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Comparison of the cytotoxic mechanisms of anti-CD20 monoclonal antibodies Rituximab and GA101 in Chronic Lymphocytic Leukemia

Lina Reslan

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L'UNIVERSITE CLAUDE BERNARD LYON 1

ECOLE DOCTORALE

BIOLOGIE MOLECULAIRE, INTEGRATIVE ET CELLULAIRE

DIPLOME DE DOCTORAT

(arrêté du 7 août 2006)

soutenue publiquement le 23 Décembre 2010

par

Melle RESLAN Lina

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Mme Catherine Thieblemont, PU-PH, Université Denis Diderot Paris 7
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Mme Christine Bezombes, Chargée de recherche, INSERM U563, Toulouse
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Abbreviations

AC: L-ascorbic acid

ADA: Adenosine deaminase

ADCC: Antibody-dependent cellular cytotoxicity

AIF: Apoptosis Inducing factor

Akt: protein kinase B

ANT: Adenosine Nucleotide translocator

Ara-C: Arabinosyl cytosine

ATM: Ataxia Teleangiectasia Mutated

ATP: Adenosine-5'-triphosphate

Bak: Bcl-2 homologous antagonist/killer protein

Bax: Bcl-2-associated X protein

Bcl-2: B-Cell CLL/Lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

BCR: B-cell receptor

Bfl-1: Bcl-2-related protein A1

BH3: Bcl-2 homology 3

Bid: BH3 domain-only death agonist protein

Bim: Bcl-2 interacting mediator of cell death

BSA: Bovine serum albumin

CAD: Caspase-activated DNase

CALGB: Cancer and Leukemia Group B

C: cyclophosphamide

CC : cladribine plus cyclophosphamide

CCM: Cladribine, cyclophosphamide and mitoxantrone

Cd: Cladribine

CDC: Complement-dependent cytotoxicity

CDRs: Complementarity-determining regions
CHOP: cyclophosphamide, adriamycin, oncovin and prednisone
CI: Cavitation index
CICD: Caspase-Independent Cell death
CLL: Chronic Lymphocytic Leukemia
CR: Complete Remission
CVP: Cyclophosphamide, Vincristine, Prednisone
CSA: ciclosporin A
CypD: Cyclophilin D
DiOC6[3]: 3,3-dihexyloxacarbocyanine iodide
DISC: Death-inducing signaling complex
DLBCL: Diffuse Large B-cell lymphoma
DNA: Deoxyribonucleic acid
EGF1: Early Growth Factor 1
EGFR: Epidermal growth factor receptor
ERK: Extracellular signal-regulated kinase
FADD: Fas associated death domain protein
FCCP: Carbonyl cyanide-p-trifluoromethoxy hydrazone
FDA: Food and Drug Administration
FISH: Fluorescence in situ Hybridization
FL: Follicular lymphoma
FLIPI: Follicular Lymphoma International Prognostic Index
FC: Fludarabine plus cyclophosphamide
5-FU: Fluorouracil
GC: Germinal center
GVHD: Graft-Versus-Host Disease
H₂O₂: Hydrogen peroxide
HD: Hodgkin's disease
HER2: Human epidermal growth factor receptor 2
HSC: Hematopoietic Stem Cells

HSCT: Hematopoietic Stem-Cell Transplantation
IAP: Inhibitor of apoptosis
ICAD: Inhibitor of CAD
Ig: Immunoglobulin
IWCLL: International Workshop on Chronic lymphocytic leukemia
JAK: Janus kinase
Lyn: src family tyrosine-protein kinase
MAb: Monoclonal antibody
Mcl-1: Myeloid cell leukemia 1
MCP: Mitoxantrone, Chlorambucil and Prednisolone
MDACC: M. D. Anderson Cancer Center
miR: micro-RNAs
Omi/HTrA2 : High temperature requirement protein A2
mtDNA: mitochondrial DNA
MMP : Mitochondrial membrane permeabilization
NAC : N-acetyl cysteine
NADPH: nicotinamide adenine dinucleotide phosphate-oxidase
NCI: National Cancer Institute
NCI-WG: National Cancer Institute-sponsored Working Group
NFκB: Nuclear factor Kappa B
NHL: Non-Hodgkin Lymphoma
iNOS: Nitric oxide synthase
IL: Interleukin
O₂⁻: Superoxide anion
O-FC: Ofatumumab-Fludarabine and cyclophosphamide
OH: Hydroxyl radical
OMM: Outer Mitochondrial Membrane
ORR: Overall Response
PBS: Phosphate-Buffer Saline
PFS: progression-free survival

PI3K: Phosphatidylinositol-3 kinase
PLC γ : phospholipase C gamma
PTPC: permeability transition pore complex
RFS: Relapse-Free Survival
RKIP: Raf-1 kinase inhibitor protein
RNA: Ribonucleic acid
ROS: Reactive Oxygen species
sCD23: soluble CD23
SEER: Surveillance, Epidemiology, and End Results
shRNA: short hairpin RNA
siRNA: small interfering RNA
SLL: Small Lymphocytic Lymphoma
SmIg: surface membrane immunoglobulin
STAT: Signal Transducer and Activator of Transcription
Syk: tyrosine-protein kinase
TK: Thymidine kinase
TLR4: Toll-like receptor 4
TNF α : Tumor Necrosis Factor α
TRAIL: TNF-related apoptosis inducing ligand
ROS: Reactive oxygen species
RTK: receptor tyrosine kinase
VDAC: Voltage-Dependent Anion Channel
VEGF: Vascular Endothelial Growth Factor
VEGFR: Vascular Endothelial Growth Factor Receptor
US: Ultrasound
WHO: World Health Organization
ZAP-70: Zeta-Associated Protein 70
Z-VAD-fmk: N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Publications

1- Comparison of the Cytotoxic Mechanisms of Anti-CD20 Antibodies Rituximab and Afutuzumab against Fresh Chronic Lymphocytic Leukemia Cells.

Lina Reslan, Stéphane Dalle, Cindy Tournebize, Stéphanie Herveau, Emeline Cros, Charles Dumontet (*In preparation for submission*)

2- Compared antitumor activity of GA101 and rituximab against the human RL follicular lymphoma xenografts in SCID beige mice

Stéphane Dalle, **Lina Reslan**, Timothée Besseyre de Horts, Stéphanie Herveau, Frank Herting, Adriana Plesa, Pablo Umana, Christian Klein, Charles Dumontet

(*Publication under revision in Molecular Cancer Therapeutics*)

3- Transfection of cells in suspension by ultrasound cavitation

Lina Reslan, Jeans louis Mestas, Stéphanie Herveau, Jean Christophe Béra, Charles Dumontet. *J Control Release*, 2010 Mar 3;142(2):251-8.

4- Understanding and circumventing resistance to anticancer monoclonal antibodies (Review)

Lina Reslan, Stéphane Dalle and Charles Dumontet

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5- In vivo model of follicular lymphoma resistant to rituximab.

Stéphane Dalle, Sophie Dupire, Stéphanie Brunet-Manquat, **Lina Reslan**, Adriana Plesa, Charles Dumontet. *Clin Cancer Res* 2009; 851 15(3)

Communications

1- Comparison of Cytotoxic Mechanisms of Anti-CD20 Antibodies GA101 and Rituximab against Fresh Chronic Lymphocytic Leukemia Cells.

Lina Reslan, Stéphane Dalle, Cindy Tournebize, Stéphanie Herveau, Emeline Cros, Charles Dumontet (Poster to be presented in the 52th ASH Annual Meeting and Exposition, Orange County Convention Center, Orlando, Florida, December 4-7, 2010)

2- Preclinical studies on the mechanisms of action of anti-CD20 antibodies in Chronic Lymphocytic Leukemia.

Lina Reslan, Cindy Tournebize, Stéphanie Herveau, Jean Louis Mestas, Charles Dumontet (Poster presented in the 3rd Scientific Journeys of the CLARA (Cancéropôle Lyon Auvergne Rhône-Alpes), Lyon, France, Mars 2010)

3- Générateur de cavitation reproductible : exemples d'application biologique in-vitro

J.-L. Mestas, J. El-Maalouf, **L. Reslan**, C. Inserra, B. Gilles, J.-C. Béra
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5- Compared Antitumor Activity of GA101 and Rituximab against the Human RL Follicular Lymphoma Xenografts in SCID Beige Mice

Stéphane Dalle, **Lina Reslan**, Stéphanie Herveau, Franck Herting, Christian Klein, Pablo Umana and Charles Dumontet (Poster in the 50th ASH Annual Meeting and Exposition, Moscone Center, San Francisco, CA, December 6-9, 2008)

6- Modèle *in vivo* de lymphome folliculaire résistant au rituximab

Stéphane Dalle, Sophie Dupire, **Lina Reslan**, Stéphanie Brunet-Manquat, Adriana Plesa, Charles Dumontet. (Poster presented in the 3rd Scientific Journeys of the CLARA (Cancéropôle Lyon Auvergne Rhône-Alpes), Lyon, France, 18-19 March 2008)

Preface

Chronic Lymphocytic Leukemia (CLL) is a malignant lymphoproliferative disorder which typically affects elderly people. It is the commonest leukemia in the Western adults, accounting for 22-30% of all leukemias and for 10% of all hematological neoplasms [1].

Monoclonal antibodies (MAbs) have revolutionized the management and treatment of B-cell malignancies [2, 3]. The first MAbs to be approved for clinical use is rituximab, a chimeric anti-CD20 MAb. The predominant mechanism(s) of action of rituximab-induced cell death is proposed to be primarily the result of antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) upon binding to CD20. In addition, part of the tumoricidal activity of Rituximab is the result of the direct activation of apoptosis via cross-linking of cell surface-expressed CD20 on malignant B cells. Rituximab has greatly improved clinical outcome of patients with many B-cell disorders including B-cell CLL [4].

Nevertheless, CLL is largely considered incurable and relapse is a common occurrence in these patients; thus, there is a continuous need to develop new treatment strategies. GA101 is the first humanized type II anti-CD20 MAb with glycolengineered Fc portion and a modified elbow hinge. GA101 has an enhanced ADCC, less CDC and superior apoptosis induction in comparison with rituximab [5].

In this thesis, we presented first an overview on the classification, incidence and epidemiology of lymphoid malignancies then we developed CLL biology and treatments. Second, we described the use and mechanisms of action and resistance of MAbs in hematologic malignancies. We focused on CD20 MAbs and their clinical use in CLL patients. Third, as CLL cells are considered among the hard-to-transfect cells, we determined the feasibility of using an ultrasound (US) cavitation (sonoporation) to transfect fresh CLL samples and the human RL follicular lymphoma cell line. Thereby, this method allowed us to investigate the influence and the mechanism of targeting *Bcl-xl* by siRNA on the sensitivity of CLL cells to rituximab and GA101.

In the section of results, we presented our work published about the transfection of cells in suspension by US cavitation, as well as our recent results that presented evidence about the mechanism of gene delivery by sonoporation through the induction of transient

membrane poration in cells in suspension. In the final section of results, we compared the mechanism of induction of apoptosis of the two anti-CD20 MAbs, rituximab and GA101 in fresh CLL samples. We studied the effects of these two MAbs on the intrinsic pathway of apoptosis.

Overall our results suggest that apoptotic signalization differ between rituximab and GA101 with a greater involvement of the mitochondrial pathway in cells exposed to GA101 as well as the inhibition of *Bcl-xL* could constitute a means to sensitize CLL cells to the apoptotic effects of anti-CD20 antibodies.

Chapter I

1. Lymphoid malignancies

1.1. Introduction

The immune system is a complex but well organized system comprising a range of white blood cells, including B-cells, T-cells, natural killer cells and antigen presenting cells. These cells can identify and eliminate any potentially harmful invaders such as bacteria, viruses, fungi or parasites, and endogenous objects such as immune complexes or transformed cells. These cells are strictly regulated by hormones and growth promoting or suppressing signals, but all of them are vulnerable to errors, potentially leading to malignant transformation.

Cancer is a class of diseases characterized by uncontrolled division of cells and ability of the cells to invade and damage normal tissues either locally or at distant sites of the body. A malignant lymphoma arises when a single B or T lymphocyte is arrested in a specific stage of cell differentiation leading to malignant transformation and clonal expression [6]. Malignancies of hematopoietic cells have been divided into myeloid malignancies and lymphoid malignancies. Lymphoid malignancies include a large number of diseases, such as Hodgkin's disease (HD) and Non-Hodgkin's lymphoma (NHL). The classification of NHL is regularly updated on the basis of molecular and cytogenetic data in order to better identify disease entities. Presently, NHL is the tenth most frequently diagnosed neoplasm in the world and ranks seventh in developed countries [7].

1.2. Classification of lymphoid malignancies

The most recent classifications of lymphoid malignancies are described in the Revised European- American Lymphoma classification system [8] and the World Health Organization (WHO) classification [9]. Recently, the WHO classification has been updated in order to better define heterogeneous or ambiguous categories of disease. This classification divides the NHL and other lymphoid malignancies into malignancies with clinical and therapeutic relevance [10, 11]. An accurate description of these diseases is based on morphologic, clinical, immunologic, and genetic information [12].

Based on immunophenotypic characteristics, NHL is grouped in two broad categories (Table 1); B and T-cell lymphomas, where mature B-cell neoplasms account for more than 85% worldwide [9] and T-cell together with NK-cell malignancies constitute approximately

12% [13]. Chronic Lymphocytic Leukemia (CLL) falls within the B-cell lymphoid diseases [9]. Clinically, lymphomas are characterized as indolent or aggressive, based upon their rate of progression [14]. Specific cytogenetic translocations have been used to differentiate subtypes of NHL, for example, t(8:14) is found in Burkitt's lymphoma, whereas t(14:18) is common in follicular lymphoma.

Table 1: WHO classification of the mature B-cell, T-cell, and NK-cell neoplasms (2008).

Mature B-cell neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic lymphoma/leukemia, unclassifiable*
Splenic diffuse red pulp small B-cell lymphoma*
Hairy cell leukemia-variant*
Lymphoplasmacytic lymphoma
Waldenström macroglobulinemia
Heavy chain diseases
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Follicular lymphoma
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma (DLBCL), NOS
T-cell/histiocyte rich large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK_ large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
Mature T-cell and NK-cell neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Aggressive NK cell leukemia
Adult T-cell leukemia/lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma

Hepatosplenic T-cell lymphoma

Subcutaneous panniculitis-like T-cell lymphoma

Mycosis fungoides

Sézary syndrome

Primary cutaneous CD30 T-cell lymphoproliferative disorders

Lymphomatoid papulosis

Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous gamma-delta T-cell lymphoma

Primary cutaneous CD8₊ aggressive epidermotropic cytotoxic T-cell lymphoma*

Primary cutaneous CD4₊ small/medium T-cell lymphoma*

Peripheral T-cell lymphoma, NOS

Angioimmunoblastic T-cell lymphoma

Anaplastic large cell lymphoma, *ALK*⁺

Anaplastic large cell lymphoma, *ALK*⁻*

Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma

Nodular sclerosis classical Hodgkin lymphoma

Lymphocyte-rich classical Hodgkin lymphoma

Mixed cellularity classical Hodgkin lymphoma

Lymphocyte-depleted classical Hodgkin lymphoma

Posttransplantation lymphoproliferative disorders (PTLD)

Early lesions

Plasmacytic hyperplasia

Infectious mononucleosis-like PTLD

Polymorphic PTLD

Monomorphic PTLD (B- and T/NK-cell types)†

Classical Hodgkin lymphoma type PTLD†

†These lesions are classified according to the leukemia or lymphoma to which they correspond.

*Provisional entities for which the WHO Working Group felt there was insufficient evidence to recognize as distinct diseases at this time.

1.3. Incidence and epidemiology of lymphoid malignancies

Globally, NHL is the tenth most frequently diagnosed form of cancer in the world. For unknown reasons, the incidence of NHL has increased in the United States and Canada (14 per 100,000 person-years) whereas China and Thailand report the lowest incidence (2 to 3 per

100,000 person-years) worldwide [7]. CLL is the most common leukemia in adults in Western countries with about 3.9 per 100,000 person-years being diagnosed each year [15].

The incidence of NHL and the patterns of expression of the various subtypes differ geographically. T-cell lymphomas are more common in Asia than in Western countries, while certain subtypes of B-cell lymphomas, such as Follicular Lymphoma (FL), are more common in Western countries [10]. The most prevalent NHL subtypes are Diffuse Large B-cell Lymphoma (DLBCL) and FL, accounting for approximately 31% and 22% of cases, respectively.

2. Chronic Lymphocytic Leukemia

2.1. Definition

Chronic Lymphocytic Leukemia (CLL) is characterized by the accumulation of morphologically mature but immunologically dysfunctional lymphocytes in the blood, bone marrow and peripheral lymphoid organs [16].

2.2. Epidemiology and etiology

CLL represents 22-30% of all leukemia cases [15]. The analysis of the Surveillance, Epidemiology, and End Results (SEER) database estimated that 14,990 men and women (8,870 men and 6,120 women) will be diagnosed with CLL and 4,390 men and women will die of CLL in 2010 in the United States population. (<http://seer.cancer.gov/statfacts/html/clyl.html>)

CLL is extremely rare in people younger than 50 years [17]. The SEER incidence noted that the median age at diagnosis for CLL was 72 years. The age-adjusted incidence rate was 4.2 per 100,000 men and women per year [18]. The male/female ratio in all populations is approximately 2:1. Studies on the racial and geographic distribution showed that CLL is 20 to 30 times more common in Europe, Australasia and North American white and black populations than in India, China and Japan [19, 20]. Hereditary and genetic links have been noted. First-degree relatives of patients with the disease are three times more likely to develop CLL [21].

CLL has generally not been associated with any environmental or external factors [15]. However, the Institute of Medicine of the National Academy of Sciences issued a report which suggested the presence of "sufficient evidence of an association" between herbicides used in Vietnam and CLL [22].

2.3. Clinical signs

In the early stages of the disease, CLL is usually asymptomatic. However, as the disease progresses, signs and symptoms become more evident. Patients may complain of generalized weakness or fatigue, anorexia, weight loss, dyspnea on exertion, may notice swollen lymph nodes, or develop recurring infections. They may also develop splenomegaly or hepatomegaly [23].

2.4. Diagnosis

During the past decade, the National Cancer Institute-sponsored Working Group (NCI-WG) on CLL has published guidelines for the design and the conduct of clinical trials for patients with CLL in order to facilitate the comparisons between different treatments and to establish definitions that can be used in studies on the biology of this disease [8, 24, 25]. The US Food and Drug Administration (FDA) also adopted these guidelines in their evaluation and approval of new drugs. However, the discovery and the availability of new prognostic factors and new treatments options prompted the International Workshop on CLL (IWCLL) to revise these guidelines [26].

CLL must be distinguished from other causes of malignant lymphocytosis, such as leukemic mantle cell lymphoma, T-CLL, prolymphocytic leukemia (B and T cell), hairy-cell leukemia, splenic lymphoma with villous lymphocytes or follicular lymphoma, on the basis of characteristic morphological and molecular features. To achieve this, it is essential to evaluate the blood count, blood smear, the morphology, the immune phenotype and in some cases the karyotype of the circulating lymphoid cells and to take into account the results of other abnormal tests.

2.4.1. Peripheral Blood

Currently, most patients are diagnosed while asymptomatic because of abnormal blood counts. The absolute blood lymphocyte threshold for diagnosing CLL is at least 5.10^9 lymphocytes/L (5000/ μ L) in the peripheral blood. The leukemic cells found in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm, a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin (Figure 1). Gumprecht nuclear shadows, or smudge cells, found as cell debris, are other characteristic morphologic features found in CLL. In addition to these typical small cells, a small proportion of cells may be found admixed with larger or atypical lymphocytes, cleaved

cells, or prolymphocytes. When “prolymphocytes” account for more than 55% of the blood lymphocytes, the diagnosis is that of prolymphocytic leukemia (B-cell PLL) [27].

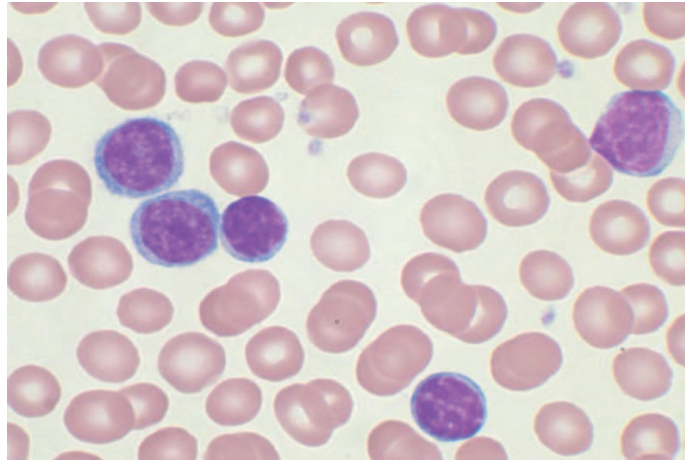


Figure 1: Morphology of lymphocytes in CLL

2.4.2. Immunophenotype

There are three major aspects of classical phenotypic findings of B-CLL lymphocytes [28, 29]:

1. The expression of B-cell-associated antigens CD23, CD19 and CD20.
2. The coexpression of CD5, a T-cell associated antigen, together with the B-cell markers.
3. The surface membrane immunoglobulin (SmIg) is commonly expressed at low levels in CLL (SmIg weak). The immunoglobulin (Ig) is most often IgM or both IgM and IgD; each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains.

The levels of surface immunoglobulin, CD20 and CD79b are characteristically low compared with those found on normal B cells [28, 30]. In addition, CLL cells are classically negative for cyclin D1, CD10, FMC7, CD22 are usually negative or weakly expressed [19].

In contrast, B-cell PLL cells do not express CD5 in half of the cases, and typically express high levels of CD20 and surface Ig [31]. In addition, the cells of mantle cell lymphoma, despite also expressing B-cell surface antigens and CD5, generally do not express CD23.

A scoring system based on five markers with a high differential diagnosis power for differentiating CLL from other B-cell lymphoproliferative diseases, known as the Matutes score, has been described (Table 2). Each of the following cellular characteristics is scored with one point [29].

Table 2: Matutes’s CLL scoring system.

This system is based on the presence or the absence of the light chain of the surface monoclonal Ig (SIg), the level of CD5, CD23, CD79b/CD22 and FMC7.

Marker	CLL	Score
SIg	weak	1
CD5	positive	1
CD23	positive	1
CD79b/CD22	weak	1
FMC7	negative	1

2.5. Clinical staging

CLL is classified by one of two cytologic staging systems, which known as Rai Classification and Binet Staging, respectively (Table 3).

2.5.1. Rai staging system

The Rai system is based upon a hierarchical grouping of disease manifestations of blood and bone marrow (lymphocytosis/Rai 0), enlarged lymph nodes (lymphadenopathy Rai I), spleen and liver (Rai II), bone marrow failure [anemia (Rai III) and thrombocytopenia (Rai IV)] [32]. The median survival times from the time of diagnosis in the series of patients studied by Rai *et al.* were 150 months for stage 0, 101 months for stage I, 71 months for stage II, and only 9 months in stages III and IV.

2.5.2. Binet staging system

The Binet staging system takes into consideration five potential sites of involvement: cervical, axillary, and inguinal lymph nodes (whether unilateral or bilateral, each area is counted as one), spleen, and liver. Patients are classified according to the number of involved sites plus the presence of anemia (hemoglobin <10 g/dL) and/or thrombocytopenia (platelets <100,000/mL) [33]:

Stage A: fewer than three involved lymphoid sites

Stage B: three or more involved lymphoid sites

Stage C: presence of anemia and/or thrombocytopenia.

Table 3: Rai and Binet staging systems for classification of CLL

System	Stage	Risk	Definiton
Rai	0	Low	Lymphocytosis only
	I	Intermediate	Lymphocytosis and lymphadenopathy
	II	Intermediate	Lymphocytosis and spleen,liver, or node enlargement
	III	High	Lymphocytosis and thrombocytopenia (platelet count<11g/dL or hematocrit<33%)
	IV	High	Lymphocytosis and thrombocytopenia (platelet count<100,000/mm ³)
Binet	A	Low	Lymphocytosis with enlargement of <3 lymphoid areas (cervical, axillary, inguinal, spleen, liver); no anemia or thrombocytopenia
	B	Intermediate	Lymphocytosis with enlargement of ≥3 lymphoid areas
	C	High	Lymphocytosis and either anemia (hemoglobin<10g/dL) or thrombocytopenia (platelet count <100,000/mm ³), or both anemia and thrombocytopenia

2.6. Evolution and complications of CLL

A majority of patients in whom disease has progressed in spite of therapy will die of infectious complications and/or cytopenias. Autoimmune complications are frequently observed and mainly directed against red cells and platelets. The highly variable clinical course of chronic lymphocytic leukemia (CLL) may be further complicated by the development of a histologically distinct aggressive lymphoma (Richter’s syndrome)[34]. The term “Richter’s syndrome” usually refers to the secondary development of a histologically aggressive lymphoproliferative disorder typically diffuse large B-cell lymphoma (DLBCL) in a patient with pre-existing CLL. Significant degrees of hypogammaglobulinemia and neutropenia may result in increased predisposition of patients with CLL to major bacterial infections. In addition, the use of purine analogs or alemtuzumab as treatment for CLL has resulted in an increased incidence of opportunistic infections (e.g., tuberculosis, candida, pneumocystis, CMV) [35].

2.7. Prognostic factors

2.7.1. Molecular cytogenetics

Genomic aberrations are common in CLL patients [36]. Dohner *et al.* showed in a series of 325 patients with CLL that chromosomal aberrations can be identified in interphase cells by fluorescence in situ hybridization (FISH) techniques in approximately 82% of CLL patients [37]. The most frequent chromosomal abnormalities observed are deletions of the long arm of chromosome 13 (del 13q14, 53%), 11q (20%), trisomy 12 (20%), deletions of 6q (9%), and deletions/mutations of the TP53 tumor suppressor gene at 17p (8%) [38].

The 17p and 11q deletions are considered as adverse prognostic factors identifying subgroups of patients with rapid disease progression and short survival times in multivariate analysis, whereas 13q deletion as the sole aberration is associated with favorable outcome [39].

The majority of 11q deletion cases show a decreased synthesis of ataxia teleangiectasia mutated (ATM), which results in TP53 dysfunction [40]. Defects in ATM/TP53 pathway correlates with reduced survival probability and may help in predicting treatment resistance. Moreover, the 17p deletion and *TP53* mutations have been associated with treatment failure [41]. The pathogenic and prognostic roles of trisomy 12 in CLL are controversial [42].

Recent studies suggest that CLL may be due to disorders in transcriptional/posttranscriptional regulation of the malignant cells' genome due to abnormalities in microRNAs (miRNAs) [43]. Calin *et al.* recorded significant differences in miRNA expression (miR-15a and miR-16-1) between normal CD5⁺ B lymphocytes and B-CLL cells. These were found expressed at high levels in normal CD5⁺ B lymphocytes, whereas downregulated in the majority of cases of CLL (about 70%) [44]. miR-15a and miR-16 expression was also found downregulated in pituitary adenomas. Reduced expression of these miRNAs correlates with a greater tumor diameter, suggesting that these genes may influence tumor growth [45], confirming their potential role as tumor suppressor genes. Fulci's team also reported a relative overexpression of miR150, miR223, miR29b and miR29c in IGHV-mutated CLL in comparison to unmutated cases [46].

2.7.2. VH Mutation status

The antibodies generated through V(D)J recombination generally have low affinity for several antigens. Somatic hypermutation is a process that serves to diversify the secondary antibody repertoire and to raise the affinity of a particular antibody to a particular antigen [47]. It occurs when B cells respond to antigen and involves the introduction of point mutations into the variable regions of immunoglobulin genes. Some of the mutagenized antibodies will have a higher affinity for the antigen. Therefore, the cells harboring these higher affinity antibodies may proliferate and survive preferentially. Successive cycles of mutation and selection lead to the generation of B cells with very high affinity antibodies, a process known as affinity maturation.

Little is known about the molecular mechanism of the mutation, but it involves targeted mutations, mainly point mutations, insertions, and deletions within a local region around the rearranged IgV region. It primarily takes place in the germinal center (GC) environment of lymph nodes following antigen recognition and co-stimulation by helper T-cells. The level of somatic hypermutation in particular B cells is evaluated by comparison of the sequence of the

rearranged variable region gene with germline sequences. Sequences with less than 98% homology to germline are considered to have undergone somatic hypermutation [48, 49]. The mutation status of the VH genes represents one of the most powerful molecular genetic parameters to dissect pathogenic and prognostic subgroups of CLL.

Patients with CLL can be divided into two groups based on the mutational status of VH genes: those with unmutated VH genes, assumed to originate from pre-germinal center cells, and those with mutated VH genes, thought to originate from post-germinal center cells. While CLL with unmutated VH follows an unfavorable course with rapid progression and an overall survival of 9-10 years, CLL with mutated VH often shows slow progression and a median overall survival of 24 years [41, 50-53].

Furthermore, CLL cases displaying mutated VH genes with Binet stage B or C had a survival similar to that of unmutated cases and significantly shorter than that of mutated stage A. Thus, the clinical heterogeneity within the VH mutated CLL group by inclusion of Binet stage data is of importance when considering surrogate marker(s) for VH mutation status [54].

As the determination of VH mutation status is technically demanding, a search for “surrogate markers,” i.e., parameters that are strongly correlated with VH mutation status has been performed.

2.7.3. CD38 Expression

CD38 is a single-chain type II transmembrane glycoprotein that is expressed by a variety of hematologic cells in an activation and differentiation-dependent manner [55]. Its cellular functions include signal transduction, cell adhesion, and lymphocyte homing [56]. In normal human B cell development, CD38 exhibits a discontinuous expression pattern where the molecule is detected at high levels in B cell precursors, germinal centre and plasma cells, whereas circulating peripheral blood and tonsillar B cells have markedly lower CD38 surface expression [57]. The potential role of CD38 in CLL pathophysiology is presently unknown. CD38 positivity was shown to correlate with unmutated VH gene and low levels with mutated V_H genes, thus considering CD38 as a prognostic marker in lieu of the mutation status. Thus, a high CD38 expression was associated with poor outcome in CLL patients. However, the clinical significance of CD38 expression as well as its value as a surrogate marker for IgVH gene mutational status still remains more controversial [51, 58, 59]. This relationship was not confirmed by several other investigators for many reasons. First, the CD38 expression can change during disease evolution, thus some patients will be classified differently depending on the time of analysis [59, 60]. Second, the definition of the best cutoff value has varied between studies ranging from 7% to 30% positive cells being reported as the best prognostic

border to use [61, 62]. Finally, the reproducibility of quantification of CD38 antigen requires certain precautions and may improve its prognostic value, especially with stage A CLL cases [63, 64].

2.7.4. ZAP-70 expression

As the determination of VH mutation status is technically demanding and may not be done in all laboratories, a search for ‘surrogate markers’ was performed. Among these surrogate markers, ZAP-70 (zeta-associated protein) is a tyrosine kinase, a molecule usually involved in T-cell receptor signaling and aberrantly expressed in some CLL cases.

The expression profiling of mutated and unmutated CLL cases revealed different expression levels of ZAP-70, which was considerably higher in unmutated than in mutated cases [65]. The levels of ZAP-70 as measured by flow cytometry separate distinct prognostic groups of Binet stage A CLL patients. In all patients, with unmutated IgVH, at least 20% of the leukemic cells were positive for ZAP-70, whereas in 21 of 24 patients with IgVH mutations less than 20% of the leukemic cells were positive for ZAP-70 [66]. Moreover, the prognostic impact of ZAP-70 expression has been confirmed in many studies [67-69]. In contrast to CD38, the ZAP-70 expression levels appear robust and stable over time [66, 67, 70]. However, there are still some technical problems in the standardization of the measurement of ZAP-70. Many studies have measured the ZAP-70 expression by flow cytometry with different antibodies and conjugation formats. For example, Crespo et al. and Orchard et al. used an unconjugated antibody whereas Rassenti et al. applied a direct-conjugated antibody [66, 67, 70]. Moreover, ZAP-70 can be measured by other methods including western blotting, reverse transcriptase-PCR and immunohistochemistry. A standardization of a valid ZAP-70 protocol with a conjugated ZAP-70 antibody and a defined cutoff for positive cases is warranted in order for this parameter to be widely used as a prognostic marker.

2.7.5. Serum parameters

Several studies have found that serum markers CD23, thymidine kinase, and β 2-microglobulin may predict survival or progression-free survival. Assays for these markers could be standardized and used in prospective clinical trials to validate their relative value to the management of patients with CLL.

2.7.5.1. β 2-microglobulin

β 2-microglobulin is the constant chain of the class I major histocompatibility complex. It is produced by nucleated cell membranes and expressed on the cell surface of many tissues.

Serum β 2-microglobulin is elevated in a variety of non-hematological disorders (rheumatic, viral, kidney) and in proliferative hematological diseases. Serum levels show a correlation with the clinical staging systems. Higher β 2-microglobulin is associated with a shorter survival in CLL patients [71]. Moreover, response to chemotherapy seems to be worse in patients with high levels of β 2-microglobulin. Recently, a prognostic nomogram based on a retrospective analysis from the M. D. Anderson Cancer Center (MDACC) has been developed including age, β 2-microglobulin, absolute lymphocyte count, sex, Rai stage, and number of involved lymph node groups. This prognostic model may help patients and clinicians in clinical decision making as well as in clinical research and clinical trial design [72].

2.7.5.2. Thymidine kinase

Thymidine kinase (TK) is a cytosolic enzyme known to be involved in the DNA synthesis. TK catalyzes conversion of deoxythymidine to deoxythymidine monophosphate. It is probably related to the number of dividing neoplastic cells, reflecting tumor mass and the rate of tumor cell proliferation. TK levels correlate with the proliferative activity of CLL cells and elevated levels of TK predict disease progression in CLL [73, 74]. TK appeared to detect a subgroup of patients with early CLL at risk for rapid disease progression and provided independent prognostic information on progression-free survival. [75]

2.7.5.3. Soluble CD23

CD23 is a 45-kDa transmembrane glycoprotein of hematopoietic cells. It functions as a low affinity receptor for immunoglobulin E (IgE) [76]. Two isotypes of CD23 exist: CD23a and CD23b. CD23a is restricted to B cells whereas expression of CD23b is induced by various stimuli (particularly IL-4) on B cells and other hematopoietic cells such as macrophages and NK cells. The two isoforms may have distinct functions and have different signaling pathways: CD23a mediates an increase in intracellular calcium, whereas CD23b is involved in upregulating cyclic AMP (cAMP) and nitric oxide synthase (iNOS) [77, 78].

Soluble CD23 (sCD23), a soluble protein found in the serum, is the 25-kDa fragment of CD23. It acts as a cytokine and together with interleukin-1 induces proliferation of normal and leukemic B lymphocytes [76, 79]. sCD23 is selectively elevated in the serum of CLL patients. Elevation of sCD23 levels above the median value predicts a significantly shorter time to disease progression and overall survival. It can provide additional information to clinical staging not only at diagnosis, but also during the course of the disease with prediction of the clinical outcome, even at an early stage [80-83].

2.8. Biology of CLL

2.8.1. Apoptosis in B-CLL cells

Apoptosis is a genetically predetermined mechanism that may be elicited by several molecular pathways including oncogene activation, genomic instability, growth factor withdrawal or DNA damage. Most or all cancer cells endure many of these stimuli and thus need to circumvent apoptosis to survive. CLL cells, characterized by a low rate of proliferation and prolonged life span, are often considered to have a defect in their apoptotic pathways rather than aberrant cell cycle progression [16, 84, 85]. The key players in the execution of the apoptotic cascade are caspases (cysteine proteases with aspartate specificity) which are activated by cleavage during apoptosis. Two major pathways of apoptosis converge on caspases: the extrinsic pathway and the intrinsic pathway (Figure 2).

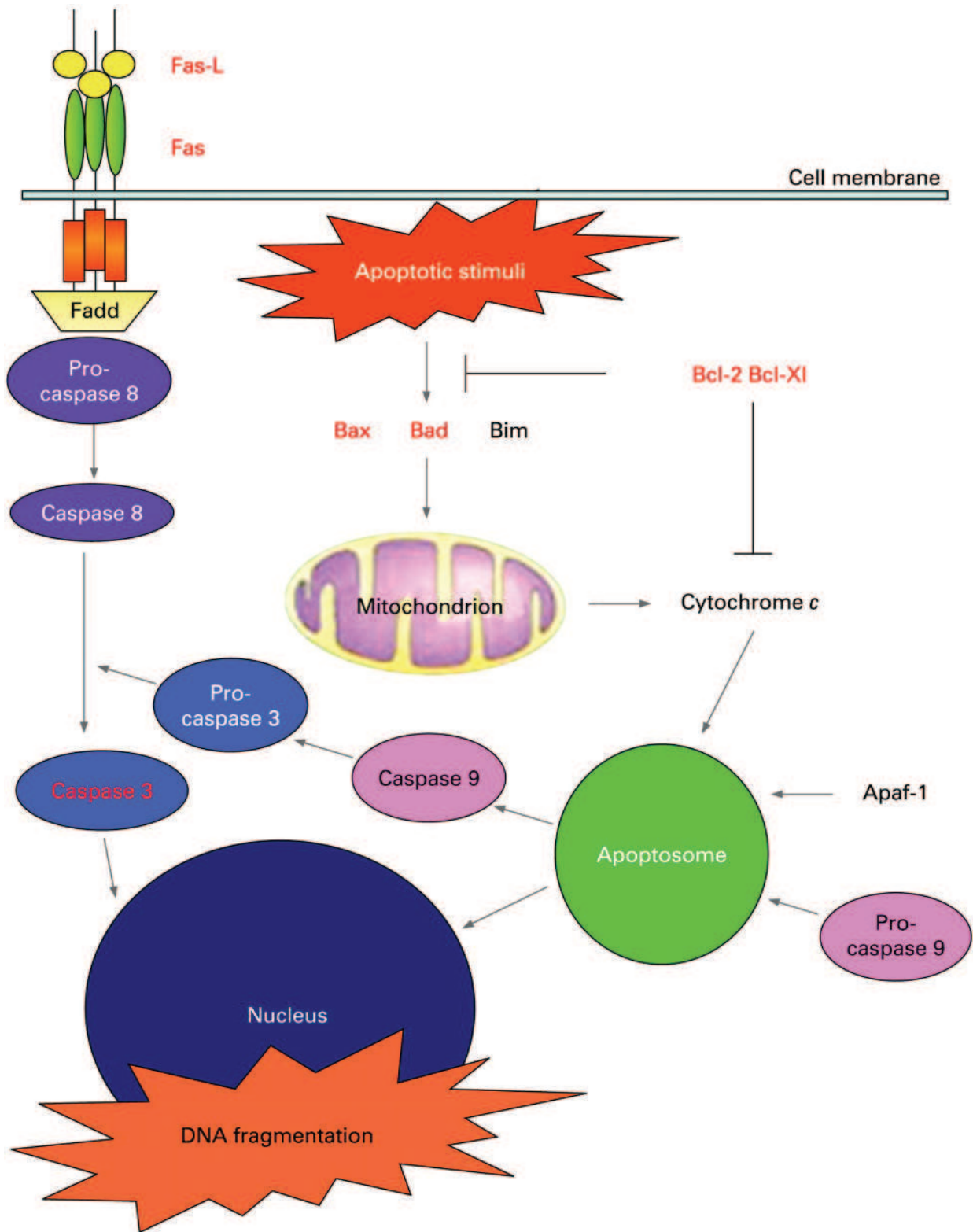


Figure 2: Apoptotic pathways.

Two major pathways lead to apoptosis: the intrinsic cell death pathway controlled by Bcl-2 family members and the extrinsic cell death pathway controlled by death receptor signaling.

2.8.1.1. Extrinsic apoptotic pathway

In the extrinsic pathway (also known as ‘death receptor pathway’), the apoptosis is triggered by the ligand-induced activation of death receptors at the cell surface. Such death receptors include the Fas receptor or CD95, the tumor necrosis factor (TNF) receptor-1, as well as the TNF-related apoptosis induced ligand (TRAIL) receptor-1 and -2. Binding of ligands causes changes in the intracellular domains of the receptors, leading to the recruitment of a pro-caspase initiator (pro-caspase -8 or -10), that leads to the formation of a death-inducing signaling complex (DISC). The local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. This process can be blocked by the protein c-FLIP. In certain type of cells, called ‘type-I’ cells, caspase-8 can cleave and activate sufficient quantities of effector caspases-3 and -7 to commit the cell to an apoptotic death. In other ‘type-II’ cells, mitochondrial amplification of the death signal is required. This involves the cleavage of the BH3-only protein BID by caspase-8. Cleaved BID engaged then the oligomerization of BAX and BAK, the mitochondrial membrane permeabilization (MMP) and the apoptosome formation.

The extrinsic apoptotic pathway in CLL cells induced by the ligation of cell surface death receptors is not readily activated [86, 87]. Indeed, B-CLL cells display little sensitivity when a TRAIL ligand is added. This TRAIL resistance in B-CLL cells may be explained by low surface expression of TRAIL receptors or a high ratio of c-FLIP to caspase-8 that prevents caspase-8 activation [87]. An alternative explanation is that the relative insensitivity of CLL cells to TRAIL is due to their preferential signaling via the TRAIL-R1 receptor, while TRAIL preferentially signals via TRAIL-R2 [88, 89]. Other studies suggested that the pretreatment of CLL cells with sensitizing agents such as valproic acid could potentiate the TRAIL response and induce apoptosis in CLL cells [90].

2.8.1.2. Intrinsic apoptotic pathway

In the intrinsic pathway (also known as ‘mitochondrial pathway’), apoptosis is initiated by signals converging from mitochondria leading to its permeabilization and the subsequent release of pro-apoptotic factors contained in the mitochondrial intramembrane space. The MMP is often considered ‘the point of no return’ in the cascade of events leading to programmed cell death from which a cell will rarely recover. The mechanisms underlying MMP are complex and probably result from the coordinate execution of several interdependent steps.

Whatever the mechanism involved, MMP leads to the release of pro-apoptotic factors in the cytosol. Among these various pathways, a great number converges towards the execution of an apoptotic program by activation of the caspase cascade or independently from these proteases. Bcl-2 family members are the major proteins involved in the intrinsic apoptotic pathway. These proteins are subdivided by function and homology into three categories: anti-apoptotic proteins such as Bcl-2, Mcl-1, Bfl-1, Bcl-xL, and Bcl-w, with Bcl-2 sequence homology (BH) at BH1, BH2, BH3 and BH4 domains; pro-apoptotic proteins such as Bax, Bak with sequence homology at BH1, BH2 and BH3 domains; and finally BH3-only pro-apoptotic proteins that share only the BH3 domain such as Bik, Bid, Noxa and Bim (Figure 3).

The main site of action of Bcl-2 family proteins is probably the mitochondrial membrane [91]. The anti-apoptotic proteins (Bcl-xL and Bcl-2) mainly reside in the outer mitochondrial membrane (OMM), where they protect mitochondria against MMP, presumably by binding to and neutralizing other pro-apoptotic proteins from the Bcl-2 family. In healthy cells, Bak is associated with the OMM, whereas Bax resides in the cytosol. The expression of at least one of the two proteins (Bax or Bak) is required for MMP, in a series of different models of apoptosis induction [92]. Upon induction of apoptosis, Bax inserts into the OMM [93] where it is thought to form supramolecular openings, alone or in association with other pro-apoptotic members such as Bak or tBid (truncated Bid). Such openings might result from the formation of homo-oligomeric Bax-containing pores or from the destabilization of the lipid bilayer, resulting in transient discontinuities within OMM. Thus, the conformational change of Bax or Bak (with exposure of their NH₂ terminus), their full insertion into mitochondrial membranes and subsequently the formation of protein-permeable pores are required for the MMP [94].

The other BH3-only members of the Bcl-2 family can exert their pro-apoptotic action by two different mechanisms. Some BH3-only proteins (the “activators”) such as Bid and Bim can activate Bax or Bak, by stimulating their allosteric change and oligomerization [95-97]. Others (the “facilitators”) such as Bad preferentially interact with the anti-apoptotic proteins, dissociating them from other BH3-only or from Bak or Bax, which in turn promote MMP. However, the molecular openings induced by Bax/Bak and/or Bax/tBid mediated cytochrome c release is still a highly controversial issue.

Some studies showed that Bax or Bak simply destabilize the lipid bilayers instead of forming specific “pores” [98]. Others reported that Bax could be engaged in a close molecular cooperation with proteins from the permeability transition pore complex (PTPC), such as Adenosine Nucleotide Translocator (ANT) and/or Voltage-Dependent Anion Channel (VDAC)

and Cyclophilin D (CypD) to induce MMP. This mechanism has been demonstrated, for instance, by electrophysiological experiments involving purified recombinant Bax and purified ANT or VDAC [99]. However experiments on isolated mitochondria and liposomes suggest that Bax can permeabilize OMM and release of cytochrome c in a fashion that does not involve any of the critical components of the PTPC, including VDAC, ANT, or CypD [94, 100].

The way the anti-apoptotic members of the Bcl-2 family inhibit MMP is also a matter of debate. Some authors suggested that anti-apoptotic members of the Bcl-2 family would simply act as inhibitors of their pro-apoptotic counterparts, without any independent effects on other mitochondrial proteins. The suppression of MMP could be either achieved by neutralizing BH3-only proteins or by direct interaction with the pore-forming members of the Bcl-2 family [97]. However, some data indicate that Bcl-2 and Bcl-xL can interact with the mitochondrial proteins including ANT and VDAC.

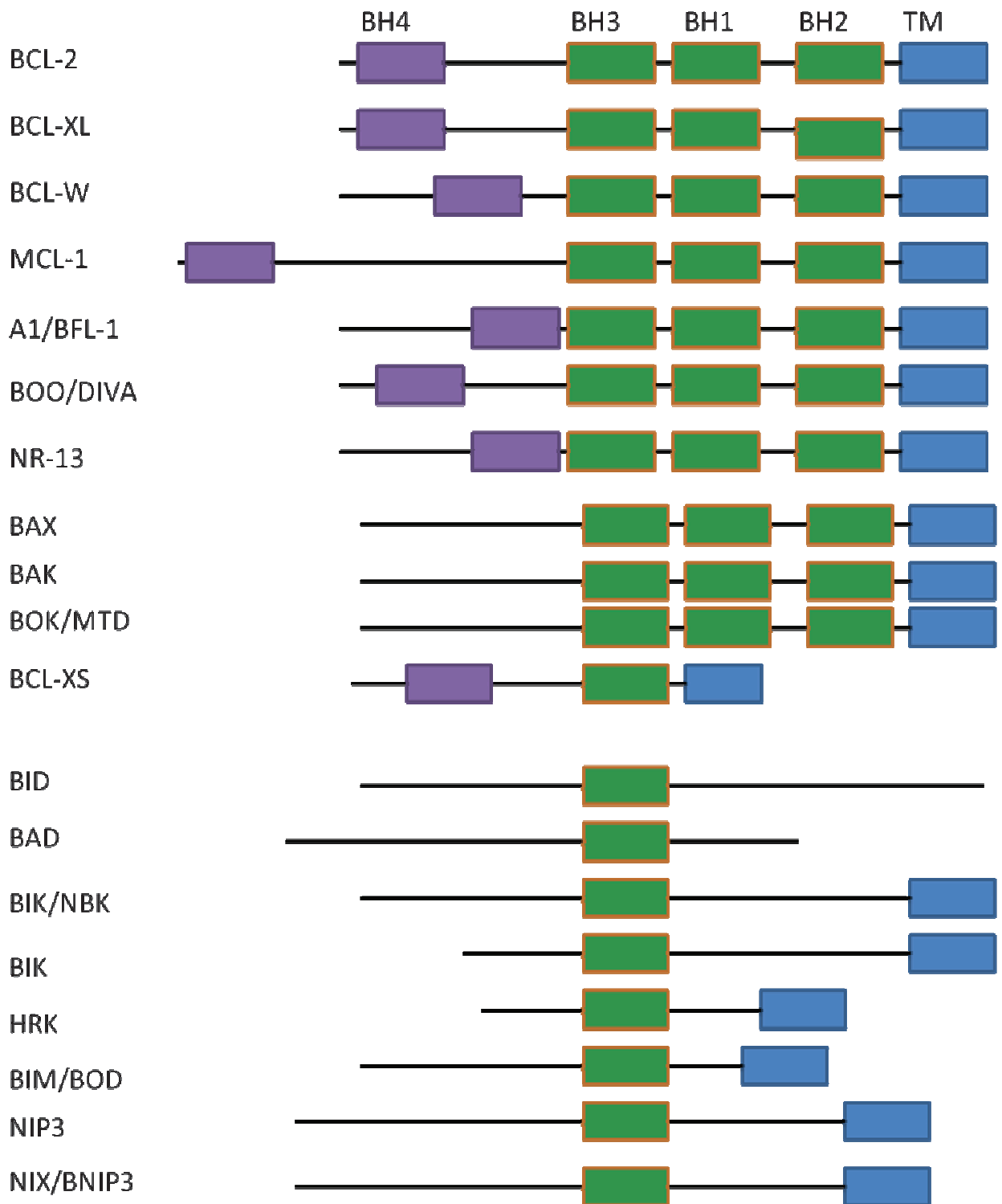


Figure 3: The Bcl-2 gene family.

