Summary and General Discussion
SUMMARY

Failure of therapy due to acquired resistance is one of the major problems in the treatment of patients with acute leukemia. Initially, most patients respond well to induction chemotherapy usually consisting of a combination of cytostatic agents including daunorubicin, vincristine, methotrexate, and cytarabine (Ara-C), 1-3 leading to 70-80% complete remissions (CR). However, despite aggressive induction and consolidation therapy, relapses of the disease frequently occur (50-60%) resulting in low overall 5-year survival rates of patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). 4,5 Those patients who relapse more than one year after diagnosis have a good chance to achieve a second remission after administration of the original induction regimen, or at least one of similar intensity. The most effective post-remission therapy, however, is high-dose chemotherapy and total body irradiation followed by allogeneic or autologous stem cell transplantation (SCT), using stem cells from a (partially) HLA-matched donor or from the patient, respectively. SCT can be considered as a rescue procedure to achieve immunologic reconstitution in patients of whom both malignant and normal hematopoiesis were destroyed. The advantage of allogeneic SCT (followed by donor lymphocyte infusions (DLI) at relapse) is that besides causing immunologic reconstitution the donor T cells in the graft may recognize and attack the residual leukemic cells, leading to a graft-versus-leukemia (GVL) effect.

Patients who never achieve a CR or who experience a relapse within 6 months (during treatment) are considered to be resistant to further cytotoxic treatment. Various mechanisms may underlie this resistance, including expression of drug efflux pumps, 6-8 and cell cycle status of the leukemic cell. 9,10 Another cause for the unresponsiveness of leukemic cells to chemotherapy has been shown to be defects in the apoptotic machinery of these leukemic cells. 11-17

Cytostatic agents (chemotherapy) and donor-derived T lymphocytes (cellular immunotherapy) kill leukemic cells via induction of apoptosis in the target cell. Aberrations in common apoptotic pathways may therefore cause unresponsiveness to both forms of therapy, as has been observed in some patients. Other patients are resistant to chemotherapy but do respond to DLI, indicating that these patients may have defects in chemotherapy-specific pathways. Assessment of potential defects in apoptotic pathways is only possible when the functional apoptotic pathways are unraveled in more detail. Although general therapy-induced apoptotic pathways have been described (Figure 7.1), actual apoptotic mechanisms are mostly much more complicated than shown in this figure. This thesis emphasizes the complexity of the apoptotic pathways that are induced in leukemic cells after treatment with conventional cytostatic drugs or with cytotoxic T cells. Moreover, several controversies that still exist in the field of apoptosis research are highlighted in this thesis, and at least partially unraveled.
The cell cycle status of leukemic cells affects their sensitivity to chemotherapy, since most chemotherapeutic agents specifically act on proliferating (S or G2/M phase) or at least activated (G1) cells, while quiescent (G0) cells are considered to be unresponsive. Incorporation of Ara-C into DNA, a process specifically occurring during S-phase of the cell cycle, is assumed to be a key event in the mechanism of killing of proliferating leukemic cells.\textsuperscript{19} Chapter 2 describes the unexpected potential of Ara-C to cause apoptosis, characterized by phosphatidyl serine exposure on the cell membrane and caspase activation, in resting (>98% in G0) primary B cells derived from four previously untreated patients with B-cell chronic lymphocytic leukemia (B-CLL). Cell cycle analysis combined with long-term analysis of cell death using CFSE-based cytotoxicity assays confirmed that these non-proliferating B-CLL cells were killed by Ara-C in G0 phase of the cell cycle. Since inhibition of DNA incorporation was not the mechanism of action of Ara-C in G0-B-CLL cells, other cellular processes must be involved. We demonstrated that increasing concentrations of deoxycytidine (dC) blocked both the formation of Ara-CTP and Ara-C-induced cell death of G0-B-CLL cells. dC is a structural analogue of Ara-C and the normal metabolite of both RNA and DNA synthesis. In contrast to DNA (repair) synthesis which was hardly detectable, unexpectedly high RNA synthesis was present in G0-B-CLL cells. This RNA synthesis was blocked by Ara-C for 30%, suggesting that inhibition of RNA synthesis is a potential mechanism of killing resting...
G₀-B-CLL cells by Ara-C. B-CLL cells are characterized by high expression levels of B-cell lymphoma-2 (Bcl-2) protein, which causes the prolonged survival of these malignant cells.²⁰ One of the initiating mechanisms for drug-induced apoptosis in B-CLL cells has been reported to be alteration of the expression levels of pro- or anti-apoptotic proteins (Bcl-2/Bax ratio ²¹,²²). Therefore, we analyzed whether addition of Ara-C resulted in alterations in Bcl-2, Bax or Mcl-1 protein levels. We showed that the survival protein Mcl-1 was downregulated in response to Ara-C, which may be a consequence of the decreased RNA synthesis. In conclusion, both downregulation of RNA synthesis and Mcl-1 expression appear to contribute to the mechanism of action of Ara-C in G₀-B-CLL cells.

