The Interplay between microenvironmental signaling and novel targeted drugs in CLL

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GENERAL DISCUSSION
Chronic lymphocytic leukemia (CLL) is a very heterogeneous disease. Most patients have a slow progression of the disease that needs no intervention for years, subsequently ending up in a terminal phase. In contrast, some patients have a more aggressive form of the disease, with rapid progression and chemo resistance. Despite the changes in treatment options over the past few years and the discovery of novel small molecule inhibitors, the main problem of CLL is the acquired drug resistance in response to treatment. Here, we discuss the limitations of standard but also novel treatment options, in relation to our own findings.

**Immuno-chemotherapy**

Standard first line treatment in fit patients with advanced CLL has become the combination of chemotherapy (fludarabine and cyclophosphamide) with the anti-CD20 monoclonal antibody rituximab (FCR), as the addition of rituximab to chemotherapy induced higher overall response rates (ORR) and longer overall survival (OS) rates compared to chemotherapy alone. The progression-free survival for patients receiving FCR was 6-7 years. Patients that benefited greatly from treatment with FCR were patients with del(13q) or trisomy 12, and life expectancy of these patients treated with FCR is comparable to age match healthy controls. As the FCR regimen is associated with increased toxicity with especially cytopenias and infections, this combination is less well suited for the elderly and the frail patients. These toxicities could be markedly reduced with the substitution of fludarabine and cyclophosphamide with bendamustine. However, the progression-free survival for patients receiving bendamustine with rituximab was shorter than patients receiving FCR.

In frail CLL patients, the first line treatment is the alkylating agent chlorambucil together with an anti-CD20 antibody such as rituximab, ofatumumab or obinutuzumab. Chlorambucil was compared to fludarabine in a clinical trial of patients aged 65 and older. Treatment with fludarabine had no significant difference in overall survival and more cytotoxicity was observed. In a recent pivotal study, the addition of the anti-CD20 antibody obinutuzumab or rituximab to chlorambucil was compared. Obinutuzumab achieved the longest progression-free survival and led to an improvement of overall survival. In addition, ofatumumab with chlorambucil showed clinically important improvements in treatment-naïve elderly and frail patients compared to chlorambucil alone.

Efficacy of antibody treatment in CLL has recently also been proved in the maintenance setting. Both ofatumumab maintenance following induction treatment of relapsed disease and rituximab maintenance following induction treatment in both relapsed and first line treatment resulted in a better progression free survival.

The immune-chemotherapies FCR and chlorambucil together with obinutuzumab are currently considered standard frontline treatment for CLL. However, relapses are common after these therapies and management of relapsed or refractory (R/R) CLL is challenging due to cytopenias, infections and worsening immune function due to prior therapy. In addition, the percentage of FCR refractory disease increases in the relapsed setting due to the selection of (sub)clones with high-risk genomic features. The prevalent genomic feature is the presence of a deletion of the short arm of chromosome 17 [del(17p)] or
mutation of the TP53 gene. P53 is important for genomic stability and an important regulator of the apoptotic pathway after DNA damage and its loss renders CLL patients resistant to major cytotoxic chemotherapeutic agents. High risk disease is also associated with 11q deletion, which contains the ATM gene. Ataxia telangiectasia mutated (ATM) is involved in repair of DNA damage, its deletion can cause mutations to accumulate during cell division. Furthermore, another mechanism that drives drug resistance is the CLL cell – microenvironment interaction. In the microenvironment, CLL cells receive stimuli that drive survival. Currently, there is much interest in drugs that inhibit the stimuli from the microenvironment.

**Kinase inhibitors**

The importance of the microenvironment and activation of the BCR is substantiated by the recent success of ibrutinib and idelalisib that target kinases in the BCR pathway. Ibrutinib and idelalisib treatment causes egress of the LN CLL cells into the blood stream and prevent migration to the microenvironment, resulting in rapid reduction of lymph node size and followed by prolonged lymphocytosis. Ibrutinib and idelalisib are FDA approved for treatment of R/R patients and patients with a del(17p) and/or TP53 mutation. Despite the clinical activity of the BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib, these drugs have limited direct cytotoxic effects on CLL cells, which poses the potential risk of remaining clones that develop mutations within the BCR pathway causing drug resistance. Especially patients with dysfunctional TP53 or ATM seem to be more prone to develop resistance to these drugs, probably due to increased genomic instability.

