at 48 h and 72 h, respectively. Of particular interest, after 72 h these IC_{50} values were much lower in Z-138 (4.5 μM), GRANTA-519 (5.1 μM), and JVM-2 cells (4.1 μM) than observed after ibrutinib treatment. Accordingly, while a short exposure to ibrutinib only marginally affected CXCL12-dependent cell migration in the UPN-1 cell line, this process was blocked up to 39% in cells cultured with IQS019 (Fig. 6b). To validate these observations in in vivo settings, MCL tumor-bearing mice were treated with a standard 25 mg/kg dose of ibrutinib [17], the equivalent dose of IQS019-2MeSO$_3$H, or vehicle. While ibrutinib allowed to a significant 25.1% reduction in tumor growth after 2 weeks of treatment (* $p = 0.049$), IQS019-2MeSO$_3$H showed superior activity, as it could inhibit the tumor outgrowth up to 42% when compared to vehicle group (Fig. 6c, ** $p = 0.006$, * $p = 0.048$). At the pharmacokinetic level, while both compounds

presented similar half-life and C_{max} values in mice after a single oral administration, the total drug exposure over time was considerably improved in the case of IQS019-2MeSO$_3$H, as shown by a 12 fold increase in the AUC value. Consequently, the global bioavailability dropped from 2 to 4% in the case of ibrutinib, to about 70% in the case of IQS019-2MeSO$_3$H (Additional file 1: Figure S4b and Table S3), thus suggesting that a better PK profile may account for the improved activity of IQS019 *vs* ibrutinib in vivo.

To unravel at the molecular level the mechanisms underlying this superior activity of IQS019 over the Btk inhibitor, we established an ibrutinib-resistant cell line designated UPN-IbruR, derived from the parental UPN-1 by repeated drug selection (Additional file 1 Methods). When compared with the parental cell line, UPN-IbruR presented approximately a 10-fold increase in the ibrutinib IC_{50} after 72 h of treatment (24.6 vs 2.4 μM for parental cells), with negligible difference in IQS019 IC_{50} (5.6 vs 2.3 μM for parental cells) (Additional file 1: Figure S5a).The ibrutinib resistance phenotype of UPN-IbruR cells was not associated to mutations in *BTK* or *PLCG2* genes, which both harbored a wild type sequence (Additional file 1: Figure S5b), but may rather be associated to the activation of non-canonical NF-κB pathway, as suggested by the overexpression of p52 (Additional file 1: Figure S5c). While a similar dose-dependent decrease in phospho-Lyn and phospho-Btk levels was found in IQS019- and in ibrutinib-treated UPN-1 cells, the expression of phospho-Syk was almost completely lost only in cells exposed to 1 μM IQS019 (Fig. 6d,e). In sharp contrast, in UPN-IbruR cells, the Btk inhibitor failed to modulate the phosphorylation of the three kinases, while IQS019 showed significant inhibitory activity of phospho-Syk and phospho-Lyn at a dose as low as 1 μM (Fig. 6d). However, the compound was unable to downregulate phospho-Btk (Fig. 6e), suggesting that in ibrutinib-resistant cells, the capacity of IQS019 to inhibit Syk and Lyn may allow the compound to maintain a significant antitumoral activity independent of the expression of a non-druggable form of Btk. Altogether, these results point out a significant superior antitumoral activity of pleiotropic BCR kinase targeting by IQS019 over the sole inhibition of Btk, in in vitro and in vivo models of B-NHL.

Discussion

BCR has recently emerged as a central oncogenic pathway that promotes growth and survival in various lymphoma subtypes [8]. Constitutive activation of the three BCR-related kinases Syk, Lyn, and Btk have been well documented in CLL [18–20], MCL [21, 22], and FL [23] cells, while chronic BCR signaling has been reported in the ABC subtype of DLBCL [5]. Consistently, BCR kinase inhibitors constitute promising therapeutic strategies in these different entities. Among these novel agents, the first-in-class Btk inhibitor ibrutinib has achieved high response rates (43–71%) in relapsed/refractory CLL, MCL and ABC-DLBCL patients, while its activity was less pronounced in FL patients (37% overall response rate) [24–27]. A small fraction of patients develop progressive disease after initial response to this agent [25, 27], in relation with the acquisition of mutations at the ibrutinib binding site (C481S) of Btk, or in the *PLCγ2* gene [9–11]. Resistance to ibrutinib may also involve a lower dependency of malignant B cells toward Btk itself, than other downstream components of the pathway, like the Syk/Lyn-dependent kinase Erk [28]. Accordingly, the Syk inhibitor fostamatinib and the Src inhibitor dasatinib have also shown efficacy in relapsed/refractory B-NHL [29, 30].

Following these observations, and in an effort to improve the therapeutic modulation of BCR signaling, we previously screened a library of compounds derived from pyrido[2,3-*d*]pyrimidines, for their capacity to bind to the active sites of Btk, Syk and/or Lyn [31]. We identified IQS019 (compound 19) as a unique molecule with affinity for the three BCR kinases [13]. In the present work, we confirm the inhibitory property of the compound against Btk, Syk and Lyn, as well as its selective antitumoral effect in B lymphoid cells, especially in MCL and FL cell lines, and independently of the response to ibrutinib. Our results suggest that IQS019 can counteract both chronic and tonic BCR signaling, as it shows similar antiproliferative activity in DLBCL cell lines from both the GCB and ABC subtype, which are respectively dependent for their survival on tonic (Syk/PI3K-mediated) and chronic (Syk/Btk-mediated) BCR signaling [8, 32–34]. This property might confer to IQS019 a greater activity than ibrutinib, which is preferentially active against tumors that rely on chronic active BCR signaling [8]. Beside Btk, the direct inhibitory activity of IQS019 towards Syk and/or Lyn phosphorylation may also explain the capacity of the compound to activate apoptosis in vitro and in vivo, as pharmacological inhibition of Syk, has been reported to elicit the apoptotic cascade in preclinical models of DLBCL and CLL [35, 36]. Also, probably thanks to its apoptogenic property and specificity, IQS019 salt is found to be significantly active and safe at a dose of 2 mg/kg/day, which is much lower than the reported active concentrations of fostamatinib, dasatinib or ibrutinib in mouse models of lymphoid neoplasms [37–39], thus predicting a probable low incidence of secondary adverse effects of the compound.

Another downstream event regulated by Btk, Syk, and Lyn is the chemokine-mediated B cell migration, a process essential to tumor B cell survival [40]. We show that IQS019 is able to impair in vitro cell migration towards CXCL12 in cell lines and primary samples, in

both basal and anti-Ig-stimulated cultures. This property may be responsible, at least in part, for the reduced infiltration of tumor cell observed in FL-bearing mice dosed with the compound. Beside this effect, IQS019-mediated inhibition of Syk, Lyn, and Btk may further impair tumor maintenance and B cell homeostasis in vivo, which are largely dependent on the coordinated activity of the three kinases [41].

Conclusions

In summary, we describe IQS019 as a new and unique BCR kinase inhibitor able to counteract both constitutive and ligand-dependent activation of the BCR pathway in in vitro and in vivo models of B lymphoid neoplasms. Thanks to the unique capacity of the compound to inhibit the three upstream BCR kinases Lyn, Syk, and Btk, this study may offer a glimpse into possible application for the treatment of the most prevalent subtypes of B-NHL, including those low responders to current BCR kinase inhibitors.

Additional file

Additional file 1: Figure S1. IQS019 tyrosine kinase inhibitory profiling. Tyrosine kinase (TK) and tyrosine kinase-like (TKL) kinome tree was elaborated on the basis of residual in vitro kinase activity upon exposure to 100 nM or 1 μM IQS019, by means of Kinome Render software (http://bcb.med.usherbrooke.ca/kinomerender.php). **Figure S2**. Sensitivity of CLL primary cases to IQS019 is independent of IGHV mutational status and involves a caspase-dependent cell death process. (a) CLL primary cells, 9 of them with ummutated (UM) and 6 with mutated (M) IGHV gene, were treated with increasing concentrations of IQS019 for 24h. Cell viability was determined by MTT method. Shown are the median values from each CLL group (UM and M), referred to control, untreated cells. (b) IQS019 induces caspase-dependent cell death in MCL (UPN-1) and in FL (DOHH-2) cell lines, as well as in two representative CLL primary cultures. Cells were exposed for 24 hours to 5 μM IQS019, in the presence of absence of the pan-caspase inhibitor Q-VD-OPh (10 μM). Apoptosis was determined by simultaneous cytofluorimetric detection of Annexin-V and caspase-3/7 activity. (c) A set of 6 CLL primary cultures were treated with IQS019 as indicated, followed by Western Blot detection of phosphohistone H3 (p-H3), using β- actin as a loading control. **Figure S3**. Flow cytometry determination of CXCR4 membrane expression in B-NHL cell lines. Four representative cell lines were stained with a PE-labeled anti-CXCR4 antibody and analyzed on an Attune cytometer. CXCR4-specific signal (black curves) and isotypic control (grey filled curve) are represented. **Figure S4**. Safety and PK properties of IQS019-2MeSO3H in mice. (a) Twenty SCID mice (10 males and 10 females) received a single intravenous injection of IQS019-2MeSO3H at a 2 mg/kg, 10 mg/kg, or 50 mg/kg dose, or equivalent volume of vehicle, and animal weight was recorded at days 1, 3, 4, 7, 11, 14, 18 and 21 post-treatment. (b) Mean plasma concentration of IQS019-2MeSO3H in ICR mice over the time, after a single p.o. administration of a 25 mg/kg dose of the compound. **Figure S5**. Comparison of parental and ibrutinib-resistant derived B-NHL cell line. (a) Dose-response of the UPN-1 parental, and UPN-IbruR derived cell line exposed for 72 hours to increasing concentrations of ibrutinib or IQS019. (b) BTK and PLCG2 exon sequencing in UPN-IbruR cells. (c) Western blot detection of the alternative NF-κB pathway component, p52, in UPN-1 and UPN-IbruR cells. β-actin was used as a loading control. (DOC 3279 kb)

Abbreviations

ABC: Activated B-cell; AGC: A, G, and C protein kinase group; BCR: B cell receptor; B-NHL: B-cell non-Hodgkin lymphoma; Btk: Bruton's kinase; CAMK: Ca2+/calmodulin-dependent protein kinase; CLL: Chronic lymphocytic leukemia; CMGC: Cyclin-dependent (CDKs), mitogen-activated, glycogen synthase and CDK-like protein kinase group; DLBCL: Diffuse large B-cell lymphoma; FBS: Fetal bovine serum; FL: Follicular lymphoma; GCB: Germinal centre B-cell; GI_{50}: Growth inhibitory 50; Lyn: Lck/Yes novel tyrosine kinase; MCL: Mantle cell lymphoma; PLCγ2: Phospholipase Cγ2; STE: Mitogen-activated protein kinase cascade component; Syk: Spleen tyrosine kinase; TK: Tyrosine kinase; TKL: Tyrosine kinase-like

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Availability of data and materials

All relevant data and materials within this work are made available in this manuscript. Any additional information can be made freely available to any scientist on reasonable request.

Authors' contributions

PB and AE-A designed the study, performed the experiments, and analyzed data and co-wrote the manuscript. JR and LJ performed the IQS019 sensitivity assays in the B-NHL cell lines. VR designed and performed the animal studies. RP performed the IQS019 synthesis. JT supervised the IQS019 synthesis, interpreted the results, and reviewed the manuscript. JGV, AC-J, AM-C, and AM provided support in the Western blot and flow cytometry analysis and in interpretation of the data. AM helped in designing the immunohistochemical and immunofluorescence assays. EC analyzed clinical data and reviewed the manuscript. AS-B supervised IQS019 kinase inhibition profiling and PK studies. JIB supervised IQS019 synthesis, interpreted the results, and reviewed the manuscript. PP-G analyzed data and co-wrote the manuscript. DC designed the study and reviewed the manuscript. GR conceived and designed the study, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

A.S.-B. is an employee of Pangaea Biotech, SL. The remaining authors have no competing financial interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

The manuscript involved the use of human and animal samples. The ethical approvals for this project, including the informed consent of the patients, the animal procedures and the handling of samples, were granted following the guidelines of the Hospital Clínic Ethics Committee (IRB, reg. num. 2012/7498) in compliance with the Animal Ethics Committee of the University of Barcelona (agreement #154/16).