The Bcl-2 gene family encodes a divergent group of proteins that regulate programmed cell death.

2.8.1.2.1. Bcl-2 family in CLL

Bcl-2 is found to be expressed in all CLL cells compared with peripheral blood lymphocytes. The mechanisms that mediate Bcl-2 expression in CLL remain unclear. Since chromosomal or genetic alterations of the *bcl-2* gene locus are rare in CLL cells, an early explanation for Bcl-2 protein overexpression was *bcl-2* gene hypomethylation; however the methylation status alone did not correlate with the amount of protein present in CLL patient cells [101].

Although it remains controversial, it has been suggested that a small proportion of patients (5%) have the t(14;18) translocation which is commonly found in follicular lymphoma [102]. Another chromosomal abnormality, the deletion of 13q14.3, which occurs in roughly 50% of CLL cases, has been linked to dysregulation of Bcl-2 protein expression [39]. This region encodes for two micro-RNAs (miR), miR-15 and miR-16. The analysis of CLL samples and normal CD5⁺ B lymphocytes showed an inverse correlation between the expression of miR-15a, miR-16-1 and Bcl-2, whereas in normal CD5⁺ B cells, the levels of both miRNAs were high, and the Bcl-2 protein was expressed at low levels [103]. Moreover, Bcl-2 downregulation by miR-15a and miR-16-1 triggers apoptosis in a leukemic cell line model [103]. However, Fulci and colleagues reported a low expression level of these miRNAs in only 12% of patients [46].

There remains a significant portion of CLL cases, probably greater than a third, in which neither miR-15 nor miR-16 levels are reduced, and no t(14;18) translocation is present, for which the mechanism of Bcl-2 upregulation remains to be elucidated.

Furthermore, the levels of Bcl-2 did not correlate with the response to chemotherapy. In a study of 235 patients no relationship between Bcl-2 expression levels and progression-free survival or clinical response to either fludarabine or fludarabine plus cyclophosphamide was observed [104]. However, the relative expression of Bcl-2 and Bax seems to be an important variable in CLL. The Bcl-2/Bax ratios were increased in 22 patients with progressive disease compared to normal controls and previously treated patients had higher ratios than untreated patients [105, 106].

The expression levels of other anti-apoptotic proteins such as Bcl-xL and Bcl-w were found respectively either minimal or weakly detectable [107, 108]. Moreover, a high expression of Bfl-1 is detected in CLL cells and is thought to contribute to the apoptosis resistant phenotype in CLL cells [109, 110]. Mcl-1 has been also studied in CLL cells. Generally, higher Mcl-1 expression has been found to correlate with decreased sensitivity to chemotherapy. An elevated Mcl-1/Bax ratio was found to correlate with inferior clinical response to rituximab, a monoclonal anti-CD20 antibody used in the treatment of CLL [111]. Moreover, the presence

of an insertion of 6 to 18 nucleotide in the MCL-1 promoter in 17/58 patients was correlated with increased Mcl-1 expression and inferior clinical response [112]. In addition, the expression of Mcl-1 has been shown to be regulated by miR-29. An enhanced expression of miR-29b reduced Mcl-1 protein levels and facilitated apoptosis [113]. Consequently, the downregulation of Mcl-1 by siRNA induces rapid apoptosis in CLL cells [114].]. Anti-apoptotic B-cell receptor (BCR) signalling is associated with a prolonged activation of the PI3K/AKT kinases and cell survival associated with the upregulated expression of the Mcl-1 [114]. These data suggest that the targets of miR-29 and miR-181 are major components of survival pathways presumably activated in CLL cells.

2.8.1.3. Cooperation between intrinsic and extrinsic pathways

The two apoptotic signaling pathways generally involve specific initiator caspases. Thus, in the extrinsic pathway, caspase-8 is activated through the activation of FADD (Fas Associated Death domain), whereas in the intrinsic pathway, caspase-9 is activated by the interaction with Apaf-1. The effector caspases, on the other hand, are generally common to both pathways. Once activated by the initiator caspases, they cleave many intracellular substrates to induce the biochemical destruction of cells. The activation of caspases-3 and -7 is accompanied by the proteolysis of the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP) [108].

Apoptosis can also be executed without the involvement of caspases, in a process known as CICD (Caspase-Independent Cell death). CICD process is due to various mitochondrial apoptotic proteins such as AIF (Apoptosis Inducing factor), [78] endonuclease G [115] and Omi/HTrA2 (High temperature requirement protein A2 [116]. These proteins are localized in the mitochondrial intermembrane space and the permeabilization of mitochondrial membranes leads to their release. AIF is translocated to the nuclear core, directly causing chromatin condensation and DNA fragmentation. The release of endonuclease G also induces the fragmentation of DNA. Moreover, the release of Omi/HtrA2, a mitochondrial serine protease, antagonizes inhibitors of apoptosis (IAP) [117]. The activation of this non classical caspase-independent apoptotic pathway was also observed in CLL cells after rituximab treatment [118].

2.8.2. Proliferation in B-CLL

The accumulation of mature B cells that have escaped programmed cell death and undergone cell-cycle arrest in the G0/G1 phase is the hallmark of CLL [119]. Although the pathogenesis of the disease remains largely unknown, many studies have focused on the

defective apoptosis of the malignant cells that seems to enhance the disease progression and chemotherapy resistance. The deregulation of cell-cycle regulatory genes might contribute to the expansion of the malignant clone in CLL cells. The overexpression of *cyclin D2* mRNA has been described in B-CLL cells as well as the expression of the cyclin-dependent kinase (cdk) inhibitor p27, a key regulator of the early phase of the cell cycle. Increased amounts of the cyclin regulator CDKN1B protein are also observed in most patients [120]. This high expression is associated with poor overall prognosis. High p27Kip1 expression in B-CLL lymphocytes seems to be associated with impairment of apoptosis, since B-CLL lymphocyte populations expressing a high level of p27Kip1 have a lower spontaneous cell death ratio in culture [120] and this expression is correlated with the level of antiapoptotic protein Bcl-2. [121]

The presence of cdk4 and cyclin E in blood cells of the majority of CLL cases studied, as well as cdk1 and cdk2 in some cases, indicates that the CLL cells are not quiescent, but are blocked in an early stage of the G1 cell cycle phase, and/or that the expression of these proteins is dysregulated [122]. Similarly, constitutively activated phosphatidylinositol-3 kinase (PI3K) has been detected in CLL cells and has been suggested as a means whereby the leukemic cells avoid apoptosis [123].

Abnormal cytokine loops may favor the survival and the expansion of the leukemic clone through the induction or inhibition of cell proliferation, protection from or induction of apoptosis, and up- and downregulation of apoptosis-related genes. The cytokines that have gained most attention are Interleukin-2(IL-2), TNF α (Tumor necrosis factor α), IL-8, IL-4 and IL-10, because of their possible involvement in CLL disease. Many of these cytokines are members of the cytokine receptor family, which utilizes a common family of signal transduction molecules; the Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) signal transduction molecules [124].

2.8.3. ROS production in CLL

In addition to the release of pro-apoptotic proteins from the mitochondrial intermembrane space, changes in intracellular concentrations of “mediators” such as reactive oxygen species (ROS) also illustrate the loss of mitochondrial membrane integrity.

Free radicals are chemical species containing one or more unpaired electrons. The unpaired electrons of oxygen react to form highly reactive species which are the Reactive Oxygen Species (ROS). Examples are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radical ($\cdot OH$). These species have not only emerged as essential signaling molecules for cell survival and carcinogenesis, but also for apoptosis [125, 126].

ROS are produced in many cellular compartments within the cell. Important contributors include the respiratory chain complex in mitochondria, proteins within the plasma membrane such as the growing family of NADPH oxidases (Nicotinamide Adenine Dinucleotide Phosphate-Oxidases), lipid metabolism within peroxisomes, as well as the activity of various cytosolic enzymes such as cyclooxygenases or xanthine oxidase. Among all these sources, 90% of ROS are produced by mitochondria.

The precise role of ROS in apoptosis has remained dubious for a long time, since ROS has been supposed to constitute only a marker of cellular stress during the apoptotic process. However, a growing body of evidence suggests that high intracellular concentrations of ROS contribute to cytochrome c release and induction of apoptosis [127, 128]. It was shown that ROS generated from the mitochondrial electron transport chain induce cytochrome c dissociation from mitochondrial particles via cardiolipin peroxidation [129]. VDAC, which regulates O^{2-} flux from mitochondria to the cytosol [130], is susceptible to high levels of O^{2-} that induce mitochondrial PTP opening and cytochrome c release [131]. Moreover, activated caspase-3 can disturb respiratory chain complexes, including a reduction in respiration rate and an increase in ROS production [132]. Thus, high levels of ROS can cause apoptosis by triggering mitochondrial PTP opening and the release of pro-apoptotic factors. Compared with normal lymphocytes, CLL cells were shown to display a substantial increase in ROS associated with oxidative DNA damage and mitochondrial DNA (mtDNA) mutations, especially in patients who had undergone prior therapy with DNA-damaging agents [133, 134].

The mitochondrial respiratory chain is the major site of ROS generation due to electron bifurcation from the transport complexes; thereby, any dysfunction of mitochondrial respiration would potentially increase electron leakage and lead to elevated ROS generation. Multiple mechanisms, including mitochondrial dysfunction and metabolic stress, likely contribute to ROS stress in CLL cells. Although the increase of ROS in cancer cells is often viewed as an adverse event due to its role in promoting genomic instability and cell proliferation [135-137], high levels of ROS can also induce cancer cell death, a desired outcome that chemotherapy attempts to achieve. This may provide a rationale to exploit the intrinsic oxidative stress to develop new strategies that turn the toxic effect of ROS against CLL cells, using proper redox-modulating agents.

2.9. Treatments of CLL

The decision to treat CLL patients should be guided by clinical staging, the presence of symptoms and disease activity [138]. In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy until they have evidence of disease progression. Patients at intermediate (I and II) or high-risk (III and IV) according to the modified Rai classification or Binet stage B or C 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.

Once the need to treat a patient is established, the next step is to choose the most suitable therapy.

2.9.1. Frontline line therapy of early stage

In early stages, the treatment of CLL patients is not necessary if no symptoms or complications such as decreased performance status, symptoms or complications from hepatomegaly, splenomegaly and lymphadenopathy are associated with the disease.

Studies from both the French Cooperative Group on CLL [139], the Cancer and Leukemia Group B (CALGB) [140] the Spanish group Pethema [141] and the Medical Research Council [142] in patients with early-stage disease confirm that the use of alkylating agents for these patients does not prolong their survival. In one study, patients treated with such early-stage CLL had an increased frequency of fatal epithelial cancers compared with untreated patients [139].

2.9.2. Frontline therapy of Advanced Stage

2.9.2.1. Monotherapy with alkylating agents

Monotherapy with alkylating agents has served as initial, front-line therapy for CLL for several decades. Chlorambucil, an alkylating agent that mainly acts by DNA cross-linking [143], has been considered the 'gold standard' for several decades. This drug remains an appropriate option for unfit, elderly patients. The advantages of this drug are its low toxicity, low cost and convenience as an oral drug; however, its disadvantages are its low to non-existent CR rate and some side effects that may occur after extended use. Higher remission rates (ORR 89%, CR 59%) had been observed when chlorambucil was administered at a fixed dose of 15 mg daily up to achievement of a CR or occurrence of grade 3 toxicity, for a maximum of six months [144].

Besides chlorambucil, cyclophosphamide (C) is another alkylating agent with activity in CLL patients. Cyclophosphamide interferes with mitosis and cell replication primarily by cross-linking DNA and RNA strands. As a single agent, it is only infrequently used when chlorambucil is not tolerated [145]. Cyclophosphamide is generally associated with vincristine and prednisone (CVP) or with doxorubicin, vincristine and prednisone (CHOP) [146, 147].

2.9.2.2. Monotherapy with purine analogs

Three purine analogues are currently used in CLL: fludarabine, pentostatin and cladribine [148, 149]. Fludarabine is the best studied compound of the three in CLL. When used as single agent, it achieves superior overall response (OR) rates and longer progression-free survival rates compared with other treatment regimens containing alkylating agents or corticosteroids [150-152]. In three phase III studies in treatment-naive CLL patients, fludarabine induced more remissions and more CRs (7–40%) as well as a longer duration of remission than other chemotherapies, including CHOP, CAP (Cyclophosphamide, doxorubicin, prednisone) or chlorambucil. Despite the superior efficacy of fludarabine, overall survival was not improved by this drug when used as a single agent [152-155].

Pentostatin is a tight-binding inhibitor of adenosine deaminase (ADA), an enzyme essential in the cellular metabolism of purines. It is active in CLL, achieving response rates between 18% and 35% in patients heavily pretreated with cytotoxic chemotherapy. Phase II studies of single-agent pentostatin in heavily pretreated patients with low- or intermediate-grade NHL report response rates between 17% and 23% [156].

Cladribine (Cd) is structurally related to fludarabine and pentostatin but has a different mechanism of action. It has cytotoxic effects on resting as well as proliferating lymphocytes and monocytes leading to the accumulation of cells at the G1/S phase junction. Cladribine monotherapy produced a higher CR rate than chlorambucil plus prednisone (47% vs. 12%) without resulting in longer survival. [157].

Finally, bendamustine, a hybrid of an alkylating nitrogen mustard group and a purine-like benzimidazole, has been used for more than 30 years in Germany. This agent appears to act primarily as an alkylator. Bendamustine metabolites alkylate and crosslink macromolecules, resulting in DNA, RNA and protein synthesis inhibition, and, subsequently, apoptosis. It was recently compared with chlorambucil in a randomized trial. Results showed that more patients showed complete responses with bendamustine than with chlorambucil (31% vs. 2%). Moreover, the median progression-free survival (PFS) was 21.6 months for bendamustine and 8.3 months with chlorambucil [158].

2.9.3. Combination chemotherapies with alkylating agents or purine analogs

Purine analogues and alkylating agents have different mechanisms of action and partially overlapping toxicity profiles. Thus, the combination of these two modalities aims to achieve synergistic effects. These combination therapies including an alkylating agent such as CVP, CAP, or CHOP did not show any advantage in comparison to the less toxic chlorambucil [159]. These regimens can be effectively used in the relapsing setting in combination with monoclonal antibodies [146, 147].

Fludarabine has been evaluated in a variety of combination regimens. The most studied combination chemotherapy for CLL is fludarabine plus cyclophosphamide (FC). Three randomized trials have shown that FC combination improves the CR and OR rates and PFS as compared with fludarabine monotherapy [160-162].

The combination of fludarabine with cytarabine appeared to be less effective than fludarabine alone, while its combination with chlorambucil or prednisone increased the hematological toxicity without improving the response rate compared to fludarabine monotherapy (response rates of 27 vs.79%) [152, 163].

The addition of FC to mitoxantrone in refractory/ relapsed CLL patients produced a higher CR rate (50%). Furthermore, the addition of cladribine to cyclophosphamide (CC) did not show any benefit in terms of PFS or response rates when compared to cladribine alone. When these two drugs are combined with mitoxantrone (CCM) compared to cladribine, CCM induced a higher CR rate (36% vs.21%). However, based on these results, cladribine combination therapies do not seem to offer a major advantage when used as first-line treatment for CLL [164].

2.9.4. Monoclonal antibodies

The use of immunotherapy is emerging as an exciting modality with significant potential to advance the treatment of B-cell malignancies. Anti-CD20 and anti-CD52 antibodies have changed the therapeutic landscape of these diseases, in particular of CLL [2].

The anti-CD20 monoclonal antibody, rituximab is less active when used as single agent than in other lymphomas such as FL, unless very high doses or denser dosing regimen are used [4, 165, 166]. However, when combined with chemotherapy, it has proven to be very efficient therapy for CLL (see section II)

Alemtuzumab, a recombinant, fully humanized, monoclonal antibody against the CD52 antigen, showed a response rate of 33-53% in patients with advanced or refractory CLL with a

median duration of response ranging from 8.7 to 15.4 months [167-169]. Furthermore, Alemtuzumab achieves response rates up to 89% with 19% CRs and 24 months of response duration in patients with previously untreated CLL[170]. It has proven efficacy even in patients with poor diagnosis, including high-risk genetic markers such as deletions of chromosome 11 or 17 and p53 mutations [171, 172]. The combination of alemtuzumab with other chemotherapeutic agents is discussed in section II.

Ofatumumab is a fully human MAb targeting an epitope of CD20 molecule distinct from rituximab. This antibody was recently been approved by the FDA for patients with CLL who were refractory to both fludarabine and alemtuzumab and those who were refractory to fludarabine and had a bulky disease unsuitable for Alemtuzumab therapy [173]. Based on encouraging phase I/II study [174], ofatumumab is now undergoing further clinical testing in patients with B-CLL patients either as monotherapy or combined with other agents.

Lumiliximab is anti CD23 macaque-human chimeric primatized MAb. In a phase I protocol with 46 previously treated and refractory CLL patients, it showed a good safety profile, but limited clinical activity [175]. The combination of lumiliximab with other agents is discussed below.

GA101, is the first humanized and glycoengineered type II monoclonal antiCD20 to enter clinical trials. A phase I study conducted on patients with relapsed or refractory diseases including CLL, DLBCL, and other NHLs showed a favorable safety profile with no dose-limiting toxicities [176]. Other phase I studies conducted on patients with relapsed or refractory CLL showed promising results [177, 178]. GA101 is currently being explored as a single agent in phase II in relapsed/refractory B-CLL and indolent/aggressive NHL, and in combination with chemotherapy in a phase Ib study.

Several novel MAbs are being clinically developed and tested in NHL and CLL to further improve outcomes in these diseases. These MAbs include veltuzumab, ocrelizumab, PRO131921, TRU-015 and AME-133v.

2.10. Hematopoietic Stem cell transplantation

Hematopoietic stem-cell transplantation (HSCT) refers to transplantation of hematopoietic stem cells from a donor into a recipient. HSC are immature cells that can develop into any of the three types of blood cells (red cells, white cells or platelets). HSCT can be either autologous (i.e., using the patient's own stem cells) or allogeneic (i.e., using stem cells from a donor). HSCT is performed in hematological malignancies to rescue patients from treatment-induced aplasia after high-dose chemotherapy and/or radiotherapy

have been administered to eliminate the cancer. Many factors affect the outcome of a tissue transplant. The individual's overall health, age and disease stage are extremely important considerations in evaluating adult patients.

2.10.1. Autologous HSCT

In autologous HSCT, the recipient's own previously harvested stem cells are reinfused. Autologous HSCT provides an alternative stem-cell source for patients who do not have a HLA-identical donor. Furthermore, it can be performed in older patients, since the conditioning regimen for autologous HSCT is less toxic than the one for allogeneic HSCT and does not create a graft-versus-host reaction (GVHD). However, the major problems after autologous transplantation remain relapsing disease, late complications such as the development of myelodysplasia and acute myelogenous leukemia, and no evidence of a plateau on disease-free survival [179]. Matched-pair analysis suggests a survival advantage for autologous transplantation in CLL. The relative efficacy of autologous HSCT must be weighed against the efficacy and toxicity of newly developing non-transplantation approaches [180].

2.10.2. Allogeneic HSCT

In allogeneic HSCT, HSC are grafted from a donor into a recipient. For the transplant to be successful, the donated cells must be similar or a match to those of the recipient. HLA typing can identify donors who may be a perfect match. Increased survival is associated with a match between the donor and recipient HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 [181].

Depletion of T cells from the transplant is associated with a significantly lower incidence of both acute and chronic GVHD [182]. However, this depletion may reduce the likelihood of a graft-versus-leukemia effect, in which the grafted cells identify the host cancer cells as foreign and eliminate them (<http://www.nlm.nih.gov/medlineplus/ency/article/003009.htm>). According to the National Cancer Institute (NCI-2008) myeloablative and nonmyeloablative allogeneic HSCT are under clinical evaluation for the treatment of CLL. Allogeneic HSCT has significant morbidity and mortality from regimen-related toxicity, GVHD and infection, but surviving patients have long-term disease control [179]. Although most patients who attain complete remission with autologous HSCT will ultimately relapse, a survival plateau for allogeneic HSCT suggests an additional graft-versus-leukemia effect (NCI, 2008). To date, the only potentially curative therapy for CLL is allogeneic HSCT [19].

Moreno *et al.* reported on outcomes of patients with advanced CLL who received either allogeneic or autologous HSCT subsequent to high-dose chemotherapy [183]. The groups differed as to the amount of tumor burden at the time of transplantation, with patients who underwent allogeneic HSCT having more advanced clinical stage and a higher degree of peripheral blood and bone marrow involvement compared to the patients who received autologous HSCT. Analysis of outcomes demonstrated a lower risk of progression and improved overall- and relapse-free survival for patients undergoing allogeneic HSCT compared to those receiving autologous HSCT.

Chapter II: Monoclonal antibodies

1. Introduction

In the late 19th and early 20th century Paul Ehrlich dreamed of a "magic bullet" [184]. He proposed that cells have specific receptors for antigens and that they can shed these receptors into the blood when being in contact with antigen. This was the first time when the nature of antibodies was suggested. In 1970's, using hybridomas, Kohler and Milstein produced their first MAb highly specific to their targeted antigens [185].

The first "magic bullet" was rituximab, a monoclonal antibody against the B-cell specific CD20 antigen, first investigated in the treatment of non-Hodgkin's lymphoma. Today, MABs represents a cornerstone in the therapeutic armamentarium for cancers and auto-immune disorders (Figure 4).

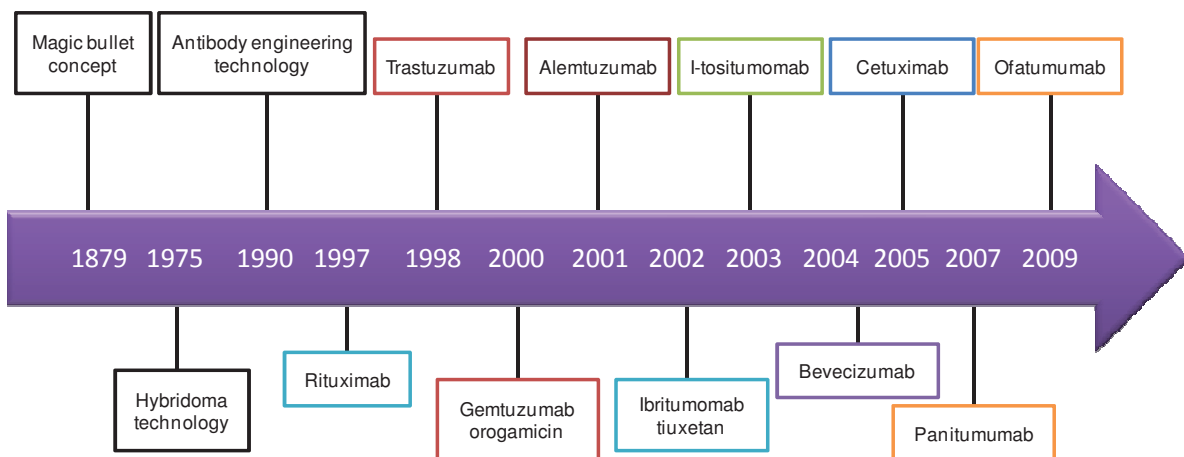


Figure 4: 100 years of progress from "magic bullets" to clinical reality.

Box outline: blue, chimeric antibody; red, humanized antibody; yellow, human antibody; green, mouse antibody

Since 1997, the success of MABs has been evident after the approval by the FDA of five unconjugated and two conjugated MABs, for cancer therapy. Rituximab (Rituxan™)(RTX), a chimerized anti-CD20 antibody, was the first MAB approved by the FDA for the treatment of cancer, specifically non-Hodgkin's lymphoma [186]. Alemtuzumab (Campath™), a humanized anti-CD52 antibody, was approved for the treatment of chronic lymphocytic leukemia [187]. Trastuzumab (Herceptin™), a humanized anti-HER-2/neu

antibody, was approved for the treatment of metastatic breast cancer [188-190]. Bevacizumab (Avastin™), a humanized anti-VEGF antibody, and Erbitux, a chimeric anti-EGFR antibody, were approved for the treatment of colorectal cancer. (http://www.cancer.org/docroot/ETO/content/ETO_1_4X_Monoclonal_Antibody_Therapy_Passive_Immunotherapy.asp?sitearea=ETO). Two MAbs conjugated to radioisotopes were also recently approved. Bexxar™ conjugated to ¹³¹I, and Zevalin™ conjugated to ⁹⁰Y, are anti-CD20 MAbs now being used to treat NHL (Table 4) [191, 192].

Table 4: Therapeutic monoclonal antibodies approved for use in oncology.

Generic name (trade name)	Target	Antibody format	Cancer Indication
Unconjugated antibodies			
Rituximab (Rituxan/Mabthera)	CD20	Chimeric IgG1	NHL
Trastuzumab (Herceptin)	HER2	Humanized IgG1	Breast cancer
Alemtuzumab (Campath/MabCampath)	CD52	Humanized IgG1	CLL
Cetuximab (Erbitux)	EGFR	Chimeric IgG1	Colorectal cancer
Bevacizumab (Avastin)	VEGFA	Humanized IgG1	Colorectal, breast and lung cancer
Panitumumab (Vectibix)	EGFR	Human IgG2	Colorectal cancer
Ofatumumab (Arzerra)	CD20	Human IgG1	CLL
Immunoconjugates			
Gemtuzumab ozogamicin (Mylotarg)	CD33	Humanized IgG4	Humanized IgG4
⁹⁰ Y-Ibritumomab tiuxetan (Zevalin)	CD20	Mouse	Lymphoma
Tositumomab and ¹³¹ I-tositumomab (Bexxar)	CD20	Mouse	Lymphoma

The evolution of MAbs from murine (human anti-mouse antibodies:HAMAs) MAbs (Suffix -momab) to chimeric (suffix -ximab), humanized (suffix -zumab), then fully human (suffix -mumab) antibodies has improved both the antigenicity and specificity of the target (Figure 5) [193].

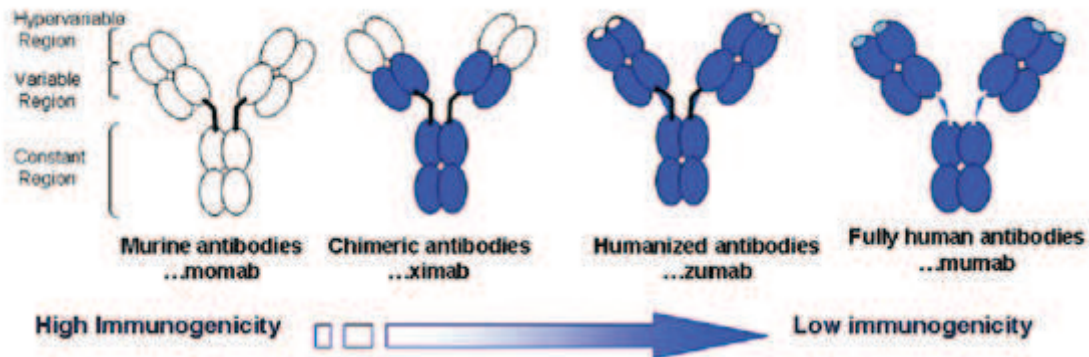


Figure 5: Evolution Monoclonal antibodies structure from murine MAbs to fully human MAbs.

There are a number of considerations that should be taken into account when using monoclonal antibodies for therapy. First, the targeted antigen selected must be presented by the tumor cells and not on normal tissues. Second, the half-life of MAb is another factor to take into consideration. Third, the immunogenicity of the monoclonal antibody itself is a concern because of how they are derived. Fourth, logistical problems such as cost and availability are general concerns for any marketable drug. Finally, a decision as to whether or not the monoclonal antibody will be used alone or if it will be conjugated (i.e. attach to radioisotopes, toxins or chemotherapeutic agents) in order to get the desired therapeutic effect.

The potential of MAbs has not been fully explored even in the cancers for which they have been approved. Over 135 MAbs are now being evaluated in clinical trials. Several studies are ongoing to elucidate their mechanisms of action, in order to ameliorate their use [194, 195]. Furthermore, applications of MAbs to other malignancies as well as autoimmunity, infectious diseases, and graft vs. host diseases are under investigation [196-198].

2. Mechanisms of action of MAbs

Monoclonal antibodies achieve their therapeutic effect through various mechanisms [199].The physiological activities of therapeutic antibodies are mediated by two independent natural immunoglobulin mechanisms:

The first therapeutic activity results from its specific and bivalent binding to the target antigen, by either blocking growth factor receptors, neutralizing the target antigen or inducing cell apoptosis; the second one results from effector functions activated only by the formation of immune complexes of the Fc region and the effector cells.

MABs can often exert their therapeutic effect by more than one of these mechanisms [200]. Furthermore, MABs can be modified to enhance their therapeutic effect by increasing their affinity or avidity, improving their binding to certain Fc receptors, improving tumor penetration, altering the half-life of the MAb, and/or conjugating them to a toxic payload such as a drug, prodrug, toxin or a radionuclide [188, 189]. We focus here on three major mechanisms of action of MABs: Antibody Dependent Cellular Cytotoxicity (ADCC), Complement-Dependent Cytotoxicity (CDC) and apoptosis.

2.1. ADCC as an effector function of therapeutic MABs

The antibody Fc region contains sites for ligands which can induce effector functions, including three structurally homologous cellular Fc receptor types (Fc γ RI, Fc γ RII, Fc γ RIII) [201, 202]. Thus, when the Fc portions of the coating antibodies interact with Fc receptors normally expressed by cytotoxic cells such as NK cells, they initiate signaling cascades that result in the release of cytotoxic granules (containing perforin and granzyme B) and the induction of apoptosis of the antibody-coated cell (Figure 6). Recently, the “effector functions” have been considered to play a key role in the clinical efficacy of therapeutic MABs. Fc γ RIIIa, a member of the leukocyte receptor family Fc γ Rs, is known to be a major triggering receptor of ADCC in natural killer (NK) cells and may thus be one of the major critical mechanisms. This was supported by genetic analysis of working polymorphisms of the receptor in patients [203-207].

Several therapeutic MABs are capable of ADCC, such as the anti-CD20 antibody rituximab (Rituxan[®]) and the anti-Her2 antibody trastuzumab (Herceptin[®]). Moreover, the ability of an antibody to produce ADCC depends on a number of factors, including the amount of antigen present on tumor cells, the type of effector cells that is activated by the antibody, as well as the subclass of the antibody.

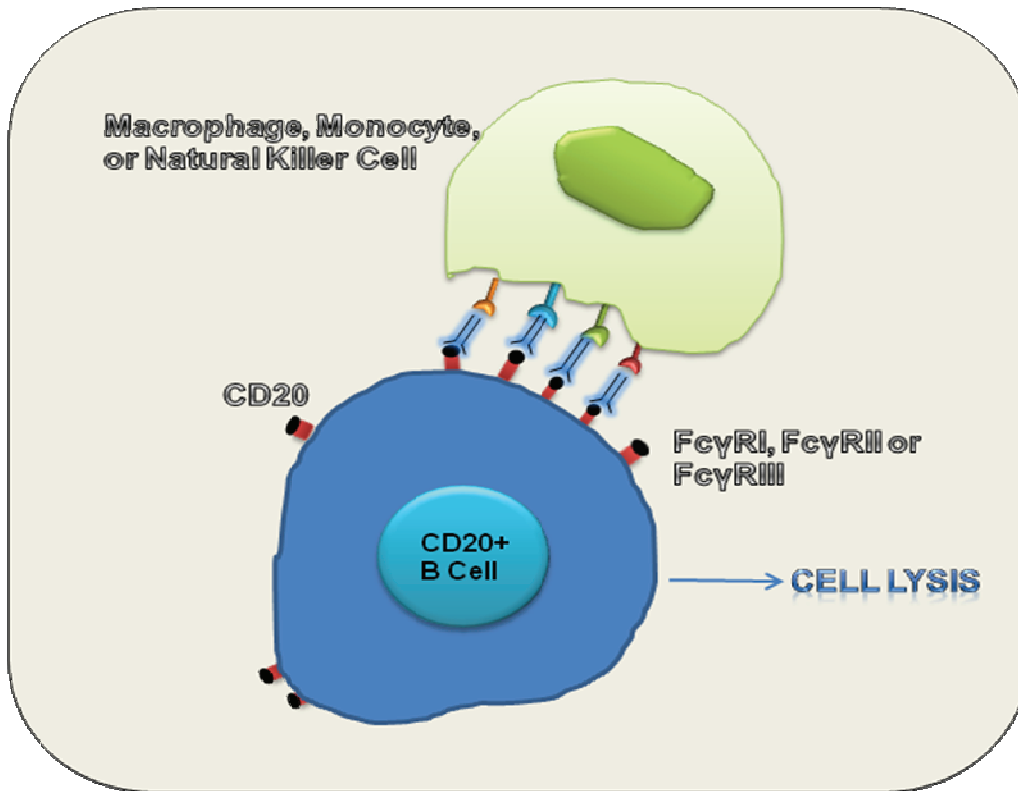


Figure 6: Antibody-dependent cell-mediated Cytotoxicity.

A CD20 MAb, targets the CD20 antigen on B cell malignancies. The Fc fragment of the MAb binds the Fc receptors found on monocytes, macrophages, and natural killer cells. These cells in turn engulf the bound tumor cell and destroy it [208-210].

2.2. CDC as an effector function of therapeutic MAbs

MAbs can also bind complement, leading to direct cell toxicity, known as complement dependent cytotoxicity (CDC). CDC is a cytolytic cascade mediated by a series of complement proteins abundantly present in serum. It is triggered by the binding of C1q to the constant region of cell-bound antibody molecules (Figure 7). For the induction of strong CDC activity, various biological and structural features of antigen molecules are required, such as relatively high expression, the presence of small or folded extracellular portions, or epitopes that retarget antigens into lipid rafts in the case of anti-CD20 MAbs [211, 212]. In addition, CDC is negatively regulated by complement-regulatory proteins (CRPs: CD46, CD55, and CD59) expressing on the cell surface [111, 213].

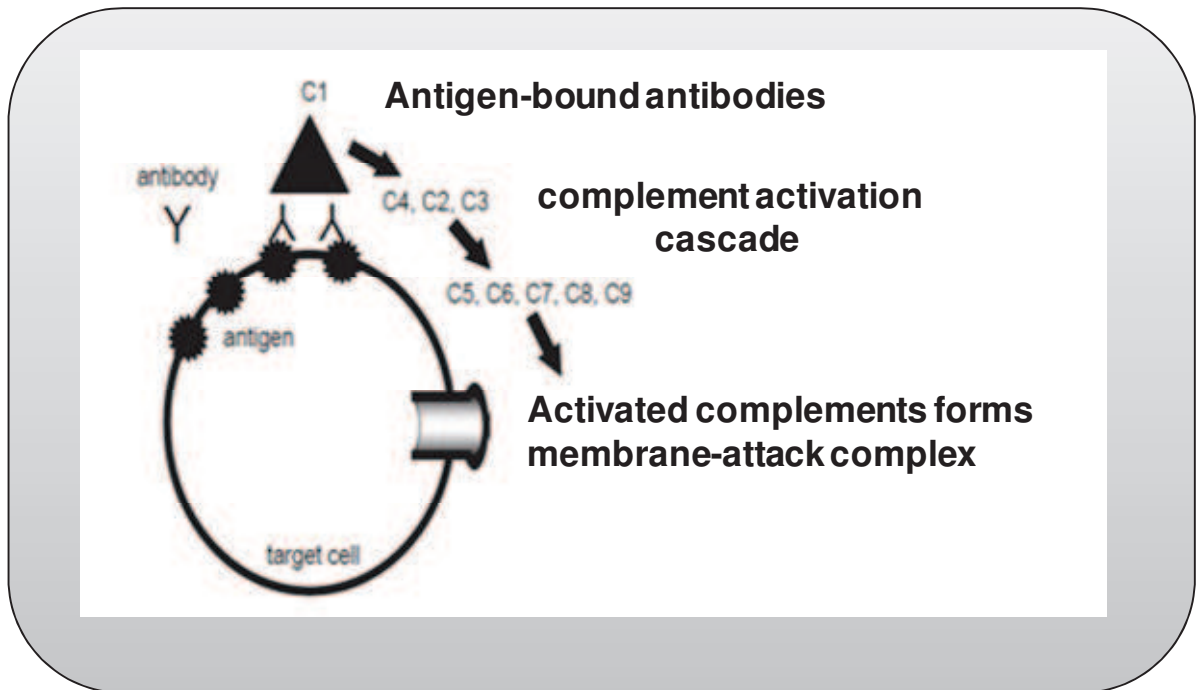


Figure 7: Complement-Dependent Cytotoxicity (CDC).

CDC is a cytolytic cascade mediated by a series of complement proteins abundantly present in serum. It is triggered by the binding of C1q, a subunit of C1, to the constant region of cell-bound antibody molecules. Finally, activated complements form membrane-attack complex, perforating membrane.

2.3. Apoptotic signalling

There are many examples demonstrating that antibodies can induce target cell killing by inducing pro-apoptotic mechanisms through the modulation of anti-apoptotic pathways (Figure 8) [214-217]. Apoptosis is controlled by both positive and negative factors. Accessory proapoptotic factors, such as Bax, Bad, and Smac/DIABLO or antiapoptotic factors such as Bcl-2 and inhibitor of apoptosis (IAPs) participate in the regulation of the apoptotic process at key steps [218]. Evidences demonstrate that these factors, by virtue of their control over the apoptotic process, are likely to be responsible for the extremes in susceptibility of tumors to conventional chemo- or radiotherapy [219, 220].

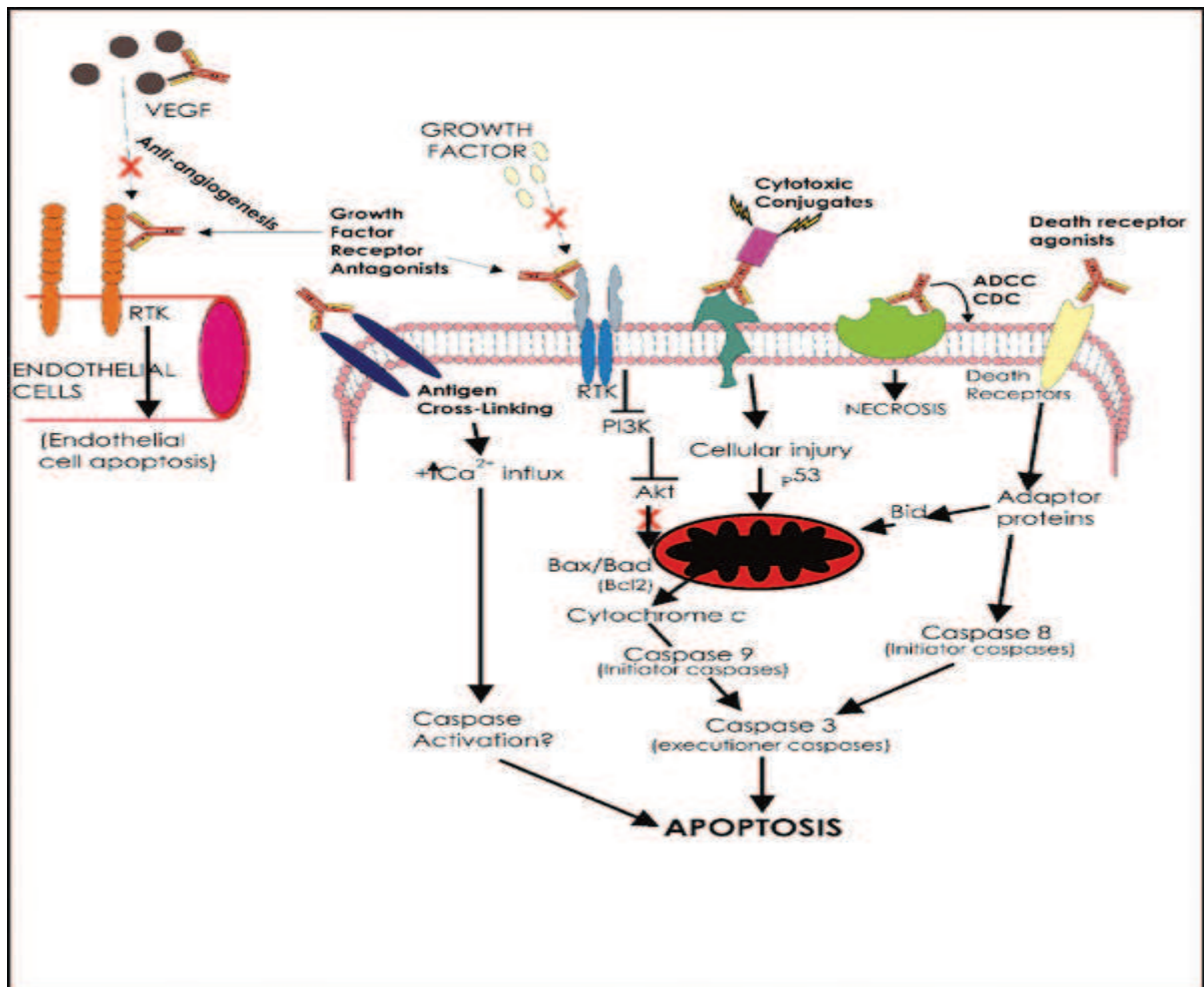


Figure 8: Signaling mechanisms of MAbs leading to the induction of apoptosis in targeted tumor cells.

There are examples of each strategy demonstrating a direct or indirect induction of apoptosis in targeted cells. First, antibodies that target growth factor receptors are capable of exerting a direct effect on the growth and survival of the tumor cell by antagonizing ligand–receptor signaling. As a result of receptor blockade, growth factor signaling mediated by receptor tyrosine kinase (RTK) autophosphorylation is inhibited, resulting in the arrest of tumor cell growth. Furthermore, because growth factor activation may also initiate antiapoptotic factors, antibodies may reduce tumor cell survival mechanisms and thus enhance the efficacy of cytotoxic agents in combination therapy. Second, antibodies can be targeted to cell surface antigens and directly elicit apoptotic signaling. Examples are antibodies that crosslink targeted surface antigen on tumor cells [215, 221] and antibody agonists that mimic ligand-mediated activation of certain receptors (death receptors [214, 222]). Third, conjugated antibodies target tumor cell surface antigens and can induce localized

tumor cell apoptosis by targeted delivery of cytotoxic agents. These antibodies have been chemically linked to toxic substances such as radioisotopes.

3. Anti-CD20 MAbs

There are a number of antigens and corresponding monoclonal antibodies for the treatment of B cell malignancies. One of the most popular target antigens is CD20. CD20 is a cell-surface glycoprotein of a natural focus for monoclonal antibody therapy. It is highly expressed in most B cell malignancies. CD20 expression begins at the pre-B stage of B-cell ontogeny and continues until the immunoblast stage; it is tightly restricted to B-cell lineage and is not expressed on either precursor lymphoid cells or the vast majority of plasma cells. Generally, CD20 does not circulate freely in the plasma thus limiting the possibility of competition for anti-CD20 antibody to CD20-positive lymphoma cells. Moreover, CD20 is not internalized, down-modulated, or shed from the surface of CD20⁺ cells following the antibody binding and has no known ligand [223]. Altogether, these characteristics permit the maintenance of antibody production during CD20 MAb therapy, facilitate the B-cell regeneration after CD20 MAb treatment, allow for sustained recruitment of natural effectors and subsequently persistent immunologic attack for as long as effectors are available [224, 225].

Despite the success in immunotherapy, the function of CD20 has not yet been fully elucidated. CD20 is involved in many cellular signaling events including proliferation, activation, differentiation and apoptosis upon crosslinking [226]. Cross-linking of CD20 by antibodies (e.g. Rituxan) has been reported to induce a rapid redistribution of CD20 into specialized microdomains at the plasma membrane, known as lipid rafts. Recruitment of CD20 into lipid rafts and its homo-oligomerization are suggested to be crucial for CD20 activity and regulation [227].

B-cell lysis is thought to occur via a number of different mechanisms, including ADCC, CDC and/or delivery of direct death signaling (apoptosis). Besides the first generation antibody rituximab, new mAb have been engineered for potential benefits. The second generation includes ocrelizumab, veltuzumab and ofatumumab where the IgG1 is humanized or fully human with an unmodified Fc region; whereas the 3rd generation includes, TRU-015, AME133V, Pro13192 and GA101 which are humanized MAbs and have an engineered Fc region designed to improve therapeutic performance by adapting their effector functions. Therefore, anti-CD20 MAbs in development for the treatment of B-cell malignancies can be broadly subdivided into two distinct types based on how B-cell lysis is achieved: type I (or

rituximab-like) MAbs appear to activate complement and effector cell mechanisms (i.e. CDC and ADCC) [14,61]; type II (or tositumomab-like) MAbs are believed to function through a combination of effector cell activation (ADCC) and apoptosis, while being relatively inactive in complement activation [224, 228, 229].

3.1. Rituximab

Rituximab has revolutionized the therapeutic approach for patients with a wide variety of B-cell malignancies, including CLL. Rituximab is a chimeric human-mouse anti-CD20 MAb. The predominant mechanism of action of rituximab-induced cell death is proposed to be primarily the result of ADCC and CDC [209, 230, 231]. Its efficiency may vary in individual patients.

In vitro studies, as well as studies in both animals and humans, suggested that the antitumor activity of rituximab was mediated by ADCC or CDC [232-234]. Rituximab also has direct anti-proliferative effects on cancer cells, and, in some instances, it induces apoptosis in lymphoma cell lines with *bcl2* gene rearrangements.

Rituximab was first approved in both the U.S. for the treatment of relapsed or refractory, low-grade or follicular, B-cell NHL [233] and in Europe, for the treatment of relapsed stage III/IV follicular NHL [232] and in Europe, for the treatment of relapsed stage III/IV follicular NHL [235]. However, the efficacy of rituximab is modest and often variable, especially when used for CLL treatment with an objective response rates ranged between 25% and 35% [4, 236].

The greatest benefit of rituximab is demonstrated when used in combination with chemotherapy. In phase III trials in patients with indolent or aggressive B-cell NHL, rituximab with CHOP or CVP as first-line or second-line therapy induced better response rates, provided lower tumor remission and increased patient survival compared to chemotherapy alone [237-240]. Likewise, rituximab induced a high overall response ORRs and complete remission (CR) rates when combined either with fludarabine/cyclophosphamide in refractory/relapsed CLL patients (73% and 25%, respectively) [238] or in those with previously untreated CLL (95% and 72%, respectively) [241] or in those with previously untreated CLL (95% and 72%, respectively) [242]. Recently, the superiority of FCR compared to FC alone was confirmed in randomized phase III trials [243, 244]. Furthermore, when combined with pentostatin and cyclophosphamide in previously untreated CLL patients,

it achieved a significant clinical activity despite poor risk-based prognoses, including achievement of minimal residual disease in some patients [245-247].

Investigations of the mechanism underlying the anti-tumor activity of rituximab as a single agent and in combination with chemotherapy are ongoing. By understanding these mechanisms, it might be possible to further enhance current cell killing strategies or develop novel agents and strategies.

3.2. Ofatumumab

Ofatumumab is a fully humanized Mab targeting a small-loop CD20 epitope distinct from that of rituximab [229]. Compared to rituximab, it demonstrates an increased target-binding affinity to CD20 and slower dissociation rates. It exhibits stronger complement-mediated toxicity and shows potent lysis of rituximab-resistant cells. In phase I/II study in relapsed/refractory CLL patients, ofatumumab achieved an ORR of 44%, however these were almost exclusively partial responses [174]. In a phase I/II dose-escalation trial, the efficacy and safety of single-agent ofatumumab (300-1000 mg) have been evaluated in 40 patients with relapsed or refractory FL. Rapid, efficient and sustained peripheral B-cell depletion was observed in all dose groups. The ORR in evaluable patients (n=36) was 43% [248].

This antibody was recently approved by the FDA for fludarabine and alemtuzumab refractory CLL patients and for fludarabine refractory patients with bulky disease. Ofatumumab was administered in these two groups with an ORR of 58% and 47%, respectively [173]. This antibody is currently being combined with other agents in CLL, including bendamustine.

A recently completed phase II trial of ofatumumab in combination with fludarabine and cyclophosphamide (O-FC) demonstrated complete responses in up to 50% of patients with previously untreated CLL, despite poor prognostic factors [173]. The median progression-free survival has not been reached with the short median follow-up of 8 months.

An international phase II trial is ongoing in FL patients to evaluate the combination of ofatumumab with CHOP (US National Institutes of Health. ClinicalTrials.gov.

<http://www.clinicaltrials.gov/ct2/results?term=ofatumumab>).

