In Vitro Drug Sensitivity and Apoptosis in Chronic Lymphocytic Leukemia

MARIA NORBERG
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Abstract

Chronic lymphocytic leukemia (CLL) is a heterogeneous malignancy displaying varying clinical outcome, where molecular markers today can divide patients into prognostic subgroups. Despite the introduction of new agents for treatment, remissions are usually not sustained in CLL and resistance towards treatment can partly be explained by aberrant apoptosis. The aim of this thesis was to find new drugs for CLL patients resistant to conventional therapy and to analyze genes involved in apoptosis within different prognostic subgroups.

In paper I-II, the *in vitro* activity of substances was investigated using the fluorometric microculture cytotoxicity assay (FMCA). When evaluating rapamycin (paper I), an inhibitor of mTOR, in 97 tumor samples from different entities, CLL was found to be one of the most sensitive tumor types. Combination experiments on patient CLL cells indicated that rapamycin acted synergistically with the CLL drugs vincristine and chlorambucil.

An investigation of 20 anti-cancer agents in cells from 40 CLL patients (paper II) revealed that prednisolone and rolipram displayed high activity in poor-prognostic patients, in particular IGHV unmutated CLL. Furthermore, when used in combination these agents were found to produce a synergistic effect.

In paper III, the anti-apoptotic BCL2 family member BFL1 was evaluated in 37 CLL cases. Levels of BFL1 were higher in fludarabine-resistant patients compared to fludarabine-sensitive patients. In addition, the high expression of BFL1 inversely correlated to fludarabine-induced apoptosis in CLL cells.

A single nucleotide polymorphism in the anti-apoptotic BCL2 gene (-938C>A) has been suggested as a novel poor-prognostic marker in CLL. In paper IV, we investigated this BCL2 polymorphism in 268 CLL patients and correlated genotypes to clinical data. However, no association could be confirmed between this polymorphism and clinical outcome or established prognostic markers.

In conclusion, this thesis has shown that rapamycin is a potential drug for treatment in CLL. Furthermore, prednisolone and rolipram were identified as interesting candidates for treatment of poor-prognostic patients. Finally, the anti-apoptotic protein BFL1 may contribute to chemoresistance and hence represents a potential therapeutic target in CLL, whereas from our data, the BCL2 -938C>A polymorphism does not appear to have any prognostic significance.

Keywords: chronic lymphocytic leukemia, *in vitro* drug sensitivity, apoptosis, prognostic markers

Maria Norberg, Department of Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden

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urn:nbn:se:uu:diva-120299 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-120299)
“The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music.”

Lewis Tomas
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<th>Definition</th>
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<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptotic protease-activating factor</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated protein X</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor repeat</td>
</tr>
<tr>
<td>BLYS</td>
<td>B-lymphocyte stimulator</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CI</td>
<td>Combination index</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>D</td>
<td>Diversity</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FC</td>
<td>Fludarabine, cyclophosphamide</td>
</tr>
<tr>
<td>FCR</td>
<td>Fludarabine, cyclophosphamide, rituximab</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FFR</td>
<td>Flavopiridol, fludarabine, rituximab</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HDMP</td>
<td>High-dose methylprednisolone</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGHV</td>
<td>Immunoglobulin heavy variable</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of NFkB</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>J</td>
<td>Joining</td>
</tr>
<tr>
<td>LDT</td>
<td>Lymphocyte doubling time</td>
</tr>
<tr>
<td>mAB</td>
<td>Monoclonal antibody</td>
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</tbody>
</table>
MCL1 Myeloid cell leukemia 1
MDAC Mithochondrial voltage-dependent anion channel
MDR Multi-drug resistance
MLPA Multiplex ligation-dependent probe amplification
MM Multiple myeloma
MRD Minimal residual disease
MTHFR Methylenetetrahydrofolate reductase
mTOR Mammalian target of rapamycin
NCI National Cancer Institute
NF-κB Nuclear factor kappa B
NK cell Natural killer cell
nPR Nodular partial response
ORR Overall response rate
OS Overall survival
PBMC Peripheral blood mononuclear cell
PD Progressive disease
PDE4 4-cAMP-phosphodiesterase
PFS Progression-free survival
P-gp P-glycoprotein
PI3K Phosphatidylinositol 3 kinase
PIP2 phosphatidylinositol 3,4-biphosphate
PIP3 phosphatidylinositol 3,4,5-triphosphate
PR Partial response
R-CHOP Rituximab-cyclophosphamide, doxorubicine, vincristine, prednisolone
RQ-PCR Realtime quantitative PCR
SBE-FP Single-base primer extension - fluorescence polarization
SCT Stem cell transplantation
SHM Somatic hypermutation
SI Survival index
sIg Surface immunoglobulin
SNP Single nucleotide polymorphism
TCL1 T-cell leukemia 1
TNF Tumor necrosis factor
TTP Time to progression
TTT Time to treatment
V Variable
INTRODUCTION

Chronic Lymphocytic Leukemia

Epidemiology and Clinical Features
The most common adult leukemia in western countries is chronic lymphocytic leukemia (CLL), which is characterized by the accumulation of small monoclonal B-cells with a mature appearance. In Sweden, approximately 500 patients are diagnosed each year with CLL, which accounts for 20% of all malignant lymphomas. The incidence rate in men is nearly twice as high as in women. CLL mainly affects the elderly population, with the median age of onset being approximately 65-70 years. The incident rate of CLL in individuals over 70 years of age is estimated to be 50/100,000 [1]. The proportion of patients with CLL under 65 years of age is 35% and the disease is rare in people under the age of 40 [1].

Most patients are asymptomatic at diagnosis and in many instances the disease is revealed at a routine blood test when a high white blood cell count is detected. CLL can be diagnosed when the clonal lymphocyte count in blood is >5x10^9/L [2] and the cells express a distinct immunophenotype (see below). An asymptomatic disease is observed in approximately 40-60% of patients at diagnosis, however duration of this asymptomatic phase varies considerably amongst patients [3]. Progression of the disease leads to various symptoms including e.g. swollen lymph nodes, recurrent infections and anemia due to organ infiltration by the malignant cells. Other symptoms of CLL include fever, weight loss and night sweats [3]. Disease-related mortality is often due to bacterial infections of the respiratory tract, skin or urinary tract [4].

The cause of CLL is still unknown. Although the majority of CLL cases are sporadic, a hereditary component exists since relatives of CLL patients have a higher risk of developing CLL than the general population; however the mode of inheritance is unknown [5].
The CLL Cell

CLL cells are present in the bone marrow, peripheral blood, lymph nodes and spleen (Figure 1). The disease is characterized by accumulation of monoclonal B-cells that are arrested predominantly in the G0 or early G1 phase of the cell cycle [6]. To date, no unique genomic aberration or chromosomal translocation has been discovered in CLL.

![Figure 1. May-Grünwald-Giemsa staining of a CLL bone marrow smear (a) and Hematoxylin-eosin staining of a section of paraffin embedded lymph node (b).](image)

However, the surface antigens that constitute the CLL immunophenotype are characteristic for the disease and are used to confirm diagnosis. The CLL cells typically express the B-cell markers CD19, CD20 and CD23 with co-expression of CD5 and faint levels of surface immunoglobulins (sIG) [7]. Cases that do not display a typical immunophenotype should be evaluated using Matutes Score [7] (Table 1). A score of 4-5 is indicative of CLL, whereas a score of 0-2 signifies another B-cell derived malignancy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>CLL</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIG</td>
<td>weak</td>
<td>1</td>
</tr>
<tr>
<td>CD5</td>
<td>positive</td>
<td>1</td>
</tr>
<tr>
<td>CD23</td>
<td>positive</td>
<td>1</td>
</tr>
<tr>
<td>CD79b/CD22</td>
<td>weak</td>
<td>1</td>
</tr>
<tr>
<td>FMC7</td>
<td>negative</td>
<td>1</td>
</tr>
</tbody>
</table>

The Origin of CLL

Since the main function of a B-cell is to recognize and bind foreign antigen, each B-cell will go through several crucial steps in order to create a functional and effective immunoglobulin (IG) molecule. The first step in generating the great diversity of antibodies in the immune system [8] is VDJ recombination of the heavy chain IG genes (IGH), where one of 46 variable (V),
one of 23 diversity (D) and one of six functional joining (J) genes are “shuffled”/recombined to create a complete IGH sequence [9-10]. Similarly, V and J light chain genes join at the IG kappa and lambda loci. Consequently, the region incorporating the VDJ junction is the most variable of the IG molecule and is known as the complementarity determining region 3 (CDR3). Even greater diversity of the CDR3 is generated by the random introduction of nucleotides by deoxynucleotidyl transferase and/or the deletion of nucleotides by exonuclease between the V-D genes and between the D-J genes [11-13]. It is the CDR3 that has the most contact with antigen and therefore confers the specificity of the IG, with each B-cell having its own unique IGH gene rearrangement.

Following encounter with antigen, a B-cell carrying a functional IG molecule, will go through somatic hypermutation (SHM) of the IG genes to further increase its affinity for the antigen [14]. This is a process by which the B-cell develops from a naïve inexperienced B-cell to a long-lived memory cell. SHM generally occurs in germinal centers (GC) of lymph nodes and requires T-cell help.

The exact cell of origin in CLL has been difficult to elucidate, since analyses of IG heavy variable (IGHV) gene sequences have shown that approximately half of all CLL cases have somatically hypermutated IGHV genes (M-CLL) whereas the other half lack mutations (UM-CLL) [15-16]. It has therefore been suggested that CLL is composed of two different entities originating from different stages of B-cell development, one that is antigen-inexperienced and one that is antigen-experienced, i.e. pre-GC and post-GC. However, studies have shown that gene expression profiles and immunophenotypes of both CLL entities resemble that of antigen experienced cells [17-19]. Moreover, since it has been shown that B-cells can gain mutations outside of a typical GC reaction and independent of T-cell help, it has been postulated that both UM-CLL and M-CLL could originate from the marginal zone [20-21]. However, the exact cell of origin in M-CLL and UM-CLL remains unknown.