Publisher's Note

Author details
[1]Division of Hematology and Oncology, Institut d'Investigacions Biomèdiques August Pi iSunyer (IDIBAPS), Barcelona, Spain. [2]Grup d'Enginyeria Molecular, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain. [3]Department of Pathology, Hematopathology Unit, Hospital Clinic, Barcelona, Spain. [4]Pangaea Biotech S.L., Quiron Dexeus University Hospital, Barcelona, Spain.

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Additional file 1

SUPPLEMENTAL METHODS

Cell line authentication

JEKO-1, MINO, JVM-2, REC-1 and JVM-13 cell lines were obtained from ATCC cell bank (LGC Standards, Teddington, UK). DOHH-2, WSU-NHL, WSU-FSCLL, SC-1, GRANTA-519, MEC-2, MEC-1, SUDHL-16, SUDHL-8 and U-2932 cell lines were purchased at DSMZ (Braunschweig, Germany). MAVER-1, UPN-1, HBL-2, Z-138, OCI-LY8 and OCI-LY10 were provided, respectively, by Dr A. Zamo (University of Verona, Verona, Italy), Dr A. Turhan (Institut Gustave Roussy, Villejuif, France), Dr M. Dreyling (University Hospital, Munich, Germany), Dr E. Ortega-Paino (Lund University, Lund, Sweden), Dr M. Raffeld (National Cancer Institute Bethesda, MD, USA) and Dr A. Staiger (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany). Cell line authentication was performed upon reception by short tandem repeat (STR) profiling, using AmpFlSTR identifier kit (Thermo Fisher), and based on available STR profiles. This analysis was then repeated every 6 months and up to 4 months prior to the submission of the present manuscript. Mycoplasm infection was routinely tested by PCR.

Apoptosis assay

Cells (4-6 x 10^5 cells/ml) were treated with IQS019 and, when specified, were preincubated for 1 hour with 10 μM of the pan-caspase inhibitor Q-VD-OPh (Merck). Apoptosis was determined by dual labeling of phosphatidylserine exposure and caspase activity by means of Annexin-V Pacific Blue and CellEvent caspase-3/7 Green (Thermo Fisher), respectively, followed by the analysis of 10.000 events on an Attune acoustic focusing cytometer.

Preparation of IQS019 soluble salt and toxicity assay in SCID mice.

IQS019-2$MeSO_3H$ was synthesized according to the following procedure: 382.8 mg (0.75 mmol) of IQS019 were dissolved in 50 ml of acetone and then 144.3 mg (1.5 mmol) of methanesulfonic acid were added. The resulting solution was stirred at room temperature for 2 hours and then cold diethyl ether was added and the resulting precipitated was filtered, washed with cold diethyl ether and dried *in vacuo* over phosphorus pentoxide to afford 456.7 mg (0.65 mmol, 87%) of 4-amino-6-(2,6-dichlorophenyl)-8-methyl-2-(4-(4-methylpiperazin-1-yl)phenylamino)pyrido[2,3-d]pyrimidin-7(8H)-one dimesylate (IQS019-2$MeSO_3H$)as a yellowish solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.53 (br s, 2H), 9.34 (br s, 1H), 8.09 (s, 1H), 7.70 (d, J = 9.1 Hz, 2H), 7.59 – 7.56 (m, 2H), 7.44 (dd, J = 8.7, 7.5 Hz, 1H),

7.43 (br s, 2H), 6.98 (d, *J* = 9.2 Hz, 2H), 3.77 (d, *J* = 13.5 Hz, 2H) 3.59 (s, 3H), 3.52 (d, *J* = 11.9 Hz, 2H) 3.24 – 3.11 (m, 2H), 2.98 – 2.84 (m, 5H), 2.34 (s, 6H).

Four groups consisting of 4 animals (2 males and 2 females per group), each received a 2 mg/kg, a 10 mg/kg, or a 50 mg/kg dose of the compound, or equal volume of vehicle (saline solution) were evaluated. Viability/mortality, motility, hair appearance and body weights of the animals were recorded during the first 30 min and at approximately 3 and 5 h after administration on test day 1, and once daily between days 2 and day 14. At the end of the observation period, no mortality or alteration of vital parameters, including body weight was recorded in animals administered with either the saline solution or the compound (supplemental Fig. S4a). Macroscopic examination of the animals after sacrifice revealed no alteration of the principal organ systems.

In vitro evaluation of BCR-related kinase phosphorylation

For the detection of phospho-Btk in cell lines, 0.5 x 10^6 cells were fixed with 4% PFA for 15 min on ice, washed once, and permeabilized with pure methanol for 10 min at -20ºC. In the case of CLL primary cells, phosphatase-mediated dephosphorylation of Btk was prevented by adding 3.3 mM hydrogen peroxide during the last 2 min of IgM stimulation, followed by a wash in cold PBS before cell fixation with PFA as before and cell permeabilization with ethanol 70% for 2 hours. Cells were then washed twice and stained for 15 min at RT with a phycoerythrin (PE)-labeled anti-Btk-phosphoTyr223 antibody (clone N35-86) or a mouse IgG1 κ isotypic control (Becton Dickinson), in PBS + 0.5% BSA, followed by analysis on an Attune acoustic focusing cytometer (Thermo Fisher).

Phospho-Syk and phospho-Lyn protein levels were analyzed by SDS-PAGE, using whole cell extracts obtained by lysing 3-5 x 10^6 cells on ice for 30 min in Triton buffer (20 mM Tris-HCl ph 7.6, 150 Mm NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Membranes were incubated with anti-Syk-phosphoTyr352 (Cell Signaling Technology, Danvers, MA, USA), anti-Lyn-phosphoTyr396 (Abcam, Cambridge, UK), and anti-β-actin (Sigma-Aldrich, Saint-Louis, MO, USA) primary antibodies, followed by appropriate anti-rabbit secondary antibody (Cell Signaling Technology). Chemiluminiscence detection was done using the ECL system (Thermo Fisher) and visualized on a mini-LAS4000 device using Image Gauge software (Fujifilm, Valhalla, NY, USA). In order to avoid signal saturation consequent to BCR ligation, exposure time of membranes containing protein extracts from anti-Ig- treated UPN-1, DOHH-2 and OCI-LY10 cells, were substantially reduced.

Detection of phospho-Syk, phospho-Lyn and phospho-Btk in OCT tumor sections and processed spleens

OCT sections from MCL tumors were fixed with PFA on glass slides and permeabilized for 15 min with a solution of saponin 0.1% + FBS 10%, followed by a 30 min incubation with an Alexa488-labeled anti-Syk-phosphoTyr352, a PE-labeled anti-Btk-phosphoTyr223 (Becton Dickinson), or a Cy5-labeled anti-Lyn-phosphoTyr397 (Bioss Antibodies, Woburn, MA, USA) antibody. Coverslips were then mounted with DAPI-containing Fluoroshield mounting medium (Sigma-Aldrich) and visualized on a Nikon H5505 microscope by means of a 20X/1.30 NA oil objective (Nikon, Amsterdam, Netherlands) with the use of Isis Imaging System v5.3 software (MetaSystems GmbH).

In the FL model, splenocytes were obtained by homogenizing harvested spleens and filtering through 70 µm nylon sieves (Becton Dickinson). Erythrocytes were lysed using ACK buffer (Quality Biological, Gaithersburg, MD, USA) and human B cells were labeled with anti-CD45-Pacific Blue (Thermo Fisher) antibody, followed by recounting on an Attune cytometer. In parallel, parts of the spleen homogenates were stained with an anti-CD45-Pacific Blue or an anti-CD20-PE (Beckman Coulter, L'Hospitalet de Llobregat, Spain) antibody, prior fixation, permeabilization and phospho-BCR kinase labeling as above, followed by analysis on either an Attune or a FACSCalibur (Becton Dickinson) cytometer.

Generation of ibrutinib-resistant cells

UPN-1 cells were initially treated for 96 hours with 1 µM ibrutinib and then were cultured in drug-free medium containing 20% FCS. After cell growth recovered, cells were treated with ibrutinib for an additional 72 hours, and the selection cycle was repeated at the same drug concentration until cell growth recoveries were obtained within 2 weeks. At this point drug concentration was increased to the next steps (2 µM, 5 µM, 7.5 µM, and, finally, 10 µM ibrutinib). After repeated rounds of selection with the 10 µM dose of ibrutinib over a period of 5 months, the established resistant cell line was designated "UPN-IbruR." At this point, this cell line was cryopreserved and cultured in the presence of the Btk inhibitor.

BTK and *PLCG2* sequencing

DNA was extracted from UPN-IbruR cells using the QIAamp DNA Mini Kit (Qiagen; Venlo, Netherlands) in automated (QIAcube) extractions according to manufacturer's instruction. We amplified by PCR the exon 15 of BTK (aa 450-522) and exon 18 of *PLCG2*. PCR products were treated with ExoSap IT (USB Corporation) and sequenced using ABI Prism BigDye terminator v3.1 (Applied Biosystems) using 5 pmol of each primer. Sequencing reactions were run on an ABI-3730 automated sequencer (Applied Biosystems). All

sequences were examined with the Mutation Surveyor DNA Variant Analysis Software (Softgenetics).

Pharmacokinetics evaluation of IQS019 in mouse

IQS019-2MeSO_3H PK study was performed at Crown Bioscience (Taicang, China). Briefly, IQS019-2MeSO_3H was administered by p.o. at a single dose of 25 mg/kg to 3 male ICR mice. Plasma was recovered after 15 min, 30 min, 1h, 2h, 4h, 6h, 8h, and 24h and mixed with acetonitrile containing 200 ng/ml tolbutamide and 200 ng/ml propranolol. After a centrifuge step, supernatant was mixed with 0.1% formic acid and injected into an API 4000 mass spectrometer (Applied Biosystems). PK parameters were calculated using mean plasma concentration time data by Phoenix WinNonlin software v6.3, using a non-compartmental model.

SUPPLEMENTAL TABLES

Supplemental Table S1.- Dose-dependent inhibitory activity of IQS019 against TK and TKL kinase subfamilies

Kinase	Family	Kinase activity inhibition upon IQS019 exposure	
		0.1 µM	10 µM
ABL1 E255K	TK	22	83
ABL1 F317I	TK	19	80
ABL1 G250E	TK	12	74
ABL1 H396P	TK	50	94
ABL1 M351T	TK	43	94
ABL1 Q252H	TK	52	94
ABL1 T315I	TK	2	38
ABL1 wt	TK	42	93
ABL1 Y253F	TK	46	93
ABL2	TK	74	100
ACK1	TK	41	100
ALK C1156Y (GST-HIS-tag)	TK	4	88
ALK F1174L (GST-HIS-tag)	TK	0	80
ALK F1174S (GST-HIS-tag)	TK	0	74
ALK L1196M (GST-HIS-tag)	TK	1	92
ALK R1275Q (GST-HIS-tag)	TK	0	84
ALK wt (GST-HIS-tag)	TK	0	88
AXL	TK	5	66
BLK	TK	34	99
BMX	TK	34	99
BRK	TK	97	117
BTK	TK	19	94
CSF1-R	TK	19	96
CSK	TK	30	99

DDR2 N456S	TK	97	95
DDR2 T654M	TK	20	74
DDR2 wt	TK	96	96
EGF-R d746-750	TK	84	99
EGF-R d747-749/A750P	TK	71	99
EGF-R d747-752/P753S	TK	78	99
EGF-R d752-759	TK	69	99
EGF-R G719C	TK	91	100
EGF-R G719S	TK	82	100
EGF-R L858R	TK	80	99
EGF-R L861Q	TK	87	100
EGF-R T790M	TK	24	94
EGF-R T790M/L858R	TK	29	96
EGF-R wt	TK	80	99
EPHA1	TK	84	100
EPHA2	TK	75	100
EPHA3	TK	21	69
EPHA4	TK	40	92
EPHA5	TK	11	92
EPHA6	TK	10	88
EPHA7	TK	11	65
EPHA8	TK	42	97
EPHB1	TK	70	100
EPHB2	TK	0	75
EPHB3	TK	5	65
EPHB4	TK	31	98
ERBB2	TK	29	96
ERBB4	TK	75	100
FAK aa2-1052	TK	4	59
FER	TK	4	86
FES	TK	10	88
FGF-R1 V561M	TK	0	78