Although ibrutinib has been shown to be effective irrespective of p53 status of cells and in patients with del(17p), many of these patients with del(17p) eventually relapse with ibrutinib-refractory disease. It was reported that 26% and 24.1% of patients had to discontinue ibrutinib treatment. Of the patients that had to discontinue therapy due to progressive disease, 4% of patients progressed with CLL, all due to mutations in the BTK binding site of ibrutinib or gain-of-function mutations in the downstream kinase PLCγ2. 6% of patients had to cease ibrutinib treatment due to Richter's transformation, which was associated with prior therapies, Bcl6 abnormalities, MYC abnormalities, presence of del(17p) and complex karyotype. Of the patients who discontinued ibrutinib therapy other than through relapse, 9% of patients had infections, which may be associated with the inhibition of T cells by ibrutinib. Surprisingly, in a trial with treatment naïve patients, 20% of patients had to discontinue ibrutinib treatment, which was correlated with mutated IgVH status. Patients that had to discontinue ibrutinib treatment had very poor outcome with only a 3 month overall survival.

Besides relapsed disease, the main reason for idelalisib discontinuation is toxicity. In a trial with treatment naïve patients, 40% had to discontinue treatment due to severe adverse events, such as colitis and pneumonitis. The mechanism for this increased toxicity is not well characterized. However, increase of T cell infiltrates was observed in the colitis from patients. A trial with idelalisib and the anti-CD20 antibody ofatumumab in treatment naïve patients showed an increase in immune-mediated hepatotoxicity, due to a decrease of regulatory T cells. We demonstrated that idelalisib inhibited proliferation of CD4+ T cells.
cells (Chapter 2). These data suggest that idelalisib blocks the proliferation of regulatory T cells, which leads to immune-mediated infections. A trial with idelalisib and the SYK kinase inhibitor entospletnib in refractory/relapsed patients was recently stopped due to excess deaths from infectious diseases. Patients with pneumonitis showed a Th1-type response in the serum. Th1-type response is associated with non-infectious pneumonitis. In contrast, we demonstrated that PI3Kδ inhibition reduces cytokine production by T cells. Cytokine production plays an important role in the protection against infections, which may suggest that the increase of infections after idelalisib treatment is related to the decrease of cytokine production by T cells.

The mechanism that drives idelalisib resistance is unknown. Idelalisib only targets the PI3Kδ isoform and acquired resistance to idelalisib might arise via a compensatory activation of other PI3K isoforms. In mantle cell lymphoma (MCL) cell lines and in patient samples, relapse occurred during idelalisib treatment by increased PI3Kα expression. In chapter 2, we demonstrated that the PI3Kα isoform is active in CLL cells as shown by treatment with a PI3Kα inhibitor, which results in partial inhibition of proliferation and adhesion. Increased expression of the PI3Kα isoform might thus be a cause for idelalisib resistance.

Venetoclax

With the development of resistance to ibrutinib and idelalisib, more treatment options for high risk patients are needed. Another treatment option is the FDA approved Bcl-2 antagonist venetoclax (ABT-199) for patients with del(17p). Venetoclax showed clinical efficacy with reduced peripheral blood cell counts and diminished lymph nodes size early after treatment. Venetoclax had an overall response rate of 79% in refractory relapsed CLL patients. However, complete remission (CR) was only reached in 16% or 8% of patients. The low percentage of complete remission could be caused by microenvironment-induced resistance. In the CLL lymph node microenvironment, upregulation of Bcl-XL, Mcl-1 and Bfl-1 can clearly be observed. Since these Bcl-2 family members are not targeted by venetoclax, it is reasonable to assume that long term application might, analogous to the situation with the kinase inhibitors, induce selection of clones or niches with decreased sensitivity for this compound. Indeed, in vitro stimulation of CLL cells with CD40L, which upregulates Bcl-XL, Mcl-1 and Bfl-1, induces resistance to venetoclax (Chapter 5). Therefore, combination therapy is needed that prevents the microenvironment-induced venetoclax resistance from coming into play, and thus may lead to long-term disease control.