Antigens in CLL Pathogenesis

Considering the huge potential for IG variation, it was surprising to observe a biased usage of certain IGHV genes in CLL, such as the IGHV1-69 [22] gene which is predominantly found in UM-CLL and the IGHV4-34 [23] and IGHV3-21 [24] genes that are mostly seen in M-CLL. Subsequent larger scale analysis of the IG genes in CLL revealed that multiple subsets of CLL patients, from different geographical regions, carried IGs with virtually identical “stereotyped” heavy chain CDR3 [23, 25-26]. Since the probability of two B-cells sharing identical B-cell receptors (BCRs) is extremely low, ap-
approximately 1 in 2.3 x10^{12} [9, 27], these findings of almost identical CDR3s indicated that antigenic selection of tumor clones may play a role in the pathogenesis of CLL [23, 28].

Moreover, it was reported that certain stereotyped subsets were associated with an indolent disease course, whereas others were associated with a poorer clinical outcome [23]. Recently, several studies have identified a number of antigens, both autoantigens and bacterial antigens, which may be important in the initiation and progression of CLL [29-33]. However, it is unclear whether antigen interaction with the CLL clone is restricted to the initiation stage of disease development or whether ongoing clonal stimulation may occur throughout the course of the disease. Furthermore, it remains to be explained why the IG specificities of stereotyped subsets result in the very different clinical outcome observed in certain patient groups.

Clinical Staging

The clinical course varies substantially among CLL patients; patients may have an indolent disease for many years without a need for therapy whilst others die within a few years of diagnosis despite receiving treatment. There are two clinical staging systems available to measure disease progression in CLL, Rai and Binet [34-35]. An overview of both scoring systems is provided in Table 2.

**Table 2. Overview of Binet and Rai clinical scoring systems [36]**.

<table>
<thead>
<tr>
<th>Binet</th>
<th>Characteristics</th>
<th>Median survival</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Less than three sites involved</td>
<td>15 years</td>
</tr>
<tr>
<td>B</td>
<td>Three or more sites involved</td>
<td>5 years</td>
</tr>
<tr>
<td>C</td>
<td>Anemia (Hb&lt;100 g/L) or thrombocytopenia (platelets&lt;100x10^9/L)</td>
<td>3 years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rai</th>
<th>Characteristics</th>
<th>Median survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Lymphocytosis</td>
<td>&gt;13 years</td>
</tr>
<tr>
<td>I</td>
<td>Lymphadenopathy</td>
<td>8 years</td>
</tr>
<tr>
<td>II</td>
<td>Splenomegaly or hepatomegaly</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Anemia</td>
<td>2 years</td>
</tr>
<tr>
<td>IV</td>
<td>Thrombocytopenia</td>
<td></td>
</tr>
</tbody>
</table>

Both systems are used to divide patients into risk-groups based on the assessment of clinical findings regarding disease burden. These staging systems are currently being applied in clinical practice since they play an important role in estimating the need for therapy and the likely prognosis and survival. However, neither of these scoring systems has the ability to identify patients that are at an early stage and whose disease will progress rapidly. Furthermore, the scoring systems fail to clearly predict the response of indi-
individual patients to specific therapies. This is further complicated by the fact that the response to specific treatments can change as the disease progresses. Therefore, owing to the heterogeneity observed in the clinical behavior of the disease, characterization of new prognostic markers that are capable of identifying patients at high risk of progressing to an aggressive disease course remains important in CLL.

Prognostic Markers

**IGHV Gene Mutational Status**

As previously mentioned, approximately 50% of CLL patients carry mutated IGHV genes, whilst the remainder carry unmutated IGHV genes [37-38]. This molecular difference has proven prognostically useful since UM-CLL have a worse prognosis than M-CLL; median survival ranging from 70-108 months for UM-CLL compared to greater than 146 months for M-CLL patients [28, 37-38]. An exception to this division is CLL cases which utilize the IGHV3-21 gene. These patients tend to have a poor prognosis despite carrying mutated IGHV genes in approximately two thirds of cases [24, 28]. The proportion of IGHV3-21 using cases varies significantly between studies and countries (3-10%), with a higher frequency reported in Scandinavia [39-40].

**Genomic Aberrations**

Fluorescence *in situ* hybridization (FISH) is used today in clinical routine diagnostics for detection of recurrent genomic aberrations. There is no single genetic abnormality present in all CLL cases, however more than 80% of cases carry known recurrent genomic aberrations [41]. The most common aberration is deletion of 13q14, which is detected in approximately 55% of patients and is an indicator of good prognosis when present as a sole aberration [41]. Trisomy 12 is detected in 11-16% of CLL patients [41-43] and is associated with intermediate prognosis and short time to treatment (TTT) [41, 43]. Deletion of 11q and 17p covering the *ATM* gene and *TP53* gene, respectively, are markers of poor prognosis. del(11q) is found in 12-18% of all patients [41-43] and is associated with enlarged abdominal lymph nodes (Figure 2). This deletion correlates with a lower response to purine analogs [44-45] as well as refractoriness to DNA-damaging chemotherapy [46]. del(17p) is detected in approximately 5-10% of CLL patients [41-43] and is associated with resistance to purine analogs, alkylating agents and rituximab [44-45]. 17p-deleted patients also have the shortest survival of all CLL cases with a median overall survival (OS) of 32 months [41]. More recently, reports have highlighted the independent prognostic value of *TP53* mutations in the absence of a deletion (approximately 3-5% of cases) [47-48]. Although patients with 17p-deletion/*TP53* abnormalities constitute a small
proportion of CLL cases, this subgroup is prognostically important since patients carrying these aberrations have the worst outcome of all CLL patients.

Figure 2. An image of a FISH analysis using an ATM-probe (green) to indicate chromosome 11 and p53 probe (red) to indicate chromosome 17. Here, the CLL cell carries a deletion of 11q.

Additional Prognostic Markers
Since both IGHV mutational status and FISH analysis are costly and time-consuming to perform, there has been a requirement for alternative prognostic markers to be discovered. As mentioned earlier, micro-array analyses have shown that most genes are expressed in a similar manner when comparing UM-CLL and M-CLL cases. Nevertheless, exceptions such as ZAP70, an intracellular tyrosine kinase involved in T-cell signaling [49], do exist and this gene has been found to be preferentially expressed in UM-CLL [19]. It had previously been assumed that ZAP70 was not expressed in normal B-cells, but it has since been shown that this kinase is indeed expressed in all B-cells although at different levels depending on differentiation stage and tissue localization [50].

In CLL, the strong association between ZAP70 expression and IGHV gene mutational status was confirmed by several studies [51-52]. Nevertheless, discordance between ZAP70 expression and IGHV gene mutational status has been observed in up to 25% of CLL patients [53-56]. However, this disparity can partly be explained by the high ZAP70 expression observed in mutated cases utilizing the IGHV3-21 gene and the low ZAP70 expression
observed in unmutated cases harboring del(11q) and del(17p) [55]. Despite this, ZAP70 has been shown to be an independent marker for TTT and OS [51-53]. However, there have been problems with standardizing ZAP70 analysis using flow cytometry [57] and this is essential before ZAP70 can be applied as a prognostic marker within the clinical setting.

Another potential prognostic marker is CD38, a transmembrane glycoprotein expressed on the surface of lymphocytes [58]. CD38 expression modulates apoptosis through its role in BCR signal transduction [59] and high expression has been correlated with UM-CLL [37]. However, subsequent studies have not been able to verify this strong association between CD38 expression and IGHV gene mutational status [60-61]. Furthermore, the cut-off value to best define CD38 positivity has been debated. The original cut-off value was set at 30% (expression of CD38 in 30% of the CLL cells), but other threshold values have been suggested such as 20% [62] [63], 7% [60] and 5% [64]. Additionally, CD38 expression may change over time in a proportion of patients [54, 65-66]. Despite these drawbacks, CD38 is considered an independent prognostic marker in CLL [67], although not the strongest one.

Additional prognostic markers have also been proposed and are under evaluation, such as expression of LPL [68], CLLU1 [69], T-cell leukemia 1 (TCL1) [70] and telomere length/telomerase activity [71].

**Single Nucleotide Polymorphisms as Prognostic Markers**

The most common form of genetic variation arises when a single nucleotide in the genome differs between individuals, referred to as a single nucleotide polymorphism (SNP). The total number of SNPs in the human genome is estimated to be ten million [72]. SNPs present within coding regions of the genome may result in amino acid changes, thereby altering the function of a protein. Additionally, SNPs present within non-coding regions of the genome, such as promoter regions and splice sites can alter gene function by affecting transcription and exon splicing, respectively [73-74].

A number of SNPs have been proposed to have prognostic impact in CLL. For instance, a SNP in the P2X7 gene correlated to a better prognosis with longer OS in CLL [75], however follow-up studies could not confirm this finding [76-77]. Moreover, within the promoter region of the BCL2–associated X (BAX) gene, a genetic variant, G(-248)A, has been associated with failure to achieve complete remission (CR) [78] and shorter OS [79]. However, these results could not be confirmed in a subsequent study using a larger patient cohort [80]. Another example of a genetic variant that has been suggested as a prognostic marker in CLL is an insertion in the myeloid cell leukemia 1 (MCL1) gene that correlates to more rapid disease progression, poor response to chemotherapy and shorter OS [81]. However, the prognos-
tic relevance of this insertion polymorphism could not be verified by other studies [82-84]. Finally, a SNP within the second promoter of the BCL2 gene (-938C>A) has been associated with disease progression and OS in CLL [85]. Furthermore, it was considered to be an independent prognostic factor for progression-free survival (PFS) in CLL.

Treatment in CLL

In the 1950s, the alkylating agents chlorambucil and cyclophosphamide, with or without corticosteroids, were introduced as a treatment for CLL with the principal aim of this therapy being relief of disease-related symptoms. This aim was frequently achieved in the majority of patients with progressive disease [86]. In the last decades, important advances have been made in the treatment of CLL. With newer drugs, such as purine analogs (e.g. fludarabine) and combinations of chemotherapeutic drugs and monoclonal antibodies (mAbs) (e.g. rituximab, alemtuzumab), a longer duration of remission and CR have been obtained. These newer and more effective drugs have altered the therapeutic approach to CLL, especially in younger patients exhibiting poor prognostic markers, with the aim being induction of long-standing remissions and prolonging survival [87]. Despite these advances, with the possible exception of allogeneic stem cell transplantation (SCT) which is only suitable for younger patients, there is currently no curative treatment available for CLL patients [88]. An overview of treatment options in CLL is shown in Figure 3.
Figure 3. Flow-chart of treatment options in CLL. Modified from the “National guidelines for investigation and treatment” by the Swedish CLL group.