FGF-R1 wt	TK	5	71
FGF-R2	TK	5	75
FGF-R3 G697C	TK	7	86
FGF-R3 K650E	TK	1	81
FGF-R3 K650M	TK	9	84
FGF-R3 wt	TK	0	86
FGF-R4	TK	0	35
FGR	TK	76	100
FLT3 D835Y	TK	1	22
FLT3 ITD	TK	4	27
FLT3 wt	TK	5	25
FRK	TK	61	99
FYN	TK	49	98
HCK	TK	50	99
IGF1-R	TK	0	67
INS-R	TK	0	37
INSR-R	TK	5	31
ITK	TK	0	79
JAK1	TK	7	9
JAK2	TK	9	43
JAK3	TK	15	39
KIT A829P	TK	2	79
KIT D816H	TK	21	87
KIT D816V	TK	23	90
KIT T670I	TK	0	57
KIT V559D	TK	4	85
KIT V559D/T670I	TK	0	63
KIT V559D/V654A	TK	0	71
KIT V560G	TK	12	92
KIT V654A	TK	1	64
KIT wt	TK	3	67
LCK	TK	81	99

LTK	TK	1	88
LYN	TK	65	99
MATK	TK	0	55
MERTK	TK	0	86
MET D1228H	TK	7	34
MET D1228N	TK	7	39
MET F1200I	TK	0	16
MET M1250T	TK	0	29
MET wt	TK	0	39
MET Y1230A	TK	6	28
MET Y1230C	TK	8	40
MET Y1230D	TK	0	23
MET Y1230H	TK	0	21
MET Y1235D	TK	0	86
MUSK	TK	14	32
NMP1ALK	TK	0	76
NMP1ALK F1174L	TK	0	56
PDGFR-alpha D842V	TK	19	76
PDGFR-alpha T674I	TK	20	31
PDGFR-alpha V561D	TK	27	94
PDGFR-alpha wt	TK	0	68
PDGFR-beta	TK	6	62
PYK2	TK	0	62
RET E762Q	TK	2	87
RET G691S	TK	0	83
RET M918T	TK	1	86
RET R749T	TK	0	88
RET R813Q	TK	0	89
RET S891A	TK	9	94
RET V804L	TK	8	65
RET V804M	TK	0	65
RET wt	TK	0	46

RET Y791F	TK	5	89
RON	TK	10	97
ROS	TK	1	20
SRC (GST-HIS-tag)	TK	59	99
SRMS	TK	1	65
SYK aa1-635	TK	17	90
TEC	TK	0	65
TIE2 R849W	TK	0	86
TIE2 wt	TK	0	90
TIE2 Y1108F	TK	10	87
TIE2 Y897S	TK	8	92
TRK-A	TK	3	72
TRK-B	TK	0	73
TRK-C	TK	0	69
TXK	TK	18	78
TYK2	TK	8	37
TYRO3	TK	29	94
VEGF-R1	TK	1	54
VEGF-R2	TK	0	37
VEGF-R3	TK	4	28
YES	TK	61	100
ZAP70	TK	12	66
ACV-R1	TKL	45	83
ACV-R1B	TKL	37	82
ACV-R2A	TKL	64	94
ACV-R2B	TKL	31	88
ACV-RL1	TKL	62	99
BMPR1A	TKL	0	49
B-RAF V600E	TKL	57	93
B-RAF wt	TKL	62	97
IRAK1	TKL	10	52
IRAK4 (untagged)	TKL	10	86

LIMK1	TKL	0	1
LIMK2	TKL	0	10
LRRK2 G2019S	TKL	6	51
LRRK2 I2020T	TKL	0	0
LRRK2 R1441C	TKL	1	0
LRRK2 wt	TKL	0	0
MLK4	TKL	9	34
RAF1 Y340D/Y341D (untagged)	TKL	55	90
RIPK2	TKL	99	92
RIPK5	TKL	3	24
TGFB-R1	TKL	5	84
TGFB-R2	TKL	12	68
ZAK	TKL	0	47

Supplemental Table S2.- Characteristics of CLL patients

Patients	Gender[a]	Age at diagnosis (years)	Cell source[b]	Tumoral cells (%)[c]	*IGHV* status[d]	*TP53* status[e]	Cytogenetic alterations
CLL n.1	F	53	PB	95	UM	wt	del(13q)
CLL n.2	M	64	PB	80	M	wt	trisomy 12
CLL n.3	M	58	PB	95	M	wt	normal
CLL n.4	M	56	PB	79	M	wt	n.d.
CLL n.5	F	52	PB	85	M	wt	del(13q)
CLL n.6	M	63	PB	97	UM	wt	del(13q)
CLL n.7	M	54	PB	92	M	wt	del(13q)
CLL n.8	M	78	PB	94	UM	wt	del(13q)
CLL n.9	M	67	PB	97	UM	wt	normal
CLL n.10	M	44	PB	97	UM	wt	del(13q)
CLL n.11	M	66	PB	96	UM	wt	(11q)del
CLL n.12	F	49	PB	94	M	wt	del(13q)
CLL n.13	F	73	PB	94	UM	wt	trisomy 12

[a] F: female; M: male

[b] PB: peripheral blood; LN: lymph node

[c] CD19+ tumor cells determined by flow cytometry

[d] *IGHV* mutational status was done according to European Research Initiative on CLL (ERIC) guidelines 42.

[e] 17p13 deletion was assessed by fluorescence in situ hybridization and *TP53* mutational status was analyzed by direct sequencing

UM indicates unmutated; M, mutated; wt, wild type; n.d., not determined; del, deletion; dup, duplication; add, addition.

Supplemental Table S3.- PK parameters and plasma concentration of IQS019-2MeSO3H vs ibrutinib

Parameter	IQS019	Ibrutinib [a and ref [42]]
t1/2 (h)	3.27	3.1
Tmax (h)	4.0	< 2.0
Cmax (μM)	2	1.07
AUC0-∞ (h x ng/ml)	6966	568
Oral bioavailability	67%	2-4%

[a] http://www.pharmacodia.com/yaodu/html/v1/chemicals/f15eda31a2da646eea513b0f81a5414d.html#pharmacokinetics1

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. IQS019 tyrosine kinase inhibitory profiling. Tyrosine kinase (TK) and tyrosine kinase-like (TKL) kinome tree was elaborated on the basis of residual *in vitro* kinase activity upon exposure to 100 nM or 1 μM IQS019, by means of Kinome Render software (http://bcb.med.usherbrooke.ca/kinomerender.php).

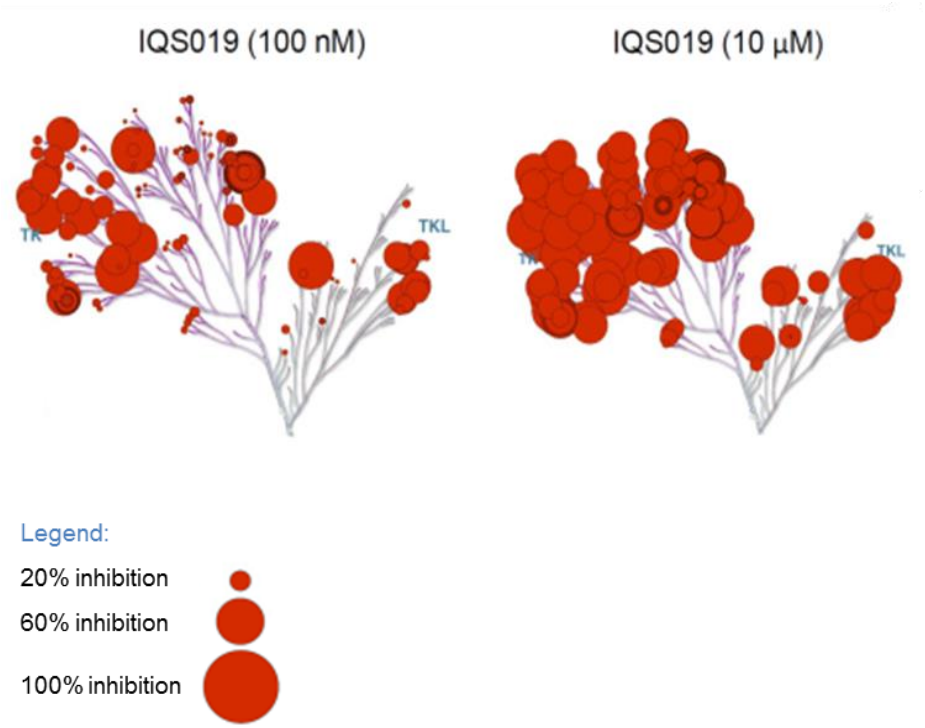
Figure S2. Sensitivity of CLL primary cases to IQS019 is independent of IGHV mutational status and involves a caspase-dependent cell death process. (a) CLL primary cells, 9 of them with ummutated (UM) and 6 with mutated (M) *IGHV* gene, were treated with increasing concentrations of IQS019 for 24h. Cell viability was determined by MTT method. Shown are the median values from each CLL group (UM and M), referred to control, untreated cells. (b) IQS019 induces caspase-dependent cell death in MCL (UPN-1) and in FL (DOHH-2) cell lines, as well as in two representative CLL primary cultures. Cells were exposed for 24 hours to 5 μM IQS019, in the presence of absence of the pan-caspase inhibitor Q-VD-OPh (10 μM). Apoptosis was determined by simultaneous cytofluorimetric detection of Annexin-V and caspase-3/7 activity. (c) A set of 6 CLL primary cultures were treated with IQS019 as indicated, followed by Western Blot detection of phospho-histone H3 (p-H3), using β- actin as a loading control.

Figure S3. Flow cytometry determination of CXCR4 membrane expression in B-NHL cell lines. Four representative cell lines were stained with a PE-labeled anti-CXCR4 antibody and analyzed on an Attune cytometer. CXCR4-specific signal (black curves) and isotypic control (grey filled curve) are represented.