3.3. GA101

GA101 is the first humanized type II anti-CD20 MAb with glycolengineered Fc portion and a modified elbow hinge [249]. The adapted Fc region gives GA101 a 50-fold higher binding affinity to FC γ RIII (CD16) compared to a non-glycoengineered antibody,

resulting in 10- to 100-fold increase in ADCC against CD20⁺ NHL cell lines via the activation of effector cells [250]. Moreover, the modified elbow hinge area also results in strong induction of direct cell death of several NHL cell lines and primary malignant B cells *in vitro* [249-251]. However, these modifications result in reduced CDC activity [252]. *In vitro* B-cell depletion assays with whole blood from healthy and leukemic patients showed that the combined activity of ADCC, CDC, and apoptosis for GA101 was significantly superior to rituximab [250, 251, 253, 254].

The enhanced efficacy of GA101 also has been shown *in vivo*. In xenograft models of DLBCL and mantle cell lymphoma, treatment with GA101 resulted in CR and long-term survival compared with tumor stasis achieved with rituximab [250]. In cynomolgus monkeys, GA101 (10 and 30 mg/kg infused on days 0 and 7) showed significantly superior depletion of B cells compared to rituximab (10 mg/kg) from day 9 to day 35 and was more efficacious at clearing B cells from lymph nodes and the spleen [252].

Initial phase I study of patients with relapsed/refractory CD20⁺ disease (n=21), including CLL, DLBCL, and other NHLs, for whom no therapy of higher priority was available (95% of patients had previously received rituximab), GA101 demonstrated a favorable safety profile with no dose-limiting toxicities [255]. The depletion of B-cell was rapid and sustained in the majority of patients. Nine of the evaluable patients responded to therapy (ORR, 43%; five CR/unconfirmed CR and four partial response), with responses observed at all dose levels and across all FcγRIIIA genotypes.

The pharmacokinetics of GA101 are generally similar to those of rituximab and dose-dependent. However, significant inter- and intra-patient variabilities have been observed, the clinical relevance of which will need further investigation [86]. Results from a phase I study in patients with previously treated B-CLL (n=13) who were given single-agent GA101 (400–2000 mg; nine infusions) showed similar safety and pharmacokinetic profiles to those observed in the previously described patients with NHL, except for an increased incidence of neutropenia [256].

GA101 is currently being explored as a single agent in phase II studies in relapsed/refractory B-CLL and indolent/aggressive NHL, and in combination with chemotherapy in a phase Ib study.

3.4. AME-133v

AME-133v is an engineered CD20 mAb with enhanced Fc affinity for FcγRIIIa (CD16). *In vitro* assays, AME-133v has shown higher binding affinity than rituximab to

FcgRIIIa (CD16) expressed in NK cells and a 10-fold increase in cytotoxicity relative to rituximab *in vitro* [249]. This high binding affinity for FcgRIIIa translates into superior activation by AME-133v of NK cells in the presence of CLL cells compared with rituximab, which exhibits minimal activation of NK cells [257].

Based on these *in vitro* encouraging preclinical results, a phase I/II dose-escalation study is currently undertaken to evaluate the efficacy and safety of AME-133v in patients with relapsed/refractory follicular NHL [249, 258].

3.5. PRO131921 (RhuMAb v114)

RhuMAb v114 is a CD20 MAb with an engineered Fc region that provides 30-fold greater binding to the low-affinity variant of FcgRIIIa (FF or FV) compared to rituximab. *In vitro* B-cell models, the enhanced affinity for FcgRIIIa results in 2- to 10-fold greater ADCC than rituximab. However, preclinical studies in cynomolgus macaques revealed that treatment with RhuMAb v114 is associated with dose-dependent reversible neutropenia and thrombocytopenia. Due to these myeloid toxicities, a phase I/II clinical trial has recently been initiated to assess the safety of escalating doses of RhuMAb v114 in patients with relapsed or refractory follicular NHL who have received prior treatment with a rituximab-containing regimen [249].

3.6. Veltuzumab (IMMU-106)

Veltuzumab is a humanized CD20 MAb (type I) constructed recombinantly on the framework regions of epratuzumab, with complementarity-determining regions (CDRs) identical to rituximab, except for a single amino acid in CDR3 of the variable heavy chain. It showed anti-proliferative, apoptotic, and ADCC effects *in vitro* similar to rituximab, but with significantly slower off-rates and increased CDC in several human lymphoma cell lines. In addition, at very low doses, given either intravenously or subcutaneously, veltuzumab showed a potent anti-B cell activity in cynomolgus monkeys and controlled tumor growth in mice bearing human lymphomas [259].

They suggested that the difference between veltuzumab and rituximab, at least with regard to off-rates, are related to a single amino acid change in CDR3-VH (Asp101 instead of Asn101), as corroborated by back-mutation studies [259]. In a phase I/II dose-escalating clinical trial in patients with recurrent NHL, the ORR for veltuzumab-treatment was 41% (33/81), including 17 patients (21%) with CR or unconfirmed CR [260]. Veltuzumab caused

B-cell depletion after the first infusion even at the lowest dose of 80 mg/m², which persisted after the fourth infusion, and was well tolerated, with no evidence of immunogenicity.

Veltuzumab is additionally being developed for subcutaneous administration, which may provide advantages for this agent versus other mAbs [261].

3.7. Ocrelizumab

Ocrelizumab is a new humanized CD20 antibody (type I) with high binding affinity for the low-affinity variants of the FcγRIIIa receptor. This has the potential to enhance efficacy in the treatment of NHL compared with rituximab, particularly with regard to improved B-cell lysis via ADCC.

A phase I/II open-label, dose-escalation study is currently ongoing in patients with relapsed refractory FL following prior rituximab-containing therapy.

The response rate with ocrelizumab at interim analysis was 36% across all treatment groups, which is encouraging in this group of patients who have previously been treated with rituximab [260].

4. Other MAb for CLL

4.1. Alemtuzumab

Alemtuzumab is a recombinant, fully humanized, MAb targeting the CD52 antigen. CD52 is expressed on virtually all lymphocytes at various stages of differentiation, as well as monocytes, macrophages and eosinophils, whereas hematopoietic stem cells, erythrocytes and platelets do not express it [262]. A high level of CD52 is found on T-prolymphocytic leukemia, followed by B-CLL, with the lowest levels expressed on normal B cells. The mechanisms of action of alemtuzumab include CDC, ADCC and induction of apoptosis [263].

The use of alemtuzumab monotherapy is approved in the United States in the first-line treatment of patients with CLL. In a pivotal phase II study in 93 patients with fludarabine-refractory disease, alemtuzumab yielded an ORR of 33% with a median OS of 16 months [246].

Alemtuzumab has been approved for the initial treatment of CLL based on randomized trial conducted including 297 patients who received either alemtuzumab or chlorambucil. The antibody induced an ORR rate of 83.2% with 24.2% CRs compared with 55.4% and 2%, respectively for chlorambucil [264]. In addition, alemtuzumab has proven efficacy even in

patients with poor prognostic factors, including high-risk genetic markers such as deletions of chromosome 11 or 17 and p53 mutations [171, 172]. The combination of alemtuzumab with fludarabine was investigated in a phase II trial with relapsed CLL patients. The ORR was 83% which included 30% CR [265]. The combination of both alemtuzumab with rituximab has been also studied in patients with lymphoid malignancies including patients with refractory/relapsed CLL, producing an ORR of 52% with 8% CR [266].

4.2. Lumiliximab

Lumiliximab is an anti-CD23 macque-human chimeric MAb with a strong similarity to the human antibody. The CD23 antigen is a low-affinity IgE receptor that is found in high levels in CLL patients [267]. Lumiliximab inhibits the IgE secretion *in vitro*, binds complement and mediates ADCC by binding Fc γ RI and RII receptors. A phase I pilot study reports a limited single-agent activity in patients with refractory/relapsed CLL [175].

Based on preclinical evidences of synergistic improvement of survival when lumiliximab was combined with fludarabine or rituximab, a phase I/II trial evaluated the safety and efficacy of lumiliximab in combination with FCR in 31 patients of relapsed CLL patients [268]. This combination regimen yielded an ORR of 65%, which was comparable to the results seen with FCR in the pivotal phase II study conducted by the M.D. Anderson Cancer Center [241, 268]. Lumiliximab/FCR appeared to double the CR rate compared to FCR alone (52% vs. 25%) without increasing the rate of toxicities. An international, randomized, phase II/III trail of FCR with or without lumiliximab was recently completed but results have not yet been presented. (<http://clinicaltrials.gov/ct2/show/NCT00391066>).

4.3. Epratuzumab

Epratuzumab is a humanized anti-CD22 MAb currently in clinical trials for treatment of NHL and autoimmune disorders [269]. Epratuzumab is selectively active against normal and neoplastic B-cells. This MAb acts as an immunomodulatory agent in contrast to rituximab which is an actually cytotoxic therapeutic antibody. *In vitro*, epratuzumab has demonstrated the ability to elicit ADCC and induce CD22 phosphorylation and signaling, both of which may contribute as potential mechanisms of action [270, 271]. Phase I/II studies demonstrated objective responses across various dose levels in both relapsed/refractory FL (24%) [272] and DLBCL (15%) [273].

Epratuzumab has also been combined with rituximab in phase II studies showing at least an additive benefit while toxicities of the combination were comparable with those of single-agent rituximab [274].

In a recent international, multicenter trial evaluating rituximab plus epratuzumab in patients with post-chemotherapy relapsed/refractory, indolent NHL, an objective response was seen in 54% FL patients including 24% with CR/unconfirmed CR (CRu) whereas 57% of small lymphocytic lymphoma (SLL) patients had ORs including 43% with CR/Cru [275]. Rituximab-naïve patients had an OR rate of 50%, whereas patients who previously responded to rituximab had an OR rate of 64%.

Thus, the combination of epratuzumab and rituximab induced durable responses in patients with recurrent, indolent NHL. Epratuzumab is also being evaluated in combination with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and as a therapy in other B-cell neoplasms [276].

5. Mechanisms of resistance

A whole array of new unanswered questions has emerged for both basic scientists and clinicians to address. What happens when an anti-tumor antibody recognizes its target? Why don't all targeted positive cells die, even if they have been recognized by the MAb? Why don't all patients respond to therapeutic MAb? Why do the durations of remissions differ so much between individual patients even if their primary responses were similar? We addressed these questions and attempted to answer them in our review entitled "Understanding and circumventing resistance to anticancer monoclonal antibodies" that was published in MABs in 2009. This paragraph on resistance to monoclonal antibodies is therefore represented by this review.

Review

Understanding and circumventing resistance to anticancer monoclonal antibodies

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Abbreviations: Akt, protein kinase B; APAF-1, apoptosis protease-activating factor-1; bcl2, B-cell lymphoma 2; CHOP, cyclophosphamide, hydroxydaunomycin, Oncovin, prednisone; CLL, lymphocytic leukemia; DLCL, diffuse large B cell lymphoma; DR, death receptor; ERK, extracellular signal-related kinase; FADD, Fas-associated death domain protein; FL, follicular lymphoma; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia sequence1; MZL, marginal zone lymphoma; NFκB, nuclear factor-κB; NHL, non-Hodgkin lymphoma; STAT3, signal transducer and activator of transcription 3; YY1, yin yang 1

Key words: monoclonal antibodies, resistance, rituximab, cetuximab, trastuzumab

With the widespread use of therapeutic monoclonal antibodies in the treatment of patients with cancer, resistance to these agents has become a major issue. Preclinical models of drug action or resistance have contributed to unravel the main mechanisms of resistance, involving both tumor-associated and host related factors. However our understanding of how a monoclonal antibody destroys cancer cells in a patient and why it one day stops being effective are still far from being complete. This review focuses on the available data on mechanisms of action and resistance to rituximab and includes some additional information for other monoclonal antibodies. Innovative approaches designed to overcome resistance, such as combination immunotherapy, costimulation with cytokines or growth factors are presented.

Introduction

Passive immunotherapy of malignancies with therapeutic monoclonal antibodies (mAbs) has profoundly modified the way clinicians consider treatment of patients afflicted with haematological malignancies or solid tumors. While some patients can benefit from mAbs administered as single agent first line therapy and/or as consolidation therapy, most patients receiving therapeutic mAbs will do so in the scope of combinations with classical antimetabolic compounds, or, in the near future, with small molecule targeted therapies.

While it is clear that mAbs have significantly contributed to improving the outcome of patients afflicted with cancer, there is no proof that mAbs have modified the curability of those types of

cancer which could not be cured by conventional treatments. In the case of lymphoma patients for example, the combination of rituximab with the CHOP regimen (cyclophosphamide, hydroxydaunomycin, Oncovin, prednisone) has demonstrated improved response rates, freedom from progression and overall survival in patients with diffuse large B cell non-Hodgkin lymphoma (NHL), a subtype which could in some patients be cured by CHOP alone.¹ Conversely in patients afflicted with follicular lymphoma (FL), an indolent yet incurable disease, rituximab has profoundly modified the way patients are treated, but does not appear to have made the disease curable. Approximately 50% of patients with relapsed/refractory CD20⁺ follicular lymphomas do not respond to initial therapy with rituximab² and close to 60% of prior rituximab responding patients will not longer benefit with retreatment with this monoclonal antibody.³ Likewise patients with solid tumors who were considered incurable with conventional therapy have not presently been shown to be cured by the addition of mAbs. Whether administered as single agents or in combination regimens, the therapeutic activity of mAbs is therefore limited by mechanisms of resistance. Whether these forms of resistance are innate or acquired, there is an urgent need to better understand why tumor cells are resistant or how they become resistant to mAbs, and which strategies could be implemented to circumvent these resistance mechanisms in patients.

Resistance to cancer therapy has mainly been explored for systemic treatments such as chemotherapy, and been designated under the term of chemoresistance. While chemoresistance was initially observed after the first unsuccessful attempts to treat leukemia patients with nucleotide analogues fifty years ago, the history of chemoresistance really starts with the discovery of the P glycoprotein efflux protein by Ling et al. in the 1970s.⁴ Lessons learned while trying to understand and circumvent the function of proteins such as P glycoprotein remain of great use in the study of newer agents, both in terms of understanding precellular (most notably pharmacokinetics) and cellular (pharmacodynamics)

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<http://www.landesbioscience.com/journals/mabs/article/8292>

resistance mechanisms. Along the same line, the large amount of data accumulated regarding resistance mechanisms to classical anticancer agents are also useful in understanding resistance to mAbs, insofar as the classical agents and mAbs share similar apoptotic effector mechanisms.

Antibodies often exhibit complicated pharmacokinetic and pharmacodynamic properties.⁵ Due to the multiple mechanisms of antibody cytotoxicity and the complex nature of the antibody disposition, the determination of these parameters will lead to improved development of monoclonal antibodies.

mAbs are similar to conventional agents in that they undergo degradation and clearance and induce apoptotic signaling, however, they differ by the fact that factors independent of the tumor cell itself strongly contribute to their anticancer effect. Complement Dependent Cytotoxicity (CDC) and Antibody Dependent Cellular Cytotoxicity (ADCC) are considered to be essential mechanisms of action of antitumor activity of mAbs, and are therefore likely to be involved in the development of resistance mechanisms. In this review, we will discuss available data regarding preclinical models of resistance to mAbs, focusing on rituximab, as well as results correlated with response to mAbs in the clinic. These data have shed some light on potential mechanisms of resistance to therapeutic mAbs, and suggest possible strategies to circumvent these resistance phenomena.

Rituximab

In 1997, rituximab became the first monoclonal antibody approved for cancer therapy.⁶ Having been used for over a decade in patients, rituximab is thus the therapeutic mAb for which there are currently the most data, both in terms of mechanisms of action, parameters associated with sensitivity or resistance, and strategies to enhance its antitumor effect. Rituximab is a chimeric anti CD20 monoclonal antibody composed of murine variable regions (Fab region) that are linked to a human Fc component, targeting the CD20 antigen. CD20 antigen is a transmembrane protein of 35 kD molecular weight, located mainly in pre-B and mature B lymphocytes but not on stem or plasma cells. Its role is still unclear, but there is evidence that it may be involved in regulating cell cycle and differentiation processes, and could behave as a calcium ion channel as well.⁷

Models used to Understand Rituximab Cytotoxicity or Resistance to Rituximab

Preclinical models of rituximab are illustrative of the difficulties involved in identifying resistance mechanisms to mAbs. As for most unlabelled mAbs, rituximab demonstrates poor cytotoxic effect per se on cell lines expressing the target antigen *in vitro*, and is much more effective when CDC or ADCC are reproduced in the test tube by the addition of fresh human serum and/or peripheral blood effector cells, respectively. Induction of apoptosis by rituximab alone has been reported in the absence of accessory cells, but has mostly been described using cell lines derived from patients with Burkitt lymphoma, a subtype of NHL for which the clinical indication of rituximab has not yet been as well documented.^{8,9}

Conversely rituximab has been shown by several groups to possess activity in murine models of xenotransplanted human CD20 positive lymphoma lines. Notwithstanding the limitations due to the use of immunocompromised mice, these models have been very informative in determining the contribution of CDC or ADCC *in vivo*, and offer the possibility of analyzing signaling pathways in tumors. Experiments with cobra venom factor, a complement-depleting agent, have shown that the antitumor effect of rituximab is at least partly CDC-dependent *in vivo*.¹⁰⁻¹² Other experiments involving the depletion of NK cells, macrophages or granulocytes have been performed, sometimes with contradictory results, but overall suggest an important role for ADCC in rituximab cytotoxic activity.¹³ Conversely there are currently few data available regarding apoptotic signaling in *in vivo* samples.

Clinical samples have been used to better understand how rituximab works using different approaches. In the “*ex vivo*” approach, fresh human samples, most commonly peripheral blood containing malignant cells, are exposed to rituximab and cell death can then be quantified.¹⁴ These models are interesting insofar as the samples have not been altered by prolonged growth *in vitro*, and that autologous effector factors (patient serum and/or accessory cells) can be used. However, these studies are difficult to generalize to patients with solid tumors for obvious reasons. Even in the context of haematological malignancies one must keep in mind the differences occurring within blood, bone marrow, lymph nodes and other tissues. Clearance of malignant cells from the blood is known to be more readily obtained than that of bone marrow or lymph nodes, suggesting that the study of blood samples might not be representative of other tissues. Clinical samples have also been used to establish correlations between the genetic makeup of the patient and response to rituximab using normal cells to study genetic polymorphisms.^{15,16} Tumor samples may also be used to analyse expression profiles and establish correlations with response to mAbs.¹⁷

Parameters Correlated with Rituximab Activity and Resistance

The mechanisms that influence rituximab efficiency include host and tumor cell-related factors. Host-related factors that possibly have an impact on rituximab are diverse, ranging from pharmacokinetic parameters to accessory effector mechanisms and intracellular signaling pathways (Fig. 1). Little is currently known regarding the pharmacokinetics of rituximab, although clinical studies have shown a large interindividual variability in rituximab exposure and its significant influence on clinical response in patients receiving similar doses of antibody.^{18,19} Dayde et al. have shown in a preclinical model that exposure to rituximab influences response and survival.²⁰ Additional investigations are clearly warranted to better define parameters influencing pharmacokinetic parameters of rituximab.

Individual variations in accessory mechanisms are also likely to influence the cytotoxic activity of rituximab. ADCC relies on the binding of the Fc portion of rituximab to Fc γ receptors on accessory cells. The relative ratio of “activating” receptors such as Fc γ RI, Fc γ RIIA, Fc γ RIII and “inhibitory” receptors

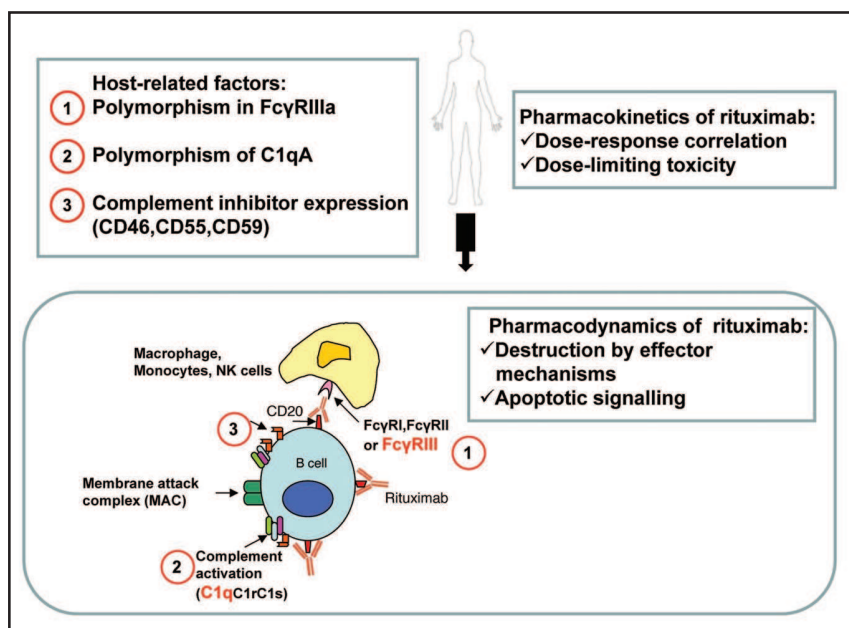


Figure 1. Summary of mechanisms that influence rituximab efficiency. These include host-related factors (including pharmacokinetics and polymorphisms of key molecules such as FcγRIIIa) and tumor cell-related factors.

such as FcγRIIB is likely to determine the net interaction with accessory cells after rituximab binding. Cartron et al. analyzed the impact of the *FCGR3A-158V/F* polymorphism by genotyping 48 patients having received single agent rituximab as first line therapy for FL. The objective response rates at 12 months was 90% in *FCGR3A-158V* homozygous patients and 51% in *FCGR3A-158F* carriers ($p = 0.03$).²¹ In murine models depletion of accessory cells such as macrophages (using liposomal clodronate) or NK cells (using specific mAbs) has been shown to reduce the cytotoxic activity of rituximab.¹² These data globally support the role of ADCC as a clinically relevant effector mechanism of rituximab in vivo.

Complement-dependent cytotoxicity is also likely to vary from one patient to another. Golay et al. investigated the role of the complement inhibitors CD35, CD46, CD55 and CD59 with blocking antibodies in FL cell lines as well as in fresh cases of FL and showed that CD55, and to a lesser extent CD59, were important regulators of complement-mediated cytotoxicity.²² These observations were further

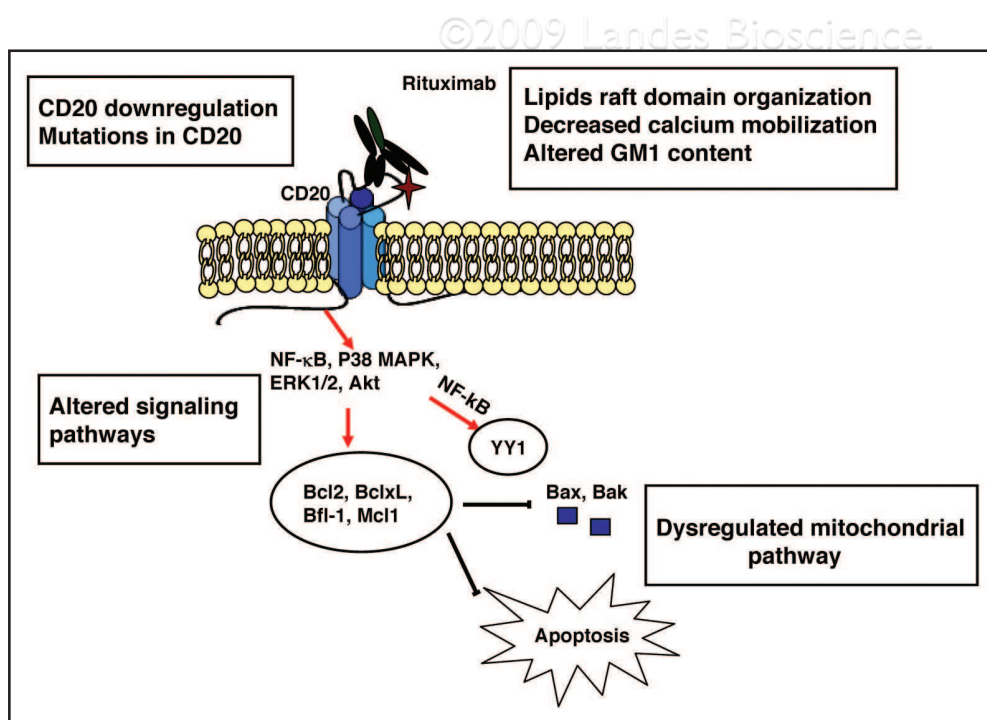


Figure 2. A schematic diagram that illustrates potential cellular mechanisms of resistance to rituximab following its interaction with CD20. Acquired resistance can be associated with significant change in CD20 antigen expression, deficient redistribution into lipids raft domains or alteration in raft components and decreased calcium mobilization. Alterations in intracellular pathways, such as those involving p38 MAPK, NFκB, ERK1/2, Akt, could be implicated in resistance. An enhanced activation of NFκB and ERK1/2 can lead to overexpression of Bcl2, Bcl-x_L and Mcl-1 thereby inhibiting apoptosis by dysregulating mitochondrial cell-intrinsic and extrinsic pathways. Moreover, the transcription repressor YY1 can negatively regulate Fas and Trail receptor expression and confer resistance to apoptosis.

supported by the results of Treon et al.²³ who found that anti-CD59 mAbs sensitized cells to rituximab cytotoxicity, and of Takei et al. who observed increased expression of CD55 and CD59 in rituximab-resistant Ramos cells.²⁴ More recently Racila et al. genotyped the C1qA([276A/G]) polymorphism in 133 subjects with FL treated with single-agent rituximab and observed a significantly different time to progression in homozygous G subjects (282 days) and in A-allele carriers (708 days, $p = 0.02$). Homozygous A subjects achieved complete response at a higher rate than heterozygous or homozygous G subjects.¹⁶

Tumor-related factors that are involved in resistance to rituximab include alteration in CD20 and lipids raft domain and regulation in signaling and mitochondrial pathways (Fig. 2).

Alterations of the CD20 antigen are prime suspects as causes of resistance to rituximab. However, there are very few data in the literature regarding CD20 mutations

and little more regarding correlations between CD20 expression and sensitivity to rituximab. Terui et al. sequenced the CD20 gene in samples from 68 NHL patients receiving rituximab and found mutations in 12 patients.²⁵ These authors reported a lower CD20 expression level in patients bearing a mutation in the C-terminal cytoplasmic domain. Reduced CD20 expression has been reported by several authors in cell lines rendered resistant to rituximab *in vitro* but have only anecdotally been reported in patients relapsing after rituximab.^{24,26,27} An *in vitro* Burkitt model resistant to rituximab developed by Jazirehi et al.⁸ has shown a 50% reduction of CD20 expression in resistant clones, and this was confirmed in another *in vitro* model of follicular lymphoma.²⁶ However, in our *in vivo* model of follicular lymphoma using the human RL line resistant to rituximab,²⁸ CD20 expression was not different in the resistant cells in comparison to the sensitive parental cells. Interestingly, there appears to be a correlation between the baseline level of expression of CD20 in various subtypes of lymphoproliferative diseases and clinical responsiveness to rituximab. Chronic lymphocytic leukemia (CLL) cells tend to have low expression of CD20, as opposed to marginal zone lymphoma (MZL) or DLCL for example.²⁹ Quantification of CD20 is however difficult to perform reliably, and flow cytometry has been reported to be more precise than immunohistochemistry.³⁰

After interaction with rituximab, CD20 has been shown to be redistributed to rafts, or detergent-insoluble microdomains.³¹ This appears to be a common finding for type I antibodies, but is not observed with type II antibodies such as tositumomab or GA101.³² Raft components and/or factors affecting redistribution of CD20 to rafts may impact on the activity of rituximab. Meyer zum Buschenfelde et al. have recently reported that the content in GM1, a raft-associated sphingolipid, in patient samples was correlated with sensitivity to rituximab.³³ Samples from patients with MZL, a subtype sensitive to rituximab, were found to have high GM1 content, while CLL samples had a lower GM1 content.³³ Deficient redistribution into rafts or alterations in the composition of rafts are thus likely mechanisms of resistance to rituximab, although this remains to be studied in greater detail. The fact that type II antibodies do not appear to require redistribution to rafts suggest that they may be active in models of resistance to rituximab.

Rituximab binding has been shown to activate a number of signalization pathways, either inducing cell death or sensitizing tumor cells to cytotoxic agents. The Bonavida group has shown that raf kinase inhibitor protein plays a key role in regulating Bcl-x_L, through NFKappaB and MAPkinase pathways.^{9,34,35} Other antiapoptotic genes, such as Bfl1, or proapoptotic genes, such as Bax or Bak, have also been found to influence sensitivity to rituximab.^{36,37} More recently it has also been found that Yin Yang and PKC (ζ) were involved in rituximab signaling.^{38,39} Suzuki et al. recently suggested that rituximab might suppress the constitutively active Akt pathway in NHL cells, without modifying unphosphorylated Akt levels.⁴⁰ The clinical relevance of apoptotic signalization as compared to that of extracellular mechanisms such as CDC and ADCC is difficult to determine. Whether apoptotic induction by rituximab *per se* occurs or not *in vivo*, it is highly likely that CD20-mediated signalization

sensitizes NHL cells to the cytotoxic activity of conventional chemotherapeutic agents.⁴¹

Both caspase-dependent and caspase-independent cell death have been reported after exposure to rituximab. Byrd et al. reported activation of caspase-9, caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage as well as significant down-modulation of the antiapoptotic proteins XIAP and Mcl-1 in CLL patients receiving rituximab treatment.⁴² More recently Stolz reported that rituximab triggers apoptosis through mitochondrial-mediated caspase pathways.⁴³ Conversely caspase-independent toxicity has also been described by various authors,^{44,45} and may involve the role of calcium.⁴⁶

Several studies have shown that resistant cells display constitutive hyperactivation of the survival pathways NFKB and ERK1/2, leading to overexpression of Bcl-2, Bcl-2-related gene and Mcl-1.⁸ In the *in vivo* resistant RL model, Bcl-X_L was also found more highly expressed in rituximab-resistant cells.²⁸ This confirms the recent results obtained *in vitro* by Jazirehi et al.⁸ showing that the phenotype of resistant cells to rituximab may be associated with a higher expression of Bcl-X_L. Moreover, we found an overexpression of YY1, a negative regulator of Fas and Trail receptor DR5 expression, that can inhibit apoptosis.⁴¹ Altered signaling pathways have been also shown to be associated with a downregulation of the pro-apoptotic Bcl2 family proteins BAX and BAK responsible for associated resistance to chemotherapy, thereby blocking initiation of apoptosis.³⁶ A low ratio of Bak (or Bax) to Bcl-2 in tumor cells was associated with increased survival in patients with follicular lymphoma⁴⁷ while a low ratio of Bax to Mcl-1 was associated with resistance to rituximab in chronic lymphocytic leukemia patients.^{47,48} These data therefore suggest that Bcl2 family proteins, involved in the regulation of apoptosis, and well-known as being involved in the sensitivity to antimetabolic compounds, are also likely to be clinically relevant in terms of sensitivity to anticancer mAbs.

Cetuximab

Cetuximab is a monoclonal chimeric antibody directed against the epidermal growth factor receptor (EGFR). EGFR is overexpressed in a variety of solid tumors, suggesting an important role in the process of neoplastic transformation. Cetuximab binds to EGFR with a 2-log higher affinity than the natural ligands TGF α and EGF.⁴⁹ Therefore, its binding deactivates many cellular pathways such as the mitogen-activated protein kinase, phosphatidylinositol 3' kinase and Akt pathways.⁵⁰ When competing with receptor binding, cetuximab induces receptor internalization and prevents ligand-mediated receptor tyrosine kinase phosphorylation. It may also exert its anti-tumor effects through ADCC via its fragment c receptor (FCR). Two polymorphisms FCGR2A-H131R and FCGR3A-V158F were independently associated with progression-free survival and may be useful as molecular markers to predict clinical outcome in metastatic CRC patients treated with cetuximab.⁵¹

It has recently been shown that patients with advanced colorectal cancer do not respond to anti-EGFR therapies such as panitumumab and cetuximab if tumors contain KRAS mutations.⁵² KRAS status was found to be an independent prognostic

factor associated with overall survival and progression free survival. Testing for KRAS mutations is fast becoming a clinically relevant predictor for patients whose disease justifies treatment with cetuximab. A BRAF V600E mutation was also detected in some patients who did not respond to neither cetuximab nor panitumumab and could be a useful biomarker for selecting patients responsive to anti-EGFR therapy.⁵³ Thus, combination therapy which can block both EGFR and BRAF in patients with BRAF-mutated tumours may be an effective therapy in non-responder patients. Other parameters, including PIK3CA mutation/PTEN expression status⁵⁴ or specific gene expression profiles, have also been suggested to influence response to cetuximab.⁵⁵

Models used to Understand Cytotoxicity of Cetuximab

To understand the molecular mechanisms of acquired resistance to EGFR inhibitors, Wheeler et al.⁵⁶ established a series of cetuximab-resistant clones in vitro following long-term exposure to cetuximab in nonsmall cell lung cancer (NSCLC; H226) and head and neck squamous cell carcinoma (HNSCC; SCC-1) cell lines. These authors report that cetuximab-resistant cells show altered EGFR internalization and degradation as well as enhanced expression of HER2, HER3 and c-Met. Benavente et al.⁵⁷ presented recently another model of resistance to cetuximab, gefitinib or erlotinib in head and neck tumor cells following chronic exposure to these agents. EGFR inhibitor-resistant lines showed increased proliferation rates and elevated levels of phosphorylated EGFR, MAPK, AKT and STAT 3, with reduced apoptotic capacity. These important observations raise the possibility that combined targeting of these pathways, using other mAbs or small molecule inhibitors of downstream pathways may enhance the antitumor activity of cetuximab.

Trastuzumab

Trastuzumab is a recombinant humanized monoclonal antibody which binds to the IV domain of the extracellular segment of HER2. The HER2 protein is involved in the regulation of normal breast growth and development.^{58,59} HER2 gene amplification/protein overexpression has been detected in 20 to 30% of human breast carcinomas and studies have indicated that HER2 amplification/overexpression plays a role in malignant transformation and tumorigenesis.⁶⁰ Cells treated with trastuzumab undergo arrest during the G₁ phase of the cell cycle, downregulate HER2 leading to disruption of receptor dimerization and signaling through the downstream PI3K and MAP (MAPK) cascades. The efficacy of trastuzumab may also depend upon its ability to induce an immune response. It can promote apoptosis in multiple breast cancer lines via antibody-dependent cellular cytotoxicity (ADCC).⁶¹ Musilino et al. showed that FcγR polymorphisms play a role in trastuzumab-mediated ADCC and may be a predictor tool for clinical outcome of patients with breast cancer treated with trastuzumab-based therapy. ADCC could therefore be an additional mechanism in the response to trastuzumab that is particularly effective in patients who are FCγR158V and/or FCγRIIIa 131H homozygous.⁶²

Several mechanisms of resistance to trastuzumab have been reported. The overexpression of MUC4, a membrane-associated

glycoprotein, can sterically hinder the antibody from binding HER2 surface receptor and may mediate a crosstalk to activate HER2, leading to tumor progression and metastasis.^{63,64} In breast cancer cell models that overexpress HER2/neu, Lu et al. showed that an increased level of IGF-IR signaling appeared to interfere with the action of trastuzumab.⁶⁵ Furthermore, the Met receptor tyrosine kinase has also been reported to contribute to trastuzumab resistance.⁶⁶ These data suggest that a variety of cell surface receptors, other than HER2, and/or its downstream signaling proteins are likely to influence sensitivity to trastuzumab. Comparing the sensitivity of 18 breast cancer lines to trastuzumab, Ginestier et al. found that sensitivity to trastuzumab was frequently associated with the expression of a phosphorylated ERBB2 protein.⁶⁷ Another potential mechanism of resistance is the accumulation of truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain. This form, known as p95HER2, is frequently found in HER2-expressing breast cancer cell lines and tumors. Scaltriti et al.⁶⁸ demonstrated that cells that expressed p95HER2 were resistant to trastuzumab, but remained sensitive to lapatinib both in vitro and in vivo.

Regarding intracellular signaling, various reports suggest that alterations in specific pathways can be associated with resistance to trastuzumab. A loss of RALT/MIG-6, a transcriptionally controlled feedback inhibitor of ErbB receptor tyrosine kinases, was found to favor resistance to trastuzumab.⁶⁹ T-DARPP, a protein associated with ERBB2, has been shown to regulate sensitivity to trastuzumab in preclinical breast cancer models.⁷⁰ In a cohort of 55 breast cancer patients, activation of the PI3K pathway, as judged by the presence of oncogenic PIK3CA mutations or low PTEN expression, was associated with poor prognosis after trastuzumab therapy.⁷¹ Interestingly these factors are similar to those identified by a genome wide scan of factors involved in resistance to lapatinib, a small molecule inhibitor of HER2 tyrosine kinase.⁷² These data also confirm previous results showing that PTEN is involved in sensitivity to trastuzumab.⁷³

Strategies to Circumvent Resistance to Monoclonal Antibodies

Current data suggest that resistance to therapeutic MAbs is multifactorial and is likely to involve, among other parameters, host-related effector mechanisms, altered interaction with the target, cross-talk between cell survival pathways and involvement of antiapoptotic proteins. It is highly likely that most resistance events downstream of the interaction with the target antigen will be redundant with those observed with small molecule tyrosine kinase inhibitors, and that several will be similar to those already reported with cytotoxic agents. Insofar as therapeutic MAbs will most commonly be used in combination regimens, avoiding or overcoming resistance will thus involve the simultaneous targeting of non-redundant death-inducing pathways, or the neutralization of compensatory mechanisms.

Several strategies have been proposed to increase rituximab activity or to revert resistance to rituximab. An elegant approach has consisted in the physical costimulation of CD20 and another cell surface antigen, either with a multivalent mAb or with a

recombinant protein. Fas, CD22 and TRAIL have thus been shown to be potential co-targets of CD20.^{74,75} Simultaneous targeting of two antigens with two antibodies is also an option and rituximab combined with epratuzumab, a CD22-directed antibody, demonstrated promising antilymphoma activity in a study conducted in patients with recurrent or refractory non-Hodgkin lymphoma.⁷⁶ Preclinical as well as clinical data suggest that simultaneous targeting of CD20 with rituximab and CD52 with alemtuzumab could also constitute a way to enhance antilymphoma activity.^{27,77,78} Another possibility is to potentiate cellular effector mechanisms using cytokines or growth factors. The feasibility of this approach, using GM-CSF, has recently been reported.⁷⁹ Other studies evaluated the combination of rituximab with interferon- α (INF α),^{80,81} interleukin-12 (IL-12),⁸² IL-2,⁸³ in order to enhance effector immune cells. Further elucidation of multiple mechanisms of action and critical signaling pathways involved in rituximab cytotoxicity will help to overcome resistance.

Novel MAbs are currently undergoing pre-clinical and clinical investigation. GA101 is a fully humanized anti-CD20 with a glyco-engineered Fc portion and a modified elbow hinge. Its glycoengineered Fc region binds with 50-fold higher affinity to human Fc γ RIII receptors compared to a standard, non-glycoengineered antibody such as rituximab. This modification has led to complete responses and long-term survival in xenograft models of diffuse large B cell lymphoma and mantle cell lymphoma⁸⁴ and has been shown to be more active than rituximab on RL xenografts at similar doses, either administered as a single agent or in combination with cyclophosphamide.⁸⁵

Novel therapeutic strategies are underway to improve response rates in HER2-overexpressing and in trastuzumab-refractory patients. Pertuzumab, belonging to a new class called dimerization inhibitors that can inhibit signaling by other HER family receptors, as well as inhibiting signaling in cells that express normal level of HER2. It can also disrupt interaction between HER2 and IGF-IR in trastuzumab-resistant cells.⁸⁶ Recently, studies have suggested that the dual HER2/EGFR tyrosine kinase inhibitor lapatinib targeted against both EGFR and HER2 inhibited the growth of HER2-overexpression breast cancer cells in patients receiving prolonged treatment with trastuzumab,⁸⁷ and inhibited insulin-like growth factor I (IGF-I) signaling in resistant cells.⁸⁸ This type of approach has potential in HER2-overexpressing breast cancers, as well as in trastuzumab-refractory patients, and constitutes a novel strategy that cotargets the IGF-I receptor and HER2 pathways. Along the same line, novel IGF-IR targeted agents and PI3K inhibitors are currently studied for potential use in trastuzumab refractory patients.

New strategies have also been developed to optimize the therapeutic effects of EGFR inhibitors, by exploring new EGFR-targeted mAbs such as panitumumab⁸⁹ or matuzumab.⁹⁰ Several clinical studies are ongoing to evaluate the combination of cetuximab with bevacizumab (anti-VEGF Mab) after the encouraging preliminary results of Saltz et al.⁹¹ Another approach involves the association of cetuximab with small molecule tyrosine kinase inhibitors (TKIs) such as erlotinib,⁹² gefitinib⁹³ and lapatinib.⁹⁴

This combined approach appears to be an effective strategy to increase the inhibition of EGFR autophosphorylation, cellular proliferation and downregulation of signaling pathways. However, the development of acquired resistance in treated patients reduces the efficiency of these agents, emphasizing the need to elucidate the molecular mechanisms of resistance.

Conclusion

Resistance to therapeutic monoclonal antibodies involves tumor-related and host-related factors. Determining clinically relevant resistance mechanisms is made difficult by the fact that several mechanisms of action are likely to be involved in the antitumor effect and that the antibodies are often used in combination with other agents. It is increasingly clear that resistance to mAbs will at least partly overlap resistance to conventional or novel small molecule anticancer agents. These findings underline the importance of understanding common resistance mechanisms and developing potent agents able to prevent the activation of survival pathways.

Issues common to several, if not all, therapeutic mAbs involve the importance of the immunocompetent status of patients receiving therapeutic mAbs. While current immunomonitoring methods are not yet sufficiently standardized to adequately evaluate immunocompetence on a routine basis, it is likely that such a pretherapeutic evaluation will be useful in the future to define which patients are most at risk to benefit from or fail mAb therapy. Another important question concerns the potential impact of medications associated with therapeutic mAbs. For example steroids, potent immunosuppressive agents, are likely to reduced the role of accessory cells. It will be important to determine whether these or other associated agents influence the cytotoxic effect of mAbs in the clinic.

In spite of these limitations, our understanding of how and why therapeutic mAbs work or fail has made tremendous progress in the short period since these agents have become available. Further investigations will contribute to the development of more potent antibodies, sensitization strategies and optimal choice of therapeutic mAbs in individual patients in clinico.

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Conflicts of interest

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Chapter III. Sonoporation

Suspension cell lines of lymphoid origin are difficult to transfect. Conventional transfection methods such as electroporation, lipofection or nucleofection have proven to be unsatisfactory in the transfection of B-CLL cells and are often associated with a poor yield. In collaboration with INSERM U556, we determined the feasibility of using an US cavitation to transfect fresh CLL samples and the human RL follicular lymphoma cell line. In this chapter, we will present an overview of all transfections methods; then, we will focus on the mechanism as well as the *in vitro* and the *in vivo* application of sonoporation.

1. Introduction

Gene therapy became a new particularly attractive approach, as it stands as a direct bridge between molecular biology discoveries and potential clinical treatments. Gene therapy is the ultimate tool in order to cure inheritable and acquired diseases in a straightforward manner by correcting their abnormalities in genes. Thus, this innovative therapy involves the introduction of genetic material into human tissues. The transferred DNA can replace a mutated or absent genes, enhance or inhibit a preexisting cell function, or introduce completely a new function into the cell. Various protocols have been tried so far to cure a disease by adding the wildtype gene or correcting the mutation in the gene. However, the success of gene therapy is largely dependent on the development of a vector that selectively and efficiently delivers a therapeutic gene in a specific cell population with minimal toxicity. The ideal vector for gene therapy would have at least the following characteristics: the specificity for the target cells, the resistance to metabolic degradation and/or attack by the immune system, the safety in gene delivery with minimal side effects and finally, an ability to express the therapeutic gene for as long as required.

2. Transfection methods

Gene therapy strategies can be roughly divided into two broad categories: viral and non-viral gene vector [277]. Viral vectors implicated viral-mediated processes referred to as infection. Retroviruses and adenoviruses are the most widely used in gene therapy applications and currently applied in clinical research [278]. They offer several advantages, but also many undesired side effects, such as the induction of toxicities, the development of

severe immunological reactions (for adenoviruses) and the potential induction of new mutations (for retroviruses) [279]. Non-viral gene transfer, or transfection, involves the treatment of cells by chemical or physical means. These are represented by lipofection, electroporation, particle bombardment and sonoporation.

2.1. Lipofection

Lipofection is one of the most widely used transfection method of transferring genetic material into living cells. Essentially, cationic lipids encapsulate the negatively charged DNA and facilitate transfer of the gene through the cell membrane.

The efficiency of lipid-mediated gene transfection is dependent on several steps, including the adsorption of the transfection complex to the cellular surface and subsequent uptake of the complex by cells, the escape from the intracellular endosomes/lysosomes, then translocation across the nuclear membrane and into the cell nucleus where transcription occurs. Of these steps, nuclear translocation of genetic material is arguably the critical and limiting step for successful transfection. Until now, lipofection allows high transfection rates with minimum cellular toxicity in some cellular types. However, efficiency and targeting remain extremely poor in some hard-to-transfect cells and do not allow the control of spatial or temporal specificity [280].

2.2. Electroporation

Electroporation is a physical process that transiently permeabilizes the cell membranes with an electrical pulse, thus permitting cell uptake of a wide variety of biological molecules [281]. The efficiency of gene transfer by electroporation is influenced by several physical (especially, pulse duration and electric field strength) and biological (including DNA concentration and conformation, cell size) factors [281, 282]. Major disadvantages of electroporation are the low transfection efficiency of primary cells and high cell mortality [278].

2.3. Nucleofection

Nucleofection is a new highly effective non-viral method for gene transfer into primary cells developed by Amaxa Biosystems [278]. This method is an electroporation-based method in which a combination of a specific nucleofector solution and specific electrical parameters achieve delivery of plasmid DNA into the cell nucleus. It was successfully applied to hematological and immunological cells [283-286] and to embryonic

and adult stem cells [287, 288]. This method is safe, easy, reproducible and fast. However, large amounts of cells (minimum 1×10^6) are needed for nucleofection, the transfection efficiency depends on the cellular type and a decrease of expression can be observed in long-term experiments [289].

2.4. Particle bombardment

Particle bombardment represents yet another way of “injecting” foreign DNA into cells, by coupling the gene to projectiles that are made to penetrate the membrane at high speed [290]. The efficiency of this delivery technique depends on several parameters, such as the loading of DNA onto the particles, the particle size, and the timing of delivery. Moreover, the final distribution of DNA-coated beads is influenced by the fine tuning of the acceleration imparted by the gene gun [291, 292]. However, this method appears to be limited to surface applications such as skin.

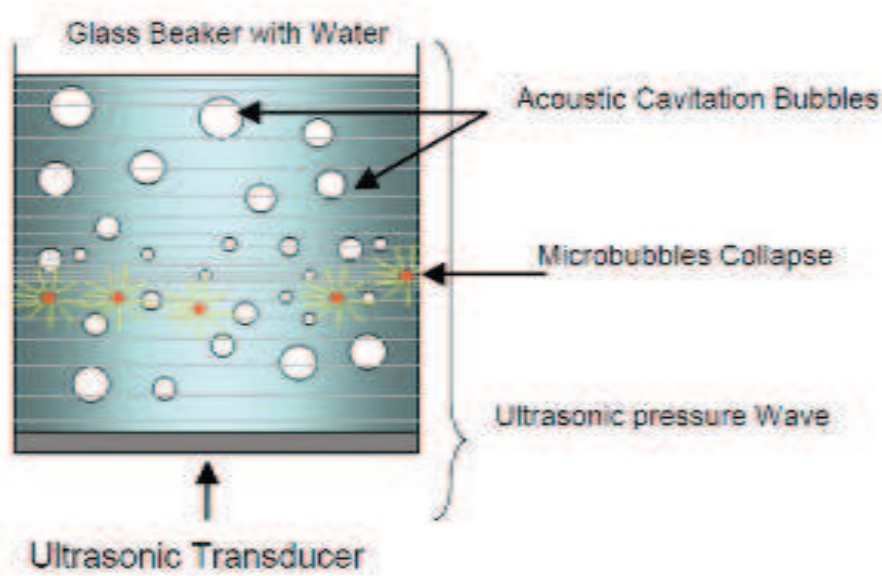
2.5. Sonoporation

Sonoporation is a relative newcomer in the field of gene transfer. It is designed to enhance cell permeability through the use of ultrasound (US). When permeabilizing the cell membrane, it allows the uptake of DNA and other molecules and can be focused on almost any location of the body. [293] Sonoporation combines the capability of enhancing gene transfer with the possibility of restricting this effect to the desired area and at the desired time; thus allowing for spatial and temporal specificity without the side effects of other transfection agents.