Indications for Treatment

Large randomized trials have demonstrated that the use of early treatment with alkylating agents does not prolong survival in CLL patients with low tumor burden [89]. Thus, newly diagnosed CLL patients with early-stage disease are monitored without therapy unless they display evidence of disease progression [2]. Although one third of CLL patients will not require therapy, studies have demonstrated that among untreated patients with early-stage disease, approximately 40% progress to advanced disease and 45% ultimately require therapy [89].

The National Cancer Institute Working Group (NCIWG) has published recommended criteria for the treatment of CLL [90]. These guidelines include the development of B-symptoms (weight loss ≥10% within six months, fever for two weeks, night sweats or extreme fatigue), worsening anemia and/or thrombocytopenia, autoimmune cytopenias, progressive splenomegaly, progressive lymphadenopathy and a lymphocyte doubling time of six months. An overview of mechanisms of action for agents used in CLL treatment is shown in Table 3
Chemotherapy

Alkylating Agents
Chlorambucil, an oral alkylating agent, has traditionally been the treatment of choice in CLL. The response rate among previously untreated patients varies between 60-90% but CR is rare (~10%) and combination with a corticosteroid such as prednisolone does not appear to increase activity [86, 91]. When chlorambucil is poorly tolerated by the patient, cyclophosphamide is an option, although it is mostly used in combination regimens [91]. For patients where the purpose of treatment is not to achieve remission, the first choice of therapy is chlorambucil. Patients who relapse after an initial response to chlorambucil may be treated with an additional course [92].

Purine Nucleoside Analogs
The purine analogs were introduced in the 1980s and fludarabine, pentostatine and cladribine are currently used in CLL treatment [93]. Fludarabine is the most extensively studied purine analog with regard to CLL treatment and was first introduced for patients suffering from either relapses or refractory disease. It was subsequently shown to be the most active agent for first-line therapy in CLL [91] and three large randomized studies have shown that fludarabine as monotherapy produces superior overall response rate (ORR), more CRs and longer PFS compared to other treatment regimens containing alkylating agents. However, fludarabine has not been shown to improve OS and it has worse side-effects than chlorambucil [93-96]. The efficiency and safety of fludarabine is the same when administered either orally or intravenously [97-98].

Combination Chemotherapy
Studies on fludarabine in combination with cyclophosphamide (FC) were initiated following a study by Bellosillo and colleagues who reported a synergistic effect between purine analogs and alkylating agents in vitro [99]. Three randomized trials have shown that FC combination improves CR, ORR and PFS when compared to monotherapy [100-102]. Patients who develop progressive disease more than 1 year after receiving F/FC and who initially responded may be treated with F/FC again [92].

Immunotherapy

Rituximab
Rituximab is a chimeric mAb that binds to the surface antigen CD20 [103]. In CLL, rituximab induces both antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity through caspase 3 activation and induction of apoptosis [104]. However, relatively low levels of CD20
are expressed on CLL cells compared to normal B cells, thereby lowering the capacity of rituximab to bind to CLL cells. Indeed, only limited activity has been observed in previously treated CLL patients using rituximab as a single agent [105] and it is not recommended as an initial treatment. Therefore, the majority of studies on rituximab over the last decade have been as combination therapy, such as fludarabine and rituximab (FR) and fludarabine, cyclophosphamide and rituximab (FCR).

**Combinations using Rituximab**

Since preclinical studies have reported synergy between rituximab and fludarabine [106], this combination has been investigated in several phase II trials. Rituximab combined with fludarabine as either a sequential or concurrent regimen has resulted in a RR of 77-90% [107] and a significantly better PFS among previously untreated CLL patients [108].

FCR has been tested in first-line, relapsed and refractory settings. The German CLL Study Group (GCLLSG) CLL8 was the largest randomized clinical trial performed in CLL and demonstrated a significant improvement in ORR (92.8% vs 85.4%), more CR (44.5 vs 22.9%) and longer duration of response with FCR compared with FC alone in a first-line setting [109]. The results of the CLL8 study led to approval of rituximab in combination with chemotherapy for CLL treatment in the United States and Europe. FCR has been more frequently associated with side effects and treatment related mortality than FC [109].

**Alemtuzumab**

Alemtuzumab is a recombinant, fully humanized antibody against CD52, a membrane glycoprotein highly expressed by both normal and malignant lymphocytes [110-111]. It is currently the only mAb approved as single agent for treatment in both first-line (if treatment containing fludarabine is inappropriate) and refractory CLL patients. Several phase II clinical trials have reported activity for alemtuzumab as a single-agent in previously treated CLL patients displaying an advanced disease, with a response rate of 33-53% [112-116]. Interestingly, alemtuzumab is also effective in fludarabine-refractory CLL patients and patients with poor-prognostic markers, including high-risk genomic aberrations such as 17p and 11q deletion [117-120]. However, alemtuzumab has been associated with side effects such as reactivation of cytomegalovirus and opportunistic infections [121].

**Combinations Using Alemtuzumab**

Administration of both fludarabine and alemtuzumab in combination and FC plus alemtuzumab have been investigated in refractory CLL patients and resulted in high response rates [122-123]. Further studies, as a first-line ther-
apy, are ongoing to confirm the results but side effects, may limit their clinical use [93].

Hematopoietic Stem-Cell Transplantation

**Allogeneic Stem Cell Transplantation**

Allogeneic stem cell transplantation (SCT) is the only potentially curative treatment in CLL and has been evaluated in young patients [86]. Bearing in mind the possibility of a graft-versus-leukemia effect and that there is a risk of transplantation–related mortality, non-myeloablative regimens have been introduced resulting in lower rates of early toxicity [124-125]. According to recent European recommendations physically fit patients with refractory CLL or with del(17p) should be considered as candidates for allogeneic SCT since their prognosis is very poor with conventional therapies [88].

**Autologous Stem-Cell Transplantation**

Despite initial high rates of complete molecular response, studies involving autologous SCT for CLL patients have failed to demonstrate a survival advantage and thus, autologous SCT is not recommended outside clinical trials [126].

**Other Therapies**

**High-Dose Glucocorticoid Therapy**

Though widely used, there is little published clinical data on high-dose glucocorticoid therapy in CLL. Glucocorticoids kill lymphocytes by apoptosis in a manner that is independent of functional p53 [127]. High-dose methylprednisolone (HDMP) was investigated in 11 patients with refractory CLL, six of the patients achieved a partial response (PR) whereas none achieved CR [128]. In another study of 25 patients, HDMP was studied alone or in combination with different chemotherapy options. Ten of the 25 cases (45%) displayed p53 abnormalities. The ORR (77%) did not differ regardless of whether HDMP was used alone or in combination with chemotherapy. Notably, five of the patients with p53 abnormalities responded, further supporting the notion that glucocorticoids are no less effective in p53-deleted patients [129]. In a study by Pettitt et al, alemtuzumab was analyzed in combination with HDMP in five clinically aggressive CLL patients with p53 defects and bulky lymph nodes. All five patients responded to therapy (three CR, two PR) [130].
In Table 3, the mechanisms of action for different treatments in 17p-CLL are presented. The table includes treatments such as Fludarabine, Cyclophosphamide, Chlorambucil, and monoclonal antibodies like Alemtuzumab and Rituximab. Abbreviations provided for understanding: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; HDMP, high-dose methyl prednisolone.

### Table 3. Mechanisms of action

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CDC</th>
<th>ADCC</th>
<th>Apoptosis</th>
<th>Activity in 17p-CLL</th>
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<tr>
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<td>HDMP</td>
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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CLL, chronic lymphocytic leukemia; CDC, complement-dependent cytotoxicity; HDMP, high-dose methyl prednisolone.

### Apoptosis

#### Intrinsic and Extrinsic Pathways of Apoptosis

There are two principally different pathways for apoptosis: the intrinsic and the extrinsic pathway [131]. An overview of both pathways is shown in Figure 4.

The intrinsic pathway may be triggered by removal of growth factors or in response to lethal stimuli from inside of the cell, including DNA damage and cytostatic drugs. Mitochondrial functionality is essential for this pathway and is controlled by the BCL2 family of proteins. These proteins are either anti- or pro-apoptotic and the net balance of these two categories determines whether the mitochondrial membrane will allow the release of cytochrome c. This molecule forms a complex with apoptotic protease-activating factor (APAF1) and activates initiator caspase 9 which will in turn activate effector caspases 3, 6 and 7 resulting in cell death. p53 is an integral part of this pathway (see below), controlling the ratio of pro- and anti-apoptotic BCL2 proteins [132-133].

The extrinsic pathway, which is p53-independent, operates on the cell surface via death receptors. It is initiated by binding of the death receptors, e.g. FAS to its ligand FASL [132, 134]. Once these receptors are activated, the intracellular domains bind to the adaptor protein FAS-associated death domain (FADD) to form the death inducing complex with recruitment of pro-caspase 8 [132, 134-135]. These molecules (ligand, death receptor, adaptor protein and caspase) form a complex that activates downstream effector proteins, e.g. caspase 3 and 7, resulting in apoptosis [136]. In the extrinsic pathway, mitochondria may also be involved as a secondary event in order to enhance apoptotic signaling of death receptors [133]. Both pathways are inter-connected through cooperation with signaling proteins, such as nuclear...
factor kappa B (NF-κB) and p53-murine double minute 2 (MDM2) and both activate caspases responsible for the morphological changes associated with apoptosis [132].

Caspases might be inhibited by inhibitor of apoptosis proteins (IAPs). These cellular cofactors have a common domain, the baculovirus inhibitor repeat domain (BIR) that binds to and inhibits active caspases. Members of the IAP family may contain many BIR domains, each with a different function [137].

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**Figure 4.** Schematic overview of the extrinsic and intrinsic pathways of apoptosis. Modified from Michael K. Rowe and De-Maw Chuang [138]
The BCL2 Family of Proteins

The BCL2 family of proteins is divided into three groups. Group I proteins are anti-apoptotic whereas groups II and III are pro-apoptotic. Group members are defined by conserved sequence motifs known as BCL2 homology domains (BH1-4). Most anti-apoptotic proteins including BCL2, MCL1, BCL2L1, BCL2L2, and BFL1 (group I), contain all four domains whereas pro-apoptotic proteins may lack one or several domains [132]. The BH3-only domain proteins (group II) include BID, BCL2L11, BIK, BAD, BMF, NOXA, PUMA and HRK. These proteins function as direct antagonists of BCL2 [134, 139]. Members belonging to the other subgroup of pro-apoptotic proteins (group III) which consists of BAX, BAK, BOK and BCLX, contain BH1-3 domains and function by activation of other pro-apoptotic family members [132, 139].

