The Interplay between microenvironmental signaling and novel targeted drugs in CLL

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GENERAL INTRODUCTION
INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in western countries, is characterized by the accumulation of mature, CD5+, CD23+ monoclonal B lymphocytes in the blood, secondary lymphoid tissues, and bone marrow. There is considerable heterogeneity in the clinical course and patients’ response to therapy. Three patient groups can be distinguished based on age, comorbidities and life expectancy regardless of the diagnosis of cancer. The first group are patients <65 years of age and physically fit without or with mild comorbidities. First line treatment for this group is chemoimmunotherapy, with fludarabine, cyclophosphamide, and rituximab (FCR). The second group are treatment-naive fit patients older than 65 years and first line treatment is the combination of bendamustine and rituximab (BR). The third group are patients with multiple or severe comorbidities and this group receives chlorambucil with a CD20 antibody as first line treatment. FCR is considered the best treatment option with a long progression free survival and 1/3 of patients that might experience long lasting disease control. However, CLL remains an incurable disease and relapses are common after FCR and the alternative regimens. Eventually, CLL cells develop drug resistance which results in a very poor prognosis. At least two mechanisms are believed to contribute to the development of resistance to drugs. First, patients carrying specific chromosomal abnormalities respond poorly to chemoimmunotherapy. Such chromosomal abnormalities are deletion of 17p or 11q, which contain the genes for the tumor suppressor p53 and the DNA damage sensing kinase ATM, respectively. Second, development of drug resistance in CLL is also strongly dependent on the lymphoid microenvironment.

Key signal transduction pathways in CLL

In CLL, two compartments can be distinguished: the blood, in which quiescent CLL cells accumulate, and the lymphoid microenvironment within the lymph nodes (LN) and spleen where proliferation occurs. In the LN, CLL cells interact with non-leukemic accessory cells, such as stromal cells, monocyte-derived nurse-like cells, and CD40L expressing T cells and provide key survival signals to the CLL cells (Figure 1). Stimulation of the B cell receptor (BCR), tumor necrosis factor (TNF) family members or cytokine receptors induce multiple downstream signaling cascades that drive survival and proliferation of CLL cells. These signaling cascades lead to proliferation, adhesion and a shift in the apoptotic balance with upregulation of pro-survival proteins. Here, we discuss key signal transduction pathways in CLL that drive adhesion, proliferation and survival.

BCR signaling

The BCR is a multimeric complex composed of a surface immunoglobulin and the BCR subunit CD79. Upon binding of an antigen to the immunoglobulin, LYN, a kinase from the BCR complex, phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tail of CD79, which then recruits Spleen tyrosine kinase (SYK) (Figure 2). As a next step in this pathway, LYN and SYK transduce the signal leading to activation of extracellular signal-regulated kinases (ERK) or Bruton’s tyrosine kinase (BTK). Activated BTK activates downstream phospholipase Cγ2 (PLCγ2) through the B-cell linker
(BLNK) adaptor, resulting in downstream effector responses, including calcium signaling (Ca\(^{2+}\)), integrin and Protein kinase C (PKC) activation\(^{19,20}\). The BCR-associated co-receptor CD19 contributes to the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway\(^ {21,22}\). Mamalian target of rapamycin complex (mTORC) 1 (Raptor) is activated by AKT, which leads to phosphorylation of downstream effectors, such as 4EBP1 and S6\(^ {23}\). In addition, mTORC2 (Rictor) activation leads to phosphorylation of AKT on the serine site\(^ {24,25}\). The PI3K/AKT/mTOR pathway is important for cellular survival, metabolism and proliferation in normal B-cells\(^ {21}\). Activation of the BCR occurs in the microenvironment, where CLL cells encounter auto-antigens or environmental antigens\(^ {16,26,27}\). In addition to antigen-dependent signaling, autonomous BCR signaling due to BCR crosslinking with BCR-intrinsic motifs has been reported\(^ {28}\).