2.5.1. Mechanism of sonoporation: Acoustic cavitation

Sonoporation is a relative newcomer in the field of gene transfer. It is designed to enhance cell permeability through the use of ultrasound (US). When permeabilizing the cell membrane, it allows the uptake of DNA and other molecules and can be focused on almost any location of the body [294, 295]. Sonoporation combines the capability of enhancing gene transfer with the possibility of restricting this effect to the desired area and at the desired time, thus allowing for spatial and temporal specificity without the side effects of other transfection agents.

a)



b)

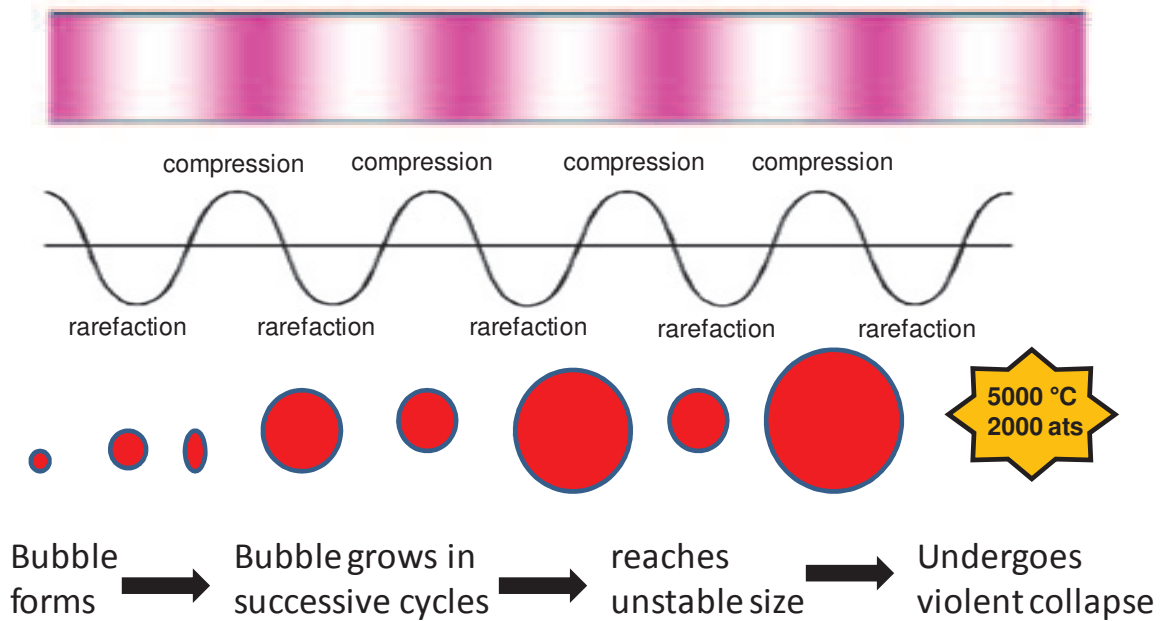


Figure 9: Acoustic cavitation bubbles.

Figure 9a shows an example of an ultrasonic transducer and glass beaker with water. Cavitation occurs when the transducer is generating sufficient amplitude of US. A range of US which can produce cavitation is within a wide range of frequencies (e.g. 0.02 to 3 MHz). Figure 9b shows the formation of acoustic cavitation bubbles in more detail. The ultrasound causes variations of pressure within the fluid. These bubbles will grow over the period of a

few cycles to an unstable size for the frequency applied. Bubble formation is affected by the acoustic pressure within the bubble when the size of bubble increases but the pressure inside the bubble will decrease [296]. If the bubbles reach a maximum radius and unstable size, the bubbles will then collapse in the following compression cycle. Cavitation creates small shock waves which can cause cell death but will potentially increase cell permeability if controlled [297].

Sonoporation is thought to induce the transient formation of small pores in the cell membrane allowing the direct transfer of genetic material into cells [298, 299]. Small compounds [300], macromolecules [301, 302] and other therapeutic compounds [300, 303], proteins [304] and DNA [305] into tissues have been delivered into cells using US. Moreover, low and high-frequency US treatment of cells in the presence of plasmid DNA has been shown to cause mammalian cell transfection *in vitro* [306, 307] and *in vivo* [308, 309]. However, the biological structure of the pores is unknown. Brayman *et al.* suggested that the cell structure may influence the degree of membrane poration and the ability of insonified-cells to survive [310]. Moreover, many studies highlighted a relationship between molecular size and uptake. Thereby, the amount of internalized molecules per cell was inversely proportional to their size, presumably reflecting the transient existence of the pores, and favouring the entry of smaller, more mobile species. In addition the pore size distribution is likely to be heterogeneous so that small pores are probably more abundant than large ones leading to more efficient intracellular delivery of small particles [299, 311].

The US generated pores on the plasma membrane must reseal to prevent the loss of intracellular contents to ensure cell survival. Furthermore, the repair of the membrane [312] serves as the triggering sources for other irreversible and reversible cellular processes such as apoptosis [313] and calcium oscillation [314]. Thus, the rate of membrane resealing is one of the key factors determining the uptake efficiency and post-US cell fate. Recent results showed that Ca^{2+} plays an important role in regulating membrane resealing in sonoporation. The pore resealing exhibited multiple Ca^{2+} dependent processes with different characteristics and rate constants, including an early fast recovery, followed by slower, late-stage recovery process [312].

Altogether, a good optimization of US parameters is a challenge in order to obtain a high transfection rate and to maintain good cell viability. Thereby, too much or too little cavitation leads to either reduced transfection efficiency or cell damage.

2.5.2. *In vitro* and *in vivo* application of sonoporation

Studies concerning the use of sonoporation have grown rapidly in recent years. The use of sonoporation systems in biotechnology and medicine has led to new methods of gene therapy, drug delivery and cancer treatment. Enhanced gene transfer is found either when the exposed bubbles are in the vicinity of the genetic material, or when the genes are encapsulated within, or bound to bubbles. Microbubbles are encapsulated of gas bubbles that can be used as drug or gene carriers, to insert gene or plasmid through the cell membrane. The shell of these microbubbles is composed of proteins, lipids or polymers. These microbubbles are efficient reflectors of US, can expand and contract in response to the pressure changes in an US wave and thereby, they have been used to enable cavitation and achieve successful sonoporation and high cell viability. US has been shown to enhance gene transfer into cells *in vitro* [306, 307, 315-323] and *in vivo* [308, 321, 324-328]. Most of the local delivery systems tested so far provide a high local-to-systemic ratio at only one particular location and treating a localized disease. However, most of the pathological processes (myocardial ischemia, tumors, etc.), would require a diffuse treatment of the affected organ that can be achieved only by enhancing the transfer of injected molecules at the microcirculation level. Sonoporation combines the capability of enhancing gene transfer with the possibility of restricting this effect to the desired area, at the desired time. For instance, sonoporation enhanced by the use of microbubbles was applied to promote pDNA/siRNA transduction to adult murine heart [329]. The ultrasound mediated with microbubble method was also applied to skeletal muscle of mice [330] rat myocardium as well as *in vivo* [326].

Chemotherapy plays a very important role in cancer treatment. However the application of anticancer agents is hampered by their adverse effect on normal tissues. US exposure can enhance the cytotoxicity of anticancer chemicals to cancer cells *in vitro*. Indeed, it has been shown that sonication can synergize the effects of chemotherapeutic drugs such as adriamycin, cisplatin, 5-fluorouracil (5-FU), arabinosyl cytosine (AraC), cisplatin and others [331, 332] in ovarian cancer cells, breast cancer cells, cervical cancer cells, leukemic cells and other cell types [333-335].

Moreover, a synergism between anticancer drugs and US exposure *in vivo* has been demonstrated. The co-administration of anticancer agents and US suppressed tumors more significantly than either drugs or US alone. Examples of drugs which could be efficiently synergized by ultrasound exposure *in vivo* are Adriamycin, 5-FU, HB, AraC and bleomycin [334-337]. Sonoporation can also trigger apoptosis in both normal and malignant cells. US-induced cell death has been confirmed in leukemia cell lines K562, HL-60, KG1a, Nalm-6

and U937 [313, 338, 339]. Finally, this field is continuously expanding and new clinical applications are being developed constantly.

PERSONAL RESULTS

Chapter IV
Transfection of B-CLL cells with
ultrasound

Objectives of this study

Gene therapy is anticipated to provide an effective treatment for various diseases, such as cancer. One critical factor that hampers the development of successful gene therapy is to design an ideal vector system allowing good transfection efficiency in the targeted tissue with minimal damage. Many different techniques have been developed including the use of viral and non-viral vectors both *in vivo* and *in vitro* and demonstrated relatively good efficacy. The retroviruses and adenoviruses are the most commonly used vectors in the viral-mediated processes; however, these systems have the potential to cause immune and/or toxic reactions. Non-viral gene transfer, or transfection, involves treatment of cells by chemical or physical means. These methods include lipofection, naked DNA injection, electroporation, particle bombardment and nucleofection. Chemical methods cover an array of complexes between DNA and diverse polycations (“polyplexes”) or cationic lipids (“lipoplexes”). This method is relatively easy, but the efficiency and targeting remain extremely poor. A naked injection, without any carrier, into local tissues or into the systemic circulation is a simple and safe ‘physical or mechanical’ approach; however, the rapid degradation by nucleases and the fast clearance by the mononuclear phagocyte system limit severely this method. Electroporation is a common physical tool that causes transient and localized destabilization of the cell membrane through its exposure to high-intensity electrical pulses. The efficiency of gene transfer electroporation is influenced by physical parameters such as pulse duration and electric field strength and biological factors such as DNA concentration and conformation, and cell size. Particle bombardment or gene gun can propell the naked DNA plasmid into target cells on an accelerated particle carrier. The major application of this technology is genetic immunization with the most obvious target being the skin [340]. Nucleofection is an electroporation-based method in which a combination of a specific nucleofector solution and specific electrical parameters achieve delivery of plasmid DNA into the cell nucleus [278]. It was successfully applied to hematological and immunological cells [283-286] and to embryonic and adult stem cells [287, 288]. However, large amounts of cells (minimum 1×10^6) are needed for nucleofection, the transfection efficiency depends on the cellular type, and a decrease of expression can be observed in long-term experiments [289].

Overall these methods are less effective for gene transfer than viral vectors, often induce transient gene expression and are also limited by issues of spatial or temporal specificity. Using these various transfection systems, a large number of adherent cell lines including some types of primary cells are easily transfected, either with plasmids or with

silencing RNA (siRNA). Conversely other lines, including a majority of suspended cell have proven hard to transfect.

While novel lipofecting agents and nucleofection have contributed to resolving this issue, the transfection of suspended cells remains difficult. The development of an efficient, spatially and temporally targeted DNA delivery method is thus clearly needed.

Recently, sonoporation has been increasingly reported in the literature as a means of stimulating cell membrane permeabilization for the purposes of transferring nucleic acids into cells. This method offers advantage over its competing technologies, primarily because of its relatively non-invasive nature and its spacio-temporel control. While the mechanisms of sonoporation are not yet completely understood, several studies have been carried out both *in vitro* [294, 315] and *in vivo* [309, 321] and have shown promising results. It is generally assumed that ultrasound-mediated gene transfer is principally due to acoustic cavitation [294]. Sonoporation may increase cell membrane permeability by inducing transient nonlethal perforations in cells and other membranes [341-343], which allow the entrance of large molecules from the surrounding medium into the cell. Under optimal conditions, the cell can reseal its membrane and survive its holes without notable damage [298, 309, 310]. This possible self-sealing mechanism [344] is thought to involve lysosomal exocytosis and Ca^{2+} release. Several factors, including cellular architecture [310] and sonoporation parameters [345-348] may influence the degree of membrane permeabilization and cell viability after sonoporation. To date, most cell lines that have been successfully transfected with ultrasound have been adherent cells [307, 349, 350]. Few attempts to porate cells in suspension have been reported [351, 352]. These latter attempts were mostly performed using microbubbles known as contrast agents and showed an enhancement in transfection efficiency. Many types of molecules, such as plasmid DNAs [309, 343], siRNAs and peptides [352] have been demonstrated to be delivered into cells by ultrasound both *in vitro* and *in vivo*.

Transfection of B-CLL cells is technically challenging and represents a major obstacle in CLL research. In my thesis project, my goal was to transfect these cells with siRNA targeting the anti-apoptotic members of the Bcl-2 family. By targeting these proteins, we can determine if these proteins exert an endogenous resistance mechanism thereby confer resistance to anti-CD20 MAbs. For this purpose, we describe the use of an US device to transfect stably or transiently cells in suspensions with nucleic acid in a human Follicular Lymphoma (FL) cell line (RL) and in Chronic Lymphocytic Leukemia (CLL) cells freshly isolated from patients. We optimized nucleic acid delivery by determining all parameters such

as US parameters, the duration of exposure, number of cells and DNA concentrations in order to obtain a high transfection efficiency and low mortality rate. Therefore, we evaluated the possibility of using US to perform transient transfection of plasmid DNA and siRNA, as well as the possibility to obtain stably transfected cells. The results from this work have been published in the article that follows on the next pages.

Article I

Transfection of cells in suspension by Ultrasound cavitation

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Transfection of cells in suspension by ultrasound cavitation

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ABSTRACT

Sonoporation holds many promises in developing an efficient, reproducible and permanent gene delivery vector. In this study, we evaluated sonoporation as a method to transfect nucleic acids in suspension cells, including the human follicular lymphoma cell line RL and fresh human Chronic Lymphocytic Leukemia (CLL) cells. RL and CLL cells were exposed to continuous ultrasound waves (445 kHz) in the presence of either plasmid DNA coding for green fluorescent protein (GFP) or fluorescent siRNA directed against *BCL2L1*. Transfection efficiency and cell viability were assessed using fluorescent microscopy and flow cytometry analysis, respectively. Knock-down of target protein by siRNA was assessed by immunoblotting. Moreover, sonoporation was used to stably transfect RL cells with a plasmid coding for luciferase (pGL3). These cells were then used for the non-invasive monitoring of tumorigenesis in immunodeficient SCID mice. Sonoporation allows a highly efficient transfection of nucleic acid in suspension cells with a low rate of mortality, both in a tumor cell line and in fresh human leukemic cells. It also allowed efficient transfection of *BCL2L1* siRNA with efficient reduction of the target protein level. In conclusion, ultrasound cavitation represents an efficient method for the transfection of cells in suspension, including fresh human leukemic cells.

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1. Introduction

Transfection of cells constitutes an essential tool for the understanding of cell biology and therapeutic modulation of gene expression. A variety of DNA delivery methods are being tested in nucleic acid therapy, both to induce expression of a deficient gene or to repress the expression of a target gene. A variety of transfection methods including viral and non viral vectors have been used to transfect nucleic acids into mammalian cells. Viral vectors such as retroviruses and adenoviruses have been shown to be efficient in transfection [1]. However, these viral vectors present some drawbacks such as lack of site specificity, potentiality for insertional mutagenesis [2], induction of immunological responses and systemic toxicity. Non viral methods [3] have also been developed such as naked plasmid DNA injection, electroporation, particle bombardment, lipofection and nucleofection. Overall these methods are less effective for gene transfer than viral vectors, often induce transient gene expression and are also limited by issues of spatial or temporal specificity.

Using these various transfection systems, a large number of adherent cell lines including some types of primary cells are easily transfected, either with plasmids or with silencing RNA (siRNA). Conversely, other

lines, including a majority of suspended cells have proven hard to transfect. While novel lipofecting agents and nucleofection have contributed to resolving this issue, the transfection of suspension cells remains difficult. The development of an efficient and if possible spatially and temporally targeted DNA delivery method is thus clearly needed.

Sonoporation is a recently developed technology enhancing cell membrane permeability which has been applied to improve the uptake of DNA and drugs by mammalian cells [4]. While the mechanisms of sonoporation are not yet completely understood, several studies have been carried out both *in vitro* [5,6] and *in vivo* [7,8] and have shown promising results. It is generally assumed that ultrasound (US)-mediated gene transfer is principally due to acoustic cavitation [6,9]. Sonoporation may increase cell membrane permeability by inducing transient non-lethal perforations in cells and other membranes [10–12], which allow the entrance of large molecules from the surrounding medium into the cell [13–15]. Under optimal conditions, the cell can reseal its membrane and survive its holes without notable damage. The self-sealing mechanism is one of the key factors that determine the transfection efficiency and post-ultrasound cell outcome. It is thought to involve lysosomal exocytosis and Ca^{2+} release [16]. This delivery of Ca^{2+} is necessary to avoid the intracellular overload of ions that might trigger many cellular processes such as apoptosis [17] and calcium oscillation [18,19].

Furthermore, several factors, including cellular architecture [20] and sonoporation parameters [21–25] may influence the degree of

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membrane permeabilization and cell viability after sonoporation. To date, most cell lines that have been successfully transfected with US have been adherent cells [26–28] whereas only few attempts to porate cells in suspension have been reported [29,30]. These latter attempts were mostly performed using microbubbles known as contrast agents and showed an enhancement in transfection efficiency. Many types of molecules, such as plasmid DNAs [7,12], siRNAs and peptides [30] have been demonstrated to be delivered into cells by US both *in vitro* and *in vivo*.

Based on the cavitation produced by US, a key advantage of this method is its potential for spatial and temporal control. Its specificity resides in combining the capacity of enhancing transfection efficiency with the possibility of restricting the effect of US to the desired area during the desired time. This study was designed to investigate the possibility of delivering nucleic acid stably or transiently with an US device in a human Follicular Lymphoma (FL) cell line (RL) and in Chronic Lymphocytic Leukemia (CLL) cells freshly isolated from patients. Using a 445 kHz transducer, we varied US parameters, the duration of exposure, number of cells and DNA concentrations to optimise nucleic acid delivery with minimal impact on cell viability. We evaluated the possibility of using US to perform transient transfection of plasmid DNA and siRNA, as well as the possibility to obtain stably transfected cells.

2. Materials and methods

2.1. Cell line and culture

2.1.1. *In vitro* studies

In vitro studies were performed on RL follicular lymphoma cells (obtained from the American Type Culture Collection) and on fresh blood specimens from CLL patients. Patients gave written informed consent after approval of the study protocol by the Institutional Review Board of the Hospices Civils de Lyon. RL follicular lymphoma cells (4.10^6) were incubated in 2 ml RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 UI/ml of penicillin and 200 µg/ml of streptomycin. All reagents were purchased from Invitrogen (Carlsbad, CA, USA).

CLL cells were isolated from peripheral blood mononuclear cells by density gradient centrifugation using Histopaque (PanColl human, PAN Biotech). Briefly, blood was diluted in Phosphate Buffered Saline (PBS) then layered over Histopaque and was centrifuged at 300 g for 20 min at room temperature. The gradient interface was harvested and was diluted 3-fold with PBS. The cell suspension was washed 3 times by repeated centrifugation at $300 \times g$ for 10 min and was resuspended in RPMI 1640 media supplemented with 10% FCS, 200 UI/ml of penicillin and 200 µg/ml of streptomycin. Cell viability was evaluated by trypan blue dye exclusion.

2.1.2. *In vivo* studies

Four week-old female CB17 SCID (Charles River laboratories, L'Arbresle, France) were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. This study was approved by the local animal ethical committee. Development of RL derived tumors in SCID mice was obtained by subcutaneous injection of 1.10^6 RL cells. The tumor volume was calculated by the formula of $4/3 (3.14 \times r^3)$. For *in vivo* imaging, the mice were anesthetized with isoflurane and oxygen. Mice were subjected to *in vivo* bioluminescence imaging using the Nightowl™ image system (Berthold, France) immediately after injection of D-luciferin solution intraperitoneally (150 µl at 5 mg/ml in PBS.). Animals were placed in the imaging cabinet and images were acquired at high resolution (8×8 Pixel

binning) for an exposure time of 2×2 min. The results were quantified using WinLight software (Berthold, France).

2.2. Cavitation device

A 20 mm diameter flat transducer (LT01 EDAP, based on a piezoceramic element P 7.62, Saint-Gobain, France) was submerged in a rectangular water bath filled with warm degassed water (20 l; O₂ concentration: 3 mg/L; temperature: 37 °C) to 14 mm above the top of the transducer aperture which was in a horizontal position (Fig. 1). The acoustic excitation was a continuous sine-wave at 445 kHz. The signal (generated by a PXI-6711 card, National Instruments) was amplified by a power amplifier (200 W, Adece) before feeding the transducer. The spatial average acoustic intensity (measured using the acoustic balance technique [31]) and the spatial peak acoustic pressure (hydrophone Lipstick GL-0200; SEA) did not exceed 1.7 W/cm² and 0.46 MPa respectively.

The bottom of each cell-containing well (12-well plates in polystyrene, 20 mm diameter wells, BD Biosciences) was aligned parallel to the transducer at 9 mm from its aperture. Its vertical position was adjusted so that the antinode plan was located at the air/culture medium interface, as described earlier [32]. The attenuation of the ultrasonic beam by the well bottom wall was less than 2%, as shown by Tata et al. [33]. The wells were exposed during time in the range of 30 to 120 s and no significant temperature increase in the medium was observed.

In order to control the bubble activity, a home-made hydrophone (cut-off frequency 10 MHz) [34] realized with a PVDF film (10 mm diameter) moulded in resin (AY103, Araldite) was placed near the ultrasound transducer pointing on the exposed medium volume (Fig. 1). As suggested by Frohly et al. [35], the cavitation index (CI) was defined as the mean of all acoustic spectrum power density points in dB over the range of 0.1 to 7.1 MHz (448 frequency points), normalized by the background noise recorded without transducer excitation. CI is slightly sensitive to harmonic peaks due to the presence of bubbles in medium, and mostly reflects the broadband noise due to inertial cavitation for CI values greater than 6. To perform the control of the bubble activity, regulation system was implemented, fixing CI to a chosen CI setpoint as follows. During sonoradiation the cavitation signal is saved by an acquisition card (PXI-5620, 14 bit resolution, 60 MHz sampling frequency, National Instruments). These data are transferred into a computer through a data bus (MXI-3 80 Mo/s, National Instruments). The CI value is calculated and compared to the desired CI setpoint. The transducer power is then readjusted by changing the excitation signal amplitude (Fig. 2). The timing is controlled by LABVIEW software (National Instruments) and

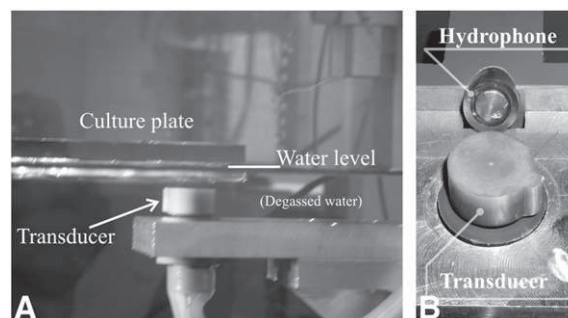


Fig. 1. Experimental setup: general design. Experimental setup was composed of a flat transducer, an acoustic sensor or hydrophone and a culture plate. A) Position of the transducer under the culture plate in a degassed water bath. B) Position and orientation of the acoustic sensor near the transducer.

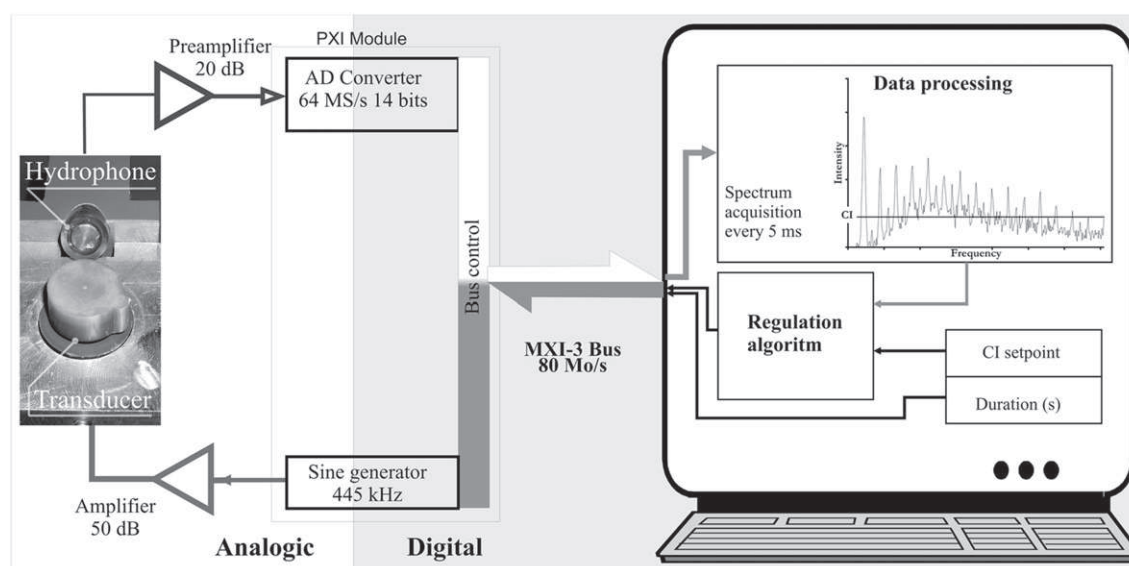


Fig. 2. Experimental setup: principle of ultrasound cavitation control. CI measure was compared to CI setpoint to adjust the acoustic power of a flat transducer by a feedback loop process.

the feedback loop rate was 200 Hz. CI oscillations were less than 10% in the present study.

2.3. Plasmid and siRNA transfection

Fluorescent siRNA directed against *BCL2L1* (sense: 5'Alexa Fluor 488–GGG UUU GGA UCU UAG AAG AAG A–3'; antisense: 5' UCU UCU AAG AUC CAA AGC C–3') and AllStars Negative siRNA were purchased from Qiagen. The siRNA were suspended in the provided buffer solution and prepared following the manufacturer's instructions. pEGFP-C2 (BD Biosciences Clontech), pGL3 (Promega) and pcDNA3 (Invitrogen) plasmids were purified using PureLink Hypure Plasmid Filter purification kits following the protocol provided by the manufacturer.

Two ml of cell suspensions (2.10^6 cells/ml in RPMI supplemented with 10% of FCS) were placed in each well of the 12-well plates. pEGFP-C2 was added to the cell suspensions of RL and CLL at a final concentration of 25 μ g/ml and *BCL2L1* siRNA at 7.5 μ g/ml.

Optimal exposure conditions that maximized cell permeability and minimized cell death were identified. CI and US exposure time were optimized for each cell type. All experiments were conducted at 37 °C. Selection of stably transfected cells began after 72 h with continuous exposure to 1.2 mg/ml of G418. Individual clones were screened for luciferase activity and selected clones were injected subcutaneously into three mice.

In conditions where lipofectin (Invitrogen, Cergy Pontoise, France) was mentioned, it was added following the manufacturer's instructions.

2.4. Analysis of transfection efficiency and cell viability

Twenty-four and forty eight hours after sonoporation of pEGFP-C2 vector, GFP-positive cells were observed using an Olympus IX50 microscope at the excitation wavelength of 488 nm and photographed at Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France).

Cells were also analysed using a fluorescence-activated cell sorter (FACS) (FACS Calibur; Becton, Dickinson and Company, NJ). Results were expressed as a percentage of GFP positive cells using the software CellQuestPro (Becton Dickinson, San Jose, CA). This percentage was calculated on the basis of the total number of cells, including dead cells. However, debris destroyed during sonoporation was not included.

The cell suspension was washed twice with PBS. In order to assess cell viability, cells were incubated with 7-amino-actinomycin D (7-AAD, BD Pharmingen) according to the manufacturer's recommendations for 10 min (10 μ l for 1.10^6 cells) prior to the FACS analysis. After incubation, cells were washed with PBS and the pellet was resuspended in 200 μ l of PBS. Cells were then transferred into the cytometer where 10,000 events were analyzed for each sample. Fluorescence of GFP and siRNA was detected in FL1 channel while 7-AAD fluorescence was detected in FL3. Furthermore, in order to quench non-specific extracellular fluorescence and to confirm the intracellular delivery of siRNAs, we added Trypan blue (TB) dye (0.2%) (Sigma Aldrich), then we proceeded immediately to flow cytometric analysis.

2.5. Immunoblot analysis

CLL cells transfected with siRNA directed against *BCL2L1* or with scrambled control siRNA (negative siRNA control) were incubated for 48 h then lysed as previously described [36]. Briefly, 20 μ g of cell lysates were resolved on a 12% SDS-PAGE using an electroblotting apparatus (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Hybond-ECL, Amersham Corp). The membrane was blocked with blocking buffer (LI-COR Biosciences, Germany) for 1 h and subsequently incubated with the primary antibodies directed against Bcl2L1 (clone S18, Santa Cruz) or Bcl2 (clone 124; Dacco, Denmark) overnight at 4 °C. The non-specific binding of antibody was removed by washing with PBS (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated with the secondary antibodies (Goat anti mouse IRDye or Goat anti-Rabbit antibody from LI-COR Biosciences, Germany) for 1 h at room temperature. After extensive washing with PBS, membranes were analysed using the Odyssey infrared imaging system (LI-COR Biosciences, Germany). The expression levels of the protein were standardized against the expression level of β -Actin (clone AC-15, Sigma).

2.6. Statistical analysis

The statistical significance of the data was determined with a Student's *t*-test. $P < 0.05$, $P < 0.001$ and $P < 0.0001$ indicate a statistically significant (*), highly significant (**) and extremely significant

difference (***) , respectively. Student's *t*-test was used to identify differences between US-exposed cells and non-sonoporated cells (NS).

3. Results

3.1. Optimization of ultrasound-mediated transfection in vitro

In order to determine the effect of US intensity on the efficiency of transfection, target population cells were subjected to a variety of parameters. Cell viability was evaluated by 7-AAD uptake. In these experiments, cells in suspension (CLL and RL cells) were exposed to a CI ranging from 12 to 20 and US exposure times ranging from 20 to 100 s. After determining all parameters including cells number per well, medium and FCS volume, plasmids or siRNA quantity, temperature, exposure time and CI, we determined the optimal conditions for each cell type.

The number of cells was set to 4.10^6 cells per well in 2 ml of RPMI supplemented with 10% of FCS. The plasmid and siRNA concentration was 25 $\mu\text{g}/\text{ml}$ and 7.5 $\mu\text{g}/\text{ml}$, respectively and the temperature was set to 37 °C.

The CI and exposure time were varied to apply to the cell a trade-off between transfection efficiency and cell viability. For RL cells, we used different CI (12, 14, 16 and 20) and different exposure times (20, 30, 40 and 60 s). The percentage of GFP-positive cells, mean fluorescence intensity (MFI) and levels of cytotoxicity were measured on the total population of cells. When applying a high CI for RL cells (e.g., CI of 20), larger fractions of cells were transfected, however, cell viability correspondingly dropped. Histogram (Fig. 3B) showed that CI of 16 is the best CI ensuring good transfection efficiency and low cell mortality ($p < 0.05$). RL cells achieved an average of 15% of transfection efficiency detected by flow cytometry and they presented less toxicity (80% of viable cells) (Fig. 3A).

For exposure time optimization, the best irradiation times were 30 and 40 s at CI of 16; however, we sonoporated RL cells during 30 s to minimize cell death (Fig. 3C).

We included the CI of 20 in these two histograms (Fig. 3B and C) to show that cells could achieve highly significant percentage of transfection, however, they suffered an extremely significant level of mortality.

For CLL cells, all CI below 20 did not yield any transfection efficiency when observed by microscopy; therefore, we used the CI at 20, and investigated US exposure time ranging between 20 and 100 s. Fig. 4A shows fluorescent GFP positive cells (green). The transfection efficiency was increased in an exposure time-dependent manner. The longer we exposed cells to US, the more we achieved GFP-positive cells. When cells were sonoporated during 60, 80 and 100 s, we found a significant difference in transfection efficiency calculated as percentage and MFI of GFP-positive cells (Fig. 4B, 4C) compared to non-exposed control; moreover, a statistically significant increase of mortality calculated as percentage and MFI of 7-AAD positive cells when exposure time was increased to 100 s compared to non-exposed cells (Fig. 4B, C).

3.2. Production of stably transfected RL clones

RL cells cotransfected with pGL3 and pcDNA3 plasmids were selected by prolonged exposure to G418. Bioluminescent resistant clones were selected in a 96 well-plates using the Nightowl imaging system immediately after adding D-luciferin into wells as shown in Fig. 5A. These positive clones were then injected subcutaneously into SCID mice. Tumors developed 21 days after implantation of bioluminescent cells, as shown in Fig. 5B. A colour enhanced overlay of the luminescent image over the photographic image demonstrates the location of the implants within the animal. These experiments demonstrate that sonoporation allows stable transfection of RL cells.

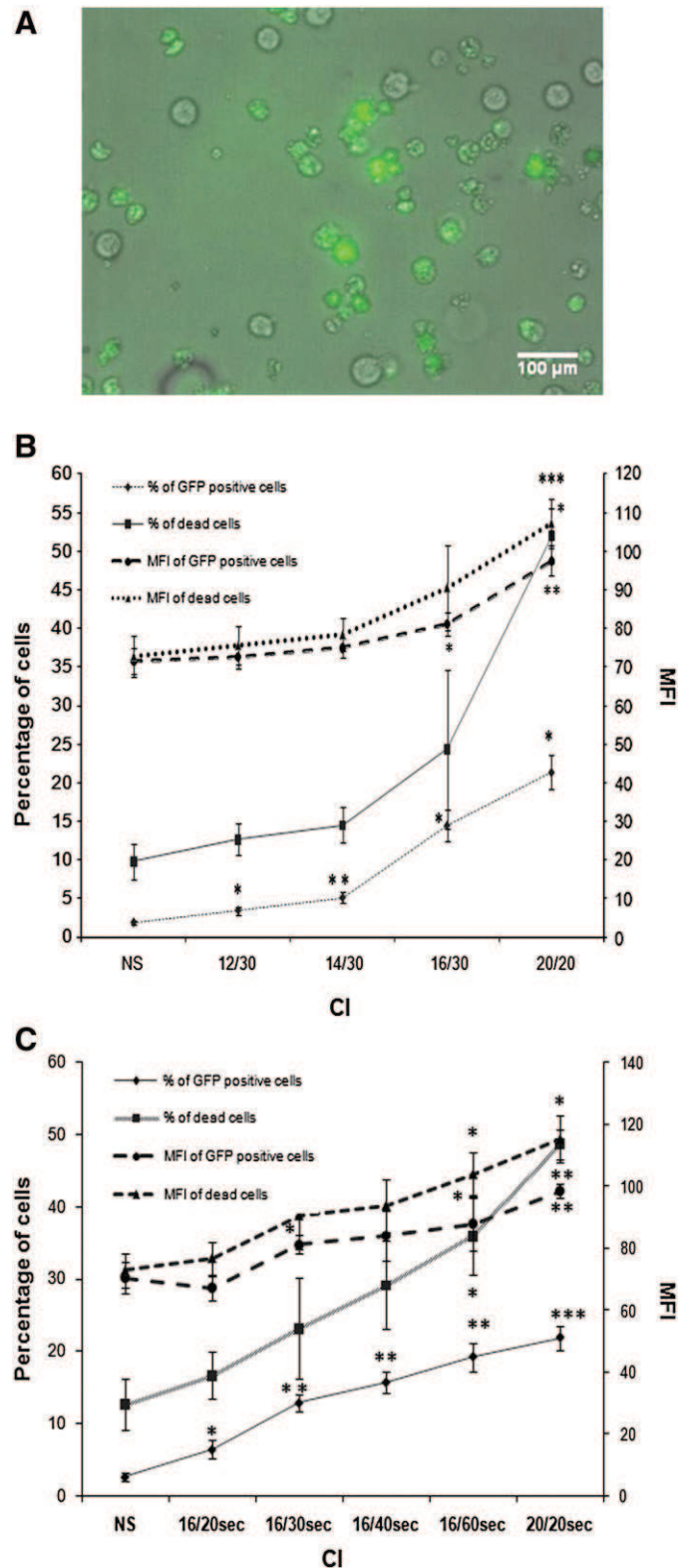


Fig. 3. Optimization of sonoporation parameters in RL cells. (A) Transfection efficiency of 50 μg of pEGFP-C2 in RL cells at a CI of 16 for 30 s at 445 kHz. The microscopy picture (objective 10 \times) corresponds to light transmission and fluorescence image taken after 48 h and overlaid using image J software (A). (B) Percentage and MFI of GFP-positive cells and dead cells at different CI during 30 s. (C) Percentage and MFI of GFP-positive cells and dead cells at different exposure times using the CI of 16. Cells were harvested after 48 h, stained with 7-AAD and analysed by flow cytometry. Each data point represents the average of 3 different measurements in RL cells. The error bars represent the standard deviations (SD). * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$.

3.3. Delivery of BCL2L1 targeted siRNA by sonoporation

After the determination of optimal transfection conditions, we investigated the possibility of using this method to introduce

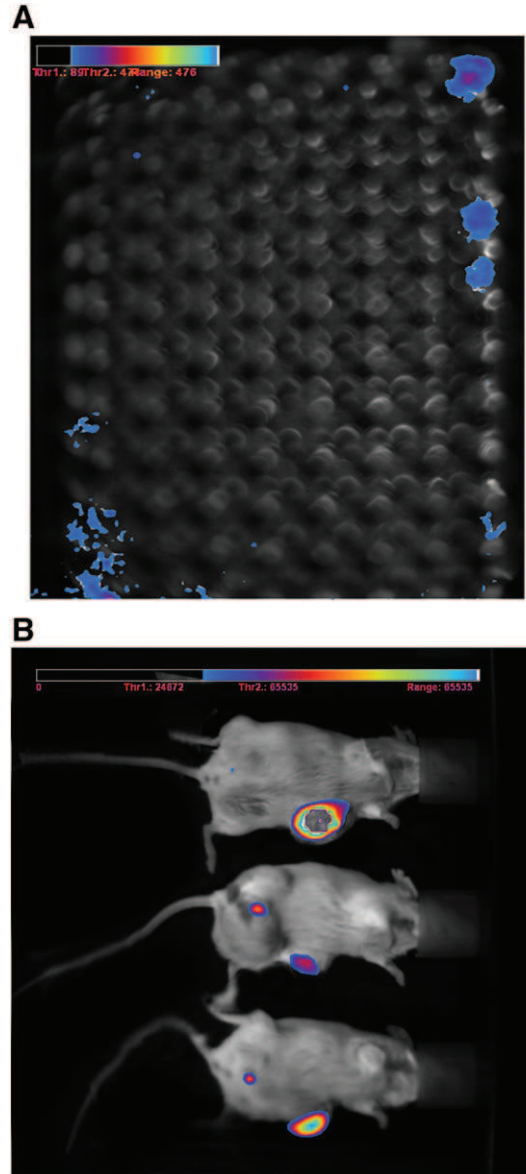
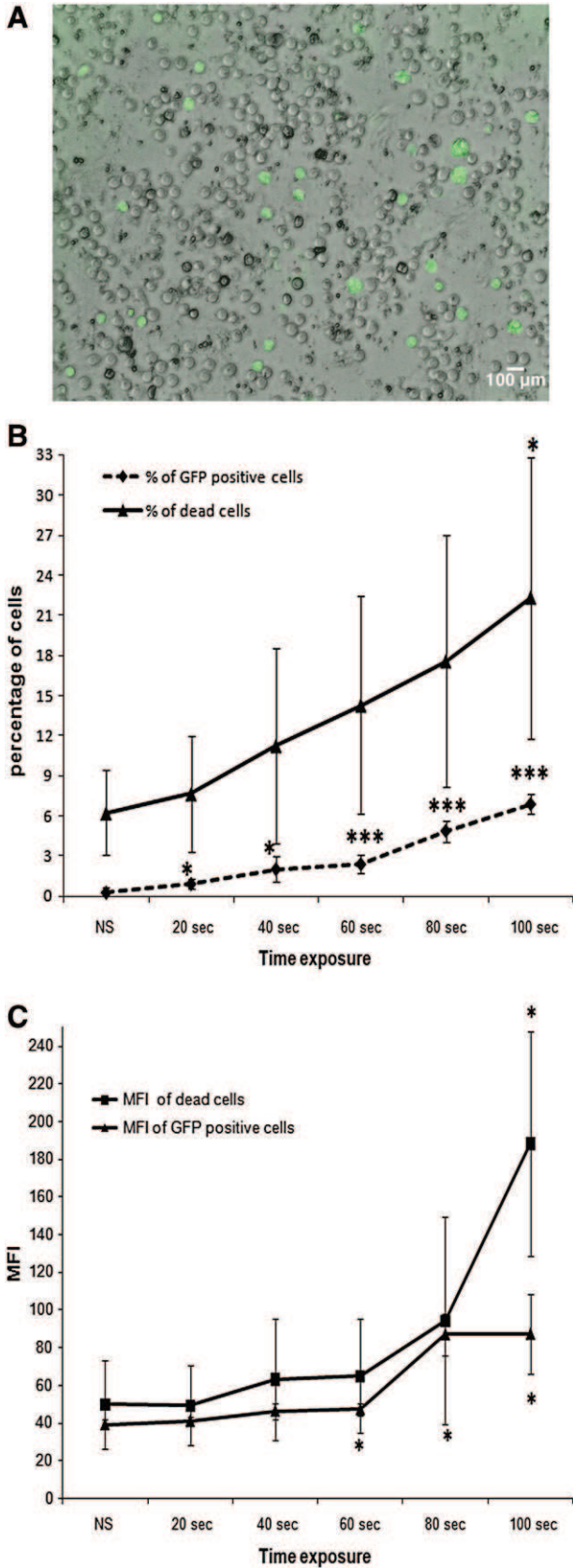


Fig. 5. Production of stably transfected RL cells by sonoporation. A Generation of stable RL-luc+ cells. RL cells were cotransfected with pGL3 and pcDNA3 plasmids. G418-resistant clones were isolated in microplate and their luciferase expression was screened using the Nightowl®. B Detection of tumor growth in 3 SCID mice. Representative images taken by the Nightowl® of bioluminescent RL implanted subcutaneously in mice.

fluorescent siRNAs into fresh CLL cells. BCL2L1, a member of the antiapoptotic BCL2 family members, was chosen as a target.

In Fig. 6A, non-sonoporated cells exposed to siRNA (panel 2) had increased fluorescence in comparison to the control (panel 1), probably due to non-specific binding of siRNA to target cell membranes. However, after exposure to US, we observed a significant increase in the count of fluorescent cells in sonoporated cells in comparison to non-sonoporated cells, confirming that sonoporation had enhanced intracellular penetration of siRNA (panel3).

Fig. 4. Optimization of sonoporation parameters in CLL cells. (A) Transfection efficiency of 50 µg of pEGFP-C2 in CLL cells at a CI of 20 for 80 s at 445 kHz. The microscopy pictures (objective 10×) correspond to light transmission and fluorescence images taken after 48 h and overlaid using image J software. (B) Percentage and (C) MFI of GFP-positive cells and dead cells at different exposure times at a CI of 20. Cells were harvested after 48 h, stained with 7-AAD and analysed by flow cytometry. Each data point represents the average of 4 different measurements in CLL cells. The error bars represent the standard deviations (SD). **P*<0.05, ***P*<0.001, and ****P*<0.0001.

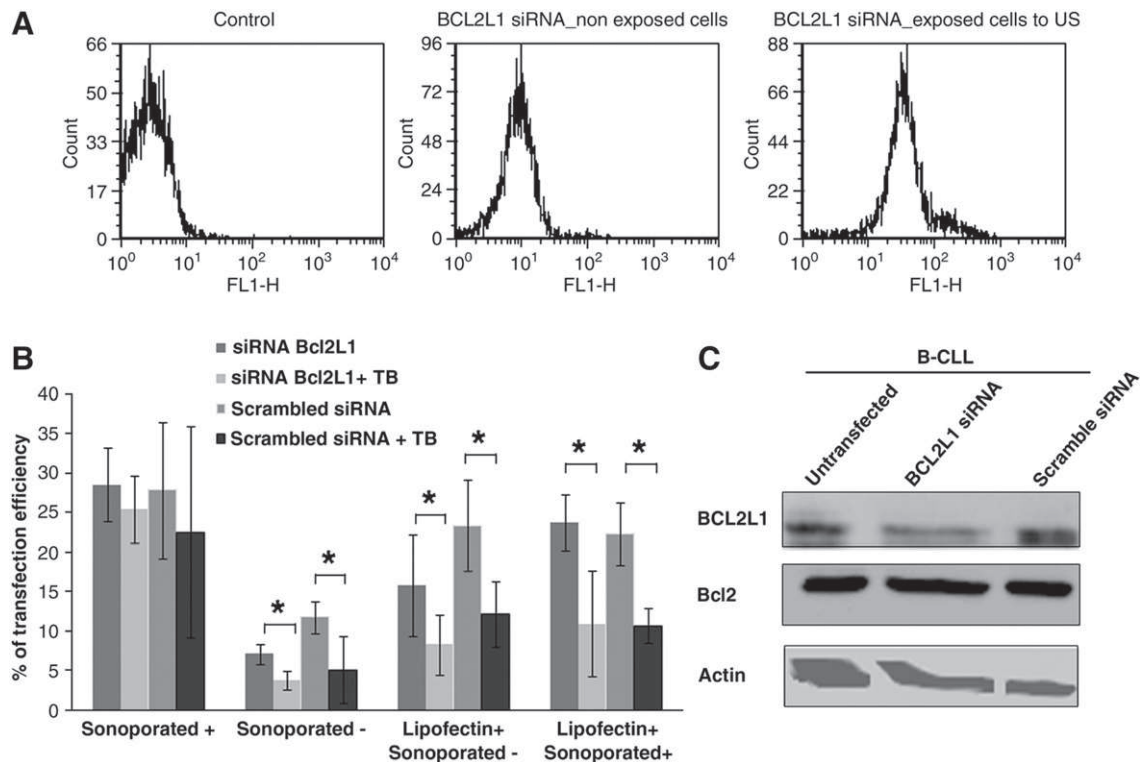


Fig. 6. Sonoporation of fluorescent siRNA into fresh CLL cells. A. Intracellular uptake of siRNA directed against *BCL2L1*. Histograms showing fluorescence (Alexa Fluor 488, FITC channel, relative units) of untreated cells (A1), non-sonoporated cells with *BCL2L1* siRNA (A2) and sonoporated cells with *BCL2L1* siRNA (A3) 48 h after sonoporation. Fluorescence was attributed to non specific binding of *BCL2L1* siRNA to target cell membranes in non sonoporated cells while enhanced fluorescence after sonoporation could be attributed to intracellular delivery of fluorescent siRNA. B. Histogram represents the transfection efficiency of cells treated with *BCL2L1* siRNA, scrambled siRNA with or without sonoporation, before and after quenching with trypan blue (TB) (0.2%), with or without lipofectin. The data indicate the mean \pm SD calculated from three different experiments. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. C. Effect of *BCL2L1* siRNA on Bcl2L1 protein content in CLL cells. Cells were sonoporated either in presence of siRNA targeting *BCL2L1* or in presence of scramble siRNA. The expression of the protein level was explored by western blot analysis using specific antibodies. Results are representative of three independent experiments.

We also determined the percentage of transfected cells after quenching the extracellular fluorescence with trypan blue (TB). These experiments were performed along with a lipofectin reagent as a classical control of transfection either alone or combined with siRNAs. Results showed that *BCL2L1* siRNA and scramble siRNA are localized extracellularly in cells because they were not quenched with TB; however, when they were combined with lipofectin either with or without sonoporation, significant decrease of the percentage of fluorescent cells were observed confirming that the fluorescence was largely localized extracellularly on cell membranes (Fig. 6B).

Confocal microscopy images further support the interpretation that cell fluorescence has an intracellular localization. Supplementary data includes a series of confocal images confirming the intracellular uptake of *BCL2L1* siRNA and scrambled siRNAs.

In order to confirm the inhibition of Bcl2L1 protein by siRNA, total cell lysates (20 μ g) were subjected to western blot analysis using specific antibody directed against Bcl2L1 in comparison with a scrambled siRNA. These experiments confirmed a reduction in Bcl2L1 protein in cells exposed to siRNA in comparison to non-exposed control cells and to cells exposed to scrambled siRNA. Moreover, to confirm the specificity of the inhibition of Bcl2L1, we studied the level of Bcl2 protein using BCL2 antibodies and found no modification of this protein after exposure to siRNA directed against *BCL2L1* (Fig. 6C).

4. Discussion

Introduction of exogenous nucleic acids into mammalian cells represents an essential method for the study of basic cell biology as well as for therapeutic manipulations. While viral-based vectors have proven to be efficient in some cases, these methods are limited by

potentially severe side-effects when applied in the clinic. In laboratories, the transfection of suspended cells is often difficult to obtain with currently available methods. Thus there are still large opportunities for the development of reliable, safe, efficient and reproducible transfection methods.

In this study, we determined the feasibility of using sonoporation to transfect a human lymphoma line as well as fresh human leukemic samples. Many approaches have been tested, including techniques developed by ourselves, to enhance the efficiency of nucleic acid transfer into these hard-to-transfect cells. However, our personal experiments with available transfection reagents such as lipofectin did not show significant transfection efficiency for RL cells and CLL cells. Moreover, new alternative methods such as nucleofection have been also tested but yielded low transfection rates in RL cells (data not shown).

The optimization of US parameters represents a major challenge for the application of sonoporation in different cell lines [8]. The effects of US on a population of cells are very heterogeneous [37]. This heterogeneity is mainly due to the random process induced by the cavitation bubble activity; thereby, cells that are located near the bubble explosion are more affected than the distant ones.