There are two proposed models regarding the function of the anti-apoptotic BCL2 family members. The BH1-3 domains of the anti-apoptotic members form a hydrophobic groove through which they can bind the BH3 domain of pro-apoptotic members [140-141], thereby blocking the integration of e.g. BAX in the outer mitochondrial membrane [142]. The role of BAX is to increase the opening of the mitochondrial voltage-dependent anion channel, which leads to loss in membrane potential and release of cytochrome c [143]. The other model suggests that BCL2 proteins inhibit BH3-only proteins from activating BAX and inducing apoptosis by binding to them at either the mitochondria or the endoplasmatic reticulum [144-145].

p53

The tumor-suppressor p53 is a key regulator of apoptosis and is mutated in more than 50% of solid tumors [146-147]. p53 functions as a transcription factor that both activates and suppresses the expression of a large group of target genes [148]. In response to DNA damage and other cellular stresses, activation of the ATM gene leads to transcriptional activity of p53. Activated p53 can lead to cell cycle arrest through expression of its target gene, p21. When the cellular damage cannot be repaired by the cell, p53 promotes apoptosis and cellular senescence [149], [132]. Due to its tumor suppressor effects, p53 has been called “the guardian of the genome” [150]. p53 activates apoptosis in multiple ways: 1) transactivation of the death-domain receptors in the extrinsic pathway [134, 151], 2) upregulation of pro-apoptotic BH3-only proteins in the intrinsic pathway [134], 3) induction of APAF1, [151]), 4) repression of BCL2 and survivin, a member of the IAP family [152] and 5) binding to the mitochondria, causing release of cytochrome c [134]. As mentioned, in CLL, del(17p), containing the TP53 gene, is detected in approximately 5-10% of the patients where the majority has a
TP53 mutation on the remaining allele, [41-43]. In addition, 3-5% of CLL patients carry TP53 mutations without del(17p) [47-48, 153].

Dysregulated Apoptosis in CLL

Several mechanisms, including various apoptosis-regulating proteins, cytokines and transcription factors have been found to contribute to resistance to apoptosis in CLL. A traditional view has been that CLL cells are unable to proliferate and that the accumulation of malignant B-lymphocytes results from dysregulated apoptosis only. However, it has recently been reported that a small fraction of the CLL clone (~0.1-1.75%) continues to proliferate [154]. The proliferating leukemic cells are presented as proliferation centres that are not observed in any other B-cell malignancy [155]. The defective apoptosis in CLL is ascribed not only to intrinsic defects of the CLL cell but also to the microenvironment. This becomes apparent when culturing CLL cells in vitro; in the absence of bone marrow stromal cells, CLL cells are difficult to maintain and die spontaneously. In contrast, CLL cells can survive in vitro once bone marrow stromal cells are added [156-157].

BCL2 Family of Proteins

Members of the anti-apoptotic BCL2 family of proteins are often up-regulated in CLL and contribute to resistance to apoptosis and poor response to treatment [158]. For instance, BCL2 is over-expressed in most CLL cases [159]. The reason for this was initially believed to be hypomethylation of the BCL2 promoter [160], but recently, it has been associated with dysregulation of miR15 and miR16, located at chromosome 13q14.3. Calin et al. showed that miR15 and miR16 are deleted or down-regulated in approximately 68% of all CLL cases [161]. Both these miRNAs negatively regulate the BCL2 gene [162]. In a study where a vector containing both miRNAs was transfected into cells from a chronic myeloid leukemia-derived cell line (MEG-01) [163] with no endogenous expression of miR15 and miR16, dramatically reduced levels of BCL2 were observed [162].

BFL1 is an anti-apoptotic member of the BCL2 family. It has been shown to protect from apoptosis induced by death-receptor ligation [164], TP53 over-expression [165], DNA-damaging agents [166] and serum deprivation [167]. In primary B-cells, BCL1 protects from apoptosis triggered by ligation of the BCR or FAS [168-169]. In humans, BFL1 is expressed in various hematopoietic cells in the bone marrow and fetal liver and in GSs of lymphoid organs [170]. Furthermore, BFL1 is a transcriptional target of NFκB [171]. Recently, BFL1 has been implicated in drug resistance in CLL (see below).
**Tumor Necrosis Factor**

Tumor necrosis factor (TNF) cytokines can provide both survival signals and induce apoptosis [172]. CD40 is a member of the TNF family of proteins. The binding of CD40 to CD40L on T-cells results in cell growth and differentiation of normal B-cells and rescues the cells from apoptosis [173-174]. A proportion of CLL cells have been found to express both CD40 and its ligand, thereby enabling an autocrine loop by which the CLL cells can promote their own survival signals [175]. BLyS (B-lymphocyte stimulator) is another member of the TNF family and critical for normal B-cell development [176]. B-cells from BLyS transgenic mice express elevated levels of BCL2 and have prolonged survival compared to normal B-cells [177]. The expression of BLyS, as well as its receptors, has been investigated in CLL cells [178] and it was found that this cytokine was expressed in 11/23 CLL patients. It was also shown *in vitro* that BLyS protects CLL cells from apoptosis. Additionally, all CLL patients expressed at least one of the BLyS receptors. These findings suggest that CLL cells can transduce survival signals through autocrine mechanisms.

**NFκB**

NFκB is a family of transcription factors that can promote survival signals through the activation of transcription of proteins involved in both the intrinsic and extrinsic pathways of apoptosis [179]. In humans, this protein family includes five members that share a homology Rel domain that is necessary for DNA binding [180]. The activity of these proteins is regulated by two pathways [181]. In the classical or canonical NFκB pathway, diverse stimuli, including TNFα and CD40L, activate specific kinases, IKKs which leads to degradation of inhibitors of NFκB (IκBs). Consequently, the heterodimers p65-p50 which were originally bound to IκBs, are released and modulate the transcription of >200 NFκB target genes [180]. This pathway is important for innate immunity and inflammation [182].

The alternative or non-canonical pathway is activated by cytokines such as BLyS. In this pathway, IKKα is activated and p100, an inhibitor of RelB is degraded followed by translocation of the heterodimers RelB-p52 and RelB-p50 and their binding to target genes [180]. This pathway is important for maturation and survival of B lymphocytes [180].

NFκB is constitutively expressed in CLL cells and can be further induced by CD40 stimulation [183]. This results in the blockage of apoptosis for the CLL cells. Several chemotherapeutic substances that induce apoptosis in CLL, including fludarabine and proteasome inhibitors, down-regulate NFκB activity [183-185].
The defects in apoptosis in CLL cells do not appear to render them inherently immortal; which until recently has been the traditional view. Instead, the accumulation of cells arises due to development of genetic abnormalities over time in CLL subclones, which alter the birth/death ratios [154]. This rationale is further strengthened by the observation that the poor-prognostic chromosomal aberrations del(11q) and del(17p) seem to develop over time since they are more common in patients with late-stage disease than patients with early disease [41].

Drug Resistance in CLL

In CLL, patients usually develop drug resistance following one or more treatment cycles. Most cytotoxic drugs act through induction of apoptosis [186-189], and the deregulation of apoptosis in CLL contributes not only to the pathogenesis of the disease but also to resistance of the cells to cytotoxic drugs [190]. It may also be the underlying cause for the disease being so far incurable by cytotoxic therapies.

BCL2 Family of Proteins

As mentioned, the expression of many members of the BCL2 family of proteins is deregulated and contributes to drug resistance in CLL. A high BCL2/BAX ratio has been associated with in vitro resistance to drug-induced apoptosis [191] and an in vitro response to many chemotherapeutic agents (e.g. chlorambucil) has been correlated with BAX expression levels [192]. In addition, the BCL2 family member MCL1 is frequently overexpressed in CLL which has been correlated with failure to achieve CR after therapy [158] and in vitro resistance to fludarabine [193]. In a study by Johnston et al [194], the sensitivity of CLL cells to in vitro chlorambucil-induced apoptosis correlated inversely with MCL1 levels. Furthermore, apoptosis induced by rituximab in vitro has been associated with reduced levels of MCL1 [195].

As discussed earlier, BFL1 is an anti-apoptotic member of the BCL2 family of proteins. In CLL, BFL1 is induced by BCR crosslinking and CD40L [196-197] and is associated with CD40L mediated resistance towards fludarabine-induced apoptosis [197]. Inhibition of NF-κB enhances the effects of fludarabine on CLL cells and induces apoptosis, which is associated with a downregulation of BFL1 in addition to other anti-apoptotic genes such as BCL2L1 [198]. In a study by Morales et al, BFL1 was found to be the most discriminating gene between fludarabine sensitive and resistant CLL cases when measuring expression of genes involved in apoptosis [199].
Although efforts have been made to investigate whether individual members of the BCL2 family contribute to drug resistance in vitro, there have been conflicting results, for instance, the correlation between reduced expression of BAX and in vitro resistance to alkylating agents mentioned earlier, could not be shown for purine analogs and corticosteroids [192].

Multi-drug Resistance

Multi-drug resistance (MDR) occurs when cells become resistant to cytotoxic substances, and this condition can be present at diagnosis or may be acquired following treatment. Resistance is usually not confined to a single agent, but to groups of drugs that may be related or unrelated. The multidrug resistance 1 (MDR1) gene product P-glycoprotein (P-gp) is a membrane transporter protein that evolved as a defense mechanism against harmful substances. It transports various substrates across membranes including chemotherapeutic agents. A mechanism by which cancer cells achieve multidrug resistance includes an increased efflux of drugs, for example by P-gp [200]. CLL cells have been found to frequently express P-gp at diagnosis [201-202] and studies have shown that P-gp expression increases with advanced clinical stage and following treatment [202-203]. Interestingly, it has been possible to inhibit MDR in vitro through the use of agents that reverse the effect of P-gp, however, the doses required are not acceptable in vivo [204].