**CD40 signaling**

In the lymph node, CLL cells are in close contact with activated CD4\(^{+}\) T cells that express the CD40 ligand (CD40L) on their surface\(^ {11}\). The CLL cells themselves produce the chemokines C-C chemokine ligand (CCL) 4 and CCL22 to attract the CD4\(^{+}\) T cells and induce the CD40L-CD40 interaction\(^ {11}\). CD40 is a member of the tumor necrosis factor receptor (TNFR) family and is expressed on the membrane of CLL cells\(^ {29}\). Upon binding...
of CD40L, the CD40 molecule trimerizes on lymphocytes and recruits the adaptor protein tumor necrosis factor receptor-associated factors (TRAFs) to the cytoplasmic domain of CD40\(^\text{30}\). TRAFs activate different signaling pathways including the mitogen-activated protein kinase (MAPK) signaling, the PI3K/AKT pathway, and the Nuclear factor-kappa B (NF-\(\kappa\)B) signaling pathway \(^\text{30}\). TRAF1, 2 and 6 are important for the canonical NF-\(\kappa\)B signaling pathway and activate the transforming growth factor-\(\beta\)-activated kinase 1 (TAK1) which leads to subsequent I\(\kappa\)B kinase (IKK) phosphorylation (Figure 3) \(^\text{31,32}\). Activated IKK, consisting of IKK\(\alpha\), IKK\(\beta\), and IKK\(\gamma\), phosphorylates I\(\kappa\)B which is part of a complex with NF-\(\kappa\)B p50 and p65. Upon phosphorylation, I\(\kappa\)B is degraded leading to the translocation of the p50/p65 dimer to the nucleus where activation of target genes occurs, including the anti-apoptotic regulator Bfl-\(\text{1}\)\(^\text{31,33}\).

TRAF2 and TRAF3 are important modulators of the non-canonical NF-\(\kappa\)B pathway. Upon CD40 activation, TRAF2, TRAF3, and cIAP1/2 are recruited to the receptor, where cIAP1/2-mediated degradation of TRAF2 and TRAF3 occurs \(^\text{34}\). Without CD40 activation, NIK is degraded through the TRAF2/TRA F3/cIAP1/2 complex. Upon CD40 activation, NIK stabilizes and induces phosphorylation of the IKK\(\alpha\) homodimers and subsequent p100 phosphorylation \(^\text{35}\). Upon phosphorylation, p100 is transformed in p52 and together with RelB will translocate to the nucleus where the p52-RelB complex activates target genes, including the anti-apoptotic regulator Bcl-X\(\text{L}\) (Figure 3) \(^\text{33,35,36}\).

In vitro CD40L stimulation is used to mimic the microenvironment and shifts the balance to a more anti-apoptotic profile in PB CLL cells as seen in LN CLL cells \(^\text{14,37-39}\). CD40 triggering causes upregulation of the anti-apoptotic proteins Mcl-1, Bfl-1, and Bcl-X\(\text{L}\) and downregulation of the pro-apoptotic protein Noxa. These changes result in increased drug resistance \(^\text{40,41}\).

### Toll-like receptor 9 signaling

The innate Toll-like receptor (TLR) 9 is activated through unmethylated cytosine guanine dinucleotide (CpG) motifs in bacterial DNA and oligonucleotides. CpG-rich hypomethylated DNA motifs are a potent activator of CLL cells \(^\text{42}\). Upon TLR9 activation,
the Toll–IL-1-resistance (TIR) domain of TLR9 engages the TIR domain-containing adaptor protein myeloid differentiation marker 88 (MyD88). MyD88 contains an IL-1R-associated kinase 1 (IRAK1) domain which interacts with TRAF6. Upon activation of TRAF6, the NF-κB, p38 MAPK and JUN N-terminal kinase (JNK) pathways are activated. CpG stimulation in unmutated IgVH CLL cells results in proliferation, while CpG stimulation in mutated IgVH CLL cells results in apoptosis. An explanation for this is that the ζ-chain-associated protein (ZAP) 70 is aberrantly expressed in unmutated CLL cells and important for the TLR9-mediated activation of SYK. Very recently, it was shown that SYK activation leads to production and secretion of autoreactive IgM and this provides an anti-apoptotic signal.

