Targets for the treatment of drug resistant chronic lymphocytic leukemia

Tonino, S.H.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Summary and general discussion
Summary

In this thesis, drug resistance in CLL is addressed. In the first part, interactions between CLL cells and the microenvironment are studied in order to identify novel targets for the treatment of (chemorefractory) CLL. The second part focuses on the exploration of p53 independent mechanisms of cell death.

One of the major determinants of drug resistance in CLL is the decreased propensity to apoptosis of CLL cells residing in the secondary lymphoid tissue and bone marrow. In the current view, the microenvironment in these niches provides a proliferative drive as well as pro-survival signaling. Activated CD4+ T cells are thought to play a key role in these interactions. Numbers of both CD4+ and CD8+ T cells are increased in CLL, but the mechanisms underlying the altered T cell homeostasis are still ill-defined. In chapter 2 the relation between CLL and the cognate T cell compartment is investigated.

We have previously found an expansion of CMV specific CD8+ effector type T cells in patients with CLL. It was unknown whether these changes are specific for CLL. We have found that in patients with CLL or other indolent B cell lymphomas (in which extensive interactions between malignant B cells and T cells are to be expected), both effector CD4+ and CD8+ T cell numbers are increased. In contrast, in patients with aggressive lymphoma and myeloma such changes were not found. On these effector T cells, we found decreased expression of PD-1, an activation-associated inhibitory member of the CD28/CTLA-4 family. In the presence of malignant B cells, upregulation of PD-1 upon T cell activation was impaired; suggesting that the abundance of B cells in patients with indolent B cell lymphoma and CLL affects PD-1 expression and the size of the effector T cell pool. These data emphasize the reciprocity of the interactions between CLL cells and the immune system and support the rationale for treatment strategies targeting these interactions.

The functional results of the interactions with the microenvironment are further studied in chapter 3. More specifically, in this chapter we studied whether interactions with the microenvironment play a role in the difference in biological behaviour (and prognosis) of IgVH mutated versus unmutated CLL. In an attempt to accurately mimic the lymph node (LN) environment, CD40 stimulation was combined with Toll-like receptor 9 (TLR-9) triggering by the oligonucleotide CpG-ODN. Natural ligands for TLR-9 are CpG motifs present in unmethylated viral and bacterial DNA, but also endogenous ligands released during cellular stress. We have found that prolonged CD40 ligation induced classical NF-κB activation followed by alternative NF-κB activation, correlating with enhanced Bfl-1/A1 and Bcl-xL levels, respectively. Upon combined CD40/TLR-9 triggering a dichotomy in NF-κB signaling could be discerned. In mutated cells this induced declining levels of p52 (a mediator of the alternative NF-κB pathway) and Bcl-xL, and reversal of chemoresistance. In contrast, unmutated cells proliferated, maintained p52 and Bcl-xL expression and remained chemoresistant. In ex vivo LN samples, p52,
p65 and Bcl-xL were highly expressed, corroborating the *in vitro* findings. These data uncover a distinction in NF-κB activation and drug susceptibility between mutated versus unmutated CLL, possibly accounting for the difference in biological behaviour between these subgroups of CLL.

In **chapter 4** we analyzed the role of CD31-CD38 interactions in the pathogenesis of CLL. Interactions of CD38 expressed on CLL cells with CD31 expressed on nurse-like cells have been proposed to result in anti-apoptotic and proliferative signaling in CLL. CD38 is primarily known as an ecto-enzyme and high expression correlates with a poor prognosis in CLL. We found a high expression of CD31 on CLL cells, irrespective of CD38 expression. However, in contrast to published reports, co-culture of CD38\textsuperscript{high} CLL with endothelial cells or CD31 transfected fibroblasts did not result in increased survival or proliferation. Analysis of gene expression of most known regulators of apoptosis revealed no influence of co-culture with CD31-expressing feeder cells. Hence, we have found no evidence for an important contribution of CD38 triggering by CD31 to the proliferative, and anti-apoptotic, state of the leukemic clone.