TP53 Aberrations and Drug Resistance

The tumor suppressor gene TP53 is a key regulator of cell death upon DNA damage [205]. Many chemotherapeutic drugs require functional p53 and accordingly, 17p-deleted CLL patients often respond poorly to chemotherapy [206]. 17p-deleted patients are usually resistant to alkylating agents [207] and fludarabine [208] but not to steroids [129], lenalidomide (see section below) [209] or alemtuzumab [119, 210]. The reason for drug resistance in CLL with del(17p) is due to the fact that the remaining allele is somatically mutated in the majority of cases [47, 153, 211]. Furthermore, the ATM gene involved in the p53 pathway has also been reported to be mutated and deleted in chemotherapy-resistant CLL patients [46, 212]. Moreover, it has recently been shown that a new target of TP53, miR34a is associated both with del(17p) and fludarabine refractory disease in the absence of del(17p) [213].

17p deletion is very common in the fludarabine/FC refractory group with an increase from 7% [214] of patients at diagnosis to 40-50% at the time of fludarabine-refractory disease [117, 215]. Patients who are refractory to fludarabine (no response to treatment or progression within six months after
termination of treatment) have a poor prognosis with a median survival of approximately one year [92]. Treatment options for this group of patients include the following: HDMP (particularly in patients with bulky lymphadenopathy), alemtuzumab, or rituximab in combination with fludarabine, with or without cyclophosphamide [92]. In addition, bendamustine [216], lenalidomide [209, 217] and flavopiridol [218] have proved to be effective in fludarabine-refractory CLL patients in phase II studies. However, despite fludarabine-refractory patients initially respond to therapy, this response is usually not long-lasting and these patients should at an early stage be considered for allogeneic SCT.

**Possible Therapeutic Pathways in CLL**

The molecular mechanisms that regulate drug sensitivity and apoptosis of CLL cells are complex. Identification of new drug targets whose modulation can inhibit or reverse disease progression may lead to improved OS and eventually a cure for CLL. In this section, examples of possible therapeutic targets in CLL are presented.

**NFκB**

As mentioned, NFκB is important for B-cell survival and is associated with high expression of anti-apoptotic members of the BCL2 family of proteins in CLL cells [219-220]. Several possibilities exist for inhibiting NFκB signaling. NFκB inhibitors currently in clinical trials act at different stages of the signaling pathway and include the following groups: IKK inhibitors, proteasome inhibitors blocking IκB degradation, inhibitors of nuclear translocation of NFκB subunits and suppressors of NFκB DNA binding [180]. One of the first NFκB inhibitors investigated in CLL was BAY-117082, an inhibitor of IκBα/IκBβ phosphorylation associated with activation of the mitochondrial apoptotic pathway [221]. In the study, CLL cells were shown to be sensitive to BAY-117082 regardless of prognostic markers such as CD38, ZAP70 or IGHV gene mutational status. Another NFκB inhibitor is BMS-345541, a selective inhibitor of the IKK subunits IKKα and IKKβ [222]. This compound was capable of inducing apoptosis in CLL cells irrespective of p53 status. In addition, the cytotoxic effect was selective for tumor cells and samples with high ZAP70 and CD38 expression were more sensitive to BMS-345541 than samples with low expression of ZAP70 and CD38 [223]. The proteasome inhibitor bortezomib is an NFκB inhibitor and may also be a suitable therapy for CLL [224]. However, in a phase II study where 19 fludarabine-refractory CLL patients were treated with bortezomib, no CRs or PRs could be observed [225]. Therefore, the potential therapeutic role of bortezomib in CLL needs to be further studied.
PI3K/AKT Pathway

The phosphatidylinositol 3 kinase (PI3K) mediates survival factors and protects cells from apoptosis. PI3K consists of a catalytic subunit p110 and a tightly associated regulatory subunit p85 [226]. When PI3K is activated, the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), is generated from the substrate phosphatidylinositol 3,4-biphosphate (PIP2). Akt is a protein kinase positioned downstream of PI3K that binds to PIP2 and PIP3 and becomes activated by phosphorylation through the action of PDK1[227]. Akt is involved in inhibition of apoptosis and promotion of cell proliferation [228]. Furthermore, it is an important mediator of resistance to cytotoxic agents [229]. The PI3K-AKT pathway is up-regulated in many types of tumors, including CLL cells which have constitutively activated PI3K [230]. There is an increasing amount of targeted agents that inhibit the PI3K/AKT signaling pathway and many are already in clinical trials. Inhibition of PI3K/AKT signaling with a specific PI3K-inhibitor, LY294002 resulted in increased apoptosis in CLL cell *in vitro* [230]. Given the important role of this pathway in cell survival, a specific PI3K inhibitor would be of interest in CLL treatment [231] e.g. enzastaurin that has been in a phase II clinical trial with relapsed mantle cell lymphoma patients [232].

mTOR

Since the proliferative nature of CLL has been accepted, cell cycle regulation has become an attractive target for CLL therapy [154]. Mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K/AKT signaling pathway. In mammals, mTOR regulates the translation of numerous factors e.g. eukaryotic initiating factor 4E and ribosomal p70 S6 kinase, that are important for translation of mRNA to proteins involved in cell cycle progression through G1, i.e. cyclin D3, cyclin E and cyclin A [233]. The mTOR inhibitor rapamycin was identified in the 1970’s as an anti-fungal agent [234] and was later approved as an immunosuppressant following renal transplantations. The demonstration of its anti-neoplastic properties led to the identification of rapamycin as a potential agent in cancer therapy [235]. It has been demonstrated that rapamycin blocks cell cycle progression in CLL cells by interfering with expression of cell cycle proteins such as cyclin A and survivin [236]. Rapamycin has also been shown to delay disease progression in mice with a lymphoproliferative disorder [237]. The rapamycin analogs temsirolimus and everolimus have recently been approved for the treatment of advanced renal carcinoma [238-239]. Temsirolimus is also approved for the treatment of mantle-cell lymphoma [240]. An overview of rapamycin inhibition of mTOR is presented in Figure 5.
**Figure 5.** In the presence of rapamycin the mTOR pathway is inhibited (large X), the translation of mRNAs is blocked and cells are arrested in G1 phase of the cell cycle. Modified from Ringshausen et al [241].

**Immunomodulatory Therapy**

Among the immunomodulatory drugs that have been developed, lenalidomide has received the most attention. This drug acts through a wide range of immunomodulatory activities such as the enhanced expression of cytokines e.g. interleukin-2 and reinforced natural killer (NK) cell cytotoxicity. Furthermore, it possesses pro-apoptotic activities through caspase 8 mediated cell death [242]. Lenalidomide has been approved for clinical use in relapsed and refractory multiple myeloma patients based on the results of two phase III clinical trials [243-244]. In a study to evaluate its effect in 45 relapsed/refractory CLL patients, the ORR was 45% (CR 9%, PR 38%) [217]. Another study demonstrated that lenalidomide was also effective in patients with del(11q) and del(17p) (ORR 31%) and fludarabine-refractory disease (ORR 25%) [209].

**Preclinical Development of New CLL Drugs**

The development of new anticancer drugs is mainly based on *in vitro* methodology with the principal aim being to investigate the antitumor activity of the compounds [245]. A compound that has demonstrated cytotoxic activity in human cancer cell lines will usually have its mechanisms of action, as well as its molecular targets clarified before initiation of *in vivo* models. Recently, the focus of anti-cancer therapy has been to search for molecular characteristics of the tumor that significantly contribute to the malignant transformation and maintenance of the tumor. Consequently, the first step is to identify molecular abnormalities of importance for tumor development and progression followed by pharmacological interventions targeting them. Examples of targeted agents are small molecule inhibitors such as flavopiridol and mAbs e.g. alemtuzumab, rituximab [245].
Models in Pre-clinical Drug Development

For pre-clinical *in vitro* evaluation, different types of cell systems may be used. One common approach used extensively by the National Cancer Institute (NCI) to characterize the antitumor activity pattern is the use of panels of cultured cell lines derived from all major human neoplasms [246]. Cell lines harboring deficiencies of each mechanism of DNA repair are also useful for defining the mode of action of new anticancer drugs [247-249]. However, for investigation of tumor-type specificity, the use of patient tumor samples is deemed to be preferable over the use of cell lines, since the cell lines may have lost some of their original tumor characteristics [250]. Another system commonly utilized involves the inoculation of xenograft tumor cells growing *in vitro* into immuno-deficient nude mice, thereby allowing a human tumor to grow. In general, xenografts from patient biopsies retain better morphological and molecular properties of the original tumor than cultured tumor cells. Disadvantages associated with xenografts are the high transplantation-failure rate, the labor required for transfer from mouse to mouse and the subsequently difficulty in establishing cell lines [245].

The development of new therapies for CLL has been impaired by the difficulties involved in establishing relevant human CLL cell lines and also in culturing primary tumor cells *in vitro*. In addition there has been a lack of murine models that adequately represent the human disease. An exception to this is the establishment of a transgenic mouse obtained by inserting the human *TCL1* gene model under the control of the IGHV gene promoter. This leads to the development of a disease with similarities to human CLL after 13-18 months [251]. Since the transgenic *TCL1* leukemia mouse has similar clinical and therapeutic response properties to human CLL, e.g. sensitivity to fludarabine, it may serve as an *in vivo* tool and be vital to the search for new therapies in CLL [252].

The establishment of xenograft models in CLL has been limited because of inefficient [253] or short-term [254] engraftments. Recently, in a study by Bertilaccio *et al*, the ability of Rag2\(^{-/-}\)γc\(^{-/-}\) mice, lacking B-cells, T-cells and NK-cells, to support the growth of the MEK1 [255] CLL cell line was investigated. This animal model had 100% engraftment efficiency and responded to fludarabine treatment. The MEK1-bearing Rag2\(^{-/-}\)γc\(^{-/-}\) mice therefore appear to be useful for testing new drugs and new therapeutic approaches in CLL with a relatively fast response [256]. Another xenograft model has been described where primary CLL cells were successfully transferred to NOD/SCID mice [257].
**In Vitro Cytotoxicity Assays**

Assays to measure sensitivity and resistance of cells to cytotoxic drugs *in vitro* can be divided into three main categories: clonogenic assays, cell proliferation assays and total cell kill assays. Clonogenic assays are based on the ability of cells to form colonies after two-three weeks of culture on agar plates, whereas cell proliferation assays measure the growth capacity of cells following a few days of culturing [258]. Today, total cell kill assays are the most commonly used and the work of this thesis is based on one such assay, the fluorometric microculture cytotoxicity assay (FMCA, see section below). As the name implies, these assays are based on an estimation of the proportion of cells killed in a tumor cell population following incubation. Total cell kill assays are considered short term assays (three-four days) with the main difference between the assays being how to measure the proportion of viable cells following incubation with cytotoxic drugs. FMCA measures esterase activity in cells with intact plasma membrane, whereas the methyl-thiaxol-tetrazolium (MTT) assay measures cell metabolism as a means to estimate cell viability. Clinically, *in vitro* cytotoxicity assays are especially useful for determining drug resistance [259]. The differential staining cytotoxicity (DiSC) assay is an *in vitro* drug sensitivity test designed specifically for use with fresh leukemia cells. In a predictive test for response to therapy in leukemia patients, 22 out of 119 tests indicated drug resistance and none of these patients responded to therapy [260].