NEW TREATMENT APPROACHES

Ibrutinib or idelalisib do not block the signal transduction pathway important for survival, which poses the potential risk of remaining resistant clones. Therefore, blocking multiple signaling pathways that control clinically relevant biological features may prevent selection of resistant clones, and fully eradicate CLL. Here, we discuss several options for blocking multiple signaling pathways, from the perspective of our results described in previous chapters.
Targeting multiple kinases

In the microenvironment, the PI3K/AKT/mTOR pathway is activated upon BCR, CD40, or chemokine and integrin receptor activation\cite{42,44}. PI3K consists of 4 isoforms \(\alpha\), \(\beta\), \(\delta\) and \(\gamma\) and is important for survival, chemotaxis and cell growth\cite{45}. Targeting all the PI3K isoforms by pan-PI3K inhibitor SAR245409 inhibited survival, adhesion and proliferation (Chapter 2). However, we demonstrated that a pan-PI3k inhibits CD4 and CD8 T cells proliferation and cytokine production to a greater extent than idelalisib\cite{33}. This observation could have negative therapeutic effects by lowering the prosurvival effects of T cells in CLL and it could lead to severe toxicities when given continuously, and pan-PI3K inhibition is therefore maybe not a good treatment option for CLL patients.

MTOR is the main downstream kinase of the PI3K/AKT pathway and contributes to survival and proliferation in CLL. In particular, mTORC1 is important for proliferation by regulating translation of proteins critical for progression from G\(_1\) into S phase and mTORC2 is important for survival by activation of AKT\cite{46,47}. DNA-PK is a kinase important for the DNA damage repair pathway and contributes to chemo-resistance\cite{48}. Targeting both mTOR and DNA-PK by the novel compound CC-115 inhibited survival and adhesion and completely blocked proliferation (Chapter 3). Even though CC-115 induced cytotoxicity, the induced cytotoxicity by itself is modest compared to a chemotherapeutic agent. In addition, blocking DNA-PK would be more beneficial in the case of double stranded breaks. Therefore, combining CC-115 with a chemotherapeutic agent may be of interest.

CC-115 with a chemotherapeutic agent

TP53- and/or ATM-defective CLL cells are more sensitive to ATR inhibition\cite{49} and have been reported to be protected from chemotherapy by DNA-PK overexpression\cite{50,53}. As peripheral blood CLL cells are in cell cycle arrest\cite{54}, it is likely that DNA repair in CLL cells predominantly depends on DNA-PK by non-homologous end joining (NHEJ). Targeting DNA-PK with small molecule inhibitors in CLL after irradiation or chemotherapy restores sensitivity\cite{55-57}. The clinical efficacy of the DNA-PK/mTOR inhibitor was tested in eight patients with relapsed/refractory CLL/SLL harboring ATM deletions/mutations (Chapter 3)\cite{58}. Although all but one patients responded, only 3 patients showed >50% reduction in lymphadenopathy. Interestingly, these patients have a bi-allelic ATM mutation, suggesting that these patients depend more on DNA-PK for DDR and are more sensitive to DNA-PK inhibition. It would therefore be of interest to combine CC-115 with a chemotherapeutic agent that causes DSBs. As is shown by in vitro data, DNA-PK/mTOR inhibition together with chemotherapy was synthetically lethal to cells\cite{58}. However, chemotherapeutics are associated with increased toxicity in patients. Inducing cytotoxicity with a compound that is more selective for CLL cells such as venetoclax will be a good strategy. We demonstrated that CC-115 will revert CD40L-induced venetoclax resistance by blocking the induction of Mcl-1, Bcl-XL and Bfl-1 (Figure 1)\cite{58}. CC-115 was also active in CLL cells obtained from idelalisib resistant patients. Furthermore, we observed that CC-115 inhibits integrin-mediated adhesion of CLL cells (data not shown). In addition to downregulation of prosurvival signals in the CLL cells, CC-115 will thus also induce egress of LN CLL cells into the bloodstream where they become susceptible to attack by venetoclax. Taken together,
the combination of venetoclax with CC-115 may be a good strategy for ibrutinib and idelalisib resistant patients.