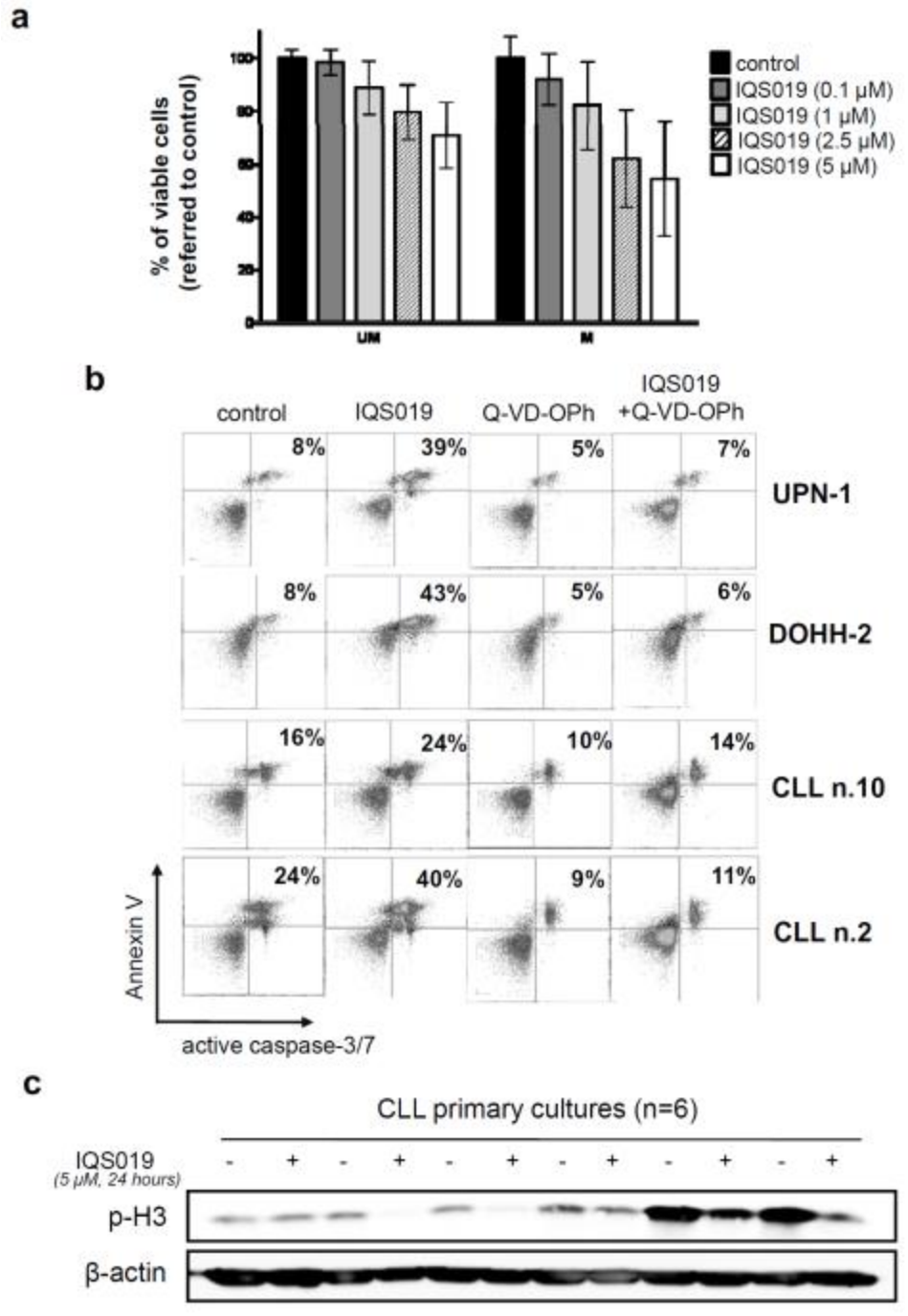
Figure S4. Safety and PK properties of IQS019-2MeSO$_3$H in mice. (a) Twenty SCID mice (10 males and 10 females) received a single intravenous injection of IQS019-2MeSO$_3$H at a 2 mg/kg, 10 mg/kg, or 50 mg/kg dose, or equivalent volume of vehicle, and animal weight was recorded at days 1, 3, 4, 7, 11, 14, 18 and 21 post-treatment. (b) Mean plasma concentration of IQS019-2MeSO$_3$H in ICR mice over the time, after a single p.o. administration of a 25 mg/kg dose of the compound.

Figure S5. Comparison of parental and ibrutinib-resistant derived B-NHL cell line. (a) Dose-response of the UPN-1 parental, and UPN-IbruR derived cell line exposed for 72 hours to increasing concentrations of ibrutinib or IQS019. (b) BTK and PLCG2 exon sequencing in UPN-IbruR cells. (c) Western blot detection of the alternative NF-kappaB pathway component, p52, in UPN-1 and UPN-IbruR cells. β-actin was used as a loading control.

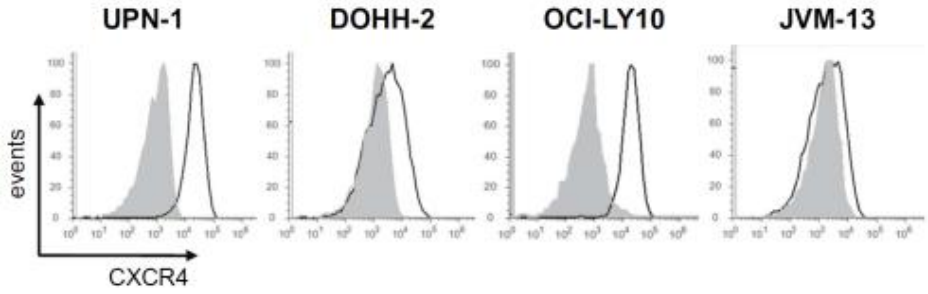
Supplemental Figure S1



Supplemental Figure S2

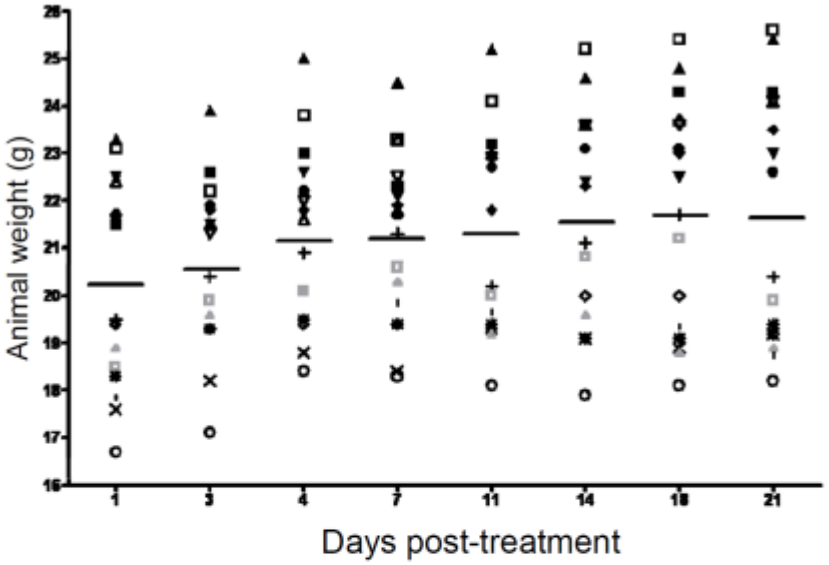


Supplemental Figure S3

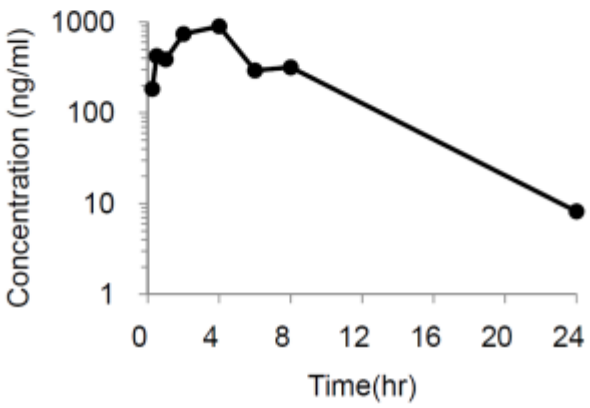


Supplemental Figure S4

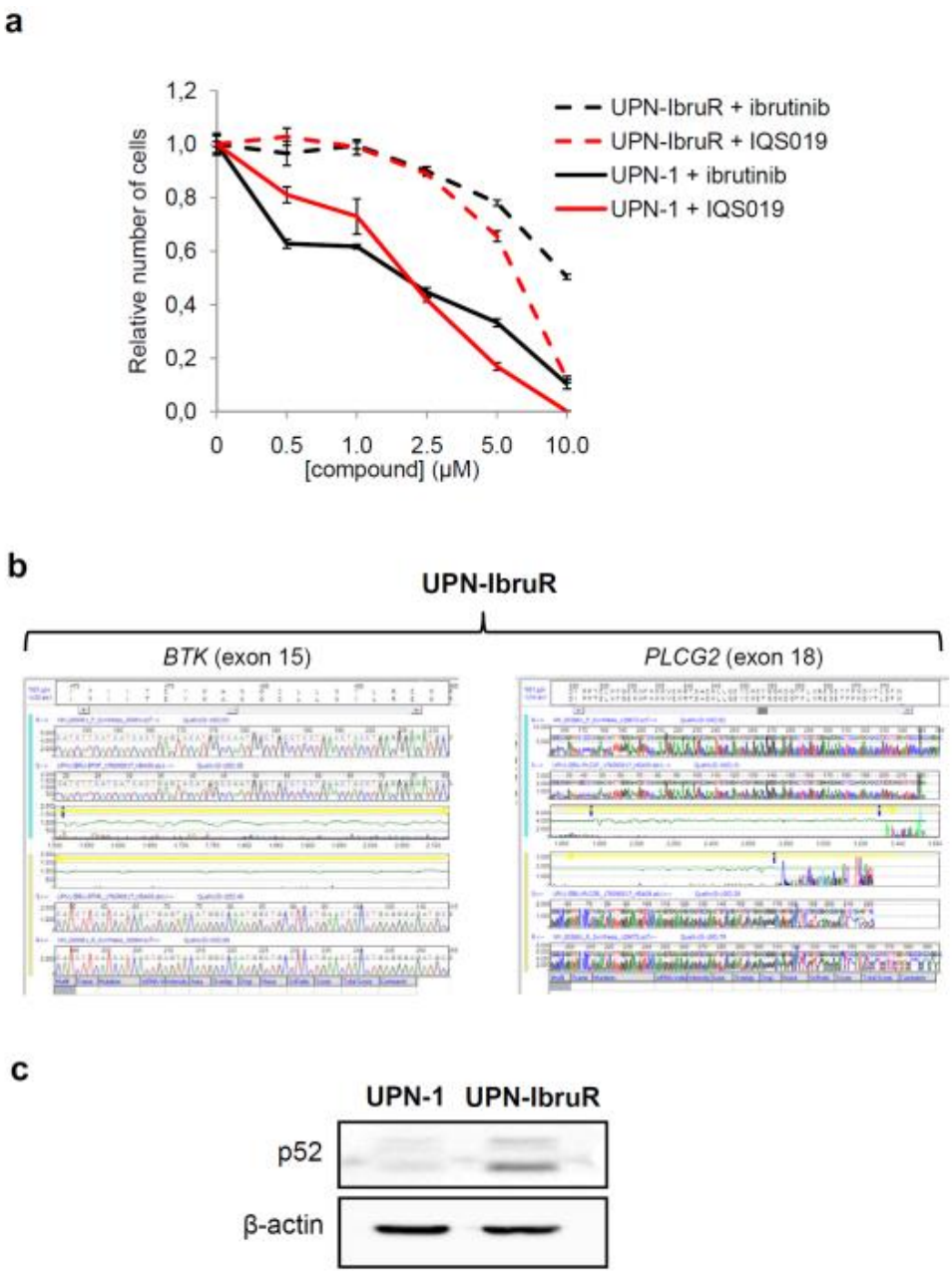
a



b



Supplemental Figure S5



SUMMARY OF RESULTS

1st paper: The BET bromodomain inhibitor CPI203 overcomes resistance to ABT-199 (venetoclax) by downregulation of BFL-1/A1 in *in vitro* and *in vivo* models of *MYC+/BCL2+* double hit lymphoma

1. Decreased sensitivity to ABT-199 correlates with upregulation of anti-apoptotic BFL-1 in DHL

GCB-DLBCL and DHL cells were exposed to different doses of ABT-199 (1-10-100nM) and cell viability measured by MTT assay at 24 and 96 hours. All cell lines were sensitive at 24 hours but DHL cells recovered part of their proliferative properties at 96 hours. This process was not related to the defective capacity of ABT-199 to displace BAX from BCL-2 in DHL cells, as BCL-2 immunoprecipitation assay showed similar degrees of BAX release from BCL-2 complexes in both DHL and GCB-DLBCL cell lines exposed to ABT-199. Also, no significant correlation was observed between the basal expression of BCL-2 proteins (BCL-2, BFL-1, MCL-1, and BCL-XL) and cell sensitivity to the BH3 mimetic. We then investigated the modulation of these four proteins with ABT-199 treatment, and observed that the upregulation of BFL-1, but not other BCL-2-like proteins, significantly correlated with the cell proliferation recovery observed in DHL cells between 24 and 96 hours, in the presence of ABT-199.