FMCA is a nonclonogenic cell viability assay developed in Uppsala, Sweden, that is used to measure the cytotoxic effect of compounds *in vitro*. The possibility to use 384-well plates makes the method suitable for large-scale drug screening but also as a test for individualization of anticancer drug therapy. FMCA can be used for preclinical studies of new substances both in cell lines and primary tumor cells. In the assay, a probe, fluorescein diacetate (FDA) will enter cells containing an intact plasma membrane. In the cells, FDA will be converted by hydrolyzation through esterases to fluorescein, the fluorescent derivative of FDA. This fluorescence can be measured and linearly related to the number of living cells. It is an assay that is easy to use, has high capacity as well as the ability to detect a small number of cells [261-262]. Furthermore, the use of robotics for drug dispensing makes it feasible to test drug combinations.

When drug resistance was studied using the FMCA in prognostic subgroups divided according to IGHV gene mutational status, it was shown that UM-CLL were more chemosensitive *in vitro* than M-CLL in untreated patients, with statistically significant differences observed for both cytarabine and prednisolone [263]. Therefore, the inferior prognosis for UM-CLL does not correlate to higher drug resistance in these cases.
Preclinical Studies of Drug Combinations

Therapeutic substances are often combined to increase the efficacy of the drugs by inhibiting several disease processes in a coordinated manner [264]. It is important to perform pre-clinical experiments on drug combinations in vitro and in animals in vivo before drugs are combined for clinical trials in humans [265]. Evaluation of combination therapy usually involves determining dose-response curves of substances individually and in combination. In the commonly used median-effect method developed by Chou and Talalay [266] linearized dose response curves are used and the interaction between two drugs is expressed as a combination index (CI). Synergism is defined as CI≤0.7 and antagonism is defined as CI>1.45, whereas CI in the range of 0.8-1.45 indicates additivity. Several studies have demonstrated sequence-specific effects when investigating drug combinations [267-268]. For instance, a study by Brown et al, revealed a sequence–dependent synergy between the FLT3 inhibitor CEP-701 and chemotherapy in two ALL cell lines [269].
The main objective of this thesis was to explore new drug therapy options in CLL and to investigate the role of apoptotic genes in CLL prognosis. The specific aims of the studies were:

- To define the *in vitro* antitumor activity of rapamycin in a cell line panel as well as in patient samples from various hematological and solid tumors to suggest diagnoses for further studies. We also investigated the combined activity of rapamycin and other cytotoxic agents in primary CLL cells.

- To characterize the *in vitro* anti-leukemic activity of 20 substances in tumor cells from CLL patients belonging to different prognostic subgroups.

- To investigate the role of *BFL1* in disease progression and to study the development of chemoresistance by determining *BFL1* levels in CLL patients and correlating the levels to clinical data.

- To investigate the role of the *BCL2* -938C>A polymorphism as a prognostic marker in CLL by correlating the genotypes with clinical data and established prognostic markers.
MATERIALS AND METHODS

Patient Tumor Samples

In paper I and II, tumor samples from 19 and 40 CLL patients from the Uppsala University Hospital were included, respectively. In paper III, 37 CLL samples were obtained from Karolinska University Hospital. In paper IV, 268 CLL samples were included from cases diagnosed at the University Hospitals in Uppsala, Huddinge, Linköping, Umeå, Sweden and Tampere, Finland. Tumor samples were mainly obtained from peripheral blood, although a proportion of samples were taken from bone marrow, lymph node or spleen. Samples typically expressed CD5, CD19 and CD23 and showed a weak expression of sIG [7]. Informed consent was provided according to the Declaration of Helsinki.

For comparison purposes, normal peripheral blood mononuclear cells (PBMC) from healthy donors were included in the analysis of paper I and II (paper I n=4, paper II n=3). Both papers also studied the activity of cytotoxic drugs in other malignancies; tumor cells were isolated from acute lymphoblastic leukemia (ALL; paper I n=10, paper II n=8), acute myeloid leukemia (AML; paper I n=13, paper II n=8), CML ( paper I n=7), NHL (paper I n=11), breast cancer (paper I n=4), colon cancer (paper I n=7, paper II n=9), NSCLC (paper I n=5), ovarian cancer (paper I n=12, paper II n=4) and renal cancer (paper I n=7).

Cell Preparation

In paper I and II, leukemic cells were isolated using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Tumor cells from solid tissue were isolated by collagenase dispersion and purified using Percoll (Amersham Biosciences) gradient centrifugation. In paper III, lymphocytes were obtained following carbonyl iron treatment and Lymphoprep (Nycomed, Oslo, Norway) centrifugation and T-cells were removed by rosetting with sheep erythrocytes. All cell preparations were cryopreserved and stored either in liquid nitrogen or at -70 °C in fetal calf serum with 10% DMSO (Sigma Aldrich Co, St Louis, MO, USA). The frozen cells were thawed and washed twice immediately before use.
Cell lines
The resistance-based cell lines used in paper I included four parental cell lines, five drug resistant sub-lines and one primary resistant cell line. The cell lines represented different mechanisms of drug resistance and they were tested regularly for maintained resistance.

Drugs and Plate Preparation
For single-agent experiments (paper I and II), all drugs were tested at five concentrations using 10-fold serial dilutions. All details regarding the drugs are found in the papers. Combination experiments (paper I) were designed following the suggestions in the CalcuSyn manual (BioSoft, Cambridge UK) aiming to achieve an equipotent ratio between the drugs. Thus, nine concentrations were tested using a two-fold serial dilution. The multiple-ratio experiments in paper I and II were designed in a similar fashion but with 80 different drug combinations (eight concentrations drug A x ten concentrations drug B). For the sequence experiment in paper I, drugs were diluted to the same concentrations as in the constant ratio experiments. Drug solutions were prepared in 384-well microtitre plates (NUNC, Roskilde, Denmark) at ten times the final drug concentration and added to duplicate wells. The programmable pipetting robot BIOMek 2000 (Beckman Coulter, U.S.) transferred and serially diluted the drugs to the plates that were stored at -70°C until use.

Fluorometric Microculture Cytotoxicity Assay
In paper I and II, cell suspensions were seeded into the drug-containing microtitre plates using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc, Winooski, VT). Plates were incubated at 37 °C for 72 h and then placed in the automated Optimized Robot for Chemical Analysis (Orca, Beckman Coulter) with the software SAMI (Beckman Coulter). Culture medium and drugs were aspirated from the plates followed by two rounds of cell wash in PBS. FDA was added and following a 50-70 min incubation, the generated fluorescence was measured at 485/520 nm using a FLUOstar Optima device (BMG Labtech GmbH, Offenburg, Germany).

Cell survival following exposure to a cytotoxic drug was presented as survival index (SI; %) defined as the mean fluorescence in duplicate test wells divided by the mean fluorescence in control wells, with blank well values subtracted. A successful assay required a ratio of >5 between the signal in the control wells and the blank wells, a coefficient of variation of <30% in
the control wells, and a tumor cell fraction exceeding 70% in the cell preparation.

**FMCA Data Analysis**

IC50 values for single drugs were calculated using non-linear curve fit by the Hills Equation using GraphPad Prism (GraphPad Software Inc, San Diego, CA). For the combination experiments, the dose-response curves were analyzed by the median-effect method [266] using the CalcuSyn software (Biosoft, Cambridge, UK). The SI values for both single drugs and combinations were submitted to the program as fraction affected (100-SI%) to linearize the dose-response curves. Drug interaction was expressed as a combination index (CI) for mutually exclusive drugs. Synergism was defined as CI ≤ 0.7 and antagonism was defined as CI > 1.45, whereas CI in the range of 0.8-1.45 indicated additivity. A refined synergy analysis was performed when evaluating the results from the multiple-ratio experiments. For each dose pair, an interaction index was determined as described earlier [264], where a positive interaction index represents a survival that is lower than expected from Loewe additivity, i.e. the expected survival value. A simplified interaction analysis was performed for the sequence experiments where the effect of the combination was compared to the effect of the most effective agent, according to the Best drug ratio method.

**IGHV Gene Analysis**

In paper I-IV, PCR amplification and sequencing of IGH gene rearrangements was performed as previously described using consensus IGHV/IGHJ primers [270-271]. PCR products were either directly sequenced using the BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer, ABI, Foster City, CA) or subcloned as detailed earlier [28, 270]. The obtained sequences were submitted to the IMGT [272-273], and GenBank/IgBLAST (National Centre for Biotechnology Information, USA) databases and aligned to the most identical germline genes. Using the IGHV identity cutoff, cases were divided into unmutated (≥ 98% identity to germline) or mutated (<98% identity).

**Cytogenetic Analysis**

In paper I-III, FISH analysis was performed for del(13q), del(11q), trisomy 12 and del(17p) using commercial probes from Abbot Vysis (Stuttgart, Germany) as previously described [41]. Two-hundred interphase nuclei were
analyzed for each probe and sample. The cut-off level for samples to have a
particular aberration was 10%. Samples showing between 10-15% cells with
del(17p) or del(11q) were considered as ‘borderline’.

Protein Expression Analysis
In paper II, a western blot was performed to investigate glucocorticoid re-
ceptor (GR) levels and the downstream signaling through NFκB activation.
Western blot using a GR and an IκBα antibody was performed on whole cell
lysates obtained from peripheral blood samples including four prednisolone
sensitive CLL cases (IC50 <2 μm) and four prednisolone resistant CLL cas-
eses (IC50 >100 μm). The concentration of soluble protein was determined
using Bradford assays. Cell lysates were run on a Nu-PAGE 4-12% Bis-Tris
gel (Invitrogen) and transferred onto a membrane filter (Amersham BioS-
ciences, HybondN+). The blots were developed using an enhanced chemoi-
luminescence reagent as substrate (Amersham ECL+ western blotting detec-
tion system).