In mild conditions of US exposure, almost all cells remain viable and only a small percentage of cells showed intracellular uptake. However, in case of strong US exposure, cells showed high transfection efficiency with high mortality rate. In order to obtain a trade-off between high transfection rates and good cell viability, we began our study by optimizing these parameters, including CI values and exposure times.

Our experiments showed that:

- The transfection efficiency was greatest at the intermediate cell concentration studied (4.10^6 cells/ml). Decreased transfection efficiency at higher cell concentration (8.10^6 cells/ml) could be

explained by cells shielding each other from nearby cavitation bubbles and could be also consistent with reduced blast radii of cavitation bubbles [37].

- Increasing the plasmid and siRNAs concentration increased the transfection outcome. The results showed that increasing the concentration of these vectors lead to an increase in the number of transfected cells in both cell types. This observation is consistent with the results of other studies [14].
- High delivery efficiency was depending on CI level. CI was found to largely influence the transfection outcome. As a compromise, we defined the CI that showed the highest transfection efficiency with minimal cell loss. The results presented here show that CI values that achieve the optimal efficiency vary from one cell type to another. Thus, a CI of 16 and 20 were considered to be the optimal CI for RL and CLL cells, respectively, within the limits of the apparatus used.
- Transfection efficiency was increased in a time-dependent manner. The longer we exposed cells to US, the more we achieved GFP-positive cells.
- Increasing temperature from 4 °C or room temperature to body temperature (37 °C) improved the percentage of transfected cells. This was consistent with previous reports which showed that low temperature decreases the membrane fluidity leading to reduced pore formation in cell membrane [38]. Moreover, this temperature may provide the necessary conditions for the cell to reseal and survive its membrane disruptions.
- The pore size distribution and their transient existence could also influence the transfection efficiency [15]. In support of this hypothesis, the transfection rates of siRNA were higher than those of pEGFP-C2 and this could be related to the size of the molecule and the distribution of pores (*BCL2L1* siRNA is 200 times smaller than pEGFP-C2). Moreover, the lower transfection rates observed as few GFP positive cells could be explained by the instability of naked DNA in the cytoplasm (presence of DNAses) [39], the half-life of GFP, and the reduced cell metabolism and growth [40].
- The combination of sonoporation with other methods or reagents, such as lipofectin did not improve the transfection rates. Many studies postulated that the combination of DNA with cationic lipids or polymers could increase the transfection efficiency [41–44]. However, our experiments failed to confirm this hypothesis and we did not find any improvement in transfection when combining these two methods, as compared with lipofection or sonoporation alone. Quenching of the extracellular binding of fluorescent siRNAs on cell membrane demonstrated that the combination of lipofectin and siRNA targeted against *BCL2L1* or scramble siRNA did not enhance the transfection efficiency and the fluorescence was largely localized extracellularly on cell membranes (Fig. 6B).
- Using this cavitation device, we were also able to generate stable transfectants that were then xenografted in mice. This will allow us in the future to study the impact of modifications of gene expression on the leukemic cells growth and response to treatments *in vivo*.

5. Conclusion

Optimal transfection parameters were achieved for RL and CLL cells using this 445 kHz transducer. We believe that the findings of this study can be used to guide optimization of DNA transfection in other suspension cultures. Sonoporation appears to be a promising method to obtain transient and/or stable transfection of nucleic acids in suspended cells. Further studies exploring this approach *in vitro* and *in vivo* are warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.10.029.

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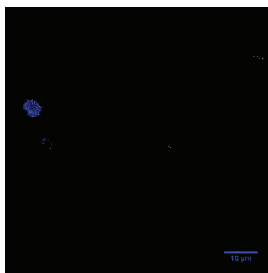
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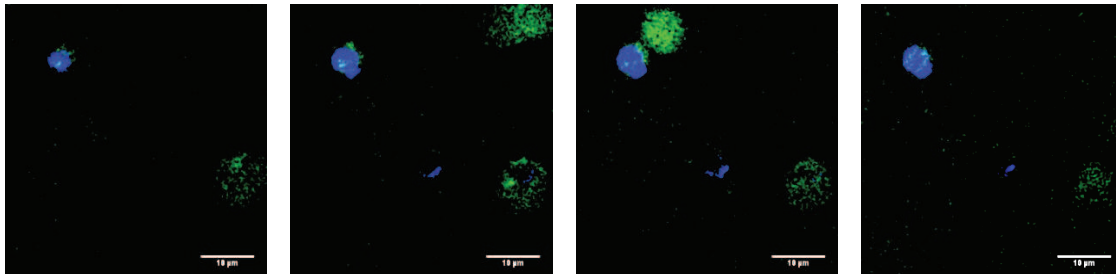
Supplementary data:

Un-exposed CLL cells (A), cells sonoporated either with *BCL2L1* siRNA (B) or with scramble siRNA (C) were imaged forty eight hours after sonoporation using a laser scanning confocal TCS Sp2 DMRXA microscope x63 objective (Leica Microsystems; Wetzlar, Germany) at the Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France). DNA staining was performed using diaminido-phenyl-indol (DAPI, Roche, Manheim, Germany).

A)



B)



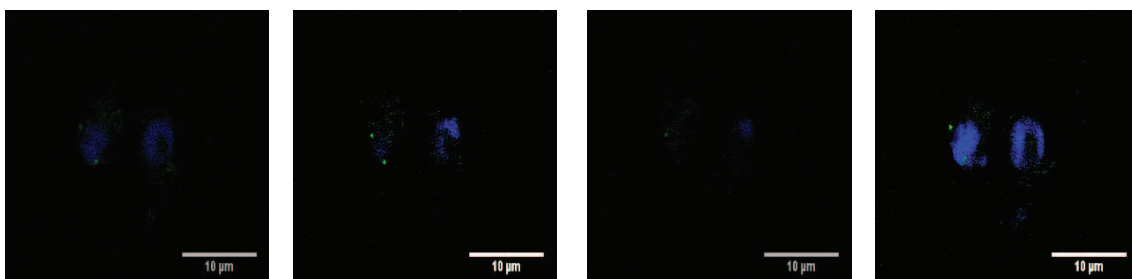
B1

B3

B5

B7

C)



C1

C2

C3

C4

Annexe 1: Intracellular uptake of fluorescent siRNA by US. Representative confocal micrographs showing a non-sonoporated CLL cells (A), cells sonoporated either with *BCL2L1* siRNA (B) or with scrambled siRNA (C). Figure B represents sequences of images numbered B1, B3, B5, and B7 out of a series of 9 images recorded by fluorescence videomicroscopy. Cells were stained with the DNA-specific fluorophore DAPI and an Alexa Fluor 488 (FITC green staining) coupled *BCL2L1* siRNA, whereas figure C represents sequences of images numbered C1, C2, C3 and C4 out of a series of 6 images recorded by fluorescence videomicroscopy for cells sonoporated with an Alexa Fluor 488 coupled scrambled siRNA. Confocal micrographs are representative from two different experiments. Scale bars are 10 μ m.

Discussion

Although many advances have been achieved in the field of gene transfer, the major obstacles that hamper the generation of an ideal vector system are poor efficiency and lack of specificity in the targeted tissue. Physical methods for gene transfer are relatively new arrivals on the scene and demonstrated their efficiency to directly transfer DNA into cells and achieve rapid expression of the transgene. However, these methods are limited by potentially severe side-effects when applied clinically. In the laboratory the transfection of suspended cells is often difficult to obtain with currently available methods. Thus, the development of reliable, safe, efficient and reproducible transfection methods is an urgent need.

Sonoporation is a physical method, in which US is applied, to potentiate pore formation into plasma membrane. In this study, we determined the feasibility of using sonoporation to transfect a human lymphoma cell line as well as fresh human leukemic samples. Many approaches have been tested, including by ourselves, to enhance the efficiency of nucleic acid transfer into these hard-to transfect cells. However, our experiments with available transfection reagents such as Lipofectamine® and HiPerFect® did not show any transfection efficiency for RL cells and B-CLL cells (data not shown). Moreover, new alternative methods such as nucleofection have been also tested but yielded low transfection rates in RL cells (data not shown).

The optimization of ultrasound parameters represents a major challenge that encounters the application of sonoporation in different cell lines [321]. The mechanism of intracellular delivery using US is believed to be initiated by local fluid dynamics generated by oscillating and/or collapsing cavitation bubbles. The impact of fluid will permeabilize the cell plasma membrane thereby allowing the internalization of molecules; the effects of US on a population of cells are typically heterogenous [353]. Under appropriate conditions, cells can survive this process, retaining large numbers of intracellular molecules and appearing to regain normal function within hours. However, in other cases, the mechanical action of the cavitation bubbles can cause cell lysis and disintegration [354]. Thus, cellular damage is too great and cells die by mechanism involving either programmed cell death or necrosis.

In order to obtain a high transfection rate and to maintain good cell viability, we began our study by optimizing these parameters, including cavitation index (CI) values and exposure times.

The results presented here show that high delivery efficiency was depending on CI level. CI was found to largely influence the transfection outcome. As a compromise, we defined the CI that showed the highest transfection efficiency with minimal cell loss. Moreover, the results presented here show that CI values that achieve the optimal efficiency vary from one cell type to another. The CI values of 16 and 20 were respectively considered to be the optimal CI for RL and CLL cells for which cell viability rates are higher than 80%.

The transfection efficiency was greatest at the intermediate cell concentration studied ($4 \cdot 10^6$ cells/ml). Decreased transfection efficiency at higher cell concentration ($8 \cdot 10^6$ cells/ml) could be explained by cells shielding each other from nearby cavitation bubbles and could be also consistent with reduced blast radii of cavitation bubbles [355].

Furthermore, increasing the plasmid and siRNAs concentration increased the transfection outcome. The results showed that increasing the concentration of these vectors lead to an increase in the number of transfected cells in both cell types. This observation is consistent with the results of other studies [356]. The transfection efficiency is also increasing in a time-dependent manner. Thus, the longer we exposed cells to US, the more we achieved a high transfection rate.

The temperature was set at 37°C since lower temperatures (room temperature or 4°C) were associated with lower transfection efficiency (data not shown) and this was consistent with previous report which showed that low temperature decreases the membrane fluidity leading to reduced pore formation in cell membranes [357].

The pore size distribution and their transient existence could also influence the transfection efficiency [299]. In support of this hypothesis, the transfection rates of siRNA were higher than those of pEGFP and this could be related to the size of molecule and the distribution of pores (*BCL2-L1* siRNA is 200 times smaller than pEGFP). Moreover, the lower transfection rates observed with plasmids could be explained by the instability of naked DNA in the cytoplasm (presence of DNAses) [358], the half-life of GFP, and the cell metabolism and growth of cell thereby reducing the quantity of GFP copies per cell [359].

Many studies postulated that the combination of DNA with cationic lipids or polymers could increase the transfection efficiency [318, 360-362]. However, our experiments failed to confirm this hypothesis and we did not find any improvement in transfection when combining these two methods, as compared with lipofection or sonoporation alone. Our experiments

showed that the quenching of the extracellular binding of fluorescent siRNAs on cell membrane with Trypan blue demonstrated that the combination of lipofectin and siRNA targeted against *BCL2L1* or scramble siRNA did not enhance the transfection efficiency and the fluorescence was largely localized extracellularly on cell membranes.

Using this cavitation device, we were also able to generate stable transfectants of RL cells *in vitro* in order to express or inhibit defined genes in the cells. Moreover, we were able to generate stable transfectants that were then xenografted in mice. This will allow us in the future to study the impact of modifications of gene expression on the leukemic cells growth and response to treatments *in vivo*.

In conclusion, sonoporation appears to be a promising method to obtain transient and/or stable transfection of nucleic acids in suspended cells. Our results demonstrate that sonoporation enhance the transfection of many vectors into cells in suspension with minimal impact on cell viability. This method becomes an important tool for the stable and transient transfection of many suspended cells in our laboratory. Further studies exploring this approach *in vitro* and *in vivo* are warranted.

Article II

Evidence of transient membrane poration induced by US exposure in cells in suspension

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(To be submitted)

Introduction and objectives

Gene delivery methods require either the use of biological vectors or the use of chemical and physical approaches. The biological vectors implicate the use of viral-mediated processes such as retrovirus or adenovirus. Non viral gene transfer involves the use of chemical and physical means. Physical methods such as microinjection, bombardment (gene gun), and electroporation have also attracted interest for their potential ability for drug and substances delivery. All these approaches offer several techniques; however, many drawbacks such as viral toxicity, host immune rejection, poor transfection efficiency, and insufficient targeting ability impair the development of gene therapy. Thus, drug delivery methods need improvement to increase drug and gene efficacy by enhancing their intracellular delivery. Sonoporation is a relatively new innovation in drug delivery. Sonoporation has shown to enhance intracellular delivery of small compounds [353], macromolecules [308], DNA [306] and therapeutics [363] by generation of nonspecific openings or pores on the cell membrane. Therefore, pore formation into cell membranes is a key-step for the introduction of foreign substances.

The induction of transient holes in the cell membrane is assumed to be caused by acoustic cavitation [294, 295]; thereby, the intracellular delivery of molecules is believed to be initiated by local fluid dynamics generated by oscillating and/or collapsing cavitation bubbles allowing the entrance of molecules inside viable cells. Many studies investigated the size of pores however accurate measurements of the spatial and temporal scales of these pores are still missed. Clearly, a better understanding of these fundamental issues is necessary to fully exploit the potential use of sonoporation for drug and gene delivery. Therefore, this study aimed to address this objective.

Materials and Methods

1. Cell culture

This study was performed on RL follicular lymphoma cells (obtained from the American Type Culture Collection) and on fresh blood specimens from CLL patients.

Patients gave written informed consent after approval of the study protocol by the Institutional Review Board of the Hospices Civils de Lyon. RL follicular lymphoma cells ($4 \cdot 10^6$) were incubated in 2 ml RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum

(FCS), 200 UI/ml of penicillin and 200 µg/ml of streptomycin. All reagents were purchased from Invitrogen (Carlsbad, CA, USA).

CLL cells were isolated from peripheral blood mononuclear cells by density gradient centrifugation using Histopaque (PanColl human, PAN Biotech). Briefly, blood was diluted in Phosphate Buffered Saline (PBS) then layered over Histopaque and was centrifuged at 300 g for 20 minutes at room temperature. The gradient interface was harvested and was diluted 3-fold with PBS. The cell suspension was washed 3 times by repeated centrifugation at 300 g for ten minutes and was resuspended in RPMI 1640 media supplemented with 10% FCS, 200 UI/ml of penicillin and 200 µg/ml of streptomycin. Cell viability was evaluated by trypan blue dye exclusion.

2. US exposure

The cavitation device used is as previously described [323]. Optimization studies were already performed to maximize cell permeability and minimized cell death. Various parameters were already identified to determine (i) the effects of various physical parameters such as frequency, duty cycle, acoustic pressure (ii) US exposure duration (30 sec to 100 sec), and (iii) cell concentration.

Therefore, CI and US exposure time were determined for each cell type. Two ml of each cell type ($2 \cdot 10^6$ cells/ml in RPMI supplemented with 10% of FCS) were placed in each well of the 12-well plates. Both cells in suspension types were insonified without internalizing materials. For RL cell line, the best irradiation time was 30 seconds at CI of 16; however, for CLL cells, it was 60 seconds at CI of 20. All experiments were conducted at 37 °C.

3. Scanning electron microscopy (SEM)

Pores induced by US cavitation in the membranes of RL and CLL cells were examined by SEM. Primary fixation involved addition of v/v of an aqueous solution of 2.5% glutaraldehyde to 2ml of the cell suspension 15 seconds (s) for RL cells and 30s for CLL cells after initiation of the US exposure (which lasted a total of 30s and 60s, respectively). Cells were incubated for 15 minutes (min) at room temperature (RT).

Then, they were washed with PBS and centrifuged at 300g for 5min. Cells were re-suspended in 2 ml of glutaraldehyde solution (v/v), and kept for 30 min at RT. After centrifugation, specimens were rinsed in 0.2 M Sodium Cacodylate buffer for 15min then washed 2 times

with this buffer. Finally, specimens were held in Cacodylate buffer at 4°C until processing for electron microscopic evaluation.

The cultures were dehydrated in graded concentrations of ethanol and immersed in sequential concentrations of acetone. Finally, 15 µl of the cell suspension was mounted on metal grids and left at RT overnight prior to microscopic observation. Observations were carried out using a Hitachi S800 scanning electron microscope at an accelerating voltage of 5 kV (Centre technologique des microstructures, UCBL, Lyon, France).

Duplicate samples were examined and at least 10 fields were observed for each replicate. Five different areas per sample were examined under different magnifications (×100, ×400, and ×800).

4. Transmission electron microscopy (TEM)

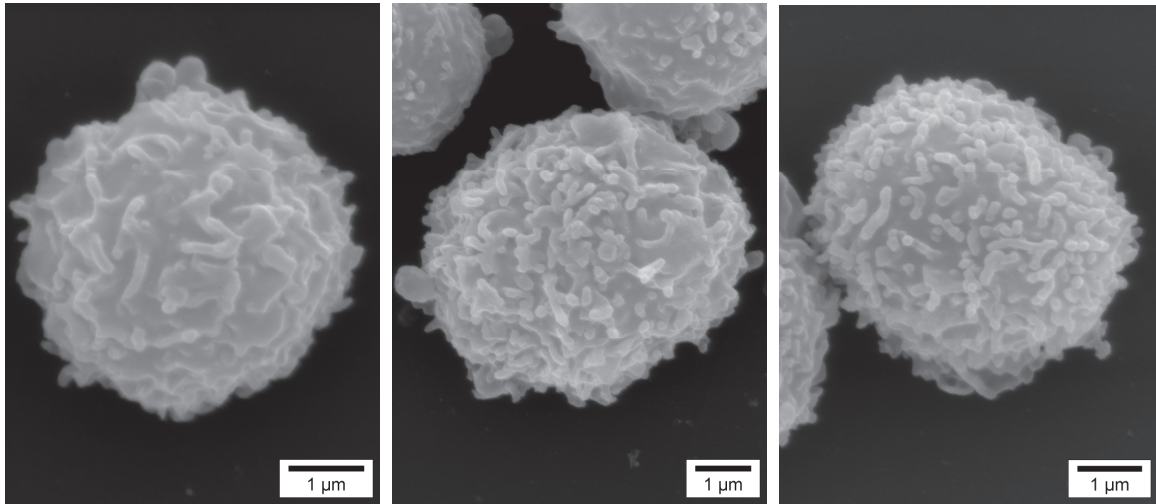
For transmission electron microscopy, RL cells were fixed overnight at RT in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate pH 7.4. After a few washes in 0.1M sodium cacodylate pH 7.4, specimens were post-fixed in 1% osmium tetroxide 0.1M cacodylate pH 7.4 for 45 min and then embedded in epoxy resin. Ultrathin sections were treated with uranyl acetate and lead citrate and then examined in a Philips CM120 electron microscope equipped with a GATAN Orius 200 2Kx2K digital camera (Centre Technique des Microstructures, Université Claude Bernard Lyon I, Villeurbanne, France).

Results

1. Direct observation of pores in the cell membrane by SEM

To directly observe the pores induced by US, the cells were observed by SEM either with or without sonoporation. Cells were treated with glutaraldehyde during insonification to fix the pores in the cell membrane. SEM images of CLL cells reveal a rough surface covered with numerous microvillousities, globular and finger-like processes (Fig. 1A). After irradiation with US, the cell surface was smoother and smaller than that of the untreated ones and the globular and finger-like processes disappeared from the cell surface. Dimple-like craters of various sizes and numbers were observed in the surface membrane in many cells as well as conspicuous pits or pores were identified in US-irradiated cells (Fig.1B)

(A)



(B)

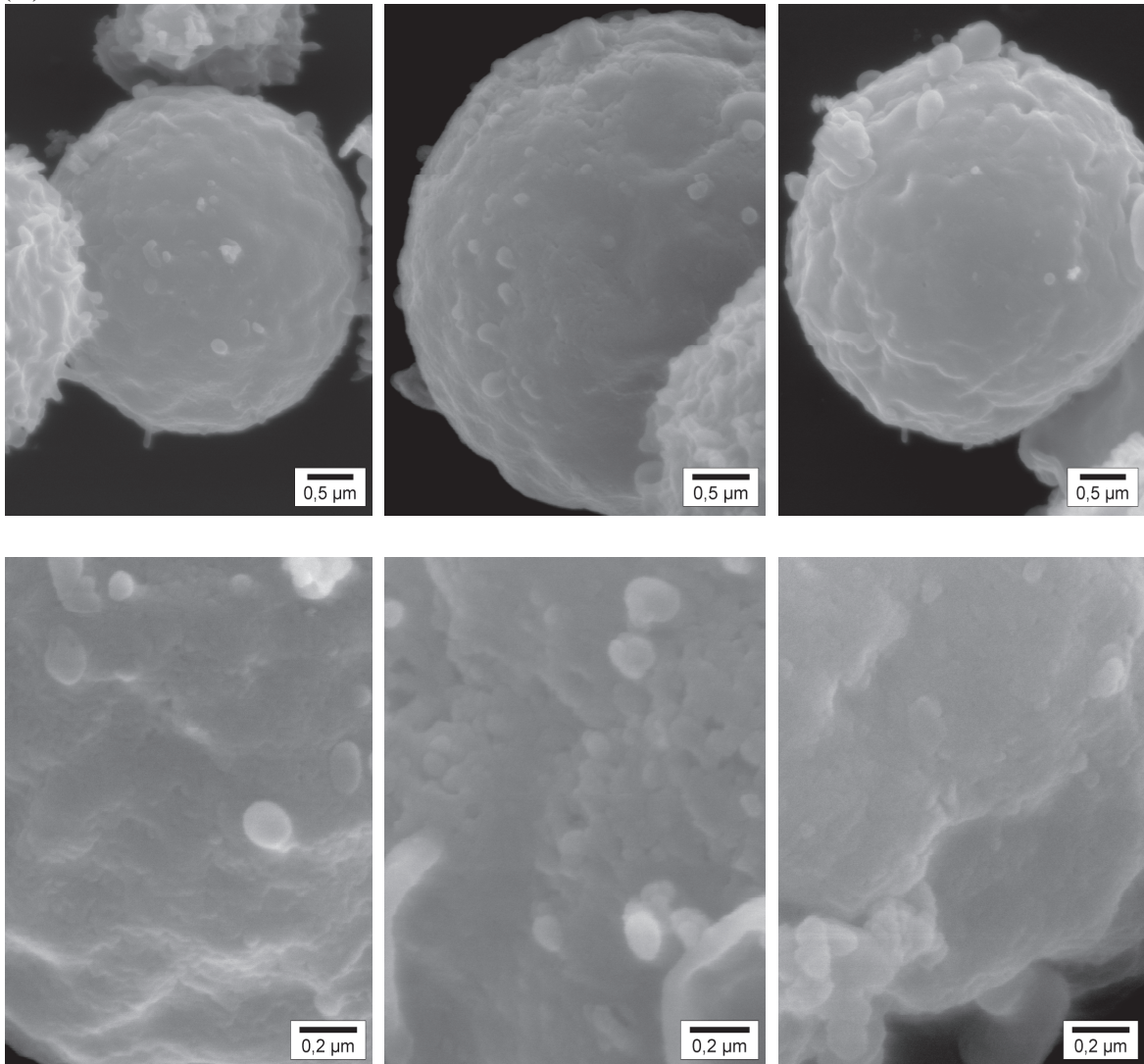
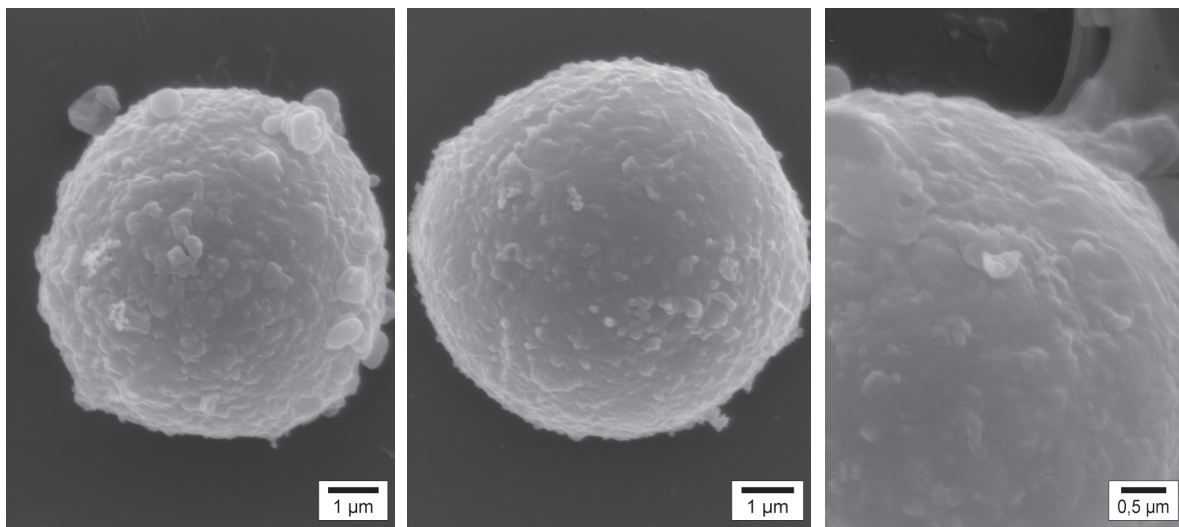


Fig.1. Direct observation of US-induced pores by SEM. CLL cells, at a concentration of $2 \cdot 10^6$ cells/ml, were either untreated (A) or insonified (B) with a focused transducer (425 kHz) during 30s then fixed immediately during sonoporation. Cells were observed with a scanning

electron microscope after sputter coating at different magnifications. (Bar, 1 μ m; 0.5 μ m and 0.2 μ m)

SEM images of RL cells reveal a rough surface with less microvillosity than CLL cells (Fig.2A). After insonification (Fig.2B), the surface was relatively smooth, with a small number of short cellular projections. Dimple-like craters of various sizes were also observed in the surface membrane in many cells.

(A)



(B)

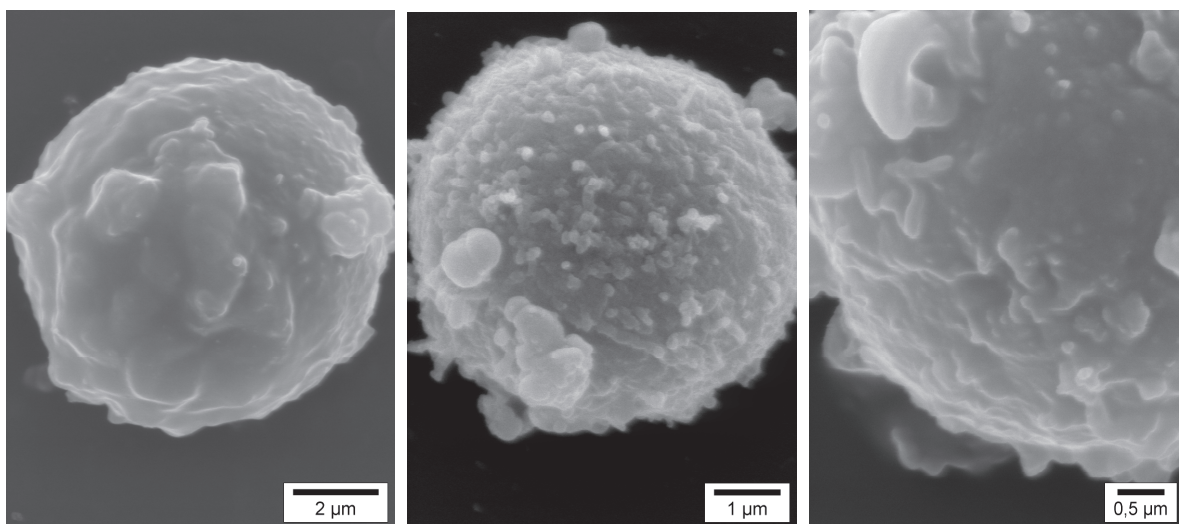


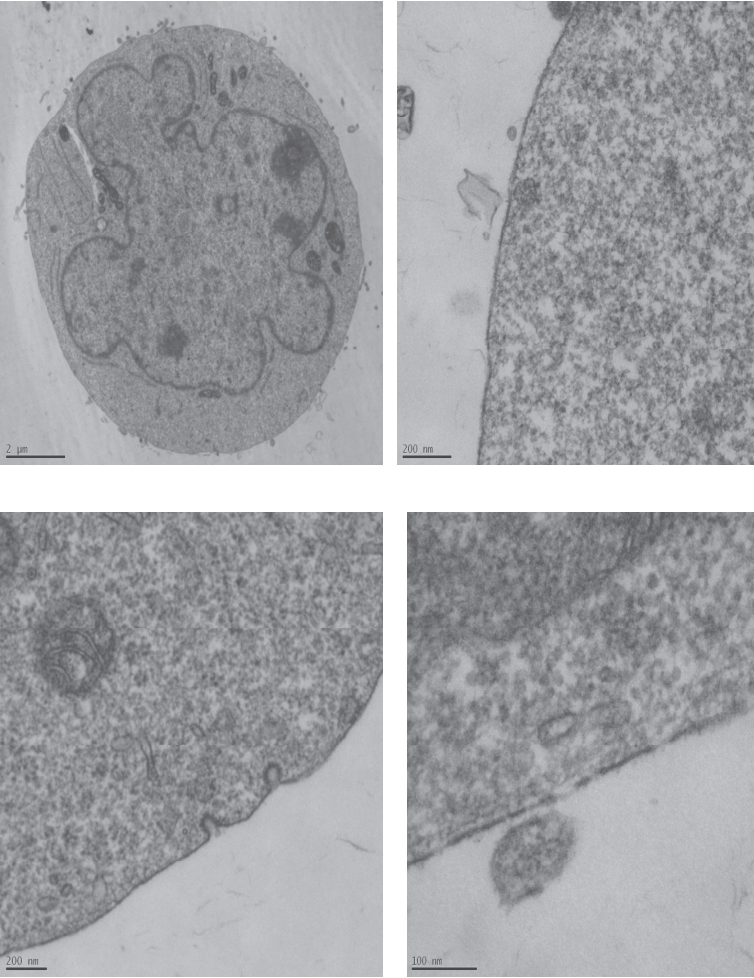
Fig.2. Direct observation of US-induced pores by SEM. RL cells, at a concentration of $2 \cdot 10^6$ cells/ml, were either untreated (A) or insonified (B) with a focused transducer (425 kHz) during 15s then fixed immediately during sonoporation with an aqueous solution of

glutaraldehyde. Cells were observed with a scanning electron microscope after sputter coating at different magnifications. (Bar, 1 μ m and 0.5 μ m)

2. Direct observation of pores in the cell membrane by TEM

To further confirm the mechanism of cell membrane porosity, we observed RL cells using TEM. The surface membrane of non-sonoporated cells was continuous in most cells (Fig.3A); however, some disruptions or pits were also identified in these cells. After US irradiation, we observed more disruptions, pores, or pits in the surface membrane of US-irradiated cells (Fig.3B).

(A)



(B)

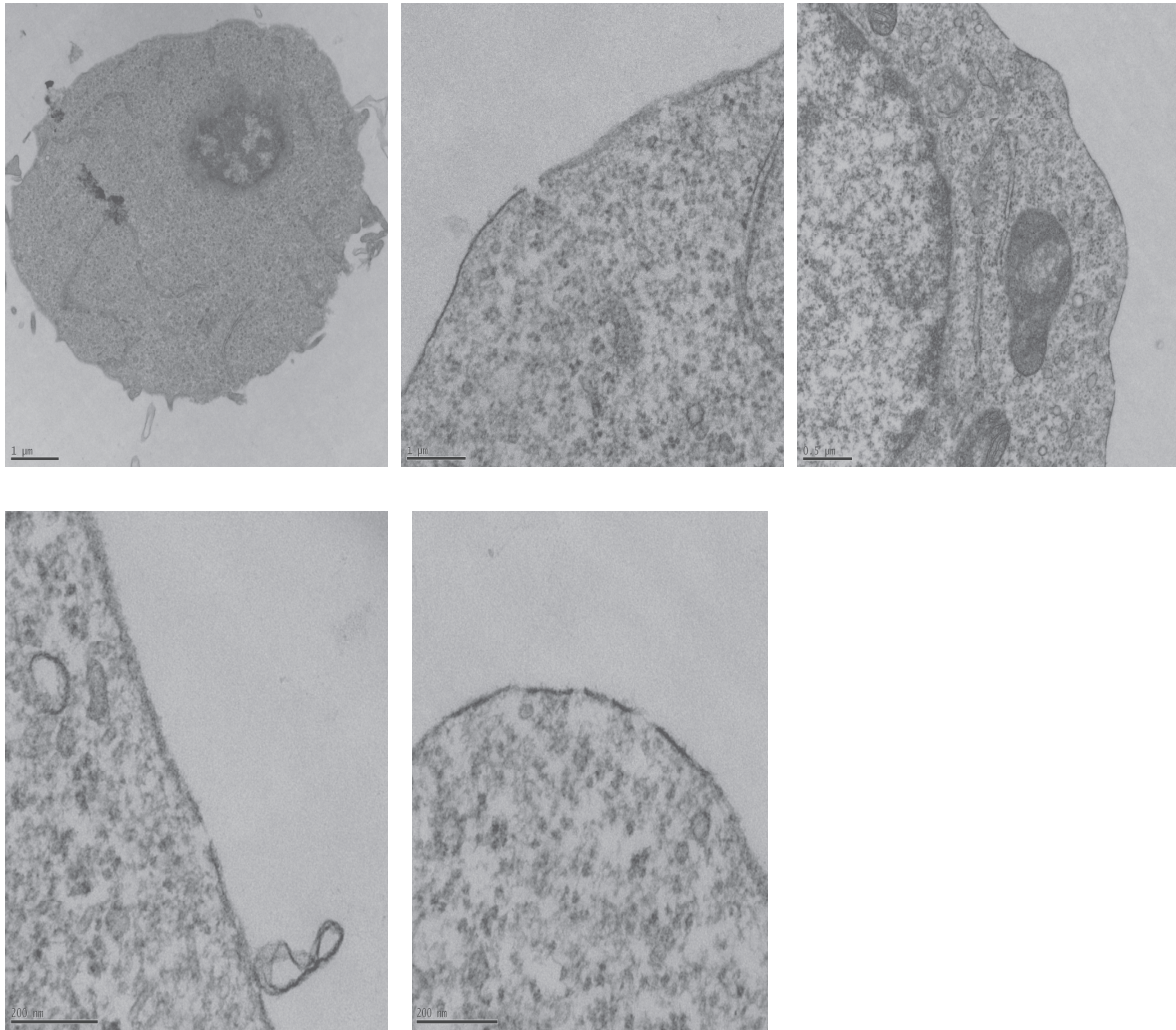


Fig.3. Direct observation of ultrasound-induced pores by TEM. RL cells, at a concentration of $2 \cdot 10^6$ cells/ml, were either left intact (A) or US-irradiated (B) with a focused transducer (425 kHz) during 15s. Cells were observed after sputter coating at different magnifications. (*Bar*, 1 μ m, 0.5 μ m and 200nm)

3. Evaluation of pore size after US-irradiation

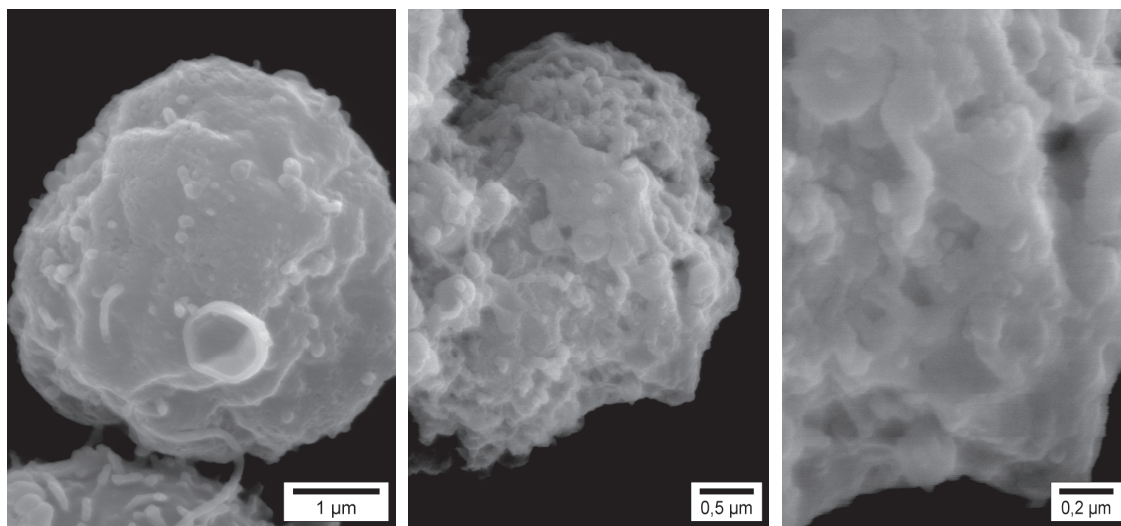
To determine whether large macromolecules can be delivered into sonicated cells, CLL and RL Cells were fixed during insonification, and then observed by SEM. The creation of pores was not always clear. The presence of microvillousities and craters on cells was conspicuous. For this reason, we visualized RL cells with TEM to further confirm the cell porosity. Large pores with an average size on the order of 47 nm can be observed. However, no small pores on the order of 1-10nm were created. Heterogeneous distribution of pores was seen within each cell and among neighboring cells. Thus, the creation of pores showed significant cell-to-

cell heterogeneity in cells exposed to the same US conditions and exhibited very different levels of pores. Unexpectedly, we observed also some pores in the non US exposed cells; however, the number of pores was limited. The pores were measured by Image J software.

4. Cell damage induced by US exposure

To identify the site and degree of damage of sonoporation to tumour cells, we studied the structure of the cell surface with SEM and TEM after US exposure. Cells exposed to US showed multiple surface pores. Dimple-like craters were seen as well as destroyed cells where the cytoplasm seemed to have extruded through the surface boundary; moreover, the cytoplasmic matrix was dense, and the cell organelles was degenerated (Fig.4).

(A)



(B)

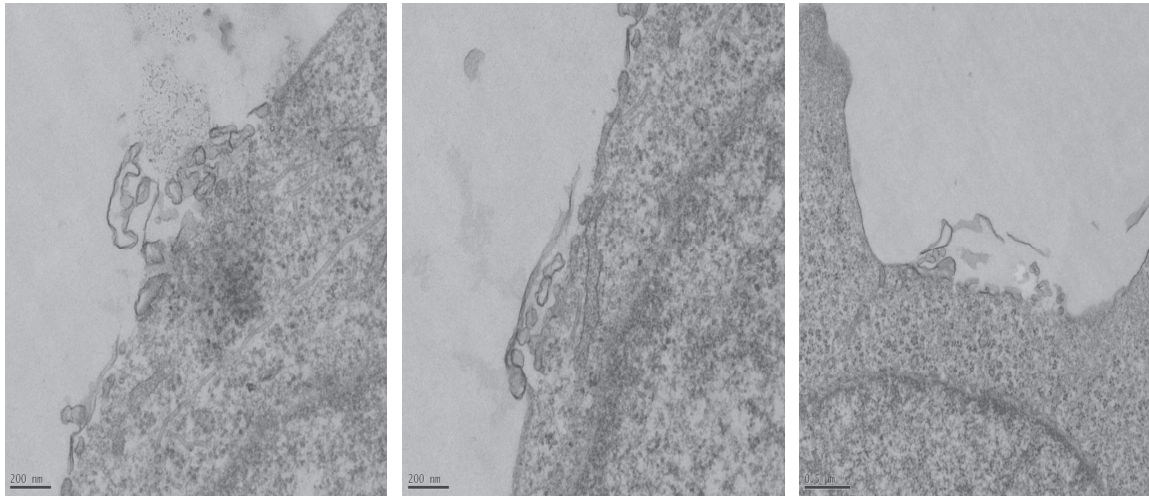


Fig.4. Cell damage induced by repeated US exposure. (A) Cells seem to have sustained more damage than non-irradiated cells shown in Fig.2A when observed by SEM as well as numerous pores of various sizes have formed in the cell membrane. (B) Cell surface membranes presented many disruptions when observed by TEM. (Bar 1 μ m, 0.5 μ m, 0.2 μ m, 200nm)

Discussion

All physical and biological parameters were already optimized in order to minimize the cell killing induced by US exposure in our previous study. Therefore, a CI of 16 and a duration of 30s was found to be optimal for RL cells, whereas a CI of 20 and a duration of 80s were optimal for CLL cells. To observe the pores induced by US, we hypothesized that pores will be open shortly after the beginning of insonification; thereby we choose to fix RL cells at 15s and CLL cells at 30s which means at half time of the US exposure.

Morphological changes in the cells occurred immediately after insonification. Consequently, the normal surface characteristics disappeared from the cell after US irradiation. The smooth aspect of the cells may be due to an ultrasonic shaving effect, by which surface macromolecules, such as glycoproteins and receptors were removed from the cell membrane. Brayman et al. also reported that insonification altered cell morphology [310]. It was demonstrated that sonoporation (using a 1 MHz focused transducer for 30s in continuous wave at a peak negative pressure of 0.5 MPa), removed the cell surface receptor CD19 from human lymphocytes. However, in our study we did find neither a loss of CD19 nor CD20 after irradiation (data not shown). Moreover, irradiation with US may cause

structural changes in the cytoskeleton of irradiated cells; thereby, it may alter the morphology and functional changes in the sonicated ones [364].

We also noticed that cell size was reduced after insonification, a finding that has been described before [365]. This could be explained by the leakage of cytosolic fluid postsonoporation, an effect that, unfortunately, could lead to partial escape of internalized molecule. Successive events of cellular change after US irradiation were not evaluated in this experiment, because the cells were fixed immediately during exposure to US.

Sonoporation is described as a transient phenomenon. Most likely, it is a combination of many bioeffects leading to a microstreaming along the cell membrane that will finally lead to physical pores, causing increased uptake of macromolecules and transgenes into cells.

Conspicuous pits or pores were identified in the US irradiated cells at the magnification we used. Moreover, the existence of smaller pores or minute membrane ruptures after US irradiation was also identified. Our study showed that the size of pores was ranged between 28nm and 72nm. This was in agreement with previous studies [299, 365, 366]. It was mentioned also that cells return almost immediately to an impermeable state after US exposure suggesting that the pore opening could last on the order of milliseconds to seconds [299, 353]. Prausnitz et al. reported similar results when cells were exposed to low frequency US (24 kHz) in the presence of fluorescent compounds having diameters of 1.2 to 56 nm [367]. Specifically, the uptake of bovine serum albumin (7.2 nm diameter) and calcein (1.2 nm diameter) was completely abolished when the compounds were added, respectively, 1 and 2 min after US exposure. Moreover, Gambihler et al. described that exposure of L210 cells to US shock waves before addition of FITC-dextran did not allow direct molecular transfer via US-induced pores [311]. Finally, the fixation procedure can by itself, close the pores, by altering the cell membrane structure via shrinkage, for instance [368]. Taken together, these results suggest that the porated cell membrane acts as a 'sieve', only allowing the entry of molecules with a size below the pore diameter and the very short opening of pores could hamper the observation of small pores.

The resealing of non-specific pores is a key factor determining both the uptake and post-US cell survival. This resealing prevents the loss of intracellular contents and limits the transfer of extracellular agents within a time before the completion of pore resealing. After US exposure, we noted visibly various-sized pores on the cell membrane in some more damaged cells. These results indicate that cell degeneration was induced by a rapid change in cell membrane porosity during sonication. These cells may not reseal and quickly lose viability. The disruption of their membranes can trigger many sources for irreversible and

reversible cellular process such as apoptosis [313] and calcium oscillation [314]. Moreover, plasma membrane disruptions in smaller mammalian cells (fibroblasts and endothelial cells) that remain open for more than approximately 15s are generally lethal [369].

The high-resolution SEM observations in the present study confirmed changes in the cell surface membrane at an ultrastructural level and the evidence of effective pore formation by US.

The results of this study demonstrated that sonoporation induced many holes in the cell membrane; thereby, the mechanism of gene delivery is realized through the induction of various pore sizes allowing the entry of molecules.

Further studies will evaluate the capacity of these cells to reseal their membrane pores and their numerous microvillousities, globular and finger-like processes after US exposure. Furthermore, as the mechanism of structural changes brought about by US irradiation is not clear, further studies such as immunocytochemical studies are needed to elucidate the molecular dynamics within cells.

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Conclusion and perspectives

Sonoporation is physical tool that temporarily permeabilizes the cell membrane allowing the uptake of DNA, drugs, and other therapeutic compounds from the extracellular environment. In our study, we showed the effect of US on RL cells and on fresh isolated B-CLL cells from patients and we demonstrated the presence of various pore sizes on their cell membranes. Further studies will evaluate the capacity of these cells to reseal their membrane pores and their numerous microvillousities, globular and finger-like processes after US exposure. Thereby, cells will be fixed immediately or many hours later after sonoporation. Then, many techniques will analyze the morphological changes of cell membrane at different times. Furthermore, as the mechanism of structural changes brought about by US irradiation is not clear, further studies such as immunocytochemical studies are needed to elucidate the molecular dynamics within cells.

Chapter V

Comparison of the Cytotoxic Mechanisms of Anti-CD20 Antibodies Rituximab and GA101 against Fresh Chronic Lymphocytic Leukemia Cells

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Charles Dumontet^{1,2,3}

(Submitted to Leukemia)

Objective of this study

The use of monoclonal antibodies, such as rituximab has revolutionized the management and treatment of B-cell malignancies, by improving the median overall survival of patients with many of these diseases.

Anti-CD20 MAbs can be broadly classified as Type I or II depending on their ability to redistribute CD20 into lipid rafts, to induce CDC or to induce cell death and homotypic adhesion. Type I MAbs (or rituximab-like) appear to activate complement and effector cell mechanisms (i.e. CDC and ADCC) whereas type II (or tositumomab-like) MAbs are believed to function through a combination of effector cell activation (ADCC) and programmed cell death (apoptosis), while being relatively inactive in complement activation [224, 370, 371].

Rituximab monotherapy shows some activity in the therapy of relapsed CLL [4, 165, 166]. Moreover, the full potential of rituximab seems to unfold when given simultaneously with chemotherapy, in particular fludarabine (cyclophosphamide). Nevertheless, relapse is a common occurrence in some B-cell disorders such as B-CLL [3].

Several novel anti-CD20 MAbs are being investigated. They have been engineered to circumvent or to avoid the resistance seen with rituximab and/or offer efficacy beyond that observed with rituximab therapy.

GA101, the first Fc-glycoengineered type II MAb, is thought to have an enhanced and superior functional activity than rituximab. *In vitro* B-cell depletion assays with whole blood from healthy and leukemic patients showed that the combined activity of ADCC, CDC, and apoptosis for GA101 was significantly superior to rituximab [250, 251, 253, 254]. The enhanced efficacy of GA101 also has been shown *in vivo*. In human lymphoma xenograft models, GA101 exhibits superior antitumor activity, resulting in the induction of complete tumor remission and increased overall survival. Moreover, in nonhuman primates, it shows superior B cell-depleting activity in lymphoid tissue, including in lymph nodes and spleen [250].

CLL is the most common leukemia in the western world and is characterized by the accumulation of the long-lived B lymphocytes [16]. It is a phenotypically distinguishable

form of B-lymphoid leukemias. B-CLL cells are B well-differentiated lymphocytes that express a constellation of surface differentiation antigens such as CD5, CD19 and CD23 and low level of surface immunoglobulins (Ig). CD20 is commonly expressed by CLL cells albeit at a lower level than in NHL cells, which might partly explain the lesser efficacy observed in CLL patients treated with rituximab [30, 372-374]

A hallmark of CLL cells is their resistance to apoptosis, leading to prolonged cell survival and development of drug resistance [375]. Apoptosis resistance may stem from a combination of microenvironmental survival signals as well as from intrinsic alterations in the apoptotic machinery within the CLL cell. The molecular mechanism involved in controlling apoptosis in CLL is influenced by many factors, including Bcl-2 family proteins.

The Bcl-2 family is a group of pro-apoptotic (Bax, Bad, Bak, Bik, Bcl-x_s, Bik and Bim) and anti-apoptotic (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bfl-1) molecules [376]. Bcl-2, Bcl-x_L and Bak are localized in the outer mitochondrial membranes whereas other members such as Bax, Bid and bad reside in the cytosol of normal cells [377-382]. Following a death stimulus, the pro-apoptotic members can undergo a conformational change that enables them to target and integrate into membranes, especially the mitochondrial outer membrane [383-385]. The resulting mitochondrial dysfunction includes a change in the mitochondrial membrane potential ($\Delta\Psi_m$), the production of reactive oxygen species (ROS), the opening of the permeability transition pore (PTP), and the release of the intermembrane space protein, cytochrome *c* (Cyt *c*). Released cytochrome *c* activates Apaf-1, which in turn activates a downstream caspase program.

The objective of this study is to compare the cytotoxic effect of Rituximab and GA101 in freshly B-cells of CLL patients. We studied the effects of these two MAbs on the intrinsic pathway of apoptosis, by evaluating the change of mitochondrial membrane potential, the generation of ROS and the expression levels, modifications and conformations of apoptosis-related proteins.