The transduction of ABT-199 sensitive cells with lentiviral particles containing BFL-1+ vector, conferred the cells a significant protection against ABT-199 treatment, resembling the acquired resistance phenotype observed in DHL cells following prolonged exposure to the drug. Other BCL-2 protein levels were not affected by the vector expression.

2. Regulation of an apoptosis gene signature by the BET bromodomain inhibitor CPI203 in DHL cells

Recent works have highlighted the pharmacological inhibition of BRD4 as an efficient strategy able to modulate *BCL2A1* gene transcription.[499] We thus assessed the cytostatic effect of GCB-DLBCL and DHL cell lines to the BRD4 inhibitor CPI203, showing a time-dependent effect in the nanomolar range in all the cell lines, independently of their sensitivity to ABT-199. We performed a gene expression profiling (GEP) analysis with DHL cell lines and primary samples, either untreated or treated with CPI203 for 6 hours. Gene set enrichment analysis (GSEA) identified a modulation of an apoptosis-related gene set. Among the main events observed in CPI203-treated cells, there was upregulation of *BCL2L11* (37% increase) and downregulation of *BCL2A1* (27% decrease), codifying for BIM and BFL-1 proteins respectively. We confirmed the CPI203-dependent modulation of BIM and BFL-1 protein levels by western blot, which showed a 70% decrease in BFL-1 levels and a BIM protein increase close to 60% after 48 hours of treatment with CPI203

treatment. BIM immunoprecipitation pointed out a displacement of BIM from BFL-1 to BCL-2 complexes in CPI203-treated cells, suggesting that the BETi could trigger a form of "priming" for death by CPI203 treatment in DHL cells.

3. CPI203 restores ABT-199 apoptogenicity in DHL cultures

The treatment of DHL cell lines with combined ABT-199 and CPI203 showed a synergistic antiproliferative effect between the drugs by MTT assay, with cell proliferation reduced by almost 60% after 48 hours. The combination also induced apoptosis, as we detected an increase of reactive oxygen species (ROS) and mitochondrial depolarization by flow cytometry. Accordingly, in DHL primary samples we observed an increase of hypodiploid sub-G1 population in cells treated with the combination of both drugs.

At the molecular level, we confirmed the activation of apoptosis in DHL cells treated with the combination, as PARP cleavage and caspase-3 processing were detected by western blot. We also observed how CPI203 efficiently counteracted both basal and ABT-199-induced expression of BFL-1.

By BCL-2 immunoprecipitation, and in accordance with the previous data, we observed an improved dissociation of BAX-BCL-2 complexes in combination-treated cells.

4. *In vivo* antitumoral activity of CPI203/ABT-199 combination involves BFL-1 downregulation and apoptosis restoration

We developed a subcutaneous mouse model to further validate the potential therapeutic effect of ABT-199 and CPI203 combination in DHL. After two weeks of treatment, the tumor growth inhibition reached 80.4% in the combination group, while CPI203 alone only achieved a 39.4% reduction in tumor burden. ABT-199 as a single-agent allowed a transient control of the tumor growth, however after the two weeks of treatment, the tumor volumes were superior to that observed in the control group (+18%). Similar results were observed with the evaluation of the intratumoral glucose uptake using a fluorescent IR800-labeled 2-deoxy glucose probe.

Immunohistochemical staining of representative tumors further demonstrated a synergistic decrease in mitotic index (phospho-histone H3 staining) and induction of apoptosis (activated caspase-3 staining) by ABT-199-CPI203 combination, together with a downregulation of MYC, BFL-1, and BCL-2. In agreement with our *in vitro* data, RNA and protein analysis from representative tumors showed the capacity of CPI203 to neutralize both basal and ABT-199-related *BCL2A1* transcripts levels and BFL-1 protein expression, as observed by RQ-PCR and western blot, respectively.

2nd paper: Activity of the novel BCR kinase inhibitor IQS019 in preclinical models of B-cell non-Hodgkin lymphoma

1. Antitumor effect of the 4-aminopyrido[2,3-d]pyrimidine IQS019 in B lymphoid cell lines and primary samples

IQS019 has been shown to be preferentially active against tyrosine kinase and tyrosine kinase-like families, having an IC_{50} in the low micromolar range for the BCR kinases LYN (0.15 μM), SYK (1.6 μM) and BTK (2.1 μM), corresponding to those kinases where the compound is able to bind the active site.[500]

In vitro, a 5μM dose of IQS019 decreased cell proliferation in CLL, MCL, FL and DLBCL cell lines, with MCL and FL cells significantly more sensitive to the compound (mean cytotoxicity at 48 hours: 67.2%) than CLL and DLBCL cells (mean cytotoxicity at 48 hours: 42%).

At 24 hours of treatment, IQS019 induces caspase-dependent apoptosis in FL and MCL representative cell lines as seen by cytofluorimetric analysis, as well as a decrease in mitotic progression was seen in CLL primary samples by western blot detection of phospho-histone H3.

2. IQS019 antagonizes constitutive and antigen-mediated BCR signaling

We analyzed the effect of IQS019 on the phosphorylation status of LYN, SYK, and BTK by western blot (LYN and SYK) and flow cytometry (BTK). A 6 hour treatment with IQS019 induced a dose-dependent dephosphorylation of the three kinases in CLL, MCL, FL, and DLBCL cell lines. In accordance with the previous results, MCL and FL cell lines were more sensitive to the drug.

The same results were observed after BCR stimulation with the corresponding anti-Ig. BCR ligation induced an increase in the phosphorylation levels of the kinases that was counteracted by the IQS019 treatment. CLL primary samples showed similar responses to the inhibitor in these conditions than the cell lines.

3. IQS019 inhibits CXCL12-mediated migration of malignant B cells

We evaluated the effect of IQS019 on the migratory capacity of malignant B cells, using a CXCL12-dependent chemotaxis assay. MCL, FL, and DLBCL cell lines with detectable levels of CXCR4 and CLL primary samples were used. The migration induced by CXCL12 was significantly inhibited by IQS019 in all the cell lines and primary samples. These results were confirmed by an actin polymerization assay in CLL primary cultures, where we observed an increase in F-actin formation after CXCL12 stimulation, which was neutralized by pre-treating the cells with IQS019.

4. IQS019 is safe and impairs tumor outgrowth and malignant B cell homing to the spleen *in vivo*

The salt form of the compound (IQS019-2MeSO$_3$H) was synthesized in order to validate our results *in vivo*, and a toxicity test was performed in healthy SCID mice. We then developed both subcutaneous (MCL) and intravenous (FL) mouse models. In the subcutaneous model, IQS019-2MeSO$_3$H inhibited UPN-1 cell line growth *in vivo* after 2 weeks of treatment, when compared to the vehicle group (63% on average). There were no significant differences between the effect of the drug at 2 and 10 mg/kg, suggesting that optimal activity was reached at the lowest dose. According to tumor volume results, glucose uptake fell to 50-52% in tumors from animals treated with IQS019-2MeSO$_3$H. By immunohistochemical evaluation of representative tumor sections, we observed a reduction of mitotic index, and a notable induction of apoptosis in tumors from animals treated with IQS019-2MeSO$_3$H.

Once inoculated intravenously, FL cells are known to migrate to the spleen.[501] Therefore, in the systemic FL model, entire spleens were processed after two weeks of treatment. IQS019-2MeSO$_3$H treatment induced a reduction in tumor cell infiltration into the spleen compared with the control group (52% on average), suggesting a blockade of tumor cell homing to the lymphoid compartment. By immunofluorescence we demonstrated that IQS019-2MeSO3H efficiently reduced the phosphorylated forms of LYN, SYK, and BTK *in vivo* in malignant CD45+ cells isolated from representative spleens.[502]

5. IQS019 shows superior anti-tumor activity than ibrutinib *in vitro* and *in vivo*

MTT assay in a panel of eight MCL and FL cell lines identified three ibrutinib-resistant cell lines, in which IQS019 was able to induce a proliferation blockade, similarly to ibrutinib-sensitive cell lines. Accordingly, IQS019 showed an improved capacity to block CXCL12-dependent cell migration when compared to ibrutinib.

A MCL subcutaneous xenograft model was developed to further validate the potential therapeutic effect of IQS019 versus ibrutinib *in vivo*. After two weeks of treatment, ibrutinib allowed a 25% reduction in tumor growth, while IQS019-2MeSO$_3$H allowed a 42% reduction in tumor volumes, thus showing a superior activity than the first-in-class BTK inhibitor. A better pharmacokinetic profile of IQS019-2MeSO$_3$H, underlying an improved bioavailability of the compound, could be at least partially involved in this differential activity.

Finally, we established an ibrutinib-resistant cell line from a parental MCL cell line by repeated drug selection. Ibrutinib was unable to modulate the phosphorylation of LYN, SYK, and BTK in this resistant cell line, while IQS019 achieved a reduction in the phosphorylated levels of LYN and SYK. Neither of the two drugs achieved a significant dephosphorylation of BTK, thus confirming the role of SYK and LYN blockade in the superior antitumoral activity of IQS019 versus ibrutinib.

DISCUSSION

DISCUSSION

Despite important progresses in knowledge of the molecular factors responsible of the development of cancer and the improvement in the OS thanks to new therapies, long-term survival is still disappointing for the majority of lymphoid malignancies. Consequently, there is a constant need to develop alternative or synergistic antitumoral approaches for these entities. The characterization of activated signaling pathways involved in survival and proliferation, together with the development of a wide pharmacological armamentarium against cancer, have facilitated the bench-to-bedside translation of new targeted therapies in B-cell malignancies. These novel therapies include two of the most relevant drugs lately approved for B-NHL: the anti-apoptotic agent venetoclax and the BTK inhibitor ibrutinib.

Venetoclax (ABT-199) is a first-in-class BH3 mimetic, FDA-approved for use in patients with R/R del17p CLL. In the clinical setting, it has demonstrated high response rates and good toxicity profiles in other subtypes of R/R NHL.

Ibrutinib is a BTK inhibitor approved for the treatment of R/R CLL, MCL, and WM, and also for first-line therapy in patients with del17p CLL. Ibrutinib has also been studied in other lymphoid malignancies, such as R/R FL and DLBCL.[327,403]

One major hurdle to their successful application is the rise of primary and acquired drug resistance, which limits the use of novel agents and pushes for the search of new therapeutic options. With this concept in mind, in this thesis we have explored new approaches to overcome the development of drug resistance.

Overcoming the acquired resistance to ABT-199 in DHL

High-grade B-cell lymphoma with *MYC* and *BCL2* rearrangements or DHL is an aggressive disease characterized by frequent failures to standard chemotherapeutic regimens.

First-line treatment of DHL patients is based on R-CHOP chemotherapy and intensive treatment strategies, but it does not produce a sustained remission in the majority of patients.[105] Among the multiple novel targeted agents currently in clinical development are the specific inhibitors of the key oncogenes, *BCL2* and *MYC*, which may offer hope for better outcomes in those patients.

The appropriate balance between pro-survival and pro-death BCL-2 family members in healthy cells is often disrupted in cancer cells, where an overexpression of anti-apoptotic BCL-2 proteins can promote oncogenesis and confer resistance to chemotherapeutic agents.[457] BH3 mimetics are the first class of drugs to target the core of the apoptosis pathway by binding directly and specifically inhibiting the anti-apoptotic BCL-2 proteins. Among these molecules, the BCL-2 inhibitor venetoclax has shown to be effective in DHL

cell lines[128] but, as we have discussed in the introduction section, drug resistance is a major issue associated to its use.

The aim of the first work derived from this thesis was to analyze the mechanisms involved in the acquired resistance to BCL-2 inhibitors in DHL cells, and explore therapeutic approaches to counteract this resistance. For this aim, a set of GCB-DLBCL cell lines were used as controls, since in the case of concurrent *MYC* and *BCL2* rearrangements, the predominant phenotype of DHL cells resembles this B-NHL subtype.