Realtime Quantitative PCR
In paper II, the expression levels of four genes related to the glucocorticoid path-
way was investigated using realtime quantitative PCR (RQ-PCR) on six
prednisolone/rolipram sensitive (IC50 <10μM) and five prednisol-
one/rolipram resistant (IC50 >100 μM) CLL cases. Total RNA was isolated
from CLL cells using the RNeasy mini kit (Qiagen, Hilden, Germany) or by
Trizol extraction (Invitrogen AB, Sweden). cDNA was synthesized using the
M-MLV RT kit (Invitrogen AB, Sweden) and RQ-PCR amplification was
performed using Stratagene Mx 3005 (AH diagnostics, Sweden). Realtime
data was calculated using the ΔΔCt method.

Competitive PCR
In paper III, BFL1 and BCL2 mRNA levels were determined by analyzing 37
CLL patients using competitive PCR. Serial dilutions of competitor frag-
ments that varied in length but utilized the same primers as the target DNA,
were PCR amplified in a single reaction along with the target DNA. The
competitor fragments used were G3PDH (Clontech, Mountain View, CA,
USA), BFL1 (Gentaur Molecular Products, Brussels, Belgium) and BCL2
(built in using composite primers and an exogenous DNA fragment BamHI-
EcoRI restriction fragment from v-erb). Densitometric analysis was per-
formed using Quantity One (BioRad, Hercules, CA, USA). Ratios of the
intensity of the PCR product pairs were plotted against the concentration of the competitor DNA in a logarithmic plot. The point of intersection, where the amounts of target and competitor DNA are equal, was used to determine the amount of cDNA in the sample.

siRNA Treatment
To investigate the effect of down-regulation of \textit{BFL1} in CLL, siRNA treatment was performed in paper III. CLL cells were transfected according to the TransIT-TKO® Transfection Reagent (Mirus Corporation, Madison, WI, USA) protocol. BFL1 specific siRNA (Dharmacon SMARTpool siRNA) and control siRNA were ordered from Dharmacon Research Inc. (Lafayette, CO, USA). At 24h and 48h after transfection of siRNA, apoptotic response was determined by Annexin V staining.

Genotyping of the BCL2-938C>A Polymorphism
In paper IV, genomic DNA was subjected to whole genome amplification and thereafter used for genotyping. Confirmation of genotype results was performed on 20 random genomic DNA samples and concordant results were obtained. Information regarding PCR and mini-sequencing primers for genotyping was obtained from the SNP assay database (http://las.perkinelmer.com/content/forms/SNPDatabase/welcome.asp). Genotyping was performed using single-base primer extension with detection by fluorescence polarization (SBE-FP) at the SNP platform, Uppsala University.

Statistical Analysis
In paper II and III, statistical differences between three or more groups were calculated using the Kruskal-Wallis test, whereas comparison between two unpaired groups was performed using Mann-Whitney U-test. In paper II, correlation analysis was performed using Pearson’s test. In paper IV, Fisher’s exact test or Chi-square ($\chi^2$) test was used to assess the significance of different genotype usage between patient groups, whereas median age at diagnosis among patients with different genotypes was compared using the Kruskal-Wallis test. Kaplan-Meier analysis was performed to calculate OS and TTT and differences in survival were evaluated using the log-rank test. OS was measured from date of diagnosis until last follow-up date or death. TTT was defined as time from date of diagnosis until date of first treatment.
RESULTS AND DISCUSSION

Paper I

Rapamycin, which originally was used as an immunosuppressant following renal transplantations, has been found to have anti-proliferative properties [274-275]. This has led to the identification of the mammalian target of rapamycin (mTOR) as a potential target for cancer treatment [276]. mTOR is involved in progression through the cell cycle and is a downstream effector of the PI3K/Akt signaling pathway, which is involved in mediating cell survival and proliferation [235]. In CLL cells, PI3K is constitutively activated and it has been reported that PI3K inhibitors can induce apoptosis in CLL cells [230, 277]. Rapamycin forms a complex with mTOR, inhibiting its kinase activity and mediating an anti-proliferative effect causing cell-cycle arrest. To further investigate the anti-tumor activity of rapamycin, a drug resistance based cell line panel as well as patient tumor samples from both solid tumors and various types of leukemias were investigated in vitro using the FMCA method.

The cytotoxic potency of rapamycin and other cytotoxic drugs was first investigated using the FMCA method in different cell lines. The cell line panel represented different mechanisms of drug resistance and included four parental cell lines, five drug resistant sub-lines and one primary resistant cell line. The activity pattern between rapamycin correlated highly with that of doxorubicin (R=0.83) and vincristine (R=0.80), with both drugs displaying sensitivity to Pgp-associated multidrug resistance. Notably, rapamycin is known to act as a Pgp substrate [278] but has also been reported to reverse resistance to other Pgp substrates [278-279].

In this study, where a total of 95 tumor samples from varying hematological and solid tumors were analyzed using the FMCA, rapamycin showed the highest cytotoxic activity in tumor cells from CLL (n=14) and ALL (n=10). This effect was significantly higher than in renal cancer (n=7) and breast cancer (n=4), where rapamycin analogs have shown clinical activity [280]. Of the CLL samples analyzed in both the single agent and the combination experiments (n=19), seven samples carried mutated IGHV genes (M-CLL) whilst twelve were UM/IGHV3-21. Regarding genomic aberrations eight samples showed del(13q) as sole aberration (including two cases with homo-
zygous del(13q)), two cases exhibited trisomy 12, one case del(11q) and five cases del(17p). There was no significant difference in rapamycin IC50 between samples from the different prognostic subgroups (M-CLL versus UM/IGHV3-21 and del(13q)/no aberration versus del(11q)/del(17p)). Although there was a tendency of higher IC50 values in the del(17p)/del(11q) group (Figure 6).

![Figure 6](image)

**Figure 6.** *In vitro* activity of rapamycin in tumor cells from CLL patients with a) no aberration/del(13q) versus del(11q)/del(17p) b) M-CLL versus UM/IGHV3-21 CLL.

Due to problems with the stability of rapamycin, three analogs with superior pharmacokinetic and biological properties (everolimus, deforolimus and temsirolimus) have been introduced into clinical trials and have displayed promising results. In phase II clinical trials, temsirolimus has shown antitumor activity in patients with advanced breast cancer [281], advanced renal cancer [282] and relapsed mantle cell lymphoma [283]. Deforolimus has also been highlighted as a candidate drug for CLL and in a phase II study, containing 51 patients (9 with CLL), produced stable disease in 19/46 evaluable patients [284]. In a phase I/II study using everolimus and 26 patients (six with CLL), one patient with myelodysplastic syndrome showed an objective response (major hematologic improvement in platelet count) and three CLL patients had a 27- 34% reduction in adenopathy after two or three cycles of therapy [285]. Everolimus has also been investigated in phase II trials in CLL patients with advanced disease [286-287] and in a study by Zent *et al*, eight out of 22 patients achieved an increased absolute lymphocyte count associated with a decrease in lymphadenopathy. This mobilization effect might be useful for combination therapy with everolimus since CLL cells within lymph nodes and bone marrow develop drug resistance more often than cells in peripheral blood [287].

Other areas of research have studied a CLL mouse model (*EμTCL1* transgenic mouse) where treatment with rapamycin prolonged the life of all animals
treated (n=5) compared to untreated animals (n=10) (100% versus 29% animals alive after three weeks of treatment) [237].

To further investigate the potential of rapamycin as a substance in treatment of CLL, rapamycin was studied in combination with a panel of other cytotoxic drugs. Vincristine, cisplatin, docetaxel and chlorambucil acted synergistically with rapamycin in 100%, 100%, 71% and 71% of seven samples analyzed, respectively. However, in combination with the standard CLL drug fludarabine, rapamycin acted antagonistically. To further evaluate the combination results, multiple concentration ratios were tested. Indeed, the combination of rapamycin/chlorambucil showed more dose combinations with positive interactions than the combination rapamycin/fludarabine. Furthermore, sequence combination experiments showed that the best effect was seen when the cytotoxic agent was added 24 h before rapamycin, which is expected from an agent inducing cell cycle arrest.

Taken together, our results warrant further clinical investigation of rapamycin as a potential therapy in CLL, especially in combination with vincristine and chlorambucil. As the aberrant activation of the AKT/PI3K/mTOR pathway is common in many hematological malignancies apart from CLL and hence, mTOR inhibitors have the potential to be effective and are being investigated in various leukemias [288].

**Paper II**

CLL is a clinically heterogeneous disease and new biological prognostic markers have been identified that divide patients into subgroups carrying varying risks for disease progression. Due to the clinical heterogeneity associated with the disease and the lack of curative treatment, it is of great importance to search for new drugs for effective treatment for CLL patients, especially those belonging to poor prognostic subgroups.

In this study, the activity of 20 substances, both clinically established (e.g. fludarabine, chlorambucil) and new drugs of potential clinical interest in CLL (e.g. PKC412, decitabine) was investigated in 40 CLL tumor samples using the FMCA. Modern prognostic markers were assessed (i.e. IGHV gene mutation status and genomic aberrations) and cases were divided into prognostic subgroups accordingly.

Patients with del(17p) demonstrate a poor response to fludarabine, alkylating agents and rituximab [44-45]. Harboring del(11q) also correlates to a lower response to purine analogs [45]. Indeed, our results were in line with this clinical experience, since in our study, chlorambucil and fludarabine dis-
played higher median IC50 in del(11q)/del(17p) (n=6) than del(13q)/no aberration (n=24) and were the only substances with a significant difference between these prognostic subgroups (Figure 7).

**Figur7. In vitro** activity of a) chlorambucil and b) fludarabine activity in tumor cells from CLL samples with no aberration/del(13q), trisomy 12 and del(11q)/del(17p).

For the majority of drugs, no significant difference in *in vitro* drug sensitivity was observed between the prognostic subgroups defined by gene IGHV gene mutational status. Interestingly, prednisolone and rolipram had significantly lower median IC50 in the UM/IGHV3-21 (n=23) subgroup compared to the M-CLL (n=16) group. This confirms previous studies that also reported that UM-CLL cases are more sensitive to prednisolone than M-CLL [263, 289]. However, the sensitivity to rolipram in UM/IGHV3-21 CLL is a novel finding. The effect of prednisolone and rolipram was also retained in some cases displaying poor prognostic genomic aberrations (Figure 8). Furthermore, both prednisolone and rolipram showed specificity for CLL cells when investigating their effect in comparison to normal lymphocytes (n=3).