In the second part of the thesis we focus on therapeutic strategies to overcome drug resistance due to p53 dysfunction. As platinum-based compounds are effective in relapsed lymphoma and also high-risk CLL, we first investigated the efficacy of a cisplatinum (CDDP) containing regimen in patients with chemo-refractory CLL. As described in **chapter 5**, we have found marked activity in 8 of 10 patients treated with the R-DHAP regimen, importantly also in patients with bulky lymphadenopathy. These results prompted us to further investigate the mechanism of action of platinum-based compounds in CLL *in vitro*. In **chapter 6** we studied whether the activity of CDDP in CLL is mediated by the p53 family member TAp73, as has been demonstrated in various solid cancer types. Increased expression of TAp73 and its downstream targets was indeed seen in PB blood derived cells from a patient with p53 dysfunctional CLL who had been treated with CDDP *in vivo*. Further studies on the role and regulation of TAp73 in the p53 dysfunctional pro-lymphocytic B cell line MEC1 revealed c-Abl dependent upregulation of TAp73 upon *in vitro* treatment with CDDP, correlating with functional effects. Although *in vitro* treatment of peripheral blood derived CLL cells with CDDP did not result in increased TAp73 expression levels, a clear induction of TAp73 was seen after stimulation of CLL cells with CD40-ligand. Also, increased expression levels of TAp73 were found in LN derived CLL cells, corroborating earlier findings that CLL cells residing in the lymphoid tissue display a distinct phenotype resulting from interactions with the microenvironment. The replicative state of the clone may play a role in this difference. We expect that the effects of CDDP in chemo-refractory CLL *in vivo* might in part be mediated by induction of TAp73.

Although CDDP did not induce apoptosis (nor resulted in upregulation of TAp73) when used as single agent, we found synergy *in vitro* between CDDP and fludarabine (F-ara-A) in CLL cells, irrespective of p53 functional status. Resistance resulting from
CD40 ligation was also overcome. As described in chapter 7, the response to treatment with this combination involved generation of reactive oxygen species (ROS), which lead to specific, p53 independent, upregulation of the pro-apoptotic BH3-only protein Noxa. ROS accumulation resulted in Noxa upregulation mainly at the transcriptional level and this was at least partly mediated by the mitogen-activated protein kinase (MAPK) p38. Noxa RNA-interference markedly decreased sensitivity to CDDP/F-ara-A, supporting a key role for Noxa as mediator between ROS-signaling and apoptosis induction. These data indicate that interference in the cellular redox-balance can be exploited to overcome chemoresistance in CLL. Finally, in chapter 8, guidelines are presented for the optimal treatment of both relapsed and drug-refractory CLL. Furthermore, some promising compounds still in experimental phase are discussed.
General discussion

In recent years considerable advances have been made in upfront treatment strategies for CLL. At present, the majority of patients will obtain long-lasting responses following combined immuno-chemotherapy. However, patients are not cured and relapse of the disease is inevitable.

A major obstacle in the management of CLL is (the development of) drug resistance. Several mechanisms contribute to drug resistance in CLL. In this thesis (1) interactions of CLL cells with the microenvironment and (2) cytogenetic alterations, especially those affecting the p53 response, are addressed.

1. Drug resistance through interactions with the microenvironment

CLL cells accumulate in vivo, but rapidly undergo spontaneous apoptosis in vitro, indicating that these cells are highly dependent upon external signals for their survival.

Central to the pathogenesis of CLL is signaling through the B cell receptor (BCR), mediated by yet unidentified (auto)antigens. Additional signaling pathways are activated upon interactions of CLL cells with the microenvironment, either through the secretion of mediators or by direct cell-cell contact.

1.1 Targeting cellular interactions

Although multiple cell types have been implied to interact with CLL cells, the precise nature of such interactions and the individual contributions to the pathogenesis of CLL, are ill-defined. In the bone marrow CLL cells mainly interact with mesenchymal stromal cells (MSCs), whereas in secondary lymphoid tissue CLL cells interact with nurse-like cells (NLCs), T cells and follicular dendritic cells. Interactions of CLL cells with NLCs and T cells have been studied most extensively.

In vitro, NLCs enhance viability of CLL cells via the secretion of stromal-derived factor-1 (SDF-1)/CXCL12, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL). Although recently interactions of CD38 expressed on CLL cells with CD31 expressed on NLCs were described to result in proliferation and pro-survival signaling, our data do not support a role for such interactions.

In the lymphoid tissue, CLL cells interact with activated CD4+ cells, mainly via CD40-CD40-ligand interactions (see Introduction). However, in addition to T cell induced anti-apoptotic signaling in CLL cells, reciprocal interactions between CLL cells and T cells are expected, as CLL patients display increased susceptibility to auto-immune phenomena and opportunistic infections. The relevance of such reciprocal interactions is supported by recent studies in T cells of untreated CLL patients, and patients with follicular lymphoma in leukemic phase, in which altered expression was found of genes, mainly involved in cell differentiation in CD4+ cells, and in cytoskeleton formation, vesicle trafficking, and cytotoxicity in CD8+ cells. Direct contact of both patient-derived and healthy control T cells with CLL cells resulted in defective immunological synapse formation.
In line with these findings, we found impaired upregulation of the inhibitory activation associated protein PD-1 on T cells activated in the presence of (malignant) B cells, possibly accounting for the increased numbers of effector T cells found in CLL.