Using ABT-199 as a single-agent, we saw a nice initial response of DHL cells to this drug but we observed that a prolonged exposure to the compound (96 hours) was associated with a decreased response of the cells, independently of the dose used. This effect was not seen in the GCB-DLBCL cell lines, where the response to the BH3 mimetic increased in a time and dose-dependent manner, suggesting the presence of some differential compensatory mechanism between GCB-DLBCL and DHL cells. Of note, a number of previous studies have also revealed the emergence of BH3 mimetic resistance in GCB-DLBCL and other hematologic malignancies after several months of drug exposure.[100,487]

Targeted inhibition of BCL-2 is likely to have a greater clinical impact when it is combined with other agents.[176] The optimal combination partners in NHL remain to be defined, and studies are underway to assess the safety and efficacy of combining venetoclax with standard chemotherapy (NCT03064867, NCT03054896), monoclonal antibodies[484] (NCT03136497, NCT03135262), B-cell receptor signaling inhibitors[503] (NCT02756897, NCT02956382, NCT03112174) or proteasome inhibitors (NCT02755597, NCT02899052).[504]

In our study, this ABT-199 resistance phenotype motivated the use of this molecule in combination with some drugs capable of sensitizing the cells to the BCL-2 antagonist. Prior to the determination of the best drug combination, we first focused on the elucidation of the molecular mechanisms involved in the emergence of drug-resistance.

Our first analysis discarded a role for ABT-199 mechanism of action in the differential responses observed between GCB-DLBCL and DHL cells, as in both lymphoma subtypes ABT-199 was able to displace BIM from BCL-2 complexes, as seen by an immunoprecipitation assay. A differential basal expression of BCL-2 family proteins was also rejected as the cause of DHL resistance to ABT-199, as we observed no correlation between the basal levels of BCL-2/BCL-2-like proteins and the inhibition of cell proliferation upon ABT-199 treatment.

Studies performed in aggressive B-cell lymphoma and leukemic mature B cells that included prolonged exposure to BH3 mimetics, highlighted a role for high MCL-1 and BCL-XL levels in cell resistance to chemotherapy[505,506] and ABT-199,[504,507] as well as a negative impact of BFL-1[100] and/or MCL-1[100,489,490] upregulation on the responsiveness to ABT-737.

The fact that MCL-1 was upregulated in both GCB-DLBCL and DHL cells after 96 hours of treatment, as well as in DHL cell lines at 24 hours, led us to discard MCL-1 as a driver of resistance to ABT-199 in DHL cells. Actually, this deregulation of MCL-1 was present in both resistant and sensitive cells. In the case of BCL-XL, although it seemed to accumulate in DHL cell lines after 96 hours of treatment with ABT-199 (when cells were resistant to the compound), there was no correlation between its modulation and the proliferation recovery. In contrast, BFL-1 was differentially overexpressed in resistant DHL versus GCB-DLBCL cell lines after 96 hours of ABT-199 treatment, suggesting that BFL-1 upregulation could represent a molecular mechanism associated to the loss of activity of ABT-199 in DHL cell lines. The same phenomenon was observed in primary DHL samples and *in vivo*, in a mouse xenotransplant model of DHL. To confirm the role of BFL-1 in acquired resistance to ABT-199, we employed a standard GCB-DLBCL cell line modified to specifically overexpress BFL-1 and further confirmed a crucial role of this protein in the acquisition of resistance to ABT-199 in B-cell lymphoma.

In light of these findings, a logical approach to counteract the protective role of BFL-1 involved the screening of rational combination strategies, which may be able to counteract this compensatory mechanism.

BFL-1 is an anti-apoptotic protein that exerts its function by sequestering pro-apoptotic/BH3-only proteins. BFL-1 bounds BAK more prominently than BAX,[508] and among the BH3-only proteins, it interacts with BIM, BID, PUMA, and NOXA, and in a weaker extent with BIK and HRK.[462,509] BFL-1 is particularly important in the hematopoietic system, where it seems to be a critical survival factor downstream of tonic as well as antigen-driven BCR activation.[510] Its expression is induced by NF-κB[508,511] and is upregulated in many human tumors in which NF-κB is involved.[512] In fact, BFL-1 is associated with different forms of leukemia and lymphoma, where probably prevents apoptosis in the advanced tumor cells.[508] Moreover, this prevention of cell death has been well associated with chemotherapy resistance and poor prognosis.[513,514] In accordance with our results, overexpression of BFL-1 in cell lines has been shown to mediate resistance to other drugs such as etoposide,[511] fludarabine[505] or cisplatin,[515] and BFL-1 knockdown was found to sensitize malignant B-cell lines to chemotherapy or rituximab.[516] Consequently, targeting BFL-1 might represent a promising strategy for the development of novel anti-cancer therapies and it might be useful to sensitize DHL cells to BH3 mimetics.

So far, very few specific and potent inhibitors of BFL-1 have been described. Recently, short peptides that mimic the interaction geometry of native BFL-1 have been developed.[517] A modified BIM peptide has also shown inhibition of BFL-1 but this approach does not provide a selective effect.[518] PUMA and NOXA modified peptides showed more selectivity against BFL-1, making them promising starting points for the development of peptide- or small-molecule therapeutics directly targeting BFL-1.[517,519] As a consequence,

given the difficulties in developing a specific BFL-1 inhibitor, indirect strategies, such as epigenetic drugs, have also been studied. Among these, the latest preclinical evaluation of BET inhibitors has offered numerous mechanistic insights of their antitumor activity, facilitating their optimal therapeutic targeting in different cancer models. Several structure/activity-based BET protein bromodomain antagonists have been developed, including JQ1, which displaces BRD4 from the acetylated chromatin.[126,127] This displacement results in the transcriptional repression of *MYC*,[520] followed by genome-wide downregulation of MYC-dependent target genes.[124] The overexpression of MYC leads to a significant increase in BFL-1 protein levels, identifying BFL-1 as a possible MYC target.[521] On the other hand, MYC is known to repress BIM via microRNA 17-92 and this repression is reversed by JQ1-mediated inhibition of the oncogen.[522,523] In fact, apart from the role of BFL-1 in the acquired resistance to ABT-199, BIM has also a crucial role in cell responsiveness to the drug. *In vitro* studies support that lower levels of BIM protein, and consequent deregulation of BCL-2/BIM expression ratio, results in the acquisition of a BH3-mimetic resistance phenotype.[524] Collectively, JQ1 treatment attenuates the levels of several anti-apoptotic members of the BCL-2 family, such as BCL-XL, MCL-1, and BCL-2, and upregulates the pro-apoptotic protein BIM.[498,520,525] These effects of JQ1 lower the threshold for apoptosis and sensitize cells to the apoptosis induced by conventional antineoplastic therapies,[131,526] as well as by ABT-199.[128,525] Of interest, the particular combination of JQ1 and ABT-199 has shown to be synergistically active in the treatment of DHL cell lines.[128]

CPI203 (Constellation Pharmaceuticals, Inc.), another BRD4 inhibitor, is structurally related to JQ1 and has a similar toxicity spectrum, together with an improved bioavailability profile in mice.[130] In accordance with the first preclinical evidence of efficacy of CPI203 in lymphomas,[131,132] our data shows a time- and dose-dependent cytostatic effect of this drug in DHL cell lines and primary samples. Similarly to JQ1, which regulates the expression of apoptotic proteins, CPI203 shows a clear trend toward downregulation of anti-apoptotic factors and upregulation of pro-apoptotic proteins in cell lines.[499] In our present work, DHL cell lines and primary samples treated with CPI203 showed a downregulation of BFL-1 and, in accordance with other studies,[522,523] an upregulation of BIM at both mRNA and protein levels. Of note, transcript levels of MCL-1, previously associated to ABT-199 resistance in NHL,[504,507] have also shown an upregulation at mRNA levels after CPI203 treatment.

Since CPI203 inhibits *MYC*, overexpressed in DHL, and considering the fact that it achieves the modulation of the *BCL2* gene family, the combination of this agent with ABT-199 has emerged as a promising, rationally based approach that may allow to counteract the up-regulation of BFL-1 observed after a continuous treatment of DHL cells with the BH3 mimetic.

Accordingly, while combining CPI203 with ABT-199, we confirmed the capacity of CPI203 to overcome ABT-199 resistance observed in both DHL cell lines and primary cultures. In *in vivo* settings, CPI203 also showed a synergistic activity with ABT-199, associated with a decrease in BFL-1 expression and a reduction in tumor burden. This phenomenon was accompanied by a decreased tumor cell proliferation and a notable increase in apoptosis rate. In addition, owing to the capacity of BET inhibitors to interfere with apoptosis-related genes,[498,499] we showed that the sensitizer effect of CPI203 was mediated, at least in part, by the blockade of BFL-1 transcription, *in vitro* and *in vivo*.

Here we introduce a clear rationale explaining the high synergistic interaction between the BCL-2 antagonist and the BET inhibitor in DHL cells and DHL-tumor bearing mice. However, synergistic interaction between CPI203 and ABT-199 treatment would not be only produced by BFL-1 downregulation but also involved the upregulation of BIM mRNA. Binding of released BIM to BCL-2-like counterparts is a known mechanism of intrinsic resistance to ABT-199.[527] In this sense, the capacity of CPI203 to increase the disbalance between intracellular pools of BIM and BFL-1 may be crucial for the capacity of the BET inhibitor to overcome ABT-199 resistance. Our immunoprecipitation analysis suggests that the events downstream transcriptional normalization by CPI203 include the redistribution of BIM from BFL-1- to BCL-2-dependent complexes, and the consequent triggering of apoptotic signaling in cells exposed to ABT-199.

We proposed a model of DHL resistance to ABT-199 in which the capacity of CPI203 to regulate the transcriptome of the cells could help to circumvent this problem (figure 22). In ABT-199 sensitive cells, the BH3 mimetic acts by displacing BIM from BCL-2 complexes, allowing the de-repression and/or direct activation of BAX and leading to an activation of MOMP. In DHL cells, a compensatory upregulation of BFL-1 would bind and inactivate the pool of BIM proteins released from BCL-2 by ABT-199, avoiding MOMP and preserving cell survival. CPI203 primes cells to death by decreasing BFL-1 and increasing BIM protein levels, and its combination with ABT-199 allows to tip the balance between pro- and anti-apoptotic signaling toward induction of cell death. This concept provides a new insight in the proposed mechanisms of resistance to BH3-mimetics in NHL cell lines, where MCL-1 and BCL-XL have been proposed as the major determinants of drug sensitivity and acquired resistance.[100,504,528,529]

Given the recent advances in our understanding of cell death mechanisms, agents that perturb the balance between pro- and anti-apoptotic proteins represent logical therapeutic candidates for being combined with ABT-199 in hematologic malignancies. Rational combination strategies will undoubtedly be required to achieve sustained responses to BCL-2 antagonists and to overcome acquired resistance to these agents. For this aim, it will be mandatory to identify the compensatory mechanisms specific for each lymphoma subtype, in order to find the best combination partner in each entity.

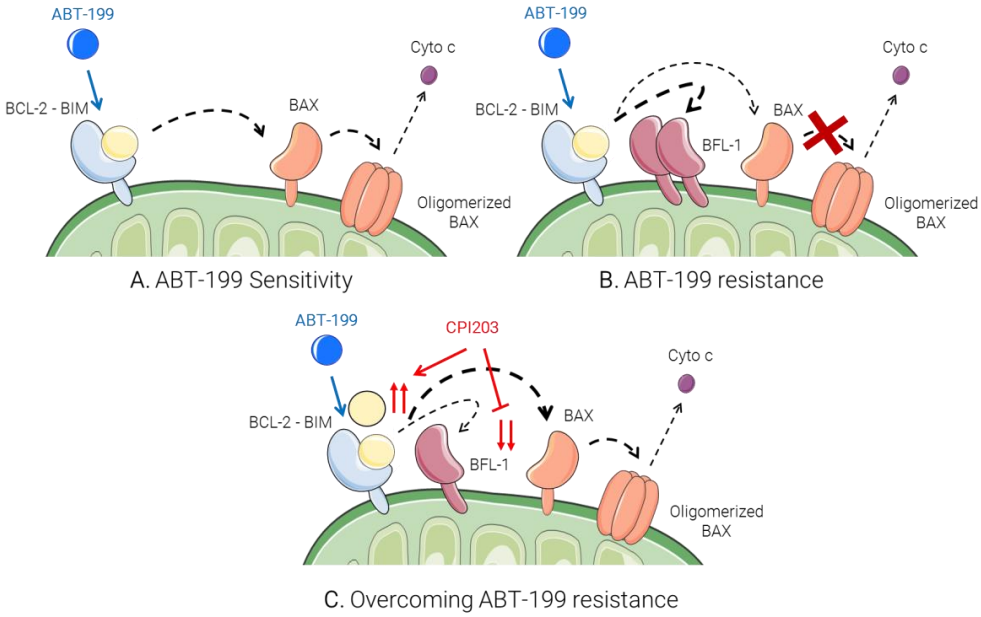


Figure 22 | **Possible mechanism of DHL cell priming by CPI203 and sensitization to ABT-199.** (A) In ABT-199 sensitive cells, the BH3 mimetic displaces BIM from BCL-2, allowing BAX oligomerization and subsequent mitochondrial depolarization and activation of caspases. (B) In DHL cells, overexpressed BFL-1 binds to and inactivates BIM, avoiding BAX activation and preserving cell survival. (C) CPI203 primes cells to death by decreasing BFL-1 and increasing BIM protein levels.