Our study suggests that apoptotic signalization differs between rituximab and GA101 with a greater involvement of the mitochondrial pathway in cells exposed to GA101.

Comparison of the Cytotoxic Mechanisms of Anti-CD20 Antibodies Rituximab and GA101 against Fresh Chronic Lymphocytic Leukemia Cells.

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Abstract

CD20 is a validated target for the immunotherapy of B lymphoid neoplasms, including Chronic Lymphocytic Leukemia. We compared the effects of rituximab and GA101 (novel Type II anti-CD20 antibody) against fresh human CLL cells *in vitro*. AnnexinV staining demonstrated induction of apoptosis after exposure to rituximab or GA101. Unlike rituximab, GA101 induced a reduction of the mitochondrial transmembrane potential, an effect which could be partially reversed by cyclosporin A and partially caspase-dependent. GA101 was also found to induce the production of Reactive Oxygen Species. Analysis of pro- and anti-apoptotic protein content after exposure to antibodies demonstrated a strong degree of heterogeneity between samples. Bax underwent conformational activation and mitochondrial translocation upon exposure to antibodies in a caspase-independent manner. GA101 but not rituximab induced cleavage of caspase-8, -9 and -3. By transfecting CLL cells with anti-Bcl-xL siRNA using a sonoporation method, we found that reduction of Bcl-xL content was associated with increased sensitivity to these antibodies. Our results suggest that apoptotic signalization pathways differ between rituximab and GA101 with a greater involvement of the mitochondrial pathway in cells exposed to GA101. Inhibition of Bcl-xL could constitute a means to sensitize CLL cells to the apoptotic effects of anti-CD20 antibodies.

Introduction

Monoclonal antibodies (MAbs) have become an essential tool in the treatment of many cancers. The use of rituximab, an anti-CD20 MAb, has changed the therapeutic landscape of B-cell malignancies, particularly in patients with non-Hodgkin's lymphoma (NHL) with more recent indications in the setting of Chronic Lymphocytic Leukemia (CLL).(1) In the field of lymphoproliferative diseases rituximab, followed by the anti-CD52 antibody alemtuzumab, has opened the way for several novel immunotherapeutic approaches. One of these approaches has consisted in the administration of radioconjugates such as ⁹⁰Y-Ibritumomab tiuxetan (Zevalin®) or ¹³¹I-tositumomab (Bexxar®).(2) Another approach has consisted in the targeting of other antigens commonly expressed in malignant lymphoid cells, such as CD19(3) or CD22.(4) However CD20 remains a validated target, prompting intensive development of antibodies targeted against this antigen.(5) Ofatumumab, a MAb directed against CD20, has recently been approved in the U.S. for the treatment of patients with CLL relapsing after fludarabine and alemtuzumab.(6) Recent improvements in the understanding of the mechanisms of action and the bioengineering of MAbs suggest that there are still untapped possibilities to improve monoclonals directed against the CD20 antigen.

CLL is the most common hematologic malignancy in the western world.(7) It is an extremely heterogeneous disease, with a highly variable clinical course. Many patients are asymptomatic and can live with CLL for decades without any therapy; however, others have rapidly progressive and fatal malignancy. CLL cells are B well-differentiated lymphocytes that express a constellation of surface differentiation antigens such as CD5, CD19 and CD23 and low level of surface immunoglobulins.(8) CD20 is commonly expressed by CLL cells albeit at a lower level than in NHL cells, which might partly explain the lesser efficacy observed in CLL patients treated with rituximab.(9) Nevertheless rituximab has demonstrated activity in the treatment of CLL in combination with chemotherapy and has recently been approved in this indication.(10)

Current treatment of CLL is based on the use of chemotherapeutic agents such as nucleoside analogues, alkylating agents and MAbs.(11) While the molecular alterations that induce apoptosis in CLL cells can potentially occur at different levels, it is becoming increasingly clear that the mitochondrion plays a key role in the induction of apoptosis in CLL. The major proteins involved in apoptotic signaling in the mitochondrion are Bcl-2 family members. This family contains both pro-apoptotic (Bax, Bad, Bak, Bik, Bcl-x_s, Bik and Bim) and anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1) molecules.(12) Bcl-2 and Bcl-xL are localized in the outer mitochondrial membranes.(13, 14) Following a death stimulus, the pro-apoptotic members can undergo a conformational change that enable them to target and integrate into membranes, especially the mitochondrial outer membrane.(15) The resulting mitochondrial dysfunction includes a change in the mitochondrial membrane potential ($\Delta\psi_m$), the production of reactive oxygen species (ROS), the opening of the permeability transition pore (PTP), and the release of the intermembrane space protein, cytochrome *c* (Cyt *c*). Released Cyt *c* activates Apaf-1, which in turn activates a downstream caspase program.

The expression of high levels of anti-apoptotic Bcl-2 protein is characteristic of CLL cells.(16) However, the role of Bcl-2 in apoptosis inhibition is unclear, since no correlation exists between apoptosis induced by exposure to agents *in vitro* and Bcl-2 content. However, many studies have suggested that an increased ratio of anti- to pro-apoptotic proteins such as Bcl-2/Bax is correlated with poor response to chemotherapy, disease progression and shorter survival.(17, 18)

GA101 is a novel type II glycoengineered IgG1 anti-CD20 MAb with enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), less complement dependent cytotoxicity (CDC) and superior cell death induction in comparison with rituximab that is currently in PhII/III clinical trials for the treatment of NHL and CLL.(19) The aim of this study was to compare the mechanism of induction of apoptosis of the two anti-CD20 MAbs, rituximab and GA101 in fresh CLL samples. We studied the effects of these two MAbs on the intrinsic pathway of apoptosis, by evaluating the change of mitochondrial membrane potential, the generation of ROS and the expression levels, modifications and conformation of apoptosis-related proteins. Our study suggests that the cell death induction described for GA101 in B-CLL involves apoptotic signalization and differs between CLL exposed to rituximab and GA101 with a greater involvement of the mitochondrial pathway in cells exposed to GA101.

Patients, materials and methods

Patient samples and cell isolation

Peripheral blood samples were obtained from patients with CLL attending the clinic at Edouard Heriot hospital. Patients provided written informed consent and this study protocol was approved by the Institutional Review Board of the Hospices Civils de Lyon. Mononuclear cells were isolated from peripheral blood samples by centrifugation on a Ficoll-Hypaque (PanColl human, PAN Biotech) gradient as previously described either used immediately or cryopreserved in liquid nitrogen in the presence of 10% of dimethyl sulfoxide.(20) Mononuclear cells were cultured at a concentration of 5×10^6 /ml in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 UI/ml of penicillin and 200 μ g/ml of streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide. All reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Reagents and Antibodies

GA101 and rituximab were obtained from Roche Glycart AG (Schlieren); Z-VAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) was obtained from Promega; N-acetyl cysteine (NAC), L-ascorbic acid (ASC), cyclosporin A (CsA) and carbonyl cyanide-p-trifluoromethoxy hydrazone (FCCP) were purchased from Sigma Aldrich. Characteristics of antibodies used in this study as well as dilutions are summarized in table 2S.

Flow cytometry assessment of cell viability, mitochondrial transmembrane potential ($\Delta\psi$) and reactive oxygen species (ROS)

Apoptosis was evaluated by Annexin V/propidium iodide (PI) staining using flow cytometry as previously described.(21) Briefly, cells were washed twice with phosphate buffer saline (PBS) then resuspended in binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂] containing 1 mg/ml FITC-Annexin V. PI (Sigma Chemicals, St Louis, MO) was added also to each sample and cells were incubated for 15 minutes in the dark prior to flow cytometric analysis.

$\Delta\psi$ was evaluated by staining with 0.5 nM 3,3-dihexyloxacarbocyanine iodide (DiOC6[3]) (Molecular Probes, Eugene, OR) as previously described.(22) ROS production was determined by staining with 5 μ M dihydroethidine (DHE) (Molecular Probes). Cells were incubated with the dyes for 15 minutes at 37°C, washed, resuspended in PBS, and analyzed by flow cytometry. FCCP, a mitochondrial uncoupler inducing dissipation of the integrity of the mitochondrial membrane was used at 10 μ M as a positive control.

For Bax intracellular staining, cells were fixed and permeabilized using IntraPrep Permeabilization Reagent (Beckman Coulter Inc., Fullerton, CA, USA); then stained with unlabelled anti-Bax monoclonal antibody (clone YTH-6A7) for 1 h at room temperature (RT) protected from light. After washing, with PBS, cells were resuspended in diluted FITC-conjugated secondary antibody and incubated for 30 minutes at RT. Cells were washed twice with PBS prior to flow cytometric analysis.

All experiments were performed in duplicate, with acquisition of 10,000 cells on a FACS Calibur flow cytometer;

Western Blotting

Cells were lysed in 20 mM Tris (tris-(hydroxymethyl)-aminomethane) HCl, pH 6.8; 2% sodium dodecyl sulfate (SDS) and processed as previously described.(23) Briefly, 30 μ g of

cell lysates were resolved on a 12% SDS-PAGE using an electroblotting apparatus (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Hybond-ECL, Amersham Corp). The membrane was blocked with blocking buffer (LI-COR Biosciences, Germany) for 1 h and subsequently incubated with the primary antibodies overnight at 4°C. The non-specific binding of antibody was removed by washing with PBS (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated with the secondary antibodies (Goat anti mouse IRDye or Goat anti-Rabbit antibody from LI-COR Biosciences, Germany) for 1 h at RT. After extensive washing with PBS, membranes were analysed using the Odyssey infrared imaging system (LI-COR Biosciences, Germany). The expression levels of the protein were standardized against the expression level of β -Actin (clone AC-15, Sigma). Mitochondrial and cytosolic proteins extracts were obtained from 50×10^6 cells per condition by means of the Apoalert cell fractionation kit (CLONTECH laboratories, Palo Alto, CA).

Sonoporation and siRNA experiments

The transfection of CLL cells was performed using an ultrasound (US) apparatus as previously described.(20)

Two ml of CLL cells (2.10^6 cells/ml in RPMI supplemented with 10% of FCS) were sonoporated in a 12-well plate with the siRNAs concentration of 7.5 μ g/ml. Optimal exposure conditions (Cavitation index and US exposure time) that maximized cell permeability and minimized cell death were identified. The CI of 20 was used in all experiments and the best irradiation time was 80 seconds.

Bcl-xL siRNA insert sequence had sense and antisense sequences as follows: Bcl-xL sense 5'-GGAGAUGCAGGUAUUGGUG-3' and antisense 5'-CACCAAUACCUGCAUCUCC-3'. The scrambled siRNA served as a control, and its sequences are 5'-GAGGAGUGUUCGAGGUGAU-3' and 5'-AUCACCUCGAACACUCCUC-3'. All siRNAs used was provided by Sigma.

Statistical analysis

To identify differences after exposure of cells to antibodies, the statistical significance of the data was determined with a Student's t-test. $P < 0.05$ and $P < 0.001$ indicate a statistically significant (*) and highly significant (**) difference, respectively.

Results

Patient Characteristics

A total of 32 samples were analysed in this study. Main patient characteristics are summarized in Table 1S. Disease was classified according to Binet stage.(24)

Induction of apoptosis of CLL cells by anti-CD20 antibodies

CLL cells were incubated up to 24 h with GA101 or rituximab at a final concentration of 10 $\mu\text{g/ml}$. Data from 9 patients are presented in Figure 1. The number of apoptotic cells assessed by AnnexinV binding in GA101-exposed cells was significantly higher in samples exposed to GA101 ($66,4 \pm 14.3\%$ and $62,9 \pm 14.3\%$) than in controls ($28.2 \pm 13.2\%$ and $41.1 \pm 18.7\%$) after 6 h and 24 h, respectively. The number of apoptotic cells in rituximab-exposed cells was statistically significant only after 6 h ($56.4\% \pm 13.66\%$). The apoptotic effect of GA101 was not correlated with sex, age, Binet stage of the disease or prior treatment. In addition, ATM mutation and ZAP-70 status which were analyzed in a subset of patients did not appear to influence the apoptotic effect of GA101. Parallel to these AnnexinV binding tests done on B-CLL cells without being purified, we studied the induction of apoptosis on a subset of patients after purifying B-CLL cells by a negative selection method using the EasySep® B Cell Enrichment Cocktail and we did not find any difference in the percentage of dead cells either with or without purification (data not shown)

GA101 induced cell death is associated with a loss of $\Delta\psi_m$ in CLL cells and an increased production of ROS

To determine whether GA101 affected the permeability of the outer mitochondrial membrane, CLL cells were incubated with GA101 or rituximab. DiOC6(3) was used to monitor the disruption of $\Delta\psi_m$. GA101 induced a decrease of DiOC6(3) staining as early as 3 h after beginning of exposure, while this was not observed in controls and rituximab-exposed cells, suggesting an early and specific effect of GA101 at the mitochondrial level (Figure 2A). The effect of GA101 on DiOC6(3) staining was similar to that of FCCP, an uncoupler of mitochondrial oxidative phosphorylation that makes the inner mitochondrial membrane permeable to protons and induces dissipation of $\Delta\psi_m$. To confirm that the dissipation of $\Delta\psi_m$ by GA101 was the result of opening of the PTP, we used CsA as a PTP inhibitor. We observed that pre-incubation with CsA for 1 h led to the restoration of $\Delta\psi_m$ (Figure 2C) and reduced GA101-induced cell death (Figure 2B) as well as the pre-incubation of cells with Z-VAD.fmk for 1h led to the restoration of $\Delta\psi_m$ (Figure 2D).

The reduction of mitochondrial membrane potential was associated with the production of ROS in GA101-exposed cells, as shown in Figure 3A. However, this increase was not statistically significant.

GA101-induced loss of $\Delta\psi_m$ was caspase-dependent

To determine whether GA101-induced loss of $\Delta\psi_m$ was caspase-dependent, CLL samples were pre-incubated in the absence or in the presence of the pan-caspase inhibitor, Z-VAD.fmk ($50\mu\text{M}$) for 1 h before the addition of GA101 or rituximab. The incubation of GA101 with Z-VAD.fmk abrogated the induction of cell death at 24 h (Figure 1B) and prevented the loss of $\Delta\psi_m$ at 3, 6 and 24 h (data not shown).

Effect of anti CD20 antibodies on apoptosis-related proteins

To compare the effect of rituximab and GA101 on apoptotic regulation in CLL cells we performed Western blot analysis after 24 h exposure to these two antibodies. We studied the content of the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 and the pro-apoptotic proteins Bak, Bax and Bad. Samples were also examined for the processing of the initiator caspase-8, caspase-9 and the effector caspase-3.

Notwithstanding the heterogeneity observed among patient samples, we observed an increase in the protein levels of Bax, Bak and Bad in most samples after exposure to GA101 or rituximab (Figure 4A). We did not observe any significant change in Bcl-2 content after exposure to either rituximab or GA101. Conversely the content of Bcl-xL and Mcl-1 was found to be increased after exposure to these antibodies in some samples (Figure 4B). Moreover, caspase-8, -9 and -3 were activated and cleaved when samples were exposed to GA101 but not to rituximab. These data, observed in 5 out of 9 patients studied, suggest that GA101 may induce apoptosis by activation of the mitochondrial pathway involving caspase-9, which then activates caspases-8 and -3 (Figure 4C). Supplemental data are provided in Figure 1S.

Bax and Bak translocation

Bax, a proapoptotic cytosolic protein, is a potent inducer of apoptosis. Bak, another proapoptotic member of the Bcl-2 family, is integrated in the mitochondria of normal cells.(25) Upon its activation, Bak is translocated from mitochondria to cytosol while Bax is translocated to the mitochondria. The cellular localization of Bcl-2 family proteins (Bax, Bak and Bcl-2) was analyzed by Western blot in mitochondrial and cytosolic protein fractions after exposure to GA101 or rituximab. In order to confirm that our mitochondrial fraction was successfully separated from the cytosolic fraction, we used cytochrome c oxidase subunit IV (Cox4) antibody as a control. Cox4 is a membrane protein in the inner mitochondrial membrane, which remains in the mitochondria regardless of apoptosis activation. The activation of Bax was studied by using an antibody that recognizes the active conformation of Bax (clone YTH-6A7). As shown in Figure 5A, the incubation of B-CLL cells with GA101 or rituximab induced a shift in the cellular localization of Bax from the cytosolic to the mitochondrial fraction, as well as a decrease of the amount of Cyt c released into the cytosol. Bak was found in the cytosolic fraction but was present in both the control and exposed groups (Bak (G-23) and Bak (H-211)). Bcl-2 behaved differently according to the type of antibody: Bcl-2 content tended to increase in a time-dependent manner in cells exposed to GA101, while large variations were observed in samples exposed to rituximab.

Bax conformational changes are caspase-independent

To establish whether the changes in Bax conformation are dependent on caspase activation, cells from 3 CLL patients were preincubated in the presence or absence of 50 μ M of Z-VAD.fmk prior to the addition of either GA101 or rituximab. As shown in Figure 5B, the increased of active Bax levels (clone YTH-6A7) induced by exposure to antibodies were not blocked by the presence of the broad caspase inhibitor Z-VAD.fmk, indicating that Bax translocation does not require caspase activation. Similar results were obtained with the use of other antibody directed against the NH₂-terminal epitopes of Bax (N-20). However, loss of $\Delta\psi_m$ and ROS production were partially reversed by the caspase inhibitor (data not shown). Similar results were obtained with Bak.

Down-regulation of Bcl-xL sensitizes CLL cells to anti-CD20 antibody-induced apoptosis

Our immunoblot studies showed that the content of Bcl-xL was increased after exposure to GA101 and rituximab in some samples. To further evaluate the role of Bcl-xL protein in CLL cells, we tested the effect of Bcl-xL knockdown by Bcl-xL-specific siRNA. The Bcl-xL protein level was greatly reduced at 48 h after treatment with Bcl-xL siRNA but not after treatment with a control siRNA (Figure 6). We investigated the influence and the mechanism of targeting *Bcl-xl* by siRNA on the sensitivity of CLL cells to anti-CD20 monoclonal antibodies such as rituximab and GA101. Bcl-xL downregulation using Bcl-xL siRNA exposure lead to decreased cell growth and apoptosis in CLL cells *in vitro* when treated with rituximab. The level of sensitization was similar to that observed with ABT-737, a small molecule inhibitor of Bcl-xL either untreated or treated with antibodies.

Discussion

Immunotherapy of lymphoid neoplasms with therapeutic MAbs is believed to involve several mechanisms of cytotoxicity, including apoptotic signaling, CDC and ADCC. The novel glycomodified type II anti-CD20 antibody GA101 has been selected for improved apoptotic signaling and ADCC activity in comparison to the first-in-class anti-CD20 antibody, rituximab. In this study we have compared the intracellular effects of GA101 and rituximab on fresh human CLL cells and found that GA101-induced cell death preferentially involves mitochondrial-mediated apoptotic mechanisms. This observation was supported by a reduction of the $\Delta\psi_m$, accumulation of ROS and translocation of Bax and Bak to the mitochondria.

Sensitivity of CLL cells to therapy has been reported to be influenced by the relative ratios of pro- and anti-apoptotic proteins. Kitada reported that patients whose cells had a high Bcl-2:Bax ratio had a lower response rate to fludarabine.(26) In this study we found that exposure to GA101 caused conformational activation and mitochondrial translocation of Bax as well as increased content of Bak. The conformational changes of these proteins have been suggested to modify the protein-protein interactions that are required for the integration of damage signals and the commitment of the cell to apoptotic death.(27) Activation of Bax or Bak was not blocked by the broad caspase inhibitor Z-VAD.fmk, thereby suggesting that Bax translocation or Bak accumulation precedes caspase activation or is caspase-independent as previously described in some experimental models using cell lines.(28) Of interest GA101-induced cell death was not associated with a decrease in Bcl-2 content and Bcl-2 protein was found to increase in a minority of patient samples after exposure to anti-CD20 antibodies. Several studies have provided evidence suggesting that pro- and anti-apoptotic molecules can function independently of one another. For instance, genetic evidence indicates that Bax and Bcl-2 are each capable of regulating death in the absence of the partner.(29) Therefore, the activation of Bax could override the protection of cells by Bcl-2 and induce a program of mitochondrial dysfunction that results in cell death.(15)

Exposure of CLL cells to GA101 was associated with the dissipation of the mitochondrial transmembrane potential. However, rituximab failed to depolarize the mitochondrial membrane and this was consistent with a previous report.(30) It has been established that Bcl-2 family proteins regulate $\Delta\psi_m$, thereby controlling the release of apoptosis-inducing proteins that are sequestered in the inter-membrane space of mitochondria such as cytochrome c.(31) Loss of $\Delta\psi_m$ was only partially prevented by Z-VAD.fmk, suggesting that the loss of $\Delta\psi_m$ is partially caspase-independent and could be secondary to the

integration of Bax into the outer mitochondrial membrane. Caspase activation could be involved in the amplification of $\Delta\psi_m$ loss after initial dissipation of the transmembrane potential by activation of Bax. However several intracellular signals can converge to cause loss of inner mitochondrial membrane potential.(32)

CLL cells accumulated larger amounts of ROS after exposure to GA101 than after exposure to rituximab. ROS play an important role in the induction of apoptosis under both physiological and pathological conditions. CLL cells have been reported to have high baseline ROS content.(33) Increased production of ROS may lead to the release of Cyt c, either by oxidation of the mitochondrial pores or by oxidation of cardiolipin, an inner mitochondrial membrane protein which binds Cyt c.(34) Although GA101 increased ROS production in CLL cells, antioxidants were unable to prevent the loss of $\Delta\psi_m$ or to circumvent GA101-induced apoptosis, suggesting that increased ROS content was not the *primum movens* of GA101-induced cell death. A similar observation has previously been reported in CLL cells exposed to gossypol, a compound reported to bind to Bcl-xL, since this compound increased production of ROS but antioxidants did not alter gossypol-induced cytotoxicity.(35) Increased ROS metabolism in cancer cells has been suggested to constitute a potential therapeutic target.(36)

The mitochondrial pathway is commonly activated by cytotoxic agents active in CLL such as fludarabine(37) and other cytotoxic anticancer drugs.(38) Shen *et al.*⁽³⁹⁾ demonstrated rituximab-induced caspase-3 activation concurrent with induction of apoptosis. Furthermore, activation of this effector caspase appears to be associated in rituximab-treated CLL cells with activation of caspase-9. Typically, caspase-9 is activated by a mitochondria-dependent pathway involving release of Cyt c from the organelles and activation of Apaf-1, which binds and activates procaspase-9.(40) Moreover, Jonna *et al.*(41) found that mitochondria and the caspase-9 activation pathway but not caspase-8 are involved in rituximab-induced apoptosis in follicular lymphoma cells. Our study confirmed the involvement of mitochondria and the subsequent cleavage of caspase-3 and -9 in most patient samples. Caspase-8 was also found to be processed in some patients samples examined. This initiator caspase is typically activated by TNF/FAS-family cytokine receptors and is often used by immune effector cells that mediate antibody-dependent cellular cytotoxicity. Several investigators have reported the involvement of the death receptor pathway in rituximab-induced apoptosis in B cell lymphoma models such as Ramos cells and thus sensitization toward Fas-induced apoptosis induction via Fas multimerization and recruitment of caspase-8 and FADD to the DISC.(42) In some cellular contexts, cytotoxic drugs can activate caspase-8 in a Fas-independent manner.(43) The involvement of caspase-8 in the intrinsic mitochondrial pathway observed in our study could result from activation of other caspases independently of Fas.

Altogether, these observations along with Moessner *et al.* (44) data confirmed that GA101 exerted stronger direct B-cell death induction than rituximab. Golay *et al.*(45) suggested that FACS analysis concerning the cell death induced by the type II anti-CD20 MAbs such as B1 (tositumomab) and GA101 should be interpreted with caution in the study of antibodies that cause strong homotypic adhesion and leading to cell aggregation; thereby, they suggested that large aggregates were probably mostly excluded from the analysis, falling outside the gate of scatter plots and only dead cells were counted within the single-cell population. Our results confirmed that the GA101-induced cell death is definitively not an artefact of pipetting and flow cytometric analysis and the response of Moesner *et al.* showed clearly with more than one technique the direct induction of cell death with type II MAbs.

In contrast to what has been shown for type II CD20 antibodies we found that GA101 exhibited more pronounced induction of apoptosis in a caspase-dependent manner and this may be the result of using fresh B-CLL samples and not cell lines.

Modulation of anti-apoptotic proteins is a promising strategy to sensitize cells to antileukemic agents. Preclinical data have shown that inhibition of Bcl-2, inhibition of the interaction between Bcl-2 or Bcl-xL and partner proteins with compounds such as ABT-737(46) or inhibition of Mcl-1 were associated with increased sensitivity to antileukemic agents.(47) Kitada *et al.* reported that high Mcl-1 levels in CLL cells were correlated with a lower complete response rate in CLL patients treated with single agent therapy.(26) As previously reported, we found that Bcl-xL was not present in most CLL samples at baseline, but increased significantly after exposure to rituximab or GA101. Bcl-xL, a mitochondrial membrane protein, promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to a variety of stimuli. Over-expression of Bcl-xL is reported to confer a multidrug resistance phenotype.(48) Moreover, inhibition of Bcl-xL expression results in an altered ratio of Bax to Bcl-xL and subsequent mitochondrial-mediated cell death.(49) Bcl-xL has been suggested to be a major actor in preclinical models of resistance to rituximab.(50) We found that decreasing Bcl-xL content by transfection of a specific siRNA sensitized CLL cells to the cytotoxic effects of rituximab or GA101. The degree of sensitization was similar to that observed with ABT-737, a small molecule inhibitor of Bcl-xL. Our data strongly supported the results obtained by Herting *et al.* when combining GA101 with Bcl-2 family inhibitors such as ABT-737 and ABT-263.(51) Thus, Bcl-xL might constitute an interesting molecular target to potentiate the antitumor effect of therapeutic MAbs.

Our results show that the targeting of CD20 antigen by two different antibodies such as rituximab and GA101 involves both common and distinct apoptotic mechanisms. Insofar as these antibodies have little activity against CLL *per se* but sensitize cells to chemotherapy, it is important to identify the pathways influenced by these antibodies in CLL cells. Anti-CD20 antibodies, as other B cell receptor regulatory molecules, are likely to decrease the threshold above which lesions induced by chemotherapy cause cell death.(52) GA101, selected for increased apoptotic signaling and ADCC, is a type II antibody which does not cause localization of CD20 antigen to membrane rafts. Further experiments should determine whether relocalization of target antigens to rafts is an important determinant of cytotoxic mechanisms of MAb-induced cell death. As the family of MAbs targeting CD20 and other lymphoid antigens is steadily growing, a better understanding of mechanisms of toxicity is required to improve the use of these antibodies and possibly to determine which patients are most susceptible to benefit from a given therapeutic MAb.

Authorship

L.R. contributed to design of the study, acquisition and interpretation of results and wrote the manuscript

S.D. contributed to acquisition and interpretation of results

S.H., E.P., C.T. contributed in technical assistance

C.D. contributed to the design, acquisition and interpretation of results and corrected the manuscript

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Legends to Figures

Figure 1. Induction of apoptosis in CLL cells by anti-CD20 antibodies

Representative histograms showing flow cytometry analysis of Annexin V-positive stained CLL cells. (A) CLL Cells obtained from 9 patients were cultured in the absence or presence of 10 µg/ml of GA101 or rituximab (RTX) for up to 24 h. (B) Cells obtained from a representative patient were incubated either alone or in the presence of GA101 or rituximab for 24 h with or without pre-incubation with 50 µM Z-VAD.fmk. The error bars represent the standard deviations (SD). *P<0.05 and **P<0.001.

Figure 2. Alterations in mitochondrial membrane potential $\Delta\psi_m$ induced by anti-CD20 antibodies in CLL cells.

Representative histogram showing flow cytometry analysis of low DiOC6(3) staining of mitochondria in cells incubated up to 24 h in the presence or absence of GA101 or rituximab (RTX) (10 µg/ml). FCCP were used as positive control. Data from 9 patients are expressed as mean \pm SD (A). GA101-induced cell death is mechanistically associated with mitochondrial dysfunction. Leukemic cells from a representative CLL patient were incubated with 5µM of CsA for 1 h prior to treatment with either GA101 or rituximab (10µg/ml) then assayed for phosphatidyl serine externalization and PI permeability (B) and loss of $\Delta\psi_m$ (C). The effect of caspase inhibition on $\Delta\psi_m$ in a CLL sample. Cells were incubated either alone or in the presence of GA101 or rituximab with or without 1 h preincubation with 50 µM Z-VAD.fmk. $\Delta\psi_m$ was measured by flow cytometry using DiOC6(3) staining after 6 h of exposure to antibodies (D). The error bars represent the standard deviations (SD). *P<0.05.

Figure 3. ROS content in CLL cells exposed to anti-CD20 antibodies.

Representative histogram showing flow cytometry analysis of DHE-positive CLL cells. CLL cells obtained from 8 patients were incubated in the absence (Ctrl) or presence of GA101 or rituximab (RTX) (10 µg/ml) for 6 h (A). A CLL sample was incubated either alone or in the presence of GA101 or rituximab without (B) or with (C) 1 h preincubation with 50 µM of ASC then assayed to phosphatidyl serine externalization and PI permeability using AnnexinV/PI, Δψ_m using DiOC6(3) staining and the production of ROS using DHE after 6 h of exposure to antibodies.

Figure 4. Effect of anti-CD20 antibodies on the expression levels of apoptotic proteins in CLL cells.

CLL cells from P13, P19 and P21 were incubated in the presence or absence (Ctrl) of GA101 or rituximab (RTX) (10µg/ml) for 24 h to study the expression levels of pro-apoptotic proteins (A), anti-apoptotic proteins (B) of Bcl-2 family members and the activation of caspase-3, caspase-8 and caspase-9 (C). Cells were lysed then immunoblotted using specific antibodies. The expression levels of the proteins were normalized against the expression level of β-Actin.

Figure 5. Effect of anti-CD20 antibodies on subcellular distribution of Bax, Bak, Bcl-2 and Cyt c in CLL cells.

Immunoblot analysis of cytosolic (C) and mitochondrial (M) fractions obtained from a representative CLL patient. CLL cells were incubated either in the absence (Ctrl) or the presence of GA101 or rituximab (10 µg/ml) for 3, 6 and 24 h (A). Cox4 antibody was used as control to confirm that our mitochondrial fraction was successfully separated from the cytosolic fraction. Flow cytometric histogram plots showing the conformational changes of Bax as determined by staining with anti-Bax (clone YHT-6A7). Representative histogram showing CLL cells from a representative CLL patient either in the absence or the presence of GA101 or rituximab for 24 h with or without 1 h preincubation with 50 µM Z-VAD.fmk (B).

Figure 6. Down-regulation of Bcl-xL by siRNA sensitizes CLL cells to the cytotoxic effect of anti-CD20 antibodies

Representative histograms showing flow cytometry analysis of AnnexinV-positive cells. CLL cells obtained from 6 patients were transfected with anti-Bcl-xL or control siRNA by

sonoporation then exposed to GA101 or rituximab (RTX) (10 μ g/ml) for 24 h (A). In parallel, cells were exposed to a positive control ABT-737 (1nM) either treated or not with GA101 or rituximab without sonoporation (B). Western blot analysis was used to confirm the inhibition of Bcl-xL proteins either in cells treated with or without GA101 or rituximab in comparison to a scrambled siRNA either treated or not with anti-CD20 antibodies 48 h after sonoporation (C). The error bars represent the standard deviations (SD). (*P<0.05; # P=0.06)

Figure 1.

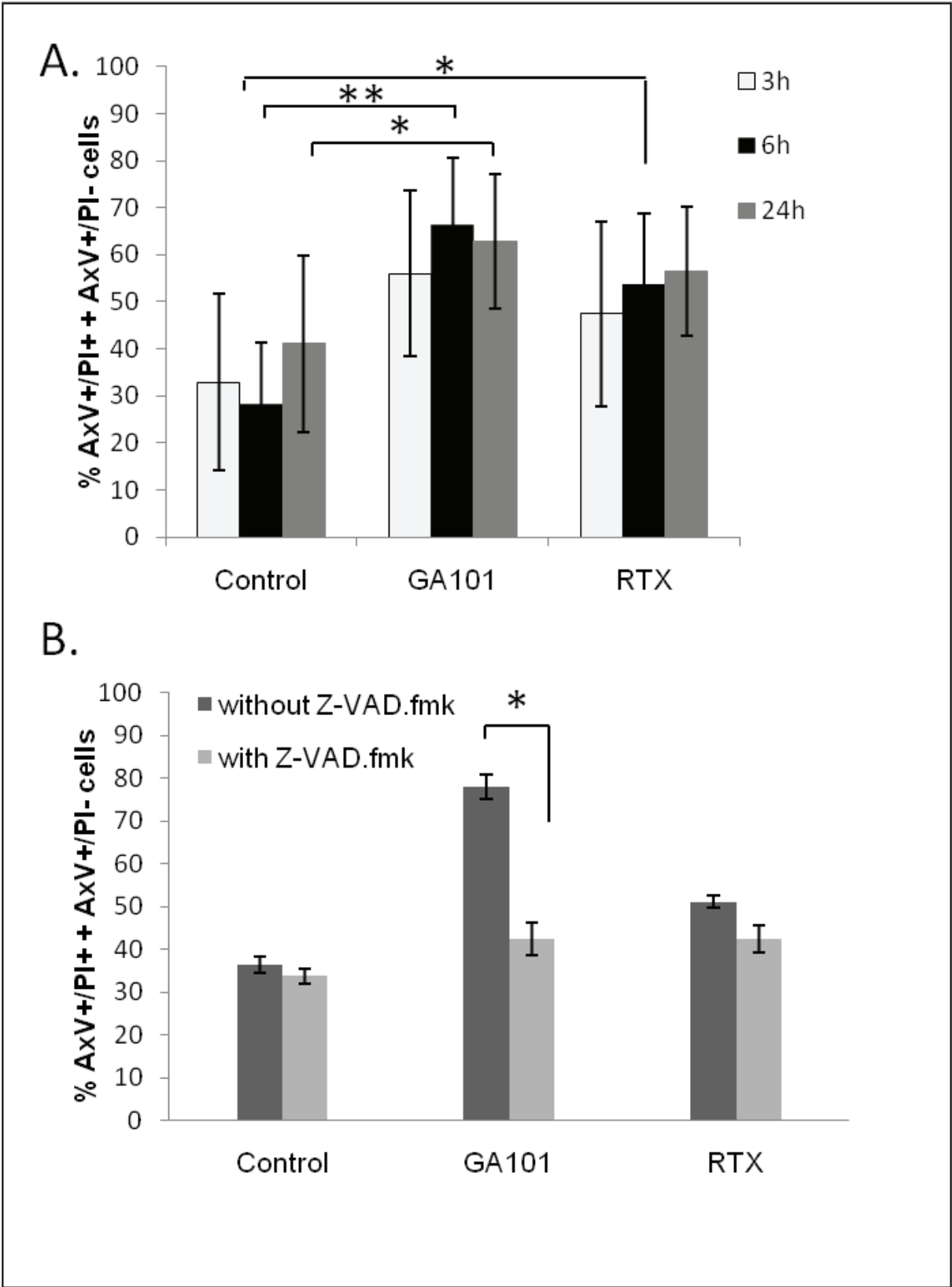
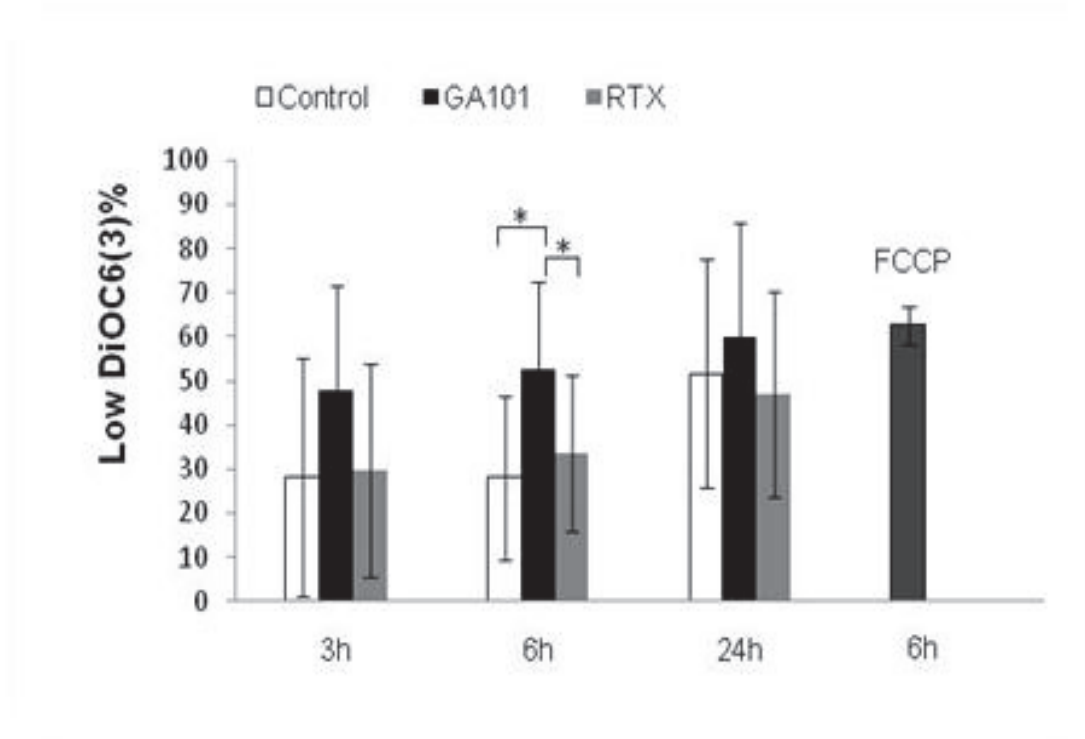
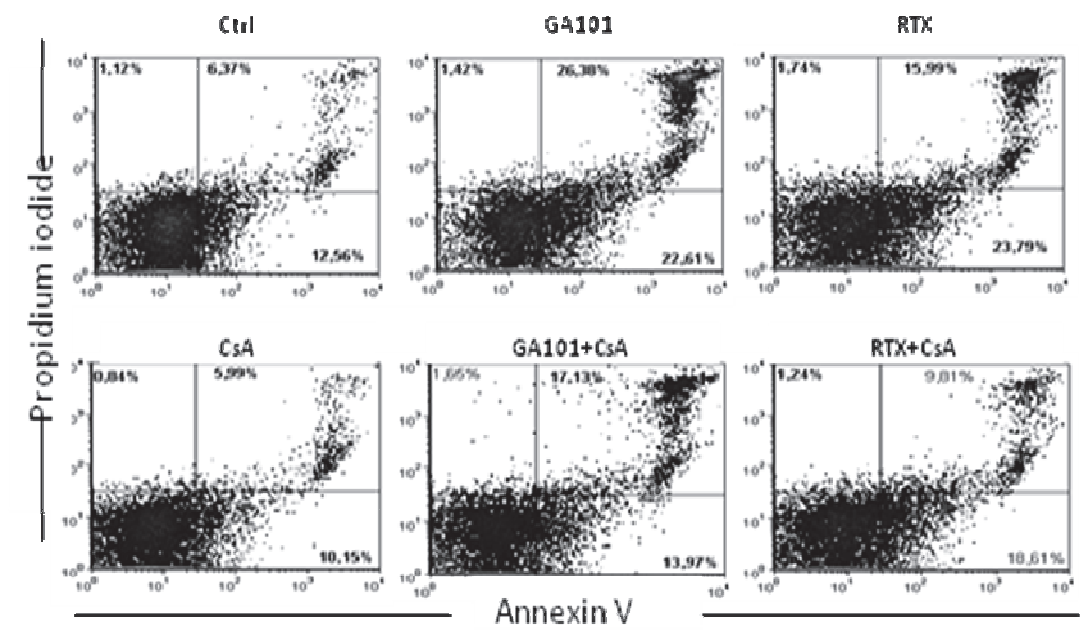


Figure 2.

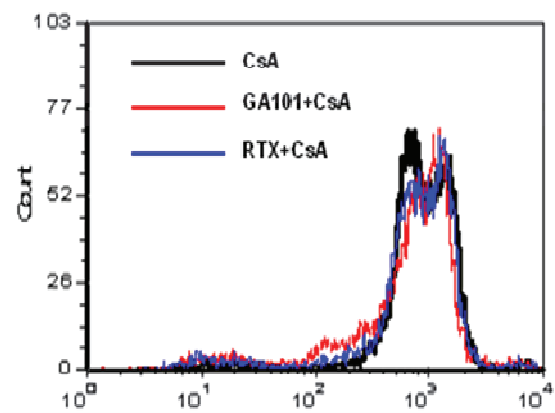
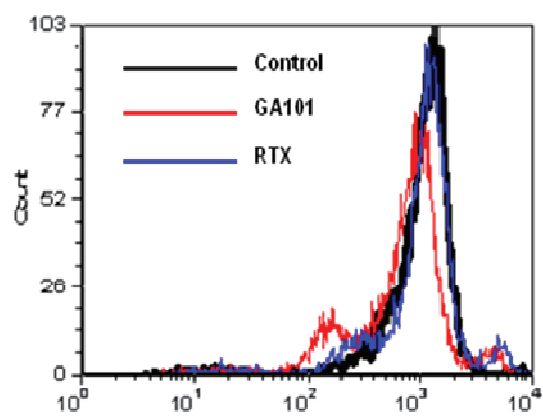
A-



B-



C.



D.

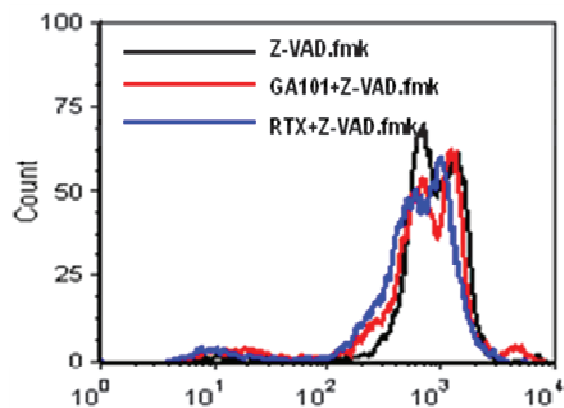
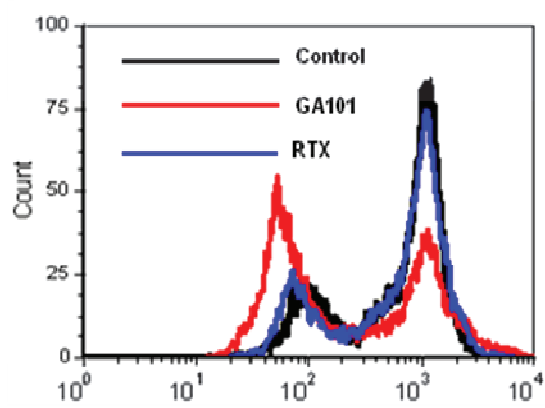


Figure 3

Figure 3.

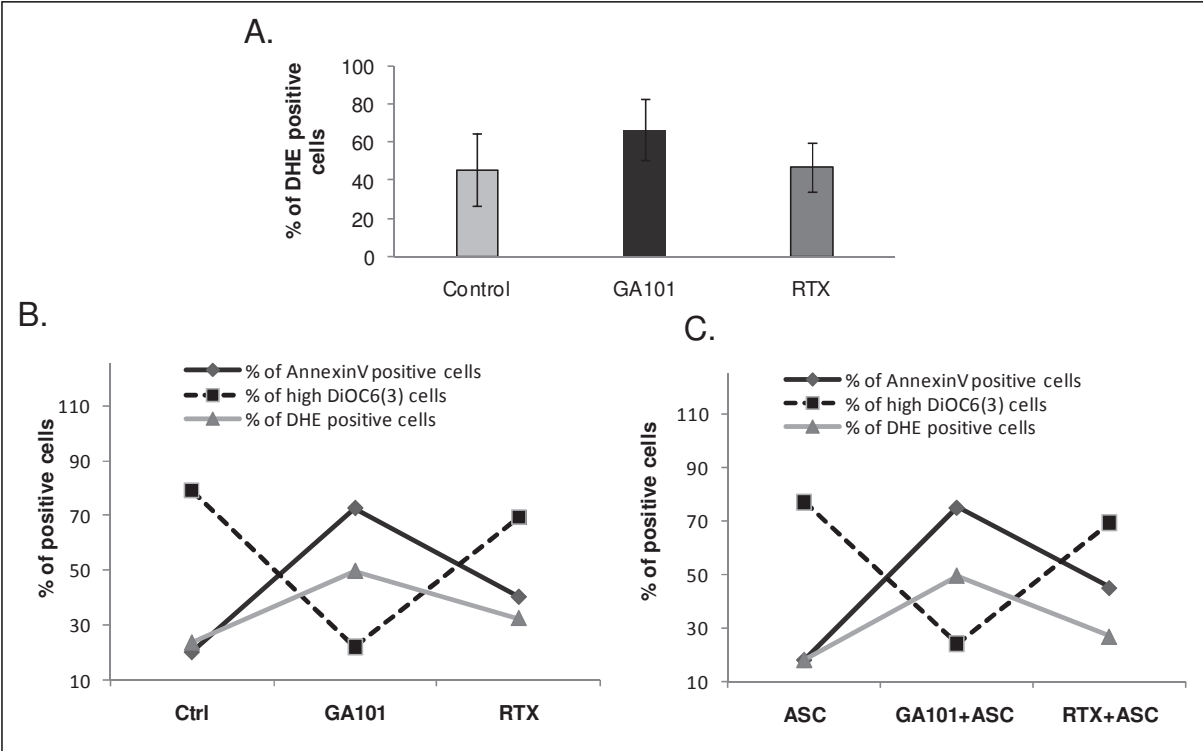


Figure 4.

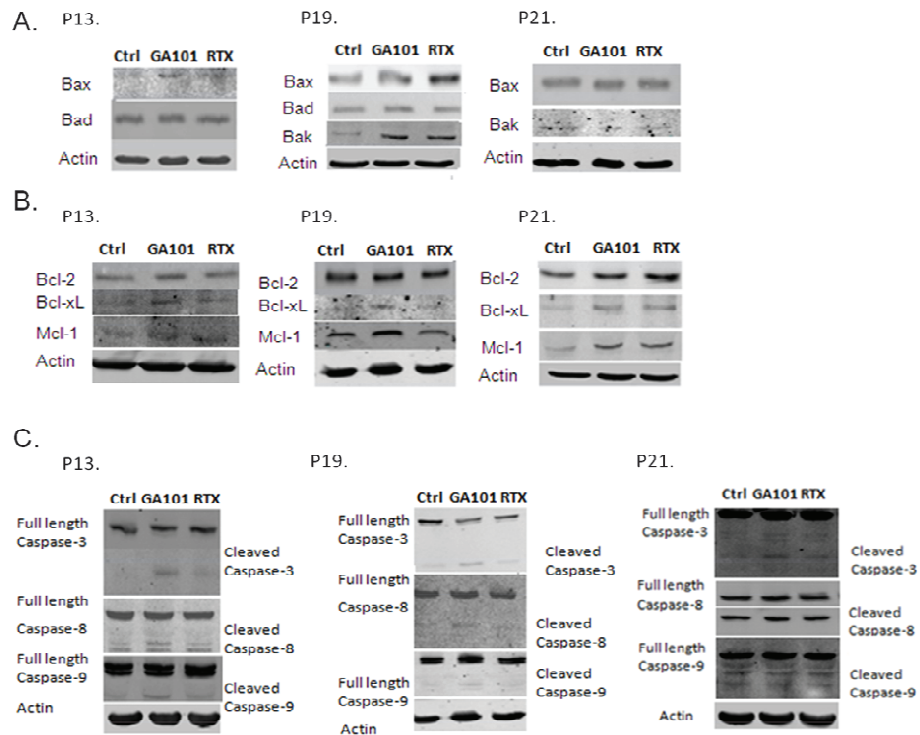


Figure 5.