**Cytokine receptor signaling**

In the microenvironment, CLL cells are in close contact with CD4+ T cells that express high levels of CXCR5. It is assumed that these T cells are T follicular helper cells (Tfh) which produce cytokines that are important for the T-B cell interaction. One major cytokine produced by Tfh cells is the class I cytokine interleukin 21 (IL-21). CLL cells upregulate the IL-21 receptor (IL-21R) after CD40L stimulation. Signaling via the IL-21 receptor complex involves activation of JAK1 and JAK3 and results in phosphorylation of STAT-1, STAT-3, and STAT-5. Our studies have shown that IL-21-mediated signaling can be found in LN samples isolated from CLL patients. In addition, CD40L and IL-21 can initiate proliferation of CLL cells in vitro. Another important cytokine involved in...
the T-B cell interaction is IL-4 produced by T cells. IL-4 receptor (IL-4R) stimulation results in JAK1 and JAK 3-mediated phosphorylation of STAT1, STAT5, and STAT6. CLL cells show increased expression of IL-4R compared with healthy B cells which correlates with increased pSTAT6 expression. In vitro IL-4 stimulation results in increased cellular survival. In CLL patients, an increased number of IL-4-producing T cells in the blood is a marker of poor prognosis.

**APOPTOSIS REGULATION**

In the CLL microenvironment, activation of key signal transduction pathways changes the balance of apoptotic regulators. The balance of apoptotic regulators in a cell is a key determinant of survival and drug resistance. Apoptosis occurs via either triggering of cell surface death receptors (i.e., the extrinsic pathway) or perturbation of mitochondria followed by cytochrome C release and caspase activation (i.e., the intrinsic pathway). Upon induction of the intrinsic apoptosis pathway, Bax and Bak form pores in the mitochondria through which cytochrome C can be released. Some pro-apoptotic BH3-only proteins can indirectly initiate apoptosis by binding to anti-apoptotic Bcl-2 proteins. These interactions prevent the anti-apoptotic proteins from inhibiting the pro-apoptotic Bax and Bak (Figure 4). Other pro-apoptotic proteins can directly induce apoptosis by binding to Bax and Bak. Most CLL patients show overexpression of the anti-apoptotic protein Bcl-2, due to deleted or silenced miR-15a and/or miR-16.1, which normally suppress Bcl-2 expression. A prominent strategy in CLL treatment is direct targeting of Bcl-2 by use of so-called BH3-mimetics. The first generation of BH3 mimetics included ABT-737/263 or navitoclax, which efficiently antagonizes Bcl-2, Bcl-XL, and Bcl-W. The clinical results were encouraging, also in terms of responsiveness in chemorefractory CLL. However, platelets also express Bcl-XL which resulted in rapid platelet death. This resulted in dose-limiting thrombocytopenia and the restriction of ABT-737 use in patients with CLL. A second generation BH3-mimetic was developed which lacked binding to Bcl-XL. This Bcl-2-directed compound ABT-199/venetoclax is highly cytotoxic for CLL cells and shows improved clinical efficacy.

**NOVEL KINASE INHIBITORS**

Targeting kinases in the key signaling pathway in CLL cells, especially in the BCR pathway, have emerged as promising treatment options. One of the drugs that emerge as a promising treatment option is ibrutinib, a selective and irreversible inhibitor of BTK. Ibrutinib has been approved by the FDA for treatment of patients with CLL associated with a poor prognosis – including those with relapsed/refractory CLL and treatment-naïve CLL with a deletion 17p or TP53 mutation. Recently, it was shown that ibrutinib induces a longer progression free survival in treatment-naïve older patients compared to chlorambucil. These data suggest ibrutinib as a promising therapeutic option for these patients with relapsed/refractory CLL.