Interactions of CLL cells with the microenvironment are not targeted by current treatment strategies. Although the mechanism of action of lenalidomide is not completely understood, this drug may exert its action through modulation of the microenvironment, possibly in part through co-stimulation of T cells. Defective synapse formation between CLL cells and T cells was reversed by lenalidomide. As single agent, lenalidomide is active in CLL. Currently, trials are conducted to study synergistic effects of lenalidomide with immuno-chemotherapy: in Germany in combination with bendamustine and rituximab in fit patients; in the Netherlands in combination with chlorambucil and rituximab in elderly/unfit patients.

1.2 Targeting intracellular signaling pathways

Binding of a ligand to its receptor results in activation of protein tyrosine kinases (PTKs). PTKs and downstream signaling pathways are commonly deregulated in malignant cells. In CLL, the PTKs Lyn and Syk (downstream targets of BCR triggering) and c-Abl are over-expressed. Consequently, also several pathways downstream of these PTKs are found to be constitutively active in CLL, including the PI-3/Akt pathway and the NFκ-B pathway. Selective inhibition of these pathways in CLL cells in vitro resulted in apoptosis of CLL cells, supporting their role in the pathogenesis of CLL.

In addition to constitutive activity, ligand-receptor interactions may further enhance signaling via these pathways. For example, we have previously shown that drug resistance upon CD40-ligand stimulation could be partly inhibited by the c-Abl specific inhibitor imatinib and completely blocked by the broad PTK inhibitor dasatinib. Specifically targeting these pathways may provide a more effective and better tolerable treatment strategy than conventional chemotherapy. Several inhibitors of PTKs and downstream targets are currently in various stages of clinical testing, including the Syk kinase inhibitor fostamatinib, the PI-3-kinase inhibitor CAL-101, the Btk inhibitor PCI-32765, imatinib and dasatinib. One potential drawback for these targeted drugs is the existence of redundancy between downstream pathways. This might be overcome by the use of dual or triple pathway inhibitors. One such compound, ARD12130 (Sanofi-Aventis) which inhibits both PI3-kinase and MTOR, will be tested in relapsed and refractory CLL within our institute.

The difference in biological behaviour and prognosis between mutated and unmutated CLL has long been thought to result from increased BCR signaling in unmutated CLL, mediated by the PTK ZAP-70 by a not yet completely understood mechanism. Unmutated CLL B cells are more dependent on environmental pro-survival signals than mutated cells, as they are more prone to spontaneous apoptosis in vitro. It has been speculated that enhanced NF-κB signaling in ZAP-70⁺ (unmutated) CLL accounts for this difference. Several ligand-receptor interactions have been found to mediate...
NFκ-B signaling, including CD40-CD40-ligand \(^{24}\), BAFF and APRIL and their various receptors \(^{25}\) as well as CpG-TLR-9 \(^{26}\). Indeed, we here uncover a dichotomy in NF-κB signaling between unmutated and mutated cells, independent of ZAP-70, upon combined CD40/TLR-9 triggering, which adds to our understanding of the difference in clinical behaviour and response to treatment between these two subgroups.

The result of aberrant signaling in CLL is apoptosis resistance through overexpression of anti-apoptotic Bcl-2 family members, such as Bcl-xL, Bcl-2, Mcl-1 and Bfl/A-1. Several drugs directly targeting these anti-apoptotic proteins are currently being investigated, including the Bcl-2 antisense molecule oblimersen \(^{27}\), the BH3-mimetic ABT-263 \(^{28}\), obatoclax, which inhibits binding of Bcl-2 family members to Bax/Bak \(^{29}\), and flavopiridol, a cyclin-dependent kinase (CDK)-inhibitor, which induces caspase 3-mediated apoptosis via a decrease in expression levels of Mcl-1 and XIAP \(^{30,31}\).

2. Strategies to overcome drug resistance; clues from clinical observations

If drug resistance develops, the prognosis rapidly worsens. Appearance of del11q and especially del17p are highly predictive for unresponsiveness to subsequent (immuno-) chemotherapy \(^{32}\). Fludarabine refractory disease infers a very poor prognosis with a median life expectancy of less than one year \(^{33,34}\). The only means by which durable remissions in patients with chemo-refractory CLL can be achieved is reduced intensity allogeneic hematopoietic stem cell transplantation (RIST). However, the long-term outcome after RIST is highly dependent upon remission status prior to transplantation as patients with high disease burden, especially bulky lymphadenopathy at time of transplantation, or poor response to last treatment, have the tendency to relapse. In addition, although morbidity and mortality have decreased with the introduction of reduced intensity (non-myelo-ablative) conditioning regimens \(^{35,36}\), transplantation is often not feasible in the elderly CLL patient with significant co-morbidity.