Taken together, our results demonstrate that for DHL cases, the combination of a BET inhibitor with a BCL-2-specific BH3-mimetic is effective and can produce synergistic antitumor activity *in vitro* and *in vivo*. This rationally-based strategy could constitute a promising therapeutic alternative for aggressive DHL cases, thus warranting its clinical evaluation in those patients with a very unfavorable prognosis.

Several questions remain unanswered regarding the role of other BCL-2 proteins in the acquired resistance to ABT-199. For example, the treatment used in this study (96 hours), is comparatively short relative to expected clinical regimens: thus other compensatory changes among BCL-2 family proteins are expected during longer therapies.

Future trials will clarify the benefit of combining BCL-2 and MYC inhibitors for the treatment of DHL patients, in both first- and second-line settings, while additional preclinical studies will be required to evaluate their antitumoral activity in other B lymphoid malignancies.

BCR kinase inhibition in B-NHL

BCR signaling has recently emerged as a key oncogenic pathway in several lymphoma subtypes.[377] The mechanisms that activate BCR signaling differ substantially among B-NHL entities, including BCR stimulation by foreign or self-antigens, or the acquisition of mutations in components of the BCR pathway that result in enhanced autonomous or antigen-induced BCR signaling. Constitutive activation of the BCR signaling has been well documented in CLL,[390,392,393] MCL,[382,530] FL,[531] and the ABC subtype of DLBCL.[79]

Consistently, the kinases involved in the cascade of effectors downstream of the BCR such as LYN, SYK or BTK, have been considered targets for possible therapeutic intervention that may play an important role in the pathogenesis of NHLs.[377] BCR kinase inhibitors induce high response rates and are transforming the therapeutic landscape in these diseases.

Dasatinib (BMS-354825; Spycell) is a multi-kinase inhibitor used to target the BCR-ABL fusion protein but it also efficiently targets SRC-family kinases (such as LYN) and BTK.[532] It was initially approved as an ABL inhibitor by the FDA in 2006 for patients with chronic-phase and R/R CML.[533] In addition, it is used in the treatment of adults with R/R Philadelphia chromosome-positive ALL.[534] More recently, in a phase II trial, dasatinib showed efficacy in R/R CLL as a single agent (ORR 20%).[400] Acquisition of mutations in ABL kinase domain, usually T315I, are responsible for the development of resistance to dasatinib.[535,536]

Fostamatinib (R788; Rigel Pharmaceuticals) is an oral SYK inhibitor that has shown efficacy in R/R B-NHL.[425,426] Despite its *in vitro* activity, it had not reached a lot of interest as attested by the lack of ongoing clinical trials for these entities.

The first-in-class BTK inhibitor ibrutinib is one of the novel agents that have received more attention in terms of B-NHL treatment. It has achieved high response rates (68%-90%) in R/R CLL,[244] MCL,[167] and FL[327] patients, while its activity was less pronounced in ABC-DLBCL patients (37% ORR)[80].[221,403] Ibrutinib has been approved for the treatment of R/R patients with CLL, MCL, and WM; and for first-line therapy in patients with del17p CLL. Despite its high level of clinical activity, acquiring of mutations or re-wiring of the BCR pathway to retain the downstream signaling appears to be a common mechanism of resistance. A small fraction of patients develop progressive disease after initial response to this agent, while others have primary resistance to the drug.[167,244,407] For example, approximately 30% of patients with MCL have primary resistance to ibrutinib treatment.[409,410] Secondary resistances are usually related with the acquisition of mutations at the ibrutinib binding site of BTK (C481S) or in the *PLCγ2* gene (R665W or L845F), which reactivates BCR signaling downstream of the kinase.[407,412,413,537] Moreover, resistance to ibrutinib may involve a lower dependency of malignant B cells toward BTK

itself. Escape mechanisms through other signaling cascades involving ERK1/2 and AKT, have been demonstrated in ibrutinib-resistant cell lines,[408] as well as dependence on the alternative NF-κB pathway, through mutations in *TRAF2*, *BIRC3* or *MAP3K14* (*NIK*).[416] Of note, those patients who progress on ibrutinib, tend to have poor outcomes with clinically aggressive disease and very short survival.[411] Accordingly, alternative approaches for the targeting of BCR signaling are under investigation.

Following these observations, and in an effort to improve the therapeutic modulation of BCR signaling, the Engineering Molecular Group from the Institut Químic de Sarrià School of Engineering in Barcelona, synthesized a library of compounds derived from pyrido[2,3-d]pyrimidines, which are pharmacologically active compounds with well-known activity as tyrosine kinase inhibitors.[538] The compounds were screened for their capacity to bind to the active sites of BCR upstream kinases, and IQS019 (compound 19) was identified as the strongest inhibitor of BTK, LYN, and SYK. As expected, IQS019 showed good antiproliferative activity against a panel of 20 NHL cell lines from the MCL, CLL, FL, and DLBCL subtypes.[500]

In the present work, we confirm the antiproliferative activity of IQS019 in different NHL subtypes, being MCL (48-78% cytotoxic effect) and FL (43-100% cytotoxic effect) cell lines significantly more sensitive to the compound than CLL and DLBCL cells (33-51% and 29-51% cytotoxic effect, respectively).

Our mechanistic studies suggest that IQS019 can counteract both chronic and tonic BCR signaling, as the drug shows similar antiproliferative activity in DLBCL cell lines from both the GCB and ABC subtypes, which are respectively dependent for their survival on tonic (SYK/PI3K-mediated) and chronic (SYK/BTK-mediated) BCR signaling.[79,377,379,381] This property might confer to IQS019 a greater activity than ibrutinib, which is preferentially active against tumors that rely on chronic active BCR signaling.[80]

We have observed how IQS019 impairs both constitutive and ligand-induced phosphorylation of BTK, SYK, and LYN in NHL cell lines and primary samples. BCR stimulation is known to increase cell viability, while BCR antagonism leads to B-cell death.[342] Upon its phosphorylation, SYK propagates BCR-derived signals by activating downstream signaling pathways, including calcium mobilization and activation of AKT kinase, ERK 1/2 and MCL-1.[539,540] Accordingly, pharmacological inhibition of SYK has been reported to abrogate the pro-survival effect of BCR stimulation, and to trigger the apoptotic cascade in preclinical models of DLBCL and CLL.[390,541,542] The direct inhibitory activity of IQS019 towards SYK phosphorylation not only decreased BCR signaling, but may also explain the capacity of the compound to activate apoptosis *in vitro* and *in vivo*. Also, likely owing to its apoptogenic property and specificity, IQS019 salt is found to be significantly active and safe at an intraperitoneal dose of 2 mg/kg/day, which is much lower than the

reported active concentrations of fostamatinib (80 mg/kg/day),[543,544] dasatinib (20 mg/kg/day),[545] or ibrutinib (20 mg/kg/day)[546] in mouse models of lymphoid neoplasms. This predicts a probable low incidence of secondary adverse effects of the compound. Then, in order to compare the antitumoral activity of IQS019 versus the first-in-class ibrutinib *in vivo*, we inoculated SCID mice with the MCL cell line UPN-1, *bona fide* ibrutinib-sensitive NHL cell line. We orally treated tumor-bearing mice with 25mg/kg/day doses of either IQS019 or ibrutinib, being it the standard oral dosage used for the BTK inhibitor in mice.[547-549] IQS019 showed to have a more potent antitumoral effect, as it reduced tumor outgrowth more effectively.

Another downstream event of BCR signaling is the chemokine-mediated B cell migration, a process essential to tumor B-cell survival. In fact, SYK inhibitors have shown to counteract the decrease in the expression of chemokine receptors like CXCR4 consequent to BCR stimulation.[541] We showed that IQS019 was able to impair *in vitro* the cell migration towards CXCL12 in cell lines and primary samples, and in both basal and anti-Ig-stimulated conditions. Moreover, this property of IQS019 may be responsible, at least in part, for the reduced infiltration of tumor cells observed in FL-bearing mice dosed with the compound.

Altogether, the capacity of IQS019 to inhibit the three upstream BCR kinases, might confer an advantage over the treatment with ibrutinib. In order to compare the antiproliferative activity of IQS019 and ibrutinib *in vitro*, we treated a panel of 8 cell lines with both drugs and calculated their IC_{50}. Our data permitted to identify three ibrutinib-resistant cell lines, all from the MCL subtype: Z138, Granta-519 and JVM-2. In contrast, IQS019 maintained a remarkable effect in these cell lines, as well as in the cells responsive to ibrutinib. As in the clinics, none of these ibrutinib-resistance cell lines have BTK mutation. Therefore, this primary resistance to the drug may not be the result of ineffective ibrutinib inhibition of BTK, but may rather involve sustained distal BCR signaling, specifically via PIK3-AKT[408,550] or alternative NF-κB pathway activation.[416] In fact, some studies that have performed transcriptome sequencing, revealed genetic lesions in alternative NF-κB pathway signaling components in association with ibrutinib resistance.[410,416,550,551] Accordingly, Granta-519 and JVM-2 are positive for Epstein-Barr virus, which is known to activate non-canonical NF-κB pathway through the expression of latent membrane protein 1,[439,440] a protein that can induce NIK-dependent p100 processing.[552] Z-138 is also dependent on alternative NF-κB for its survival, especially on NIK protein expression. Mechanistically, Z-138 harbors a nonsense mutation in the *TRAF2* gene, a negative regulator of alternative NF-κB signaling which interacts with BIRC2 and BIRC3 to downregulate NIK, promoting the processing of p100 into the active p52 isoform.[416] As a consequence, Z-138 shows high levels of p52,[416,553] similarly to Granta-519 and JVM-2, conferring to these cell lines a constitutive non-canonical NF-κB signaling.[416]

In an attempt to recapitulate such a phenotype of resistance to ibrutinib, we generated an ibrutinib-resistant cell line derived from the parental UPN-1 cells by long term culture (over 5 months) in the presence of increasing concentrations of ibrutinib. The resulting cell line (UPN-IbruR) was 10-fold more resistant to ibrutinib in comparison to the parental cells. Both ibrutinib and IQS019 were unable to inhibit BTK phosphorylation in these resistant cells, suggesting the role of BTK kinase in this secondary resistance acquisition process, and that the unavailability of the BTK kinase domain was affecting the two drugs in the same extent. Nonetheless, sequencing studies did not reveal any mutations in *BTK* or *PLCγ2* genes, consistent with whole exome sequencing data from MCL cases with primary resistance to ibrutinib.[412,554] With this in mind, we hypothesized that the UPN-IbruR cell line could be dependent on the alternative NF-κB pathway. Accordingly, we observed an overexpression of p52 in this cell line, suggesting an association of the resistance with the activation of the non-canonical NF-κB pathway.