Clinical activity of ibrutinib in CLL is attributed to attenuated retention and homing of cells to the CLL microenvironment due to impaired BCR-controlled integrin-mediated adhesion to fibronectin and CD49d/vascular cell adhesion molecule 1 (VCAM-1). Fibronectin and
VCAM-1 both play an important role in the homing of CLL cells to the microenvironment. Ibrutinib also inhibits chemokine-controlled migration. The inhibitory effects of ibrutinib on adhesion and tissue homing correlates with the clinical efficacy. Ibrutinib treatment causes a rapid reduction in the lymph node size followed by a prolonged lymphocytosis. Idelalisib is another kinase inhibitor that has emerged as a promising treatment option. This FDA-approved drug targets PI3Kδ, a kinase in the BCR pathway. Similar to ibrutinib, idelalisib abolishes both chemotaxis towards stroma and BCR-controlled integrin-mediated cell adhesion. This causes a rapid egress of leukemic cells from their protective microenvironment.

The prolonged lymphocytosis as the result from kinase-inhibitor treatment appears to have no clinical disadvantage. However, prolonged lymphocytosis could enhance the chance of patients accumulating resistant clones. Acquired resistance to ibrutinib was reported in patients with a C481S mutation in the binding pocket of BTK or activating mutations in kinases downstream of BTK. However, in patients that acquired resistance to idelalisib, no mutations were observed in the CLL cells. Resistance to idelalisib may occur via a compensatory activation of other PI3K isoforms. Because ibrutinib and idelalisib have limited direct cytotoxic effects on CLL cells, continuous treatment is needed to control the residual disease until patients relapse. The long-term clinical application of ibrutinib and idelalisib can result in toxicities that can affect the quality of life. Chronic diarrhea has been reported as a severe toxicity for idelalisib treatment and for patients on ibrutinib treatment fatigue was the major toxicity reported. Because of these toxicities, patients have to stop treatment, which can result in the outgrowth of clones that are resistant to ibrutinib or idelalisib. Another issue related to continuous treatment with ibrutinib or idelalisib is the high costs that can burden the healthcare systems. Therefore, there is still a major clinical need for development of novel more effective targeted and/or combination therapies that result in deep remission and allow for discontinuation with the drugs.
Various companies are currently developing novel inhibitors, which act either upstream (such as SYK-inhibitors) or more downstream (such as mTOR inhibitors) of the BCR and TNF-receptor family pathway. For all these novel emerging therapies, it is of utmost importance to understand the signaling pathways in CLL leading to clinical-relevant biological features in these cells, in order to understand how we can avoid the development of drug resistance, for example through combination of agents with different modes of action.

OUTLINE OF THIS THESIS

There is great interest in novel therapies and significant progress has been made in the development of targeted therapy options in CLL, in particular through the development of kinase inhibitors and BH3 mimetics. However, none of these therapies do fully eradicate the disease and resistance has been reported. In this thesis, we studied mechanisms that drive drug resistance and we investigated combination therapy of inhibitors that targeted different signaling routes in the CLL cells. By combination therapy, we hope to overcome resistance mechanisms from coming into play and eventually lead to long-term disease control and discontinuation with drugs.

In chapter 2, a pan-PI3K inhibitor was compared with the more selective PI3Kα and PI3Kδ inhibitors, on the impact of induction of apoptosis, and inhibition of cell adhesion, CD40-induced survival and proliferation in primary patient derived CLL cells. In chapter 3, we evaluated the applicability of a novel dual TORK and DNA-PK inhibitor in primary CLL samples of different prognostic risk subgroups. The potency of a dual TORK and DNA-PK inhibitor was analysed with respect to induction of cytotoxicity, and blocking of CD40-mediated chemo-resistance and proliferation. Furthermore, clinical efficacy of DNA-PK/mTOR inhibitor was tested in a clinical trial with CLL patients. As most kinase inhibitors exert an effect on the proliferation of CLL cells, we studied the mechanism underlying antigen-independent proliferation in chapter 4. We studied two distinct antigen-independent proliferation routes in CLL: CD40 + JAK/STAT pathway and the CpG + BCR pathway.

CLL cells are highly sensitive to the Bcl-2-selective BH3-mimetic ABT-199; however, in the microenvironment, pro-survival signals can upregulate Bcl-2 members. In chapter 5 we analyzed if CD40-stimulated CLL cells can become resistant to ABT-199. In chapter 6 we studied the regulation of Bcl-XL; the main regulator of drug resistance in CLL.
REFERENCES