**Venetoclax with an anti-CD20 antibody**

Other therapies combined with venetoclax may also fully eradicate the disease. Anti-CD20 monoclonal antibodies display increased capacity to induce non-apoptotic cell death in CD40L-stimulated CLL cells, and this counteracts resistance to fludarabine\(^{59,60}\). CLL cells in the blood are not sensitive to anti-CD20 mAbs killing, but readily undergo apoptosis by venetoclax. Conversely, CLL cells in the microenvironment are resistant to venetoclax but are sensitive to crosslinked rituximab or GA101 (obinutuzumab) (Figure 1). We demonstrated that resistance to venetoclax could at least partially be counteracted by anti-CD20 antibodies (Chapter 5)\(^{61}\). This combination has now entered clinical trials. In the first clinical trial of venetoclax with rituximab, 41% of patients achieved CR and CR with incomplete bone marrow recovery (CRi)\(^{62}\). Venetoclax with rituximab is now compared to bendamustine with rituximab in a phase III clinical trial.

**Figure 1.** Schematic representation of combined action of BH3 mimetics and kinase inhibitors/CD20 antibody or lenalidomide. Ibrutinib, SAR409 or CC-115 will induce release of lymph node CLL cells into the blood (upper right) where they become susceptible to rapid elimination by venetoclax. CLL cells in blood are prevented from (re-)entering lymph nodes. In the blood CLL cells are sensitive for venetoclax and in the LN CD40L stimulation will sensitize CLL cells to cell death by anti-CD20 mAbs. Dasatinib and CC-115 inhibits protective signals of the LN microenvironment and sensitize CLL cells to venetoclax in situ (bottom left). A non-canonical NF-κB inhibitor blocks the upregulation of the anti-apoptotic protein Bcl-XL, preventing resistance mechanisms from coming into play and sensitize CLL to venetoclax. Lenalidomide will induce IL-21 production by T cells. IL-21 stimulation downregulates Bcl-XL and reverts venetoclax resistance.
Venetoclax with kinase inhibitors

Another treatment strategy to obtain long-term and complete remission in vivo could be the combination of other kinase inhibitors with venetoclax. In chapter 5, we demonstrated that the BTK and ABL inhibitor dasatinib blocks the induction of Mcl-1, Bcl-XL or Bfl-1 by microenvironmental signals, rendering the CLL cells more sensitive to venetoclax in the LN (Figure 1). Since dasatinib also is a BTK inhibitor it will induce release of the lymph node CLL cells into the bloodstream where they become susceptible to attack by venetoclax. However, the clinical efficacy of dasatinib in combination with fludarabine was modest with an overall response rate of only 18% in refractory CLL patients. Combination of venetoclax with ibrutinib or idelalisib will be a better treatment strategy, since these kinase inhibitors achieved a higher overall response rate as monotherapy. Ibrutinib and Idelalisib both inhibit adhesion of CLL cells in the microenvironment and homing to the LN. This will lead to the release of CLL cells in the blood where they become susceptible to rapid elimination by venetoclax (Figure 1). The combination of ibrutinib or idelalisib with venetoclax is expected to work for patients with a del(17p) and/or TP53 mutation, but for ibrutinib or idelalisib resistant patients other combinations are needed. In chapter 4, we showed that JAK inhibition blocks CD40L/IL-21 induced BCR-independent proliferation, while SYK inhibition blocks CpG/CD40L induced proliferation. The interplay between TLR triggering and BCR signaling can induce proliferation. CpG activates SYK and induces the production and secretion of autoreactive IgM and this together with TLR triggering leads to proliferation. Combination of a JAK or SYK inhibitor together with venetoclax will be a good strategy, venetoclax will induce cytotoxicity and a JAK or SYK inhibitor will prevent accumulation of the CLL cells.