**Figure 8. In vitro** activity of a) prednisolone and b) rolipram activity in tumor cells from CLL cells displaying no aberration/del(13q), trisomy 12 and del(11q)/del(17p).

To compare the CLL activity of rolipram and prednisolone with the effect in other malignancies, these agents were further investigated in ALL (n=8), AML (n=8), ovarian cancer (n=4) and colon cancer (n=5). CLL proved to be
the most sensitive cell type for both substances, which is in line with the clinical use of prednisolone in both ALL and CLL. Connections in the activity pathways between prednisolone and rolipram have been suggested [290]. Rolipram is an inhibitor of 4-cAMP-phophodiesterase (PDE4) and positive interactions between PDE4-inhibitors, such as rolipram, and glucocorticoids in primary CLL cells have been reported in vitro [291]. In our study, combination experiments with prednisolone and rolipram showed positive interactions with the drugs, with synergism in particular at lower prednisolone concentrations. Based on this, gene expression analysis on genes involved in the glucocorticoid pathway (i.e. \textit{HSPCA}, \textit{NR3C1}, \textit{PKA} and \textit{BAG1}) was performed on prednisolone/rolipram sensitive (n=6) and resistant (n=5) samples. However, there was no correlation between sensitivity to prednisolone/rolipram and expression of genes involved in the glucocorticoid pathway.

A well known glucocorticoid target is NF-κB [292], which is constitutively active in CLL cells [184, 293]. In this study, prednisolone sensitive cases displayed lower IκB-α than resistant cases, possibly resulting in a higher degree of apoptosis in sensitive cases. This is an indication that altered signaling downstream of the GR may be important for glucocorticoid resistance in CLL.

Although widely used there is little data reported on HDMP in CLL. In CLL, patients who relapse following HDMP often respond to further treatment with the same treatment [92]. Furthermore, HDMP can result in remissions in CLL patients with \textit{TP53} abnormalities [129]. HDMP has been investigated in combination with alemtuzumab [130] and rituximab [294] with promising results. No clinical data on rolipram in cancer treatment is available today, however, studies in vitro and in vivo in xenograft models suggest a therapeutic role for rolipram in the treatment of brain [295] and colon [296] cancer.

In summary, of the 20 anti-leukemic agents studied, prednisolone and rolipram showed high CLL specificity, higher activity in CLL cells with unmutated/IGHV3-21 versus mutated IGHV genes and a retained effect in some patients with 17p/11q deleted cells. These data make prednisolone and rolipram interesting candidates for further studies of treatment in CLL patients with inferior prognosis.

**Paper III**

As mentioned, although CLL patients may respond initially to treatment with alkylating agents and purine analogs, relapse and eventually chemotherapy
resistance is common. Members of the BCL2 family of proteins appear to play a role in the development of this resistance [158, 199].

In a previous study by Morales et al, gene expression analysis of apoptosis-regulating genes in indolent CLL patients with cells sensitive to fludarabine in vitro were compared to CLL cells resistant to fludarabine in vitro. Results from this study revealed that BFL1 was upregulated in fludarabine-resistant patients and it was also the strongest discriminating gene amongst the two CLL subgroups that were analyzed. [199].

In the present study, BFL1 and BCL2 mRNA levels were measured in 37 CLL patients by competitive PCR in order to evaluate the role of these apoptotic genes in disease progression and development of chemoresistance. Among the patients included in this study, BFL1 and BCL2 levels were significantly higher in the group that did not respond to therapy (n=13) compared to those who responded or did not need therapy (n=24). The strongest difference between responding and non-responding patients was seen when BFL1 and BCL2 were considered together, with high expression in at least one of the anti-apoptotic genes in the resistant group and low expression of both genes in the responding group. These results indicate that BFL1 and BCL2 expression may be good predictors of chemotherapy response. However, these results do not indicate whether high levels of BFL1 can predict chemotherapy response or if they occur as a result of treatment. High BFL1 levels could be a result of treatment since most untreated patients had low BFL1 levels and most treated patients had received many rounds of treatment, responding initially.

No correlation was found between high expression of BFL1 and progressive/advanced stage of disease or IGHV gene mutational status. In a study by Aleskog et al [263], induction of apoptosis by chemotherapeutic agents was higher in UM-CLL cases. These results suggest that the prognostic difference between M-CLL and UM-CLL cannot be explained by differences in sensitivity to apoptosis. Unexpectedly, a higher expression of BFL1 was seen in patients with del(13q)/no aberrations compared to trisomy12/del(11q)/del(17p) patients. Others have found higher BCL2 expression in CLL cells with del(13q) or normal karyotype compared to those with trisomy 12, del(11q) and del(17p) [297]. A possible explanation for this may be the fact that miR15 and miR16 (see above), located at the deleted region of chromosome 13q14.3, inversely correlate to BCL2 expression in CLL and function as negative regulators of BCL2 [162]. Further studies are needed to investigate the relationship between chromosomal aberrations and apoptosis-regulating genes in CLL.
Furthermore, samples with high \textit{BFL1} expression showed lower degree of \textit{in vitro} fludarabine-induced apoptosis than cases with low \textit{BFL1} expression. This correlation was not observed with chlorambucil, although \textit{BFL1} expression did correlate with \textit{in vivo} response to chlorambucil. These contradictory results between \textit{in vitro} and\textit{ in vivo} response to chlorambucil suggest different mechanisms for apoptosis regulation \textit{in vitro} and \textit{in vivo}.

When \textit{BFL1} was targeted with siRNA in fludarabine-resistant CLL cases with high expression of \textit{BFL1}, apoptosis was clearly induced in the transfected cells. This shows that \textit{BFL1} has a protective role against apoptosis in these cells and may be important for the prolonged survival of CLL cells. Since BCL2 family members are considered promising therapeutic options in CLL treatment and have been investigated in clinical trials [298], our results suggest that a therapeutic approach targeting \textit{BFL1} also deserves to be further explored.

**Paper IV**

As discussed, CLL is a disease with molecular alterations of apoptosis control and the anti-apoptotic \textit{BCL2} gene is over-expressed in most cases [159]. This over-expression of \textit{BCL2} has been correlated with clinical outcome since high \textit{BCL2} expression was associated with poor prognosis [299]. The \textit{BCL2} gene is located on chromosome 18q21.3 and is composed of three exons and two promoters [300]. The first promoter (P1) enhances the expression of \textit{BCL2} whereas the second promoter (P2) regulates the activity of P1 and down-regulates \textit{BCL2} expression. [301].

A SNP positioned in P2 of the \textit{BCL-2} gene (\textit{BCL-2} (-938C>A) has recently been suggested to be a prognostic marker in CLL [85]. In this study, the -938A/A genotype had increased levels of \textit{BCL2} and was correlated to shorter median OS and shorter TTT when compared to the -938AC/CC genotypes. Furthermore, the -938AA genotype was suggested as an independent prognostic marker for PFS in CLL.

In order to further investigate the \textit{BCL2}-938C>A polymorphism in CLL, we genotyped this SNP and correlated these results to clinical data in 268 CLL patients. Among the patients studied, 33% displayed the A/A genotype, whereas 46% carried the A/C genotype and 21% were homozygous for the C/C genotype. In contrast to the study by Nuckel \textit{et al}, no correlation could be observed between the -938A/A genotype and clinical outcome, OS or TTT. In addition, analysis within prognostic subgroups as defined by Binet stage or IGHV mutational status did not show any difference in OS or TTT between patients with different genotypes.
Furthermore, from our MLPA data on BCL2 mRNA expression, we could not confirm that an increase in BCL2 expression was associated with the 938A/A genotype, as reported in the previous study [85]. Therefore, based on our data, we conclude that the BCL2 938A/A genotype does not have any functional effect on the second regulatory promoter on the BCL2 gene, at least not at the transcriptional level.

We have received support for our results from another group [302] that genotyped the BCL2-938C>A polymorphism in 276 CLL patients. The data was correlated to both clinical and prognostic parameters e.g. treatment status, survival, genomic aberrations and IGHV gene mutational status. No correlations between BCL2-938C>A genotype usage and clinical outcome were found. They also performed protein expression analysis of BCL2 and correlated their results to BCL2-938C>A genotype data, however, no association could be found between the promoter SNP and BCL2 protein expression.

To conclude, we do not support the suggestion that the BCL2-938C>A polymorphism can be used as a prognostic marker in CLL.
CONCLUDING REMARKS

Since CLL is a clinically heterogeneous disease, an important advance has been the identification of molecular markers that can predict prognosis. Due to development of resistance to cytotoxic drugs in the poor prognostic subgroups it is also important to search for new treatment strategies effective in these subgroups. Defective apoptosis contributes substantially to treatment resistance in CLL and therefore, a deeper knowledge about the apoptotic pathways involved in CLL pathogenesis is desired.

In paper I-II, we have investigated the effect of several cytotoxic substances on CLL cells using the FMCA, both as single agents and in drug combinations. From paper I, we conclude that the mTOR inhibitor rapamycin has more effect in hematological malignancies than solid tumors where CLL was one of the most sensitive tumors. Furthermore, combination experiments showed a synergistic effect between rapamycin and the cytotoxic drugs chlorambucil and vincristine in CLL.

In paper II, out of 20 cytotoxic substances, the corticoid steroid prednisolone and the phosphodiesterase inhibitor rolipram were the only substances that displayed a higher effect in IGHV UM-CLL compared to M-CLL. Also, in a combination experiment with prednisolone and rolipram a synergistic effect of the substances was revealed.

In paper III we showed that BFL1 and BCL2 expression levels were higher in fludarabine resistant CLL patients. We also showed that apoptosis clearly was induced when targeting BFL1 expression with siRNA, which supports the idea that BFL1 has a protective role against apoptosis in CLL.

In paper IV, our studies on the BCL2 -938C>A polymorphism did not show any correlation between genotype and clinical outcome in CLL. These results argue against the usage of this polymorphism as a prognostic marker in CLL.

Altogether, this thesis has shown that rapamycin could be a potential drug for CLL treatment, especially in combination with chlorambucil or vincristine. Moreover, prednisolone and rolipram are interesting candidates for the treatment of poor prognostic CLL patients. It has also provided further sup-
port to the notion that *BFL1* is involved in the aberrant apoptosis and drug resistance in CLL. Finally, this thesis has underscored the importance of performing independent investigations of any potential SNP with postulated prognostic impact in large cohorts.
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