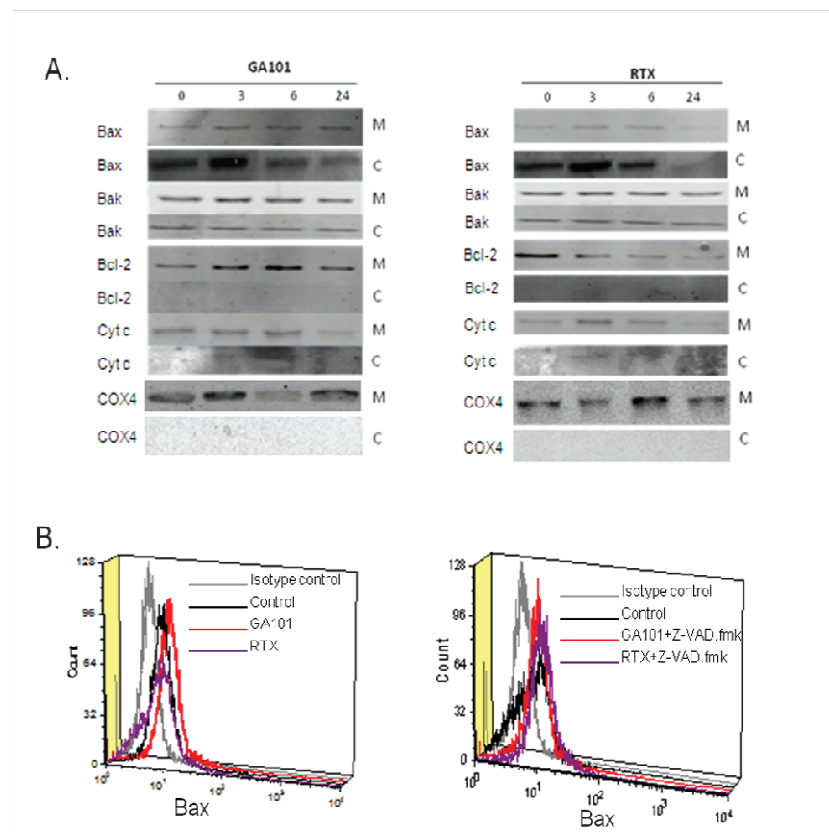
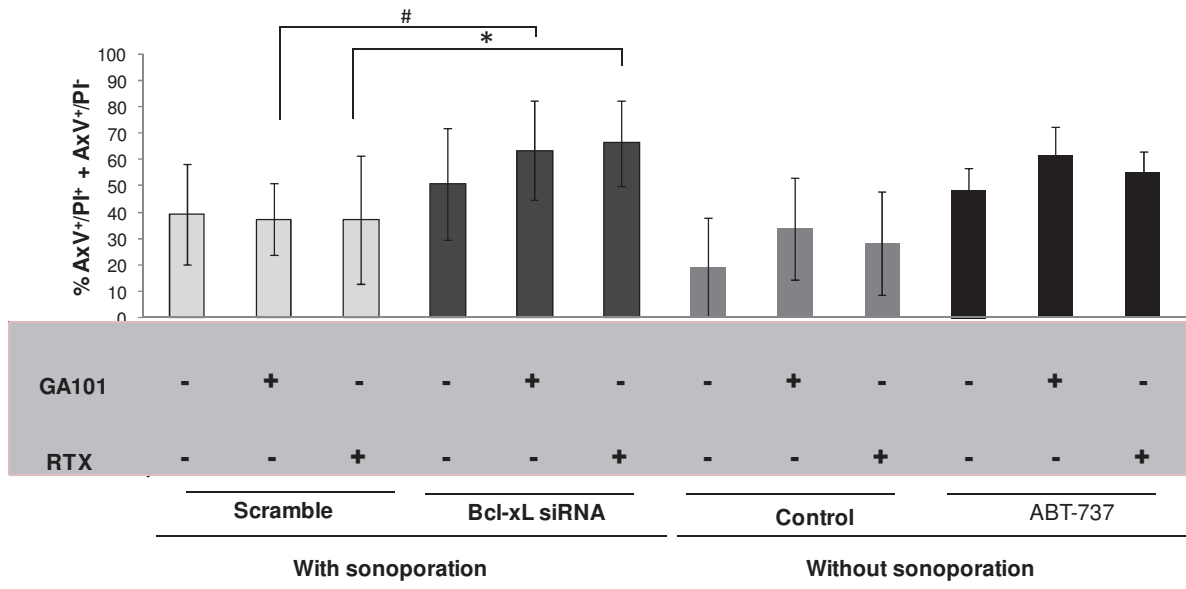


Figure 6.



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Supplemental data

Table 1S. Clinical characteristics of CLL patients

Sex was defined as male and female, and age is expressed in years. Staging was according to the Binet's staging system. Treatments (if any) were received at least 3 months before cell sampling.

Abbreviations: ATM, Ataxia-telangiectasia-mutated; f, female; CLB, chlorambucil; FC, fludarabine and cyclophosphamide; m, male; ND, not determined; RFC: rituximab, fludarabine cyclophosphamide; TP, Tumor Protein 53; ZAP-70, Zeta-chain-associated protein kinase 70.

Patient number	Age (yrs)	Sexe	Binet stage	Nb of prior therapy	Absolute lymphocyte	%CD38/CD19+	ZAP-70 status	ATM
P1	61	F	A	0	15000	0	0	ND
P2	74	M	C	CLB,FC	10915	11	NR	ND
P3	98	M	C	FC autograft	45420	99	NR	(ATM, D12Z3, D13S319, TP53)X2
P4	78	M	C	FC autograft	54540	71	NR	(ATM, D12Z3, D13S319, TP53)X2
P5	69	M	B	RFC	48110	3	31	(ATM, D12Z3, TP53)X2, D13S319X1
P6	75	F	A	0	4890	0	6	ND
P7	65	F	D	CLB,FC	50000	0	NR	ND
P8	69	F	A	0	15470	82	NR	ND
P9	69	F	A	CLB,FC	5910	92	NR	47
P10	61	M	A	U	23440	98	4	ND
P11	73	M	B	RFC	8910	8	ND	46
P12	70	M	A	0	20010	ND	ND	ND
P13	87	F	A	0	ND	ND	ND	ND
P14	59	F	A	0	27080	0	NR	48
P15	75	M	B	FC	5830	88	NR	ND
P16	78	M	A	0	4640	88	NR	ND
P17	61	M	A	0	17780	0	NR	ND
P18	79	M	C	RFC	53750	0	0	46
P19	68	F	A	0	33460	71	NR	ND
P20	75	M	C	RFC	3070	3	0	ND
P21	74	M	B	RFC	10590	93	32	ND
P22	70	F	B	0	1077	0	NR	ND
P23	77	M	A	0	13680	4	22	ND
P24	53	M	A	0	17040	2	9	(ATM, D12Z3, D13S319, TP53)X2
P25	71	M	A	0	12180	0	NR	ND
P26	81	M	A	0	18930	85	0	ND
P27	72	M	A	0	18820	0	15	ND
P28	68	M	B	0	5830	97	NR	ND
P29	69	F	B	FC	20050	0	0	ND
P30	71	F	A	0	1830	0	15	ND
P31	74	M	A	0	15100	30	NR	ND
P32	72	M	A	U	12970	0	25	ND

Table 2S. All antibodies used in this study are summarized in this table.

Target	Type	Clone	Provider	Dilution - Technique
Bcl-2	mouse monoclonal	124	Dako	1/1000
Bcl-xL	mouse monoclonal	2H12	Sigma	1/500
Mcl-1	mouse monoclonal	22	BD Pharmingen	1/1000
Bax	mouse monoclonal	YTH-6A7	Trevigen, Gaithersburg, MD	1/500
Bax (N-20)	rabbit polydonal		Santa Cruz	1/1000
Bak (H-211)	rabbit polydonal		Santa Cruz	1/1000
Bak(G-23)	rabbit polydonal		Santa Cruz	1/1000
Bad	mouse monoclonal	APHP4/5	Serotec	1/500
caspase-3	mouse monoclonal	19	BD transduction laboratories	1/1000
caspase-9	mouse monoclonal	(96-2-22)2-23	Upstate	1/1000
caspase-8	mouse monoclonal	12F5	Biosource	1/1000
cox4	mouse monoclonal	s2207	Clontech	1/500
cytochrome c	rabbit polydonal		Clontech	1/100
β-Actin	mouse monoclonal	clone AC-15	Sigma	1/1000

Figure 1S :

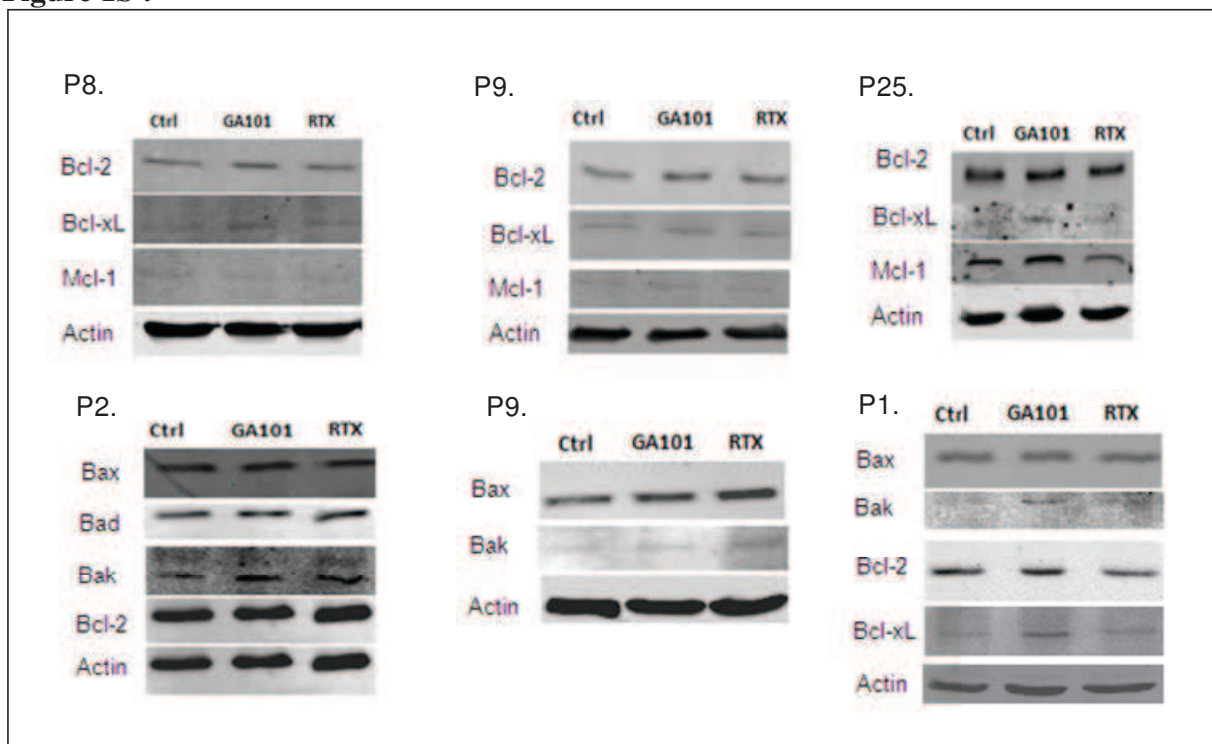


Figure 1S. Effect of anti-CD20 antibodies on the expression levels of apoptotic proteins in CLL cells. CLL cells were incubated in the presence or absence (Ctrl) of GA101 or rituximab (RTX) (10 µg/ml) for 24 h to study the expression level of pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 family members.

Discussion

In the present study, we developed tools allowing us to transfect fresh CLL cells using sonoporation then applied this approach to investigate the cytotoxic mechanisms of two anti-CD20 MAbs, rituximab and GA101.

Sonoporation is an original method creating temporary pores in cells, allowing penetration of complex molecules such as plasmids. Our experiments show that this method can be adapted to cells in suspension, both continuous cell lines and fresh CLL cells. Thanks to our collaboration with INSERM U556 (JL Mestas, JY Chapelon), we performed both transient and stable transfections. This method has proved invaluable for the study of fresh human samples since conventional methods such as electroporation, lipofection or nucleofection are often associated with a poor yield. Since our initial publication with CLL cells we have successfully applied this method to fresh myeloma cells and will explore its application to fresh acute leukemic cells in the near future. Additional experiments performed in murine models have confirmed that this method can also be applied *in vivo* by US treatment of established tumors. Sonoporation has therefore emerged as a powerful tool for the *ex vivo* evaluation of hematological malignancies with potential applications in the clinical setting as well.

Rituximab is the first MAb approved by the FDA that revolutionized the treatment of B-cell malignancies and increased the median overall survival of patients with many diseases including the B-CLL. GA101, a novel Fc-glycoengineered type II MAb, is thought to have an enhanced and superior functional activity than rituximab [250, 251, 253]. The rationale underlying the development of GA101 was that ADCC and apoptotic signaling are key properties of the antitumor effect *in vivo* whereas CDC is believed to be involved in side-effects of MAb therapy. While data is accumulating that ADCC and apoptotic signaling are important in the clinic, the question of the role of CDC in toxicity or efficacy of MAbs is still controversial.

In this study we chose to focus on potential differences in apoptotic signaling induced by rituximab and GA101 in fresh CLL cells. The comparison of cytotoxic activity of GA101 to rituximab on freshly isolated CLL cells showed that GA101 induces significantly apoptotic cell death after 6 and 24 hours; whereas, rituximab induces significantly apoptosis only after 24 hours. These observations confirmed our previous results on the human transformed FL RL cells where GA101 induced significantly more apoptotic cell death in RL cells *in vitro* than rituximab after exposure times ranging from 6 to 24 hours (article in revision in

Molecular Cancer Therapeutics). These differences in the kinetics of apoptotic induction suggest possible differences in mechanisms of action. We found that GA101 induces apoptosis preferentially through the mitochondrial intrinsic pathway. Upon exposure to GA101, the resulting mitochondrial dysfunction includes a change in the mitochondrial membrane potential ($\Delta\psi_m$), the production of ROS, the opening of the PTP, and the release of the Cyt *c*. The release of Cyt *c* led to cleavage of caspase 3, 8 and 9 in CLL cells exposed to GA101.

The major proteins involved in apoptotic signaling at the mitochondrion are Bcl-2 family members which had been well documented to affect the mitochondrial outer membrane permeability [99]. The study of the expression levels of the pro- and anti-apoptotic proteins of this family in our series showed a strong expression variation in the content of these proteins among samples. Overall we did not observe significant alterations in Bcl-2 content; whereas, we found large variations in the expression levels of Bcl-xL and Mcl-1. Moreover, the content of pro-apoptotic proteins Bax, Bak and Bad increased in most samples after exposure either to rituximab or to GA101. Thus, we suggested that the exposure of cells to either rituximab or GA101 may induce a change in the ratio of pro- and anti-apoptotic proteins leading mostly to the activation of apoptosis. However we did not have clinical data of sensitivity to Mabs since many of these patients were not due to received therapy after sampling, and correlations with *in vitro* sensitivity data were available only for a fraction of samples.

In our previous RL model *in vivo*, there were no differences concerning the expression levels of Bim, Bak, Bcl-2, Bcl-xL, caspase-8 and caspase-9 on xenografts samples after exposure of mice to MAbs. However, caspase-3 protein was found to be more strongly expressed in cells exposed to GA101 than in cells exposed to rituximab. This was in agreement with our observation about the cleavage of caspase-3 when CLL cells were treated with GA101.

The conformational changes of some pro-apoptotic proteins have been suggested to modify the protein-protein interactions that are required for the integration of damage signals and the commitment of the cell to apoptotic death [398]. The study of conformational activation and mitochondrial translocation of Bax and Bak upon exposure of CLL cells to antibodies showed that Bax is translocated and that Bak accumulates in the mitochondria. This activation was not blocked by the broad caspase inhibitor Z-VAD.fmk, thereby suggesting that Bax translocation or Bak accumulation precedes caspase activation or is

caspase-independent as previously described in some experimental models using cell lines.[399]

Targeting the Bcl-2 family is a promising strategy to sensitize cells to antileukemic agents. Bcl-xL has been suggested to be a major actor in preclinical models of resistance to rituximab [420, 422]. As previously reported, we found that Bcl-xL was not present in most CLL samples at baseline, but increased significantly after exposure to rituximab or GA101, a finding consistent with observations made by the Bonavida group in cell lines [423]. The inhibition of *Bcl-xL* using *Bcl-xL* siRNA exposure leads to decreased cell growth and apoptosis in CLL cells exposed to rituximab or GA101 *in vitro*. This synergism was similar to that observed with the BH3 mimetic compound, ABT-737, that synergizes with a range of cytotoxic chemotherapy agents in CLL [416]. Thus, Bcl-xL might constitute an interesting molecular target to potentiate the antitumor effect of therapeutic MAbs.

There are still many unknowns regarding the apoptotic signaling induced by anti-CD20 antibodies. Bezombes *et al.* recently reported that rituximab could cause a time-dependent inhibition of the BCR-signaling cascade involving Lyn (src family tyrosine-protein kinase), Syk (tyrosine-protein kinase), PLC gamma 2 (phospholipase C gamma), Akt (protein kinase B), and ERK (Extracellular signal-regulated kinase), and calcium mobilization [283]. The inhibitory effect of rituximab was found to correlate with decrease of raft-associated cholesterol, complete inhibition of BCR relocalization into lipid raft microdomains, and down-regulation of BCR immunoglobulin expression. BCR signalling has emerged as a key prosurvival pathway not only for normal but also for neoplastic B cells [424]. Given the fact that rituximab induces redistribution of CD20 antigen to raft microdomains but GA101 does not, the comparison of these two antibodies in terms of consequences on BCR signalling will be of particular interest.

Pangenomic studies are another way to explore signaling pathways elicited by MAbs. We recently analysed the effects of exposure to rituximab or GA101 on the transcriptome the RL line, both *in vitro* and *in vivo*. These experiments showed that it was very difficult to compare these two situations since very few differentially expressed genes were found in both conditions (data not shown). However this approach allowed us to identify potentially interesting signaling pathways, involving Early Growth Factor 1 (EGF1) or Toll Like Receptor 4 (TLR4), the expression of which was increased after exposure to both antibodies. Similar approaches using pangenomic approaches are underway in fresh CLL samples exposed to rituximab or GA101. Notwithstanding the limitations due to heterogeneity between patient samples and the requirement to analyse as large a cohort as possible we hope

that this approach will yield insight regarding intracellular modifications induced by exposure to anti-CD20 antibodies.

Overall data from the literature and from this study suggests that apoptotic signaling induced by therapeutic MABs is complex, may depend on the epitope targeted or the MAB studied, and may be quite different from one patient to another. Practical ways to deal with this heterogeneity may be either to define the smallest common denominator, i.e. alterations commonly found in a significant number of patient samples, and exploit them therapeutically, or to take into account this heterogeneity in order to tailor therapy according to the biological profile of disease of each patient.

Conclusion and perspectives

These studies have allowed the comparison of type I and type II anti-CD20 antibodies and identified both common and distinct apoptotic mechanisms elicited by these MAbs (Figure 10). Our results provide preclinical evidence that GA101 may constitute a promising new therapy for the treatment of B-cell malignancies including NHL such as the RL follicular lymphoma model and CLL. These data are in keeping with preliminary clinical data analyzing the effect of GA101 in patients with lymphoproliferative diseases [255, 425, 426]. As the family of MAbs targeting CD20 and other lymphoid antigens is steadily growing, a better understanding of mechanisms of toxicity is required to improve the use of these antibodies and possibly to determine which patients are most susceptible to benefit from a given therapeutic MAb.

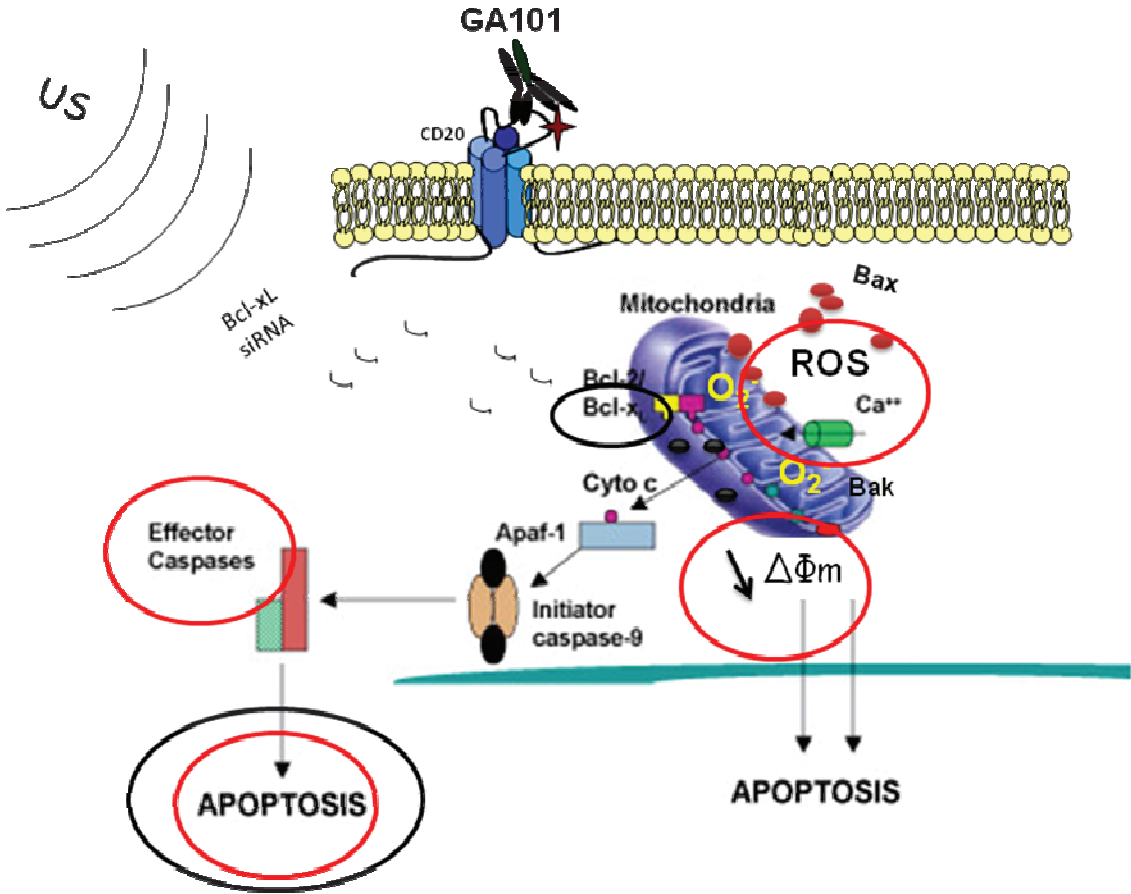


Figure 10: The schematic diagram illustrates the apoptotic signaling pathway triggered by rituximab and GA101 (red circle) following its interaction with CD20 as well as the enhancement of MAb mediated apoptosis after the inhibition of *BclxL* with *BclxL* siRNA using US

Apoptotic signaling induced by anti-CD20 antibodies appears to be complex. Previous experiments on CLL cells in our laboratory showed an induction of Raf-1 kinase inhibitor protein (RKIP) expression after treatment with GA101. RKIP has previously been identified as an inhibitor of Raf1 kinase in the Raf-MEK-ERK signaling pathway by interacting with Raf1 and MEK (MAPK/Erk kinase) thereby disrupting the interaction of Raf1 and MEK by competing with their binding. RKIP was also found to play a pivotal role in NF- κ B (Nuclear factor-kappa β) pathway by inhibiting I κ B α (Inhibitor of NF- κ B) degradation and the p50 and p65 nuclear translocation [427-429]. Rituximab treatment of Ramos and Daudi NHL-B cells significantly up-regulated RKIP expression by interrupting the ERK1/2 signaling pathway through the physical association between Raf-1 and RKIP, which was concomitant with Bcl-xL downregulation [430]. Therefore, the study of the NF κ B and ERK1/2 signaling pathways after exposure to these antibodies will be interesting.

Our results suggest that *Bcl-xL* could constitute a potential therapeutic target in cancer cells. Future studies will combine small molecule inhibitors of Bcl-xL (such as ABT-737) with MAbs *in vivo*. Other approaches will consist in the *in vivo* administration of *Bcl-xL* siRNA to tumor bearing animals, if possible in combination with sonoporation of established tumors. Our hypothesis is that inhibition of *Bcl-xL* could sensitize B-cell lymphoma to anti-CD20 MAbs *in vivo*. An important limitation of our current approach is the scarcity of preclinical models of CLL. Our group is currently exploring the possibility of reproducibly establishing tumors from fresh CLL samples in profoundly immunodepressed mice, such as the NOG model.

Considerable work will be required to understand which cytotoxic mechanisms are relevant in the clinic or in a given patient in particular. Current data has essentially been derived from cells lines or observational studies in patients. Limitations in cell line models are due to several factors including the fact that ADCC is poorly reproduced *in vitro*, common assays consisting in short co-incubations between target cells and “effector cells” in a simplified environment which does not reproduce the complexity of the bone marrow or the lymph node microenvironment. Current efforts by our group are aimed at understanding how the tumor microenvironment may influence ADCC cytotoxicity. Similarly it may be overly simplistic to distinguish between apoptotic signaling, CDC and ADCC. In the clinical setting it is likely that these three mechanisms interact or synergize to induce cell death.

A second line of studies will concern the use of sonoporation as a laboratory tool or a therapeutic modality. The INSERM U556 has been a pioneering group in the development of

US therapy in cancer. Our ongoing collaborations aim to create a prototype allowing routine sonoporation of cells by research groups and will determine whether sonoporation can be used to specifically activate certain therapeutic formulations in tumors. These studies will also require additional studies on the biological consequences of ultrasound exposure, both in normal and in cancer cells.

ANNEXE

Article IV

*In vivo Model of Follicular lymphoma resistant to
Rituximab*

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In vivo Model of Follicular Lymphoma Resistant to Rituximab

Stéphane Dalle,^{1,2} Sophie Dupire,¹ Stéphanie Brunet-Manquat,¹ Lina Reslan,¹ Adriana Plesa,² and Charles Dumontet^{1,2}

Abstract Purpose: Follicular lymphoma (FL) is the most common subtype of indolent lymphomas. Rituximab is widely used alone or in combination therapy for the treatment of FL. Despite its well-established clinical efficacy, a subpopulation of patients does not respond to rituximab and most patients will relapse after therapy. The mechanisms of action and resistance to rituximab are not fully understood.

Experimental Design: To study these mechanisms we developed an *in vivo* model of FL resistant to rituximab. This model was developed using the human RL line, isolated from a patient with FL, grown as xenotransplants in severe combined immunodeficient mice, exposed weekly to rituximab *in vivo*, followed by serial reimplantation and reexposure to rituximab, until a resistant phenotype was obtained.

Results: RL-derived tumors unexposed to rituximab were grown as controls and compared with the resistant tumors. Although the expression of CD46 and CD55 antigens were not differently expressed in the resistant cells, the complement inhibitor CD59 was overexpressed in a subpopulation and CD20 was found to be expressed at a lower level in a minority of cells. Bcl-X_L and YY1 were also found more highly expressed in rituximab-resistant cells.

Conclusion: This model provides insight on potential *in vivo* resistance mechanisms to rituximab and could help contribute to the development of novel therapies in rituximab-refractory diseases.

Rituximab was the first commercially available monoclonal antibody for the treatment of lymphoma. It is now a treatment of choice for a variety of lymphoproliferative disorders including low- and high-grade non-Hodgkin's lymphomas (NHL; refs 1–5). Various *in vitro* and *in vivo* experiments have shown that elimination of CD20+ cells by rituximab involves complement-dependent cytotoxicity (6–13), the recruitment of effector cells leading to antibody-dependent cellular cytotoxicity (14) and direct apoptotic signaling (15).

Follicular lymphoma (FL) is the most common subtype of indolent lymphoma. Rituximab is now widely used either alone or in combination with multiagent chemotherapy for

the treatment of FL, either at diagnosis (16, 17), at relapse (18–20), or for maintenance therapy (2, 21, 22). However, despite its well-established clinical efficacy, a subpopulation of patients does not initially respond to rituximab and most patients will relapse after rituximab therapy (23, 24).

To date, the mechanisms of action of rituximab are not fully understood and are a matter of debate. The complement regulatory proteins CD46, CD55, and CD59 have been shown to inhibit rituximab-mediated cell kill by interfering with complement activation (8, 10, 13). *In vivo* studies show that FcγR receptor expression is necessary to eradicate NHL in a murine animal model (14), suggesting that antibody-dependent cellular cytotoxicity may play a significant role in the activity of rituximab. Furthermore, certain polymorphisms in the FcγRIIIa gene have been associated with variable clinical and molecular responsiveness to anti-CD20 monoclonal antibody therapy in patients with indolent NHL (25). Several studies have addressed whether the level of CD20 expression level or other surface markers may be linked to the efficacy of rituximab but the results remain controversial (23, 26–28). *In vitro* analysis of FL cells revealed no correlation between CD20 expression level and complement-dependent cytotoxicity sensitivity (7, 8). On the other hand, Czuczman et al. (28) have recently developed an *in vitro* resistant model, in which they showed that the CD20 gene and protein were down-regulated during the acquisition of the resistant phenotype.

Moreover rituximab is known as a sensitizer for drug-induced apoptosis by interference with several intracellular antiapoptotic pathways. The transcription factor Yin Yang 1 (YY1) has recently emerged as a negative regulator for the expression of Fas and the Trail receptor DR5. The action of rituximab has been suggested to result in a decreased

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Sophie Dupire contributed to acquisition and interpretation of results.

Stéphanie Brunet-Manquat contributed to acquisition and interpretation of results.

Adriana Plesa contributed to acquisition and interpretation of results.

Lina Reslan contributed to acquisition and interpretation of results.

C. Dumontet contributed to the design and submission to animals ethical committee of the study, acquisition, and interpretation of results and corrected the manuscript.

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Translational Relevance

Rituximab is now a standard of care in the treatment of follicular lymphoma patients. But *de novo* or acquired resistance to this anti-CD20 monoclonal antibody is common. Dalle et al. established an *in vivo* model of follicular lymphoma resistant to rituximab. This model should prove to be quite useful to identify novel mechanisms of resistance to rituximab *in vivo* and in the development of novel monoclonal antibodies or therapeutic strategies, including drug combinations designed to overcome resistance to rituximab.

expression of YY1 and sensitization of tumor cells to Fas and Trail-induced apoptosis (29).

There remains a large discrepancy between the results obtained *in vitro* using FL-derived cells (low level of cytotoxicity) and results observed in the clinical setting (high level of efficacy). Most lymphoma lines do not exhibit high sensitivity to rituximab *in vitro*, and most of the preclinical data on rituximab has been produced using Burkitt lines. Recently, Jazirehi et al. (30) developed a model of Burkitt lymphoma clones resistant to rituximab, whereas Czuczman et al. developed (28) follicular and Burkitt resistant cells *in vitro*. These clones were shown to have altered cellular signaling dynamics and to exhibit different phenotypic properties when compared with parental cells. The resistant clones were shown to have a reduced CD20 surface expression, failed to respond to rituximab-mediated inhibition of cell growth and apoptosis after cross-linking. However *in vivo* models of resistance to rituximab are needed to follow the progressive modifications of tumor cells induced by rituximab and their mechanisms of adaptation in the host.

We thus chose to develop such a model of FL resistant to rituximab *in vivo*. We then studied the characteristics of this model by focusing on the mechanisms of resistance to rituximab previously described *in vitro*. We also explored opportunities to overcome resistance to rituximab in this model using combination therapy.

Materials and Methods

Cell lines and culture. The RL cell line (derived from a human FL) was maintained in culture medium consisting of RPMI (Life Technologies), 10% FCS (Integro), 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). All cells were cultured at 37°C in a 5% CO₂ atmosphere (31).

Animals. Female CB17 severe combined immunodeficient mice purchased from Charles River Laboratories were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. All mice used in this experimental study were 5- to 6-wk-old at the time of tumor implantation. This study was approved by the local animal ethical committee.

Lymphoma xenograft experiments. Lymphoma cells (1×10^6 ; RL) were serially passaged in mice exposed or unexposed to rituximab. Cells were injected s.c. on day 1. To determine sensitivity of RL cells to rituximab *in vivo*, mice were first divided into three experimental groups of 3 to 5 mice per group: control mice receiving no treatment, an "early

treatment" group with rituximab started on day 2, and a "late treatment" group with rituximab started on day 15 after injection of the lymphoma cells. Rituximab 10 mg/kg (clinical formulation; Roche) was injected i.p. weekly. Mice were weighed and the tumor size measured twice a week with an electronic caliper. The tumor volume was estimated from two dimensional tumor measurements by the formula: tumor volume (mm³) = length (mm) × width²/2. Animals were euthanized either when tumor volume exceeded 2 mL to avoid animal discomfort or if conditions suggested the potential for animal suffering. The tumors were then gently flushed in culture medium to recover the tumor cells in the suspension. The cells were then centrifuged and counted, using trypan blue exclusion staining.

To establish the resistant model, tumor cells obtained from mice having received rituximab were reinjected the same day to a new group of mice that were then treated with the "early treatment" rituximab regimen described above.

In experiments evaluating the role of complement-dependent cytotoxicity in rituximab inhibition of tumor growth, complement inhibition was obtained by weekly i.p. injection of cobra venom factor (2 µg per mouse; Quidel Corporation).

Immunohistochemistry of tumors growing in severe combined immunodeficient mice. S.c. tumors obtained from tumor-bearing severe combined immunodeficient mice were analyzed by pathologic examination. Unstained paraffin-embedded tissue sections were used for detection of CD20 antigen by immunohistochemistry. Sections (4- to 6-µm thick) were deparaffinized by incubation at 60°C for 1 h followed by immersion in xylene. Slides were then treated with serial dilutions of alcohol (100%, 90%, and 70% ethanol/distilled water) and rehydrated in PBS. No antigen retrieval was necessary. All samples were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase. Protein blocking was performed using horse serum for 20 min, followed by a 30-min incubation with mouse antihuman CD20 clone L26 labeling an intracytoplasmic epitope of CD20 (DAKO) at a 1:50 dilution.

Flow cytometry analysis. Cell surface antigen expression of RL cells was performed on a FACS Calibur flow cytometer (Becton Dickinson). Analysis of the data were performed with the Cell Quest software program (Becton Dickinson). Mouse fluorochrome-conjugated isotype control antibodies, phycoerythrin 5-coupled anti-CD19, phycoerythrin-coupled APC anti-CD20, FITC-coupled anti-CD59, and phycoerythrin-coupled anti-CD55 were purchased from Immunotech. FITC-coupled anti-CD46 was purchased from Becton Dickinson. Mean Fluorescence intensity was determined by subtracting the signal of isotype-matched antibody staining from the staining observed with the specific primary antibody.

Western blot protein analysis. Protein expression was determined by Western blot analysis in rituximab-naïve and rituximab-resistant tumors as previously described. Briefly, cell lysates were resolved by 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Hybond-ECL; Amersham Corp). The blots were then incubated with the appropriate dilution of primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. For this analysis, 10×10^6 cells were pelleted and proteins fractionated by SDS-PAGE (12-15% gradient gels) and transferred to a polyvinylidene difluoride membrane using an electroblotting apparatus (Bio-Rad). The loading of equal amounts of protein was verified by Ponceau staining of the polyvinylidene difluoride membranes. The membrane was blocked with 5% nonfat, dry milk for 1 h and subsequently incubated with the primary antibody at a dilution of 1:1,000 for 1 h at room temperature. Antibody directed against Bcl2 was purchased from Dako (clone 124), YY1 from Active Motif and Bcl-x_L (clone S18) from Santa Cruz, BAX from Santa Cruz (clone SC 493), BAK from Santa Cruz (clone SC 7873), BAD from Serotec (clone AHP475), BIM from Santa Cruz (clone SC 8265), CD59 from Serotec (Clone mem-43), CD55 from Abcam (MEM-118), and CD20 from Abcam (clone L26). Unbound antibody was removed by washing with PBS (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated

with the secondary antibody [anti mouse peroxidase-conjugated antibody (Sigma) at a dilution of 1:6,000] for 1 h at room temperature. After extensive washing with PBS, proteins were detected after addition of the staining substrates enhanced chemiluminescence (Amersham). Proteins were detected by chemiluminescence using Kodak film (Eastman Kodak Company). The Western blot analyses were performed for each animal from the different groups of animals.

Cytotoxicity assays. Tumor cells were obtained from tumors in severe combined immunodeficient mice and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (32). The IC_{50} was defined as the drug concentration resulting in 50% loss of cell viability relative to untreated cells. Assays were performed in triplicate in at least three separate experiments for doxorubicin, cytarabine, and cisplatin.

Statistical analysis. The data were expressed using Student's *t* test. Analyses were performed with Statistica 6.0 (StatSoft, Inc.).

Results

Induction of *in vivo* resistance to rituximab. Unimpeded growth of RL tumors under these experimental conditions showed palpable tumors after 17 to 18 days during the first passages, with a tendency to earlier growth during after passages. Spontaneous tumor growth required euthanasia of animals around day 30. During the first passages, mice receiving early treatment (starting on day 2) were highly sensitive to rituximab therapy when compared with the control group (Fig. 1). Early treated mice and control groups were respectively euthanized at day 35 and 46 after injection with a statistical different tumor growth at day 35 ($P = 0.004$). Conversely, in mice receiving delayed treatment (from day 15 onward), we did not observe a significant difference in tumor growth between treated and untreated groups ($P = 0.958$; Fig. 1). After four cycles of consecutive reimplantation and early treatment, the re injected cells progressively became resistant to rituximab. The growth of the resistant tumors in mice exposed to early rituximab therapy was comparable with that of the rituximab-naïve control group, whereas the cells serially implanted in mice but never exposed to rituximab remained highly sensitive (Fig. 2A and B).

CD20 antigen expression in resistant lymphoma cells. Immunohistochemistry showed that the lymphoma cells were strongly CD20 positive both in the treated and nontreated

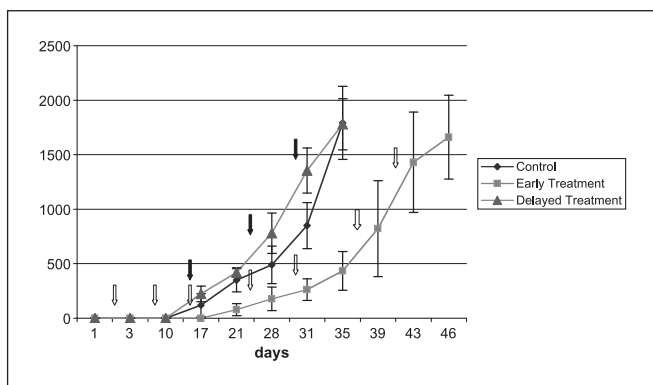


Fig. 1. Effect of treatment of rituximab on tumor growth of sensitive RL cells. Three different groups of mice were defined as follows: the first group of mice receiving early rituximab treatment (initiated 24 h after the injection of tumor cells), a second group for which the rituximab was beginning at day 15 (delayed treatment group), and a third control group. *White arrow*, rituximab treatment in early-treated group. *Black arrow*, rituximab injections in delayed treatment group.

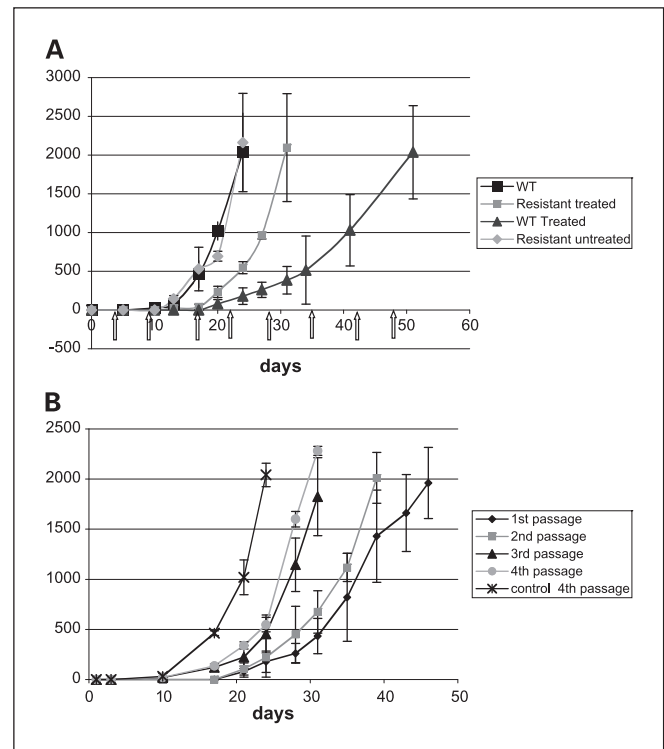


Fig. 2. A, acquisition of a rituximab-resistant phenotype after consecutive *in vivo* passages. Tumor growth after four sequences of injection/treatment in consecutive groups of mice: the lymphoma cells became progressively resistant to rituximab. *↑*, days of treatment for treated groups. B, acquisition of a rituximab-resistant phenotype during the consecutive *in vivo* passages; *arrows*, days of injection.

groups. Flow cytometry analysis did not show a global difference in CD20 expression levels between parental and resistant RL cells (mean fluorescence intensities of 215 and 252, respectively). But when comparing the resistant group and the control group, a small fraction of resistant cells had become CD20 negative (4%). This suggests that iterative treatment with rituximab did not result in a global decrease of surface CD20 expression in this model, but a subset of treated cells lost this expression during the acquisition of resistance (Fig. 3A, B, C, and D). This subset of CD20⁻ cells was not different in the expression of CD55, CD59, and CD46. When CD20 content was evaluated by immunoblots performed on lysates of control and resistant tumors, no difference was observed (data not shown). These results suggest that most of the cells exposed to repeated injection of rituximab did not lose the CD20 expression. Nevertheless, a small counterpart of treated cells did. These results were not distinguishable on the Western blot analysis that has been made with the entire population.

Role of complement in resistant lymphoma cells. Complement-dependent cytotoxicity seemed to play a role in the efficacy of rituximab in our model. When the rituximab was given in combination with cobra venom factor, we observed a significant loss of antitumor activity ($P = 0.027$; Fig. 4). To determine the possible role of complement inhibitors in the antitumor effect of rituximab, we analyzed the surface expression of CD46, CD55, and CD59. Two-color flow cytometric staining using CD19 gating and monoclonal antibodies against CD46, CD55, and CD59 was performed on cells obtained from tumors. Taking in account median fluorescence

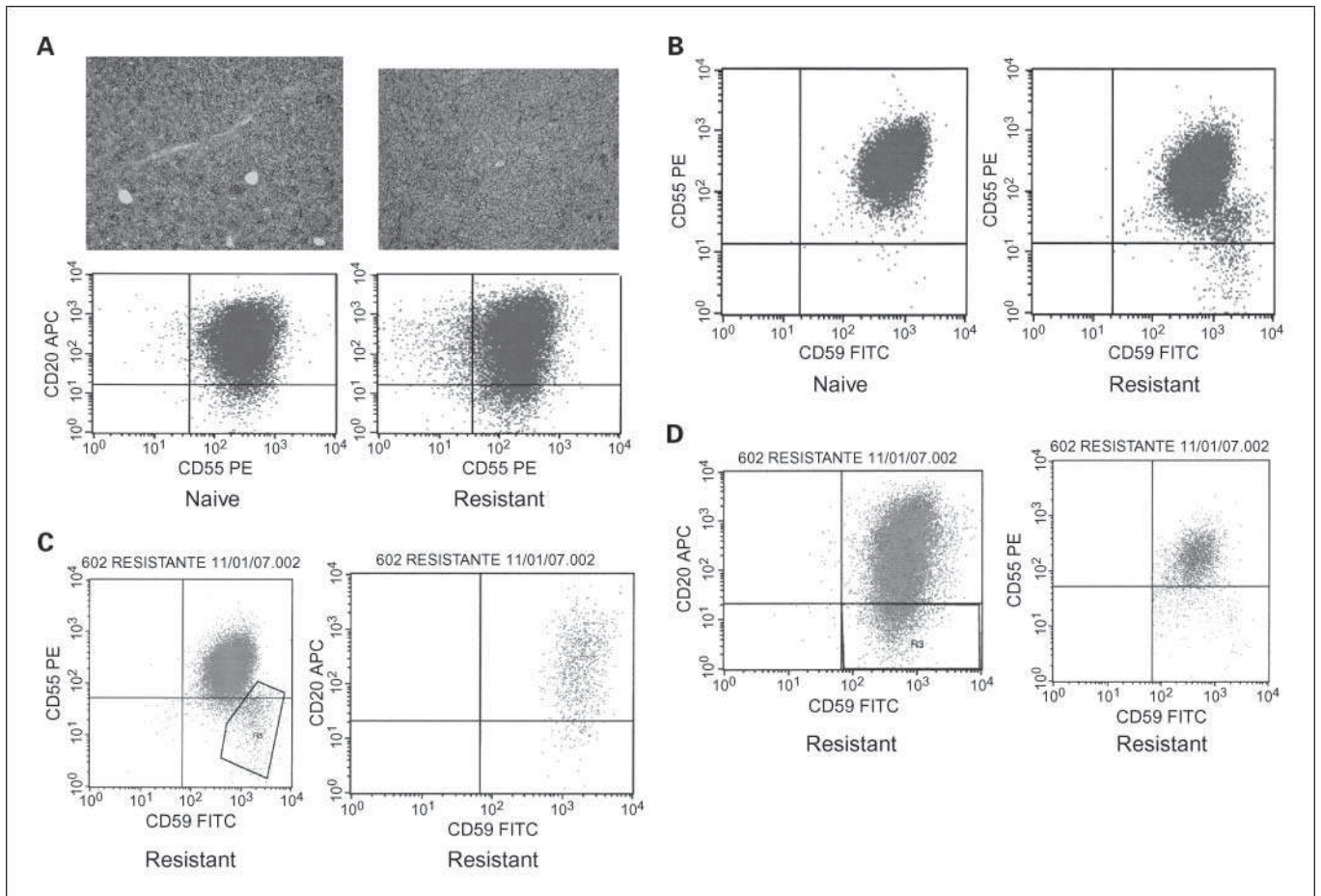


Fig. 3. A, expression of CD20 in sensitive and resistant cells by immunohistochemistry and FACS Analysis. B, expression of complement inhibitors in sensitive and resistant cells. CD55 and CD59 were analyzed by flow cytometry on fresh tumor samples. C, expression of CD20 on CD59 higher subpopulation cells. D, expression of CD55 and CD59 on CD20⁻ cells.

intensity, we did not observe altered expression of CD46 in resistant cells compared with parental cells. Conversely analysis of CD59 in resistant cells showed a subpopulation over-expressing this complement inhibitor (Fig. 3B). This population also showed a down-regulation of the CD55 expression and was globally CD20⁺. We subsequently attempted but failed to sort this population of interest by flow cytometry. In western Blot analysis, the protein contents of CD55 and CD59 were not different between naïve and resistant cells (data not shown).

Overexpression of Bcl-X_L in resistant lymphoma cells. Previous findings have established Bcl-X_L as being involved in resistance to rituximab *in vitro*. We therefore evaluated Bcl-X_L and Bcl-2 levels in the sensitive and resistant tumors. Immunoblotting showed that resistant cells expressed higher Bcl-X_L protein levels compared with wild-type cells (Fig. 5). Conversely, there was no significant difference concerning the level of expression of Bcl-2. The proapoptotic factors of the Bcl-2 family BAX and BAK were also tested on Western blot analysis. Although there was no difference in BAX protein expression, BAK was found to be down-regulated in resistant cells. In the same way we did not observe differences in protein expression for the BH3-only Bcl-2 family proteins BIM and BAD (data not shown).

Expression of YY1 in naïve cells and resistant cells. YY1 has been suggested to be involved in the sensitivity of NHL cells to

apoptosis. It has already been shown that expression of YY1 is decreased in lymphoma cells after exposure to rituximab, resulting in a sensitization to chemotherapeutic agents. In our experiments, the protein content of YY1 was indeed found to be strongly decreased in naïve cells after exposure to rituximab (data not shown). Conversely, expression of YY1 was increased

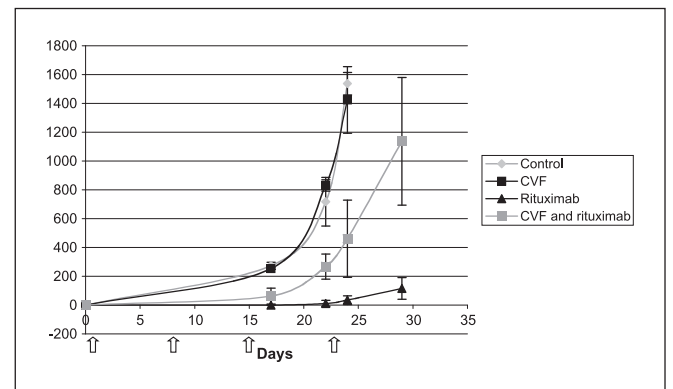


Fig. 4. Inhibitory effect of cobra venom factor on the antitumor activity of rituximab *in vivo*. Arrow, days of treatment by rituximab and/or cobra venom factor, respectively.

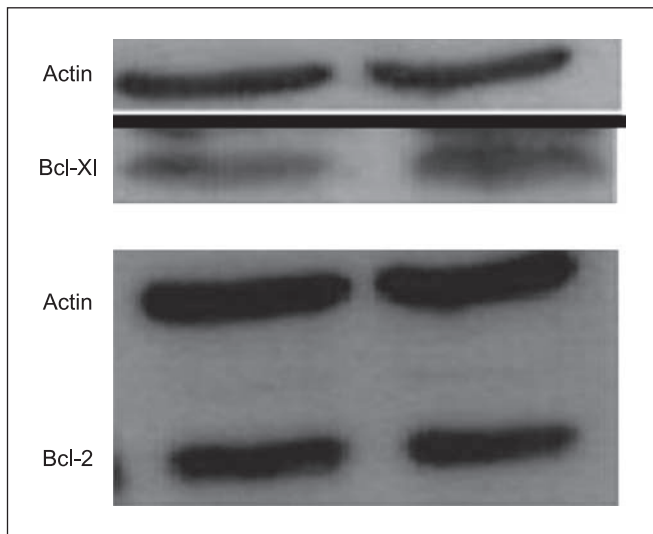


Fig. 5. Bcl-xL and Bcl-2 expression in sensitive and resistant cells. Bcl-xL and Bcl-2 were analyzed by immunoblotting on tumor samples.

in resistant cells after *in vivo* treatment in comparison with the control group (Fig. 6).