Venetoclax with lenalidomide

We showed that the anti-apoptotic protein Bcl-XL is the main regulator that drives drug resistance in CLL cells, including resistance to venetoclax (Chapter 6). Knock down of Bcl-XL in CD40L-stimulated CLL cells increases susceptibility to most drugs. So targeting Bcl-XL will prevent resistance mechanisms from coming into play. Previously it was shown that Bcl-XL expression correlates with the activation of the non-canonical NF-κB pathway. We demonstrated that knock down of non-canonical NF-κB NIK inhibits Bcl-XL expression (Chapter 6). Therefore, it would be interesting to combine a non-canonical NF-κB inhibitor together with venetoclax (Figure 1).

An endogenous signaling pathway that decreases Bcl-XL expression is the IL-21R pathway. IL-21 stimulation decreases Bcl-XL transcription via the JAK/STAT3 pathway which makes CD40L-stimulated CLL cells more vulnerable to venetoclax treatment. IL-21 stimulation was shown to promote apoptosis in CLL cells. In a phase I trial, recombinant IL-21 together with anti-CD20 antibody rituximab was tested and the ORR was 42%. However, the anti-CD20 antibody obinutuzumab alone achieved an ORR rate of 62%, so it cannot be stated that the achieved responses are due to the addition of recombinant IL-21 to the treatment of an anti-CD20 antibody. The addition of IL-21 to other therapies was not further explored, probably because IL-21 also promotes proliferation in CLL cells. The immunomodulatory agent lenalidomide targets the microenvironment of CLL. Despite the mild induction of direct apoptosis in CLL cells by lenalidomide, it decreases
the survival of CLL cells by interfering with microenvironmental signals\textsuperscript{72,73}. Lenalidomide activates NK and T cells and enhances NK and T cell-mediated anti-tumor activity\textsuperscript{74-77}. Furthermore, lenalidomide reduces proliferation of CLL cells by upregulation of p21\textsuperscript{78}. Recently, it was demonstrated that lenalidomide induces the production of IL-21 by T cells and upregulates the IL-21R on CLL cells\textsuperscript{79}. It would therefore be interesting to combine lenalidomide with venetoclax as lenalidomide will induce the production of IL-21 by T cells, which in turn will downregulate Bcl-XL expression in CLL cells and enhance the sensitivity to venetoclax of CLL cells in the microenvironment (Figure 1).

\section*{CONCLUSION}

CLL remains an incurable disease due to the acquired resistance in response to immuno-chemotherapy or small molecule inhibitors. Immuno-chemotherapy and venetoclax induce cytotoxicity, while microenvironmental survival signals are not blocked. BCR kinase inhibitors cause egress of CLL cells from the microenvironment into the blood stream, while these inhibitors do not induce cytotoxicity. Therefore, combination therapy is needed to completely block all survival signals and to fully eradicate the disease. Venetoclax induces CLL cell specific cytotoxicity and is therefore less toxic than chemotherapy. Combination therapy of venetoclax with a drug that prevents the microenvironmental survival mechanisms could be an effective treatment. Our data showed that the combination of venetoclax and an anti-CD20 antibody, lenalidomide or CC-115 may be a good strategy. Venetoclax will target the cells in the blood and an anti-CD20 antibody will target the CLL cells in the microenvironment. Lenalidomide shifts the balance to a less tumor supportive microenvironment and induces sensitivity to venetoclax. CC-115 inhibits proliferation and adhesion of CLL cells in the microenvironment. Furthermore, CC-115 inhibits the upregulation of Bcl-XL and sensitizes CLL cells to venetoclax. Further clinical evaluation of these combination therapies seems warranted to demonstrate which combination therapy is curative and if patients with different molecular and biological characteristics benefit from different combination therapies.
REFERENCES