2.1 Treatment options in refractory CLL

At present, two drugs are registered for the treatment of fludarabine refractory CLL: the anti-CD52 monoclonal antibody alemtuzumab and the anti-CD20 monoclonal antibody ofatumumab. In phase II trials, alemtuzumab has shown modest response rates in patients with refractory CLL \(^{37,38}\). However, the use of alemtuzumab is hampered by the high incidence of opportunistic infections. Furthermore, this drug is less active in patients with bulky disease \(^{39}\). Ofatumumab has shown activity in patients with CLL refractory to both fludarabine and alemtuzumab and in patients with fludarabine-refractory bulky disease, but activity appears to be rather low in patients with del17p \(^{40}\). Responses have also been obtained following treatment with the CDK-inhibitor flavopiridol \(^{41}\) and lenalidomide \(^{8}\).

We found activity of a regimen containing cisplatin (CDDP) in this category of patients, as a response was achieved in 8 of 10 patients. Moreover, 6 of these 8 responding patients could proceed to allogeneic stem cell transplantation. These
findings are in line with results of a previous study, in which 2 courses of DHAP followed by alemtuzumab were used as conditioning regimen prior to autologous transplantation in chemo-refractory CLL 42. In another small phase I-II trial, the OFAR-regimen (oxaliplatin, F-ara-A, cytarabine, and rituximab) induced a response in 7 of 20 patients with documented del17p 43.

2.2 The TAp73 pathway may circumvent p53 dysfunction

The major challenge in the development of treatment strategies for drug resistant CLL is the identification of p53 independent apoptosis pathways. A candidate may be the TAp73 pathway. The role of TAp73 in CLL has not yet been fully elucidated, but functional relevance of this protein in apoptosis regulation and response to drug treatment in CLL has been described 44. Recently the activity of some novel drugs for the treatment of CLL has been described to involve upregulation of TAp73, including HDAC-inhibitors 45, lenalidomide 7 and the purine nucleoside phosphorylase forodesine 46.

The activity of platinum-based compounds is mediated by TAp73 in various solid tumour types 47-49. We found that the response to CDDP treatment in the p53 dysfunctional cell line MEC1 was mediated by TAp73. Also, TAp73 expression was found both in CLL cells exposed to CDDP treatment in vivo and in LN derived CLL cells, indicating that the activity of CDDP in chemo-refractory CLL might at least in part be mediated by induction of TAp73 (Figure 1). Further exploring the role and regulation of TAp73 in CLL may provide clues for the development of novel treatment strategies for p53 dysfunctional and drug refractory CLL.

2.3 Redox balance, the Achilles’ heel of CLL?

Studies in various cancer types, including CLL, have shown that malignant cells are under increased oxidative stress due to enhanced ROS formation 50;51. The relation between ROS and the pathophysiology of cancer is bipartite; ROS are involved in many signalling pathways and thereby contribute to oncogenic transformation. However, when ROS levels exceed a critical threshold death-pathways are initiated. Therefore, the balance between ROS and anti-oxidant mechanisms is especially critical in cancer cells, and hence presents an attractive target for therapeutic intervention (reviewed in 52).

The potential relevance of reactive oxygen mediated (ROS)-dependent apoptosis in drug responses is supported by the recent observation that F-ara-A resistant cells are highly sensitive to beta-phenylethyl isothiocyanate (PEITC), a compound that induces ROS accumulation by disabling the glutathione system 53. Furthermore, the cytotoxic activity of various novel drugs in CLL was linked to p53 independent ROS production, including bendamustine 54, HDAC-inhibitors 55;56 and proteasome inhibitors 57-59. We observed that the response following treatment with CDDP and fludarabine in PB derived CLL cells, was mediated by the generation of ROS and subsequent Noxa-dependent initiation of apoptosis, irrespective of p53 function. Noxa is a p53 response gene in many cell-types 60, but this is not the case in CLL 61. We have found that, at least in CLL,
Noxa is the only BH3-only member that is induced upon ROS signaling. These findings are of particular interest as they constitute an apoptosis pathway in CLL which does not depend upon p53 (Figure 1).

3. Conclusion

As advances are made both in upfront treatment strategies and salvage regimens, and the standard of supportive care improves, the emergence of drug resistant disease will be an increasing challenge in the care for patients with CLL. Although reduced intensity conditioning regimens will make allogeneic stem cell transplantation accessible to a growing number of patients, many will still experience progressive disease for which no effective treatment is available. Therefore, a continuous quest for novel treatment targets is warranted. Targeting CLL at its loci minoris resistentiae, i.e. the dependency on interactions with the microenvironment and the disturbed redox balance, may be the key to long term control of drug resistant disease.
Reference List


21. Coscia M, Pantaleoni F, Riganti C et al. IGHV unmutated CLL B cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL B cells. Leukemia 2011;25:828-837.