Sensitivity to chemotherapy *in vitro*. Enhancing the cytotoxic effects of anticancer agents is an established property of rituximab. However, these results obtained *in vitro* using Burkitt's lymphoma cell lines are not reproducible on FL lines or other models of NHL. When naïve RL cells were exposed *in vitro* to rituximab, the cytotoxic effect was very low (>95% of cells stay alive) even with the adjunction of human serum as source of complement or accessory cells to enhance antibody-dependent cellular cytotoxicity. On the other hand, these cells were initially highly sensitive to doxorubicin (IC_{50} , 12,3 nmol/L). After having acquired resistance to rituximab *in vivo*, these resistant cells also proved to be significantly less sensitive to doxorubicin *in vitro* (IC_{50} , 153,3 nmol/L). These cells were also significantly less sensitive to cytarabine and less sensitive to gemcitabine, cisplatin, vincristine, and maphosphamide, but these later differences were not significant (Table 1). Thus, *in vivo* resistance to rituximab seemed to be associated with *in vitro* resistance to a variety of chemotherapeutic agents.

Discussion

This is the first report of a human FL model resistant to rituximab *in vivo*. Comparison with the sensitive parental cells has allowed the identification of a subpopulation with increased expression of the complement inhibitor CD59, whereas the expression of CD20 was also reduced in a distinct subpopulation of resistant cells. Rituximab-resistant RL cells were found to express higher levels of Bcl_{xL} and YY1 protein than their sensitive counterparts.

It has been shown that FL cells can be effectively destroyed by single agent rituximab in patients, whereas B-cell chronic lymphocytic leukemia cells show a poor response. A major difference between these two diseases is the higher level of CD20 expression in FL cells in comparison with that of chronic lymphocytic leukemia cells (8, 9, 33). Several studies have addressed the question of whether CD20 expressions level

could be used to predict response to rituximab (7, 8, 10, 13, 34). The results remain controversial. *In vitro* analysis of FL cells found no correlation between CD20 expression and sensitivity to rituximab-induced complement-dependent cytotoxicity (7, 10, 12). In contrast, a strong correlation was reported in two other studies involving patients with a variety of B-cell malignancies (9). Interestingly, in a Burkitt model resistant to rituximab *in vitro*, Jazirehi et al. (30) have shown that the resistant clones displayed a 50% reduction in CD20 expression. This was recently confirmed in another *in vitro* model by Czucman and al (28). In our *in vivo* model the surface expression of CD20 was not globally diminished. Nevertheless a subpopulation representing 4% of the resistant cells became CD20- during the consecutive passages. This subpopulation has emerged only in the treated group and not in the control group. It is therefore possible that longer exposure could increase the percentage of CD20- cells. Further work is needed to elucidate this point.

In our model of xenograft tumor, the complement system seems to play a critical role because its inhibition by cobra venom factor provokes a clear loss of efficacy of rituximab. Complement lysis is regulated by complement inhibitors such as CD46, CD55, and CD59. The initial expression of these inhibitors does not seem to be a predictive factor of response to rituximab therapy (8). Conversely, the blockade of these inhibitors can increase the *in vitro* response of rituximab through complement-dependent cytotoxicity. Increased expression of CD55 and CD59 was associated with a resistant phenotype in Burkitt's lymphoma cells derived *in vitro* by repeated exposure to rituximab. It has also been shown that the increased expression of the complement regulator CD59 was associated with resistance to rituximab-mediated complement lysis of multiple myeloma and NHL cell lines. Treon et al. (35) reported that complement inhibitors, particularly CD59 is present on various B-cell tumors and is associated with resistance to rituximab in patients (8). Our results tend to confirm that increased expression of CD59 is associated with the acquisition of a resistant phenotype. Conversely we did not observe modifications in CD46 expression in our resistant model. Surprisingly, the CD55 expression was down-regulated in our cell subpopulation overexpressing CD59.

Several signaling pathways have been described to be involved in rituximab-induced apoptosis. Cross-linking of

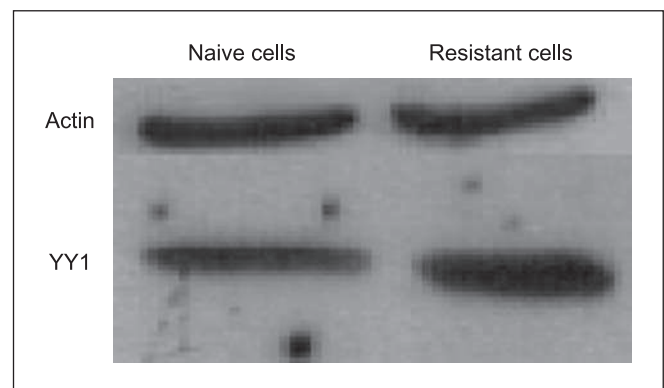


Fig. 6. YY1 is overexpressed in resistant cells. YY1 was analyzed by immunoblotting on tumor samples.

Table 1. *In vitro* sensitivity of sensitive and resistant cells to anticancer compounds

	Mean IC ₅₀ (naive cells)	Mean IC ₅₀ (resistant cells)	Resistance ratio	P
Doxorubicin	12,3 nmol/L	153,3 nmol/L	12,46	0.049
Vincristine	16,25 pmol/L	24 pmol/L	1,48	(P = 0.08)
Cisplatin	3,75 μmol/L	12 μmol/L	3,2	(P = 0,12)
Cytarabine	6,3 nmol/L	9,3 nmol/L	1,47	(P = 0,043)
Gemcitabine	0,86 ng/mL	1,26 ng/mL	1,46	(P = 0,07)
Maphosphamide	8 μmol/L	16 μmol/L	2	(P = 0.08)

NOTE: *In vitro* sensitivity was evaluated using an MTT cytotoxicity assay. Rituximab-resistant cells also displayed a significant resistance to doxorubicin and cytarabine with a trend toward reduced sensitivity to vincristine, gemcitabine, maphosphamide, and cisplatin. *In vitro* sensitivity to chemotherapeutic compounds of cells obtained from rituximab-naïve and rituximab-resistant tumors. The resistance ratio is the ratio of the mean IC₅₀ in resistant cells and in naive cells.

rituximab, for example via FcR-positive cells, leads to the activation of an intracellular signaling cascade, which results in B-cell death by apoptosis (15, 36). Cross-linking of anti-CD20 antibodies on B cells leads to translocation of CD20 to lipid rafts, followed by activation of complement activation (37, 38). In addition, CD20 cross-linking results in a rapid up-regulation of members of the src family of tyrosine kinases, increased intracellular Ca²⁺ concentrations, up-regulation of the proapoptotic protein Bax, increase in RNA levels of *c-myc* and Berg, activation of mitogen-activated protein kinase family members p44 and 42, and increased activator protein DNA binding activity (39). Other antiapoptotic signaling pathways, including the extracellular signal-regulated kinase 1/2 and the nuclear factor-κB pathway, have been reported to be inhibited by rituximab, resulting in the sensitization of various B-cell lines to chemotherapy (40–42) and Fas receptor-mediated apoptosis induction (43). Interestingly, the protein expression of the transcription regulator YY1 was modified in our model. Although a treatment with rituximab resulted in a decreased expression of YY1 in antibody naive cells, we observed an overexpression of this protein in antibody resistant cells. This rituximab-induced overexpression of YY1 could be implicated in the resistance to combined therapy including rituximab and chemotherapeutic agents. Although it is generally believed that the mitochondrial pathway, activated via caspase-9, is the main apoptotic cascade induced by rituximab, other routes, activated via caspase-7 and caspase-8, have been reported as well (44, 45).

Several recent reports have shown that rituximab can induce a chemosensitization of NHL cell lines via the down-regulation of Bcl-X_L (30, 40, 41, 46). p38 mitogen-activated protein kinase and signal transducers and activators of transcription 3 protein activity have been reported to be inhibited as a result of CD20 cross-linking by rituximab, subsequently down-regulating the antiapoptotic proteins Bcl-xL, Bcl-2, and inducing apoptosis protease activating factor 1 (46–48). In keeping with these observations, our data show increased Bcl-X_L content in the

resistant cells compared with the sensitive parental cells and confirm the recent results obtained *in vitro* by Jazirehi et al. (30), showing that the phenotype of resistant cells to rituximab may be associated with a higher expression of Bcl-X_L. The onset of a rituximab-resistant phenotype has recently been shown to be associated with a down-regulation of the proapoptotic Bcl2 family proteins BAX and BAK responsible for associated resistance to chemotherapy (49). Conversely, we have not observed altered Bcl-2 or BAX content in our resistant line, whereas BAK levels were reduced. Multidrug-resistance phenotype (MDR) associated with the overexpression of transmembrane efflux pumps such as P-glycoprotein (MDR1) was not observed in the RL cells, either rituximab-naïve or resistant (data not shown).

In conclusion, we have established and begun the characterization of a model of human FL resistant to rituximab *in vivo*. Two phenotypically different subpopulations emerged from these repeated exposures: a CD59+ and a CD20- population. The YY1 expression was also found to be increased in resistant cells. This model has a certain number of limitations, including its fast growth rate due to the fact that it is a transformed FL and is thus not representative of indolent disease, or its lack of immune human effector cells. In spite of these limitations, this model and others currently under development should prove to be quite useful to identify novel mechanisms of resistance to rituximab *in vivo* and in the development of novel monoclonal antibodies or therapeutic strategies, including drug combinations designed to overcome resistance to rituximab.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Article V

*Preclinical studies on the mechanism of action
and the anti-lymphoma activity of the novel anti-
CD20 antibody GA101*

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Molecular Cancer Therapeutics



Preclinical Studies on the Mechanism of Action and the Anti-Lymphoma Activity of the Novel Anti-CD20 Antibody GA101

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Preclinical Development

Preclinical Studies on the Mechanism of Action and the Anti-Lymphoma Activity of the Novel Anti-CD20 Antibody GA101

Stephane Dalle^{1,2}, Lina Reslan¹, Timothee Besseyre de Horts¹, Stephanie Herveau¹, Frank Herting³, Adriana Plesa², Thomas Friess³, Pablo Umana⁴, Christian Klein³, and Charles Dumontet^{1,2}

Abstract

GA101 is a novel glycoengineered Type II CD20 monoclonal antibody. When compared with rituximab, it mediates less complement-dependent cytotoxicity (CDC). As expected for a Type II antibody, GA101 appears not to act through CDC and is more potent than the Type I antibody rituximab in inducing cell death via nonclassical induction of apoptosis cytotoxicity, with more direct cytotoxicity and more antibody-dependent cell-mediated cytotoxicity. We evaluated the antitumor activity of GA101 against the human-transformed follicular lymphoma RL model *in vivo* in severe combined immunodeficient mice (SCID) mice. GA101 induced stronger inhibition of tumor growth than rituximab. Combination of GA101 with cyclophosphamide *in vivo* confirmed the superiority of GA101 over rituximab. Neutralizing the complement system with cobra venom factor partially impaired the antitumor activity of rituximab, but had no impact on the efficacy of GA101. *In vitro* GA101 more potently induced cell death of RL cells than rituximab. The expression of a limited number of genes was found to be induced by both antibodies after exposure *in vitro*. Among these, early growth response 1 and activation transcription factor 3 were confirmed to be increased at the protein level, suggesting a possible role of these proteins in the apoptotic signalling of anti-CD20 antibodies. These data imply that GA101 is superior to rituximab not only as a single agent, but also in combination with chemotherapy. These data suggest the presence of novel signalization pathways activated after exposure to anti-CD20 antibodies. *Mol Cancer Ther*; 10(1); 178–85. ©2011 AACR.

Introduction

Rituximab, directed against the CD20 antigen on B cells, was the first commercially available monoclonal antibody (mAb) for the treatment of lymphoma. It is the current treatment of choice for a variety of lymphoproliferative disorders including low and high grade B-cell non-Hodgkin's lymphomas (NHL; refs 1–5). Follicular lymphoma (FL) is the most common subtype of indolent lymphoma. Rituximab is now widely used either alone or in combination with multi-agent chemotherapy for the treatment of FL, either at diagnosis (6, 7), at relapse (8–10), or for maintenance therapy (2, 11, 12). However,

despite its well-established clinical efficacy, a subpopulation of patients does not initially respond to rituximab and most patients will relapse after rituximab therapy (13, 14). Thus, there is still a need either for more efficient rituximab combination therapies or for novel CD20-specific monoclonal antibodies with increased efficacy (15).

Various *in vitro* and *in vivo* experiments have shown that elimination of CD20+ lymphoma cells by rituximab involves complement-dependent cytotoxicity (CDC; refs 16–23), direct induction of apoptotic signalling (24–26), as well as the recruitment of effector cells leading to antibody-dependent cell-mediated cytotoxicity (27). Nevertheless, the *in vivo* mechanism of action of and resistance to rituximab are not fully understood (28). Depending on their ability to redistribute CD20 into lipid rafts and to induce CDC, or to induce cell death and homotypic adhesion, anti-CD20 mAb can be classified as Type I or II CD20 antibodies. Schematically, Type I antibodies (such as rituximab) induce CDC with redistribution of CD20 into lipid rafts, whereas type II mAb (such as the murine antibody tositumomab, or the humanized antibody GA101) are believed to act primarily through the direct induction of nonclassical cell death and exhibit low CDC activity (29).

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Rituximab as a chimeric mAb belongs to the first generation of CD20 antibodies recognizing a Type I epitope. While the second generation of humanized (ocrelizumab, veltuzumab, AME-133, Immu-106) or fully human (ofatumumab) anti-CD20 antibodies recognize a Type I epitope, GA101 represents a novel generation that in addition to being humanized recognizes a type II epitope and is glycoengineered using GlycoMAB technology leading to bisected, afucosylated fragment crystallizable (Fc) region carbohydrates resulting in enhanced affinity for the human FcγRIIIa receptor on human effector cells such as NK cells, macrophages, and dendritic cells (29). GA101 was obtained by grafting CDR sequences from the murine mAb B-ly1 on framework regions with fully human IgG1-κappa germline sequences.

In this study, we compared the effect of GA101 and rituximab on the human follicular RL lymphoma model, both *in vitro* and *in vivo*.

Materials and Methods

Cell lines and culture

The RL cell line, derived from a human transformed FL sample, was purchased from American Type Culture Collection within the 6 months before experimentation and routinely characterized before and during the experimentation regarding the CD19; CD20; HLA DQ, and HLA DR expression. Cells were maintained in culture medium consisting of RPMI-1640 (Life Technologies), 10% of fetal calf serum (Integro), 100 units/mL of penicillin and 100 µg/mL of streptomycin (Life Technologies). All cells were cultured at 37°C in a 5% CO₂ atmosphere.

In vivo studies

Six-week-old female CB17 severe combined immunodeficient mice (SCID) mice purchased from Charles River laboratories (l'Arbresle) were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. This study was approved by the local animal ethical committee.

For xenograft experiments, 1×10^6 RL cells were injected subcutaneously on day 1. Mice were randomized when a tumor became palpable in groups of 10 and treatment was initiated. In a first set of experiments, rituximab and GA101 were used as monotherapy at different dosages twice weekly. The 5 different groups of 10 mice were: control group receiving vehicle (NaCl 0.9%), rituximab (30 mg/kg), GA101 (10 mg/kg), GA101 (30 mg/kg), and GA101 (100 mg/kg). The treatment was administered intravenously twice a week. The mice were closely monitored regarding weight and general status. In experiments evaluating the role of CDC in rituximab inhibition of tumor growth in groups of 3 mice, comple-

ment inhibition was induced by weekly intraperitoneal injection of cobra venom factor (CVF; 2 µg/mouse, Quidel Corporation).

Combination studies were done with cyclophosphamide. In these combination studies, the treatment was administrated weekly. The control group received vehicle (NaCl 0.9%), whereas the treated groups received rituximab (30 mg/kg), GA101 (30 mg/kg), rituximab (30 mg/kg) + GA101 (30 mg/kg), cyclophosphamide (50 mg/kg), rituximab + cyclophosphamide (50 mg/kg), and GA101 + cyclophosphamide (50 mg/kg). Rituximab and GA101 (30 mg/kg) were provided by Roche, whereas cyclophosphamide was obtained from Baxter. The mice were injected intravenously in the tail vein, once a week. They were weighed and the tumor size was measured twice a week with an electronic calliper. The tumor volume (TV) was estimated from two dimensional tumor measurements by the formula: tumor volume (mm³) = length (mm) × width²/2. Median tumor growth inhibition (% TGI) was calculated according to the NCI formula: $1 - ([TV_{\text{treated}}(\text{day } 34 - 20) \times 100 / TV_{\text{control}}(\text{day } 34 - 20) \times 100])$.

Flow cytometry analysis

Cell surface antigen expression of RL cells was performed on a FACS Calibur flow cytometer (Becton Dickinson). Analysis of the data was done with the Cell Quest software program (Becton Dickinson). Mouse fluorochrome-conjugated isotype control antibodies, phycoerythrin 5 (PE5)-coupled anti-CD19, phycoerythrin coupled APC anti-CD20, fluorescein isothiocyanate (FITC)-coupled anti-CD59, and PE-coupled anti-CD55 were purchased from Immunotech. FITC-coupled anti-CD46 and FITC active caspase-3 apoptosis kit were purchased from Becton Dickinson. Mean fluorescence intensity (MFI) was determined by subtracting the signal of isotype-matched antibody staining from the staining observed with the specific primary antibody. RL exposed to rituximab, GA 101 and CVF were firstly evaluated *in vitro*, but were also evaluated *ex vivo*, immediately after the extraction of tumor cells from animals. These experiments were done after exposure of 50,000 cells in 6-well culture plates in 2 mL of complete medium to rituximab or GA101 with or without CVF. These assays were done after 1, 2, 4, 6, and 24 hours of culture.

Annexin V/Propidium iodide staining

To evaluate the induction of apoptosis and the reduction of cell viability in cells exposed to antibodies with or without CVF, 10^6 cells were resuspended in 300 µL of human serum and 700 µL of culture medium with or without 100 µg/mL rituximab at 37°C for 6, 15, and 24 hours. Dead and viable cells were discriminated by Annexin V/propidium iodide (PI) staining using flow cytometry. Briefly, the cells were washed and resuspended in binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂] containing 1 mg/mL FITC-Annexin V PI (1.25 mg/mL) was also

added to the samples to distinguish between early apoptosis and secondary necrosis.

Western blot protein analysis

Protein expression was determined by Western blot analysis in rituximab-naïve and rituximab-resistant tumors as previously described (30). Briefly, cell lysates were resolved by 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-ECL). The blots were then incubated with the appropriate dilution of primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. For this analysis, 10^7 cells were pelleted and proteins fractionated by SDS-PAGE (12–15% gradient gels) and transferred to a PVDF membrane using an electroblotting apparatus (Bio-Rad). The loading of equal amounts of protein was verified by Ponceau staining of the PVDF membranes. The membrane was blocked with 5% nonfat, dry milk for 1 hour and subsequently incubated with the primary antibody at a dilution of 1:1,000 for 1 hour at room temperature. Antibody directed against Bcl2 was purchased from Dako (clone 124), YY1 from Active Motif, and Bcl-x_L (clone S18) from Santa Cruz; BAX from Santa Cruz (clone SC 493), BAK from Santa Cruz (clone SC 7873), BIM from Santa Cruz (clone SC 8265), CD59 from Serotec (Clone mem-43), CD55 from Abcam (MEM-118), CD20 from Abcam (clone L26), early growth response 1 (EGR1) and activation transcription factor 3 (ATF3) from Santa Cruz, and caspase-3 from BD Biosciences (clone CPP32). Unbound antibody was removed by washing with phosphate buffered saline (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated with the secondary antibody (anti mouse peroxidase-conjugated antibody [Sigma] at a dilution of 1:6,000) for 1 hour at room temperature. After extensive washing with phosphate buffered saline, proteins were detected after addition of the staining substrates ECL (Amersham). The proteins were detected by chemiluminescence using Kodak film (Eastman Kodak Company) or using the Odyssey infrared system (LI-COR Biotechnology). The Western blot analyses were done for each animal from the different groups of animals.

Gene expression profiling

To determine which genes were differentially expressed in cells exposed to rituximab or GA101, RL cells were exposed *in vitro* and *in vivo* to these antibodies then analysed by pangenomic profiling using Agilent 44K chips in the Laboratoire de Caractérisation Moléculaire des Tumeurs (LCMT). Briefly, 1-color labeled cRNAs were generated from 200 ng of total RNA using the Low RNA Input Amplification Kit (Agilent Technologies) according to the instructions of the manufacturer. Labeled cRNA were hybridized overnight to Whole Human Genome 4 × 44K microarrays (ref Agilent G4112F) containing 45,015 features representing 41,000 genes. Each probe is a 60-mer, synthesized *in situ*. After washing, microarrays were

scanned using the Agilent model G2505B microarray scanner, and data were extracted by Feature Extraction software, version 9.5. The default settings, as 2 photomultiplier values (XDR high 100% and XDR low 10%), were used to scan the microarrays. Data were normalized using the quantile normalization method (31). Each sample was done in triplicate. Analyses of differentially expressed genes and Gene Ontology pathways (Gene Ontology Consortium, 2000, <http://www.geneontology.org/>) were done using GeneSpring 7.0. Determination of differentially expressed genes was done using a parametric test, with a false discovery rate of 0.01. Quantitative RT-PCR confirmation of selected genes was done as previously described (30).

Statistical analysis

For the evaluation of tumor growth, calculations started at staging (day 34) until termination for the control group and the group receiving therapy. Values were documented as medians and standard deviations (SD). Median (%) TGI for volume (*T/C*) was calculated according to the NCI formula: $1 - ((TV_{\text{treated}}(\text{day } Y) - 100) / (TV_{\text{control}}(\text{day } X) - 34)) \times 100$

Briefly, in a randomized 2-sample design the treatment-to-control ratio:

$$TCR = \frac{\bar{V}_{\text{treated}}}{\bar{V}_{\text{control}}}$$

and its 2-sided nonparametric (1- α) confidence interval according to Fieller (1954)/Hothorn and Munzel (2000) were estimated. The calculations were done with the special SAS program TUMGRO (version 3) using version 8.1 (SAS Inc. Cary, 2000).

Results

Inhibition of tumor growth *in vivo* by rituximab or GA101

The efficacy of GA101 and rituximab was compared in the RL model that we recently described (30). In the first study GA101 was administered *i.v.* twice weekly at 3 dosages (10, 30, and 100 mg/kg), whereas rituximab was given at fixed dose of 30 mg/kg twice weekly (Fig. 1). Both antibodies were administered as intravenous injections, for a total of 5 injections. As shown in Figure 1, we observed that the new CD20 antibody GA101 was more active than rituximab administered at similar doses on established RL tumors. The antitumor effect of GA101 against RL xenografts was dose dependent in terms of TGI. TGI was calculated using NCI formula at day 34 and showed values of 25, 75, and 85% for the 10, 30, and 100 mg/kg dosages of GA101, respectively, whereas the 30 mg/kg dose of rituximab induced a TGI of 43%. The higher doses of 30 and 100 mg/kg of GA101 significantly inhibited the growth of RL tumors and resulted in some complete tumor remissions (10% and 30%, respectively), whereas no complete tumor remissions were observed in the rituximab group. Taken together, the antitumor

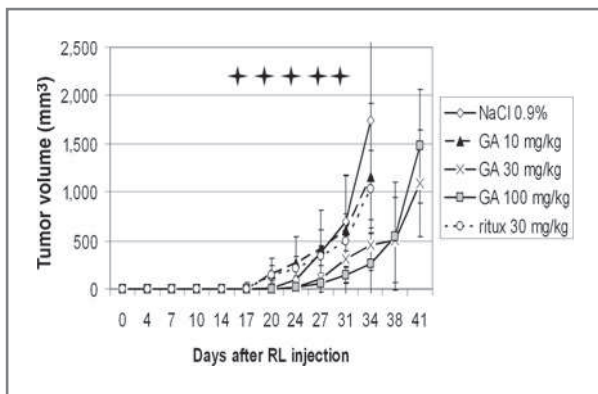


Figure 1. Inhibition of tumor growth *in vivo* by rituximab or GA101. Mice injected with RL cells subcutaneously were treated by IV infusion twice a week starting on day 17 and ending on day 31 (black crosses). The tumors were measured twice a week. The mice were euthanized when the tumor volume reached 2 cm³. The difference between GA (30 mg/kg) and rituximab (30 mg/kg) was significant ($P = 8.10^{-5}$)

activity of rituximab against RL xenografts was inferior to an equivalent dosing of GA101. Tolerability of GA101 with these regimens was excellent and no significant modification of body weight was observed. Since there was no significant difference between the 30 mg/kg and 100 mg/kg doses of GA101, the 30 mg/kg was used for subsequent combination studies.

Combination of cyclophosphamide with rituximab or GA101 *in vivo*

In a separate series of experiments, rituximab 30 mg/kg and GA101 30 mg/kg were administered once weekly *i. v.* for 4 weeks, either with or without cyclophosphamide 50 mg/kg administered once weekly *i. p.* for 4 weeks. As shown in Figure 2, this study confirmed the previous finding that the new anti-CD20 antibody GA101 was more active against established RL tumors than rituximab administered at similar doses. TGI values at day 42 were 79% for GA101, 35% for rituximab, and 93% for cyclophosphamide administered as single agents when compared with untreated controls. When groups receiving combination therapy were compared with the groups receiving the corresponding single agent antibody, cyclophosphamide increased antitumor efficacy with TGI values of 83% at day 42 and 55% at day 66 for rituximab and 94% at day 42 and 88% at day 66 for GA101, respectively. Taken together, the GA101-cyclophosphamide combination was significantly better than the rituximab-cyclophosphamide combination in this setting. Thus, when using a suboptimal dose of the classical antilymphoma alkylating agent cyclophosphamide, the combination of either antibody with cyclophosphamide was more active than either agent alone, and the most active combination was GA101 in combination with cyclophosphamide. In all cases, the administration was well

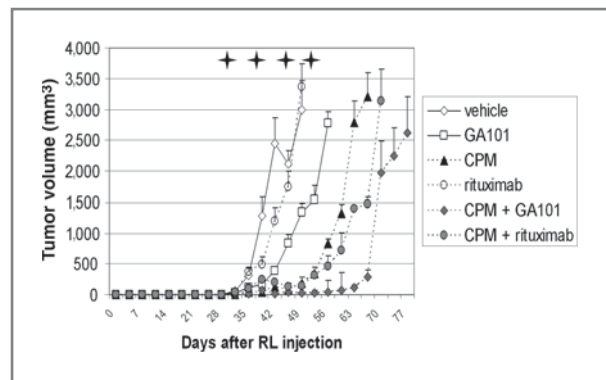


Figure 2. Effect of combination therapy of GA101 or rituximab with cyclophosphamide. Mice-bearing established SC RL tumors were treated weekly (on days 31, 38, 45 and 52 (black crosses)). The control group received vehicle (NaCl 0.9%), whereas the treated groups received one of the following: rituximab (30 mg/kg), GA101 (30 mg/kg), rituximab (30 mg/kg) + GA101 (30 mg/kg), cyclophosphamide (CPM; 50 mg/kg), rituximab + CPM (50 mg/kg), GA101 + CPM (50 mg/kg). The difference between rituximab + CPM and GA101 + CPM was significant ($P = 0.05$)

tolerated with no toxic deaths, nor loss of body weight greater than 10% (data not shown).

Role of complement in the antitumor effect of antibodies *in vivo*

Complement-dependent cytotoxicity appears to play a key role in the efficacy of rituximab in the RL model (30). When GA101 or rituximab were administered in combination with CVF, we observed a significant loss of antitumor activity in the rituximab group, whereas we did not observe a loss of efficacy in the GA101 group (Fig. 3). No difference was observed between the rituximab and GA101 groups, we assumed that it was induced by the small number of mice in each group in these experiments. When RL cells were exposed to rituximab or GA101 *in vitro*, the addition of 30% human serum as a source of complement increased the apoptotic fraction in the case of rituximab but not in the case of GA101 (data not shown), thus supporting the lack of CDC in the case of GA101-mediated cytotoxicity.

Flow cytometry and Western blot analyses of tumor cells exposed to antibodies *in vitro* and *in vivo*

To assess the direct effect of GA101 on RL cells, we did PI/Annexin testing. We observed more apoptotic cells (early and late apoptosis) in cells *in vitro* exposed to GA101 than in cells exposed to rituximab. This difference was seen after time exposure varying from 6 to 24 hours (Fig. 4), and disappeared after 48 and 72 hours (data not shown). For instance apoptotic cells (early and late apoptosis) represented 6.70%, 10.72%, 17.35%, respectively, in untreated and rituximab- and GA101-treated cells after 15 hours of exposure to the treatment. As a consequence, the percentage of live cells was more strongly reduced in the GA101 group compared with the rituximab group (Fig. 4). In agreement with this, procaspase-3 protein was

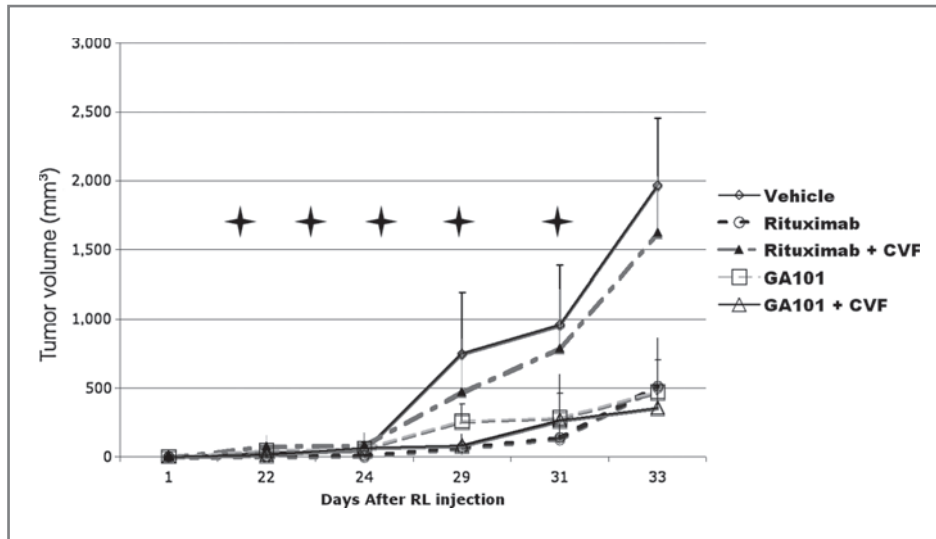


Figure 3. Effect of CVF on the *in vivo* antitumor efficacy of antibodies. The mice (3 animals per group) were treated by IV infusion two times a week starting on day 17 with rituximab (30 mg/kg) and GA101 (30 mg/kg) with or without weekly injection of CVF (2 μ g/mouse). The difference between rituximab and rituximab + CVF was significant ($P < 0.05$), whereas the difference between GA101 and GA101 + CVF was not statistically significant.

found to be more strongly expressed in cells exposed to GA101 than in cells exposed to rituximab from 6 hours after the treatment start (data not shown). Although cleaved form of caspase-3 was not detected in the early

phase (6 hours), it became detectable on FACS analysis from 12 to 48 hours after exposure (Fig. 5). There were no differences concerning Bim, Bak, Bcl2, Bcl-x_L, caspase-8, and caspase-9 by western-blot analysis and CD20

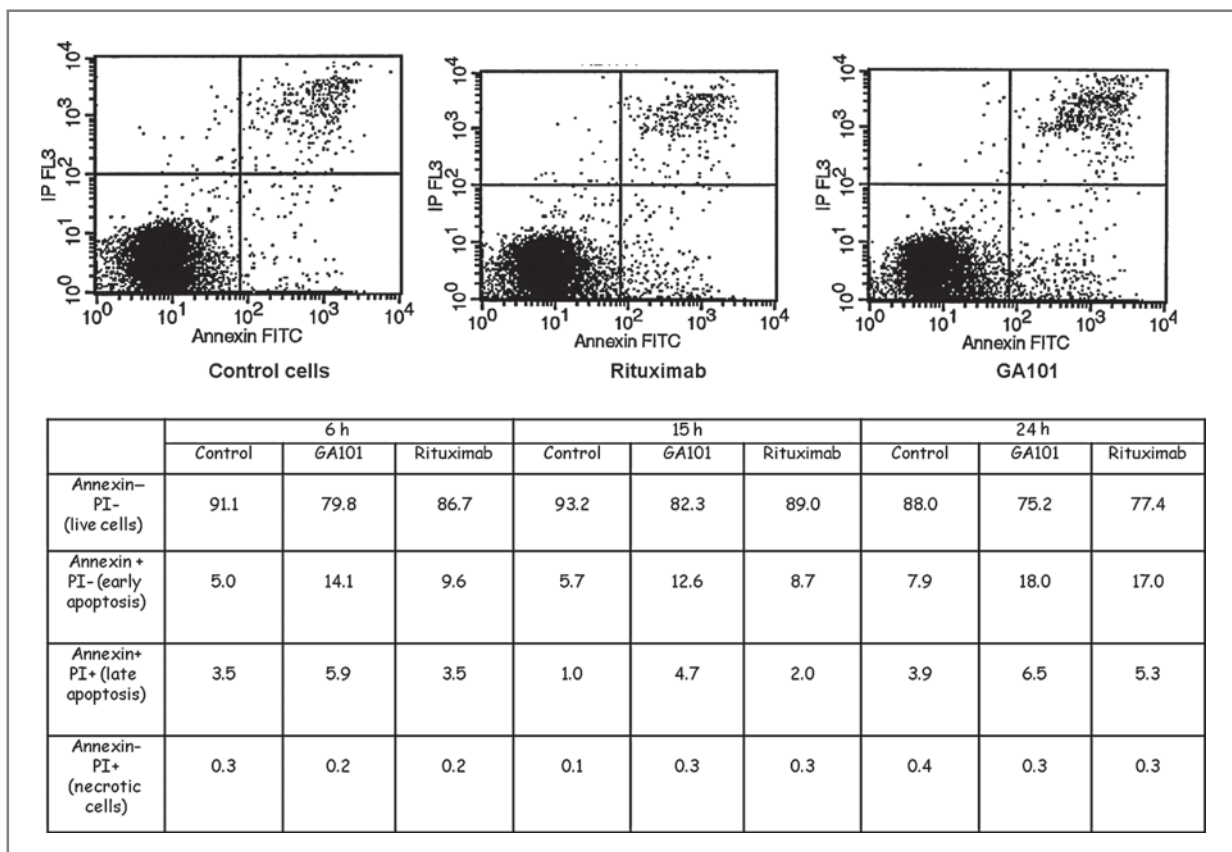


Figure 4. Induction of apoptosis by rituximab and GA101 on RL cells. Induction of apoptosis by rituximab and GA101 on RL cells was evaluated by flow cytometry. Apoptosis was evaluated at various time points after exposure to rituximab or GA101 followed by staining with Annexin V and PI. The dot plots represent the PI and Annexin V expression for untreated and rituximab- and GA101-treated cells after 15 hours of *in vitro* exposure. The table shows the results observed in each groups (alive, early, and late apoptotic, necrotic cells) in percentage of 10,000 analyzed cells per condition.

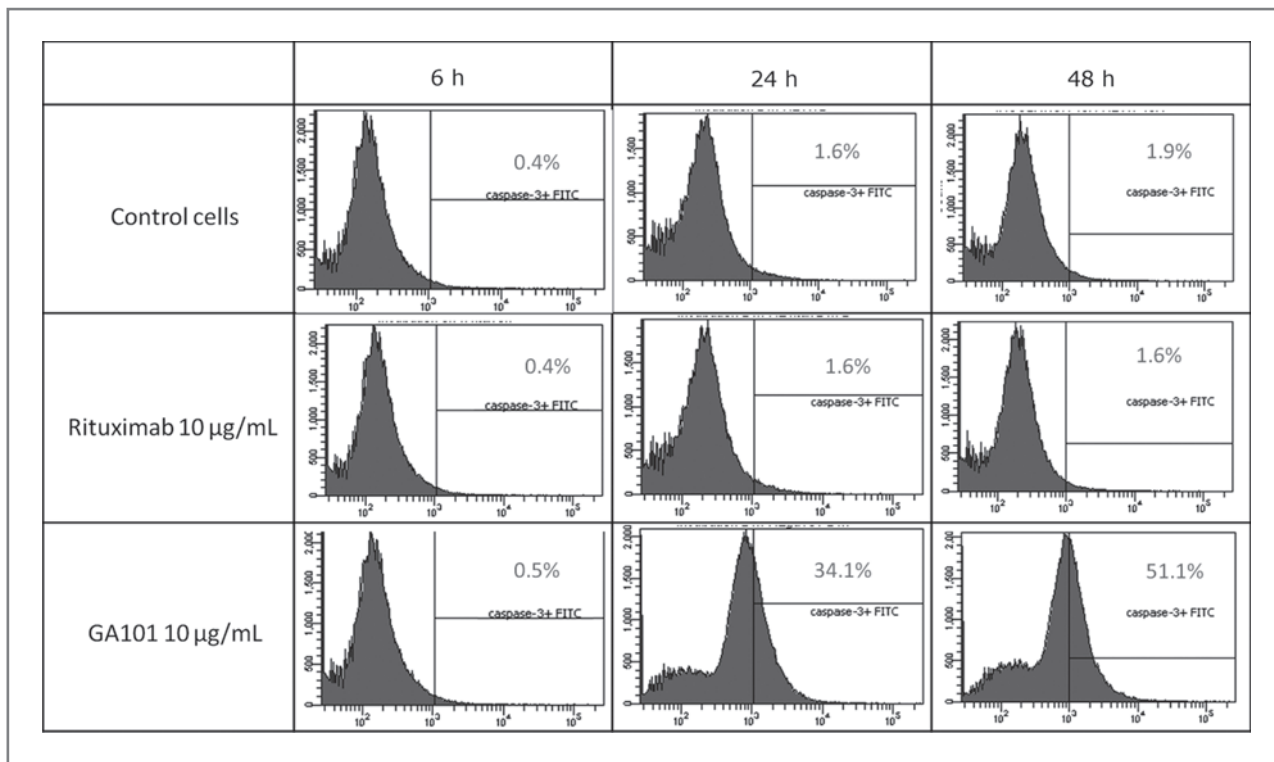


Figure 5. Effect of rituximab and GA101 on caspase-3 expression in RL cells *in vitro*. Cleaved caspase-3 expression was evaluated *in vitro* by FACS analysis after 6, 24, and 48 hours of exposure to either GA101 or rituximab. Percentage represents the number of positive cells.

expression both by FACS and Western blot analysis (data not shown). When cells were co-incubated with antibodies and CVF, the quantities of caspase-3 appeared to be decreased in the case of rituximab-exposed cells but not in the case of GA101-exposed cells.

The expression levels of CD19, as well as of complement inhibitors CD46, CD55, and CD59, on RL cells were studied after 1, 2, 4, 6, and 24 hours *in vitro* exposures to GA101 or rituximab, in the presence or absence of CVF. There was no change in the CD19 expression after exposure to rituximab or GA101 (data not shown). In addition, we did not observe any upregulation of the CD46, CD55, and CD59 antigens in tumor cells exposed *in vivo* to either antibody. Although procaspase-3 was found slightly increased by Western blot analysis in the GA101-treated group in comparison with untreated or rituximab-treated mice 12 hour after exposure, we did not observe cleaved form of caspase-3 expression following these *in vivo* experiments (data not shown).

Gene expression arrays

In RL cells exposed to mAb *in vitro* for 6 hours, a total of 867 genes were induced by rituximab and 664 by GA101, including 152 genes induced by both antibodies (See Supplementary Table 1). A series of genes found to be significantly overexpressed after exposure both to ritux-

imab and to GA101 were analysed by RT-PCR (data not shown). Among these, overexpression of EGR1 and ATF3 were confirmed in 3 independent experiments. EGR1 was increased up to 10-fold and ATF3 to 4-fold in RL cells after exposure to antibodies ($P < 0.01$). Increased expression of the corresponding proteins was also documented by immunoblotting (Fig. 6). We did not observe increase in Bax protein level expression.

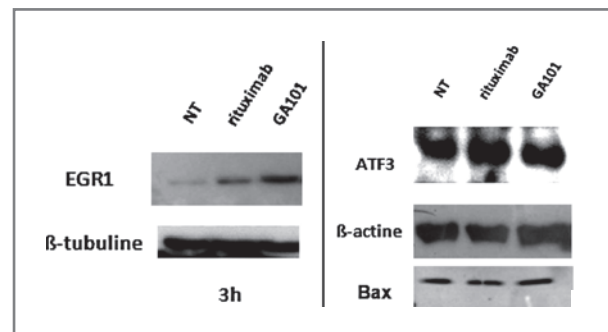


Figure 6. Effect of rituximab and GA101 on EGR1, Bax, and ATF3 expression in RL tumors. EGR1, Bax, and ATF3 protein levels were evaluated by Western blotting after exposure of RL cells to rituximab and GA101 *in vitro*.

Gene ontology pathways of the genes induced by rituximab and GA101 are presented in Supplementary Tables 2 and 3. Interleukin 12 (IL-12) biosynthesis, glucose import, and ribosome biogenesis were among the pathways most significantly enriched after exposure to rituximab. Response to biotic stimulus or parasites, immune activation, and IL-1 biosynthesis were among the pathways most significantly enriched after exposure to GA101. Several pathways were found to be enriched with both antibodies, including IL-1, IL-6, IL-13 biosynthesis as well as negative regulation of cell differentiation and osteoclast differentiation. It thus appears that the pathways activated by these 2 anti-CD20 antibodies partially overlap but differ significantly in other aspects.

Discussion

This preclinical study was initiated to compare the *in vivo* efficacy of rituximab, the first in class anti-CD20 mAb, and GA101, a novel generation CD20 antibody, in a preclinical model of human NHL. The results show the superior antitumor activity of GA101 in a model of human FL RL grown as xenografts in SCID mice, either as a single agent or in combination with cyclophosphamide, in comparison to rituximab. The effect of GA101 did not appear to involve complement, whereas the effect of rituximab in this model was at least partially complement dependent. Tolerability of GA101 with these regimens was excellent with no toxic deaths and no significant modification of body weight.

The role of complement in anti-CD20 treatment is now a matter of debate. CDC is clearly involved in the action of rituximab both *in vitro* and in preclinical mouse models (21, 22, 32–34). Manches et al. have also reported a correlation between *in vitro* sensitivity to CDC of various lymphoma subtypes and the likelihood of response to rituximab in the clinic (17). Conversely, it has been suggested that deposition of inactivated C3b on rituximab-coated cells could diminish the interaction between the Fc region and CD16 on NK cells. Our *in vivo* results using rituximab corroborate the previously reported data and support the role of complement in rituximab-mediated antitumor activity. However, we did not observe variations in the expression of the complement inhibitors CD46, CD55, or CD59 in our model. These antigens have been reported to be altered in cells exposed to antibodies or to be correlated with response to anti-CD20 antibodies (20). In the case of GA101, the role of CDC is clearly less important both *in vitro* and *in vivo* (29).

In the SCID mice model, it is not expected that a major contribution to efficacy comes from the optimized interaction of the glycoengineered Fc-part of GA101 with the murine FcγRIV receptors expressed on macrophages/monocytes. Thus, the direct induction of cell death is likely to play an important role in GA101-mediated cytotoxicity. In support of this hypothesis, we observed

a greater induction of early cell death as well as higher expression of procaspase-3 and Bad protein after exposure to GA101 than after exposure to rituximab. Although the activated form of caspase-3 was not detected in our animal model euthanized 12 hours after last exposure to treatment, we observed in additional *in vitro* kinetic experiments that the cleaved-caspase-3 form was mainly detectable after 24 and 48 hours of exposure.

The signalization pathways involved in anti-CD20 antibody-mediated cytotoxicity have been explored by several groups. Bonavida and Jazirehi have reported the importance of RKIP as a central regulator of rituximab-induced cytotoxicity, regulating BclX-L (35). Leseux et al. have found that PKCζ was involved in cytotoxicity of rituximab in the RL line (36). In this study, we identified 2 novel proteins potentially involved in CD20-mediated cytotoxicity. ATF3 and EGR1 were overexpressed both after exposure to rituximab and to GA101, suggesting that they may be involved in CD20-mediated signaling. EGR1 or early growth response 1 is a zinc finger protein that has been reported to possess both oncogenic and tumor suppressor properties. Zheng et al. have shown that NFκB-induced EGR1 transcription allowed survival of prostate tumor cells, whereas other authors have suggested a proapoptotic role by induction of Bax (37, 38). ATF3, or activating transcription factor 3, is a leucine zipper protein involved in cellular stress pathways. ATF3 has been reported to behave as an oncogene in murine mammary carcinogenesis, but has also been found to be involved in apoptosis in prostate cancer (39, 40). Additional experiments are required to show whether the overexpression of these genes after exposure to anti-CD20 monoclonal antibodies is required for cytotoxicity.

In conclusion, these results show that GA101 used as a single agent or in combination with cyclophosphamide is more active than rituximab on human lymphoma RL xenografts. As expected for a Type II antibody, GA101 appears not to act through CDC, and is more potent than the Type I antibody rituximab in inducing cell death via nonclassical induction of apoptosis.

Disclosure of Potential Conflicts of Interest

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ABSTRACT

CD20 is a validated target for the immunotherapy of B lymphoid neoplasms, including Chronic Lymphocytic Leukemia (CLL). We compared the activities of rituximab and GA101 (novel anti-CD20 antibody) on fresh human CLL cells *in vitro*. AnnexinV staining demonstrated induction of apoptosis after exposure to rituximab or GA101. Unlike rituximab, GA101 induced a reduction of the mitochondrial transmembrane potential, an effect which could be partially inhibited by cyclosporin A and which was partially caspase-dependent. GA101 was also found to induce the production of Reactive Oxygen Species. Analysis of pro- and anti-apoptotic protein content after exposure to antibodies demonstrated a strong degree of heterogeneity between samples. Bax underwent conformational activation and mitochondrial translocation upon exposure to antibodies in a caspase-independent manner. GA101 but not rituximab induced cleavage of caspase-8, -9 and -3. By transfecting CLL cells with anti-Bcl-xL siRNA using a sonoporation method, we found that reduction of Bcl-xL content was associated with increased sensitivity to these antibodies. Our results suggest that apoptotic signaling pathways differ between rituximab and GA101 with a greater involvement of the mitochondrial pathway for GA101. Inhibition of Bcl-xL could constitute an approach to sensitize CLL cells to the apoptotic effects of anti-CD20 antibodies.

TITRE en français

Comparaison des mécanismes de cytotoxicité des anticorps monoclonaux anti-CD20 Rituximab et GA101 dans les cellules fraîches de la Leucémie Lymphoïde Chronique

RESUME

CD20 est une cible thérapeutique validée pour l'immunothérapie des néoplasmes lymphoïdes des cellules B, incluant la Leucémie Lymphoïde Chronique (LLC). Nous avons comparé les effets de rituximab et de GA101 (nouvel anticorps anti-CD20) contre les cellules LLC fraîches *in vitro*. Le marquage avec AnnexineV a démontré une induction de l'apoptose après l'exposition au rituximab et GA101. Contrairement au rituximab, GA101 induisait une réduction du potentiel transmembranaire mitochondrial, un effet qui peut être partiellement inhibé par la cyclosporine A et qui est partiellement caspase-dépendant. GA101 induisait aussi la production des espèces d'oxygènes réactives. L'analyse du niveau d'expression des protéines pro- et anti-apoptotiques après exposition aux anticorps a démontré une forte hétérogénéité entre les échantillons. Bax subissait une activation de conformation et une translocation mitochondriale suite à l'exposition aux anticorps d'une manière caspase-indépendante. GA101, mais pas rituximab, induisait le clivage des caspase-8, -9 et -3. En transfectant les cellules LLC avec un siRNA ciblant Bcl-xL utilisant la sonoporation, nous avons trouvé que la réduction du niveau d'expression de Bcl-xL est associée à une augmentation de la sensibilité aux anticorps. Nos résultats suggèrent que les voies de signalisation apoptotiques diffèrent entre rituximab et GA101 avec une implication de la voie mitochondriale avec le GA101. L'inhibition de Bcl-xL peut constituer une façon pour sensibiliser les cellules LLC aux effets apoptotiques des anticorps anti-CD20.

DISCIPLINE : Pharmacologie

KEYWORDS / MOTS-CLES

Chronic Lymphocytic Leukemia, Rituximab, GA101, apoptosis, sonoporation

Leucémie Lymphoïde Chronique, Rituximab, GA101, apoptose, sonoporation

INTITULE ET ADRESSE DU LABORATOIRE :

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