In summary, we describe IQS019 as a new BCR kinase inhibitor able to counteract both constitutive and ligand-dependent activation of the BCR pathway. Our results point out a significant superior antitumoral activity of pleiotropic BCR kinase targeting by IQS019 over the inhibition of BTK alone, in *in vitro* and *in vivo* models of B-NHL, being of special importance for the treatment of those patients with non-canonical NF-κB activation.

Outlook

Resistance to therapy is observed in many cases of B-cell malignancies. This phenomenon significantly limits the utility of the current therapeutic strategies, and remains a substantial challenge for the clinical management of patients with advanced cancers. Resistance comes in two flavors: intrinsic resistance (also known as innate or *de novo* resistance) and acquired resistance, resulting from the clonal evolution of resistant variants.

The mechanisms of action of several antineoplastic drugs involve interactions between the drug and an essential intracellular protein, resulting in an alteration or inhibition of their normal functions. Quantitative or qualitative changes in these protein targets can compromise drug efficacy. Actually, alterations of the drug target, modulation of compensatory proteins or activation of alternative pathways, have become recognized as mechanisms of resistance to newer molecularly targeted chemotherapy. Recognition of these resistances paved the way for the development of exciting new strategies to overcome them. The studies included in this dissertation perfectly fit into this priority area in lymphoma research.

On the one hand, we have contributed to the elucidation of the BCL-2 family proteins role in the compensatory mechanisms underlying ABT-199 resistance in DHL, proposing epigenetic modulation as an effective approach to restoring therapeutic response. On the

other hand, we have shown activation of alternative NF-κB signaling as one of the mechanisms involved in ibrutinib resistance, and how the simultaneous targeting of multiple BCR kinases can become a valid strategy for the treatment of both, sensible and low responders to ibrutinib.

Therefore, the development of innovative therapeutic approaches that permit to overcome drug resistance opens a window to important therapeutic advances that will help researchers move forward in the fight against cancer.

CONCLUSIONS

CONCLUSIONS

The main conclusions derived from this thesis are as follows:

Overcoming the acquired resistance to ABT-199 in DHL

The BET bromodomain inhibitor CPI203 has the capacity to overcome ABT-199 resistance in DHL by regulating the BCL-2 family members, BIM and BFL-1.

1. BCL-2 targeting by ABT-199 is effective in GCB-DLBCL cells, while acquired resistance to this agent arises in DHL cells after 96 hours of treatment.
2. The stabilization of BFL-1, rather than BCL-XL or MCL-1, is involved in ABT-199 resistance, and determines the sensitivity of DHL cultures and xenografts to this drug.
3. Epigenetic modifications by the BET bromodomain inhibitor CPI203 have cytostatic activity in DHL cells.
4. CPI203 regulates the apoptosis gene signature in DHL cells, inducing an upregulation of BIM and a downregulation of BFL-1.
5. BFL-1 overexpression caused by ABT-199 treatment in DHL can be counteracted *in vitro* and *in vivo* by CPI203, allowing the redistribution of BIM from BFL-1-dependent to BCL-2-dependent complexes, and the consequent triggering of apoptotic signaling.
6. ABT-199 and CPI203 combination therapy shows synergistic antitumor activity in *in vitro* and *in vivo* models of DHL, becoming a promising strategy for the treatment of DHL patients.

BCR kinase inhibition in B-NHL

IQS019 has good antitumor and safety profiles in B-NHL with constitutive activation of BCR signaling, including those low responders to current BTK inhibitors.

7. IQS019 is able to counteract both constitutive and ligand-dependent activation of the BCR pathway in *in vitro* models of B lymphoid neoplasms by inhibition of BTK, SYK and LYN phosphorylation.
8. IQS019 impairs CXC12-dependent chemotaxis, and has the capacity to induce caspase-dependent apoptosis in B-NHL cell lines and primary samples.
9. *In vivo*, IQS019 is safe and impairs tumor outgrowth and malignant B cell homing to the spleen.
10. The co-inhibition of LYN, SYK and BTK can counteract BCR signaling and exert an improved antitumoral activity in *in vitro* and *in vivo* models of B-NHL, when compared with the sole inhibition of BTK by the first generation inhibitor, ibrutinib.
11. The co-inhibition of LYN, SYK and BTK by IQS019 achieves antitumoral response in B-NHL cases with alternative NF-κB activation.

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ANNEXES

ANNEX 1: PAPERS IN COLLABORATION

A mouse model of disseminated mantle cell lymphoma highlights a lack of activity of estrogen receptor β agonists toward tumor burden

Body S[1], Esteve-Arenys A[2], Recassens-Zorzo C[2], Troussard X[1,3], Roué G[2,4], Sola B[1]

1 Normandie Univ, INSERM U1245, UNICAEN, UNIROUEN, Caen, France.
2 Division of Haematology and Oncology, IDIBAPS, Barcelona, Spain.
3 Laboratorie d'Hématologie Biologique, CHU Côte de Nacre, Caen, France.
4 Molecular Genetics Unit, Department of Hematology, Vall d'Hebron University Hospital, Barcelona, Spain.

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Cytoplasmic cyclin D1 controls the migration and invasiveness of mantle lymphoma cells

Body S[1], Esteve-Arenys A[2], Miloudi H[1], Recasens-Zorzo C[2], Tchakarska G[1], Moros A[2], Bustany S[1], Vidal-Crespo A[2] , Rodriguez V[2], Lavigne R[3], Com E[3], Casanova I[4,5], Mangues R[4,5], Weigert O[6], Sanjuan-Pla A[7], Menéndez P[5,8], Marcq B[9], Picquenot JM[1,9], Pérez-Galán P[2], Jardin F[1,10], Roué G[2,11], Sola B[1].

1 Normandie Univ, INSERM U1245, UNICAEN, UNIROUEN, Caen, France.
2 Division of Haematology and Oncology, IDIBAPS, Barcelona, Spain.
3Plateforme protéomique - Protim, Biogenouest, Rennes, France.
4 Grup d'Oncogènes i Antitumorals, Institut d'Investigacions Biomèdiques Sant Pau and Centro de Investigación Biomédica en Red, Barcelona, Spain.
5 Josep Carreras Leukaemia Research Institute, Department of Biomedicine, School of Medicine, University of Barcelona, Barcelona, Spain.
6 Medical Department III, University Hospital, Ludwig Maximilians University, Munich, Germany.
7Hematology Service, Hospital Universitario y Politécnico La Fe, Valencia, Spain.
8 Institució Catalana de Recerca i Estudis Avançats, and Centro de Investigación Biomédica en Red de Cáncer, ISCIII, Barcelona, Spain.
9 Service d'anatomie et cytologie pathologiques, Centre Henri Becquerel, Rouen, France.
10Département d'hématologie clinique, Centre Henri Becquerel, Rouen, France.
11Laboratory of Experimental Hematology, Department of Hematology, Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, Barcelona, Spain.

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ABSTRACT

Mantle cell lymphoma (MCL) is a hematologic neoplasm characterised by the t(11;14)(q13;q32) translocation leading to aberrant cyclin D1 expression. The cell functions of cyclin D1 depend on its partners and/or subcellular distribution, resulting in different oncogenic properties. We observed the accumulation of cyclin D1 in the cytoplasm of a subset of MCL cell lines and primary cells. In primary cells, this cytoplasmic distribution was correlated with a more frequent blastoid phenotype. We performed immunoprecipitation assays and mass spectrometry on enriched cytosolic fractions from two cell lines. The cyclin D1 interactome was found to include several

factors involved in adhesion, migration and invasion. We found that the accumulation of cyclin D1 in the cytoplasm was associated with higher levels of migration and invasiveness. We also showed that MCL cells with high cytoplasmic levels of cyclin D1 engrafted more rapidly into the bone marrow, spleen, and brain in immunodeficient mice. Both migration and invasion processes, both in vivo and in vitro, were counteracted by the exportin 1 inhibitor KPT-330, which retains cyclin D1 in the nucleus. Our data reveal a role of cytoplasmic cyclin D1 in the control of MCL cell migration and invasion, and as a true operator of MCL pathogenesis.

Dual targeting of MCL1 and NOXA as effective strategy for treatment of mantle cell lymphoma.

Höring E[1,2,3], Montraveta A[3], Heine S[1], Kleih M[1], Schaaf L[1], Vöhringer MC[2], Esteve-Arenys A[3], Roué G[3], Colomer D[3], Campo E[3], Ott G[4], Aulitzky WE[2], van der Kuip H[1].

1 Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology and University of Tuebingen, Stuttgart, Germany.
2 Department of Haematology and Oncology, Robert-Bosch-Hospital, Stuttgart, Germany.
3 Haematopathology Unit, Hospital Clínic - Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain.
4 Department of Clinical Pathology, Robert-Bosch-Hospital, Stuttgart, Germany.

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ABSTRACT

Imbalances in the composition of BCL2 family proteins contribute to tumourigenesis and therapy resistance of mantle cell lymphoma (MCL), making these proteins attractive therapy targets. We studied the efficiency of dual targeting the NOXA/MCL1 axis by combining fatty acid synthase inhibitors (NOXA stabilization) with the CDK inhibitor Dinaciclib (MCL1 reduction). This combination synergistically induced apoptosis in cell lines and primary MCL cells and led to almost complete inhibition of tumour progression in a mouse model. Apoptosis was NOXA-dependent and correlated with the NOXA/MCL1 ratio, highlighting the importance of the NOXA/MCL1 balance for effective cell death induction in MCL.

ZEB1-induced tumourigenesis requires senescence inhibition via activation of DKK1/mutant p53/Mdm2/CtBP and repression of macroH2A1.

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1 Group of Transcriptional Regulation of Gene Expression, Department of Oncology and Hematology, IDIBAPS, Barcelona, Spain.
2 Lendület Cancer Biomarker Research Group, MTA TTK and 2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary.
3 Department of Pathology, Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain.
4 Department of Pathology, Hospital Clínico San Carlos, Madrid, Spain.
5 Lymphoma Group, Department of Oncology and Hematology, IDIBAPS, Barcelona, Spain.
6 Department of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, Spain.
7 Department of Oral Immunology and Infectious Diseases and Center for Genetics and Molecular Medicine, University of Louisville, Louisville, Kentucky, USA.
8 Institute of Metabolic and Digestive Diseases, Hospital Clínic, Barcelona, Spain.
9 Gastrointestinal and Pancreatic Oncology Team, Biomedical Research Networking Centers in Hepatic and Digestive Diseases (CIBERehd), Carlos III Health Institute (ISCIII), Barcelona, Spain.
10 Molecular Targets Program, James Graham Brown Cancer Center, Louisville, Kentucky, USA.
11 ICREA, Barcelona, Spain.

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ABSTRACT

Objective

Understand the role of ZEB1 in the tumour initiation and progression beyond inducing an epithelial-to-mesenchymal transition.

Design

Expression of the transcription factor ZEB1 associates with a worse prognosis in most cancers, including colorectal carcinomas (CRCs). The study uses survival analysis, in vivo mouse transgenic and xenograft models, gene expression arrays, immunostaining and gene and protein regulation assays.

Results

The poorer survival determined by ZEB1 in CRCs depended on simultaneous high levels of the Wnt antagonist DKK1, whose expression was transcriptionally activated by ZEB1. In cancer cells with mutant TP53, ZEB1 blocked the formation of senescence-associated heterochromatin foci at the onset of senescence by triggering a new regulatory cascade that involves the subsequent activation of DKK1, mutant p53, Mdm2 and CtBP to ultimately repress macroH2A1 (H2AFY). In a transgenic mouse model of colon cancer, partial downregulation of Zeb1 was sufficient to induce H2afy and to trigger in vivo tumour senescence, thus resulting in reduced tumour load and improved survival. The capacity of ZEB1 to induce tumourigenesis in a xenograft mouse model requires the repression of H2AFY by ZEB1. Lastly, the worst survival effect of ZEB1 in patients with CRC ultimately depends on low expression of H2AFY and of senescence-associated genes.

Conclusions

The tumourigenic capacity of ZEB1 depends on its inhibition of cancer cell senescence through the activation of a herein identified new molecular pathway. These results set ZEB1 as a potential target in therapeutic strategies aimed at inducing senescence.