Besides investigating the mechanisms that are involved in chemotherapy-induced apoptosis of resting leukemic cells, it is very important to gain more insight into the apoptotic pathways induced by cytostatic agents in proliferating leukemic cells, since resistance of leukemic cells to chemotherapy-induced apoptosis frequently occurs in patients with acute leukemia. One of the controversial topics in chemotherapy-induced apoptosis is the pathway via which cell death is initiated. Treatment with chemotherapy may lead to specific activation of the mitochondrial pathway, in which cytochrome C release and caspase-9 activation have been reported to play a central role. (Figure 7.1)²³,²⁴ Alternatively or perhaps additionally it may activate the death receptor pathway of apoptosis, in which formation of a Death Inducing Signaling Complex (DISC) and caspase-8 (FLICE) activation are key events.²⁵ In Chapter 3 of this thesis, the role of the death receptor pathway in chemotherapy-induced apoptosis of human derived myeloid and lymphoblastic leukemia cell lines is described. The rapid and substantial activation of caspase-8 that we observed after treatment with camptothecin, combined with the effective inhibition of chemotherapy-induced apoptosis by caspase-8 inhibitor, strongly suggested involvement of the death-receptor pathway in apoptosis induced by cytostatic agents. This assumption was examined by introducing retroviral constructs encoding Bcl-2 or FLICE inhibitory protein (FLIP) into the target cells, and subsequently analyzing the sensitivity of these cell lines for various cytostatic drugs. Enhanced Bcl-2 expression was used to specifically block the mitochondrial pathway, whereas FLIP selectively prevented death-receptor-induced apoptosis. No inhibitory effect of elevated expression of FLIP on camptothecin-induced apoptosis or caspase-8 activation was observed, while introduction of Bcl-2 resulted in almost complete inhibition of camptothecin-mediated cell death and caspase-8 activation in all three cell lines studied, indicating that in these cell lines chemotherapy-induced apoptosis is completely dependent on the mitochondrial pathway. Moreover, caspase-8 apparently plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia.
Not only controversy existed about the role of the death receptor pathway in chemotherapy-induced apoptosis, the involvement of this pathway in the elimination of leukemic cells by cytotoxic T lymphocytes (CTLs) was also largely unclear. In Chapter 4 we studied whether in addition to cell death mediated by perforin (PFN) and granzyme B (GrB) death receptor-induced apoptosis contributes to the elimination of human tumor cells by CTLs. In most studies that have been published, CTL-mediated target cell death was only analyzed after 4 hrs of interaction between target and effector cell. Since death-receptor-mediated apoptosis is slow compared to PFN/GrB-induced cell death, in this study we used T cell clones with slow and rapid kinetics of killing derived from a patient with chronic myeloid leukemia (CML), and analyzed cell death in various time intervals. The three CTL clones used in this study all recognized primary CML cells from the patient. Because for these types of studies insufficient leukemic cells can be isolated from the patient, EBV-LCL from the patient were used as target cells in this study. To determine the involvement of the death receptor pathway, a retroviral construct encoding FLIP was introduced into these target cells. A CTL clone capable of killing 50% of the target cells within 2 hrs of incubation primarily acted by release of PFN and GrB (no inhibition by FLIP was observed). In contrast, two CTL clones showing slower target cell killing kinetics partially used the death receptor pathway (~30% inhibition by FLIP). Thus, not only PFN/GrB release but also the death receptor pathway can play a role in the execution mechanism of cytotoxic T cells.

Whereas the anti-apoptotic protein FLIP specifically blocks the death-receptor pathway of apoptosis, proteinase inhibitor-9 (PI-9) has been reported to be a specific inhibitor of GrB-induced cell death, and should therefore not affect Fas-induced apoptosis. However, in Chapter 5, we demonstrate that PI-9 is less specific than previously expected. Two different cell lines transduced with retroviral constructs encoding FLIP or PI-9 together with their wild type counterparts were exposed for 5 or 24 hrs to Fas apoptosis-inducing antibodies. In both types of cell lines we showed substantial and significant inhibition of Fas-induced apoptosis by enhanced PI-9 expression, although the levels of inhibition were lower than in the FLIP-expressing cell lines. PI-9 did not affect chemotherapy-induced apoptosis, showing that PI-9 interferes with the death receptor pathway but not with the mitochondrial pathway.

Cytotoxic T cells are considered to selectively kill target cells after specific TCR-mediated recognition of target antigen in the context of the appropriate HLA. In Chapter 6, we describe another T cell-mediated mechanism of killing target cells which is non MHC-restricted and is independent of TCR-triggering. HLA-A2-restricted CTL clones showed cytolytic activity to HLA-A2-expressing target cells via classical TCR/MHC-dependent interactions resulting in 40-70% lysis after 4 hrs of incubation. As a consequence of this specific TCR triggering, the T cells internalized their
TCRs, and produced interferon (IFN)-\(\gamma\). This MHC-restricted target cell death was completely inhibited by pre-incubating the T cells with the calcium chelator EGTA, which inhibits all secretion pathways of the CTL, showing that the lysis was mediated by PFN and/or GrB. In addition the same CTL clones exerted non MHC-restricted target cell death (10-30%) after 10 hrs of coculture, which was not accompanied by TCR downregulation and IFN-\(\gamma\) release. This lysis could be specifically blocked by FLIP expression in the target cells, demonstrating death-receptor-induced apoptosis. A similar non MHC-restricted mechanism of killing is used for the removal of the surplus of T cells after clearance of an infection to prevent collateral damage.\(^{29}\)

The results presented in this thesis emphasize the complexity of the mechanisms involved in apoptosis induction in leukemic cells after treatment with chemotherapy or cellular immunotherapy.
GENERAL DISCUSSION

Background
Defects in the apoptotic machinery of leukemic cells have been shown to cause resistance of these cells to chemotherapy and/or cellular immunotherapy.  

11-17 Apoptosis is induced and regulated by a complex network of proteins that are connected via various signal transduction cascades. Although the exact pathways are not precisely known, induction of apoptosis eventually leads to death of the target cell. In the last decades a lot of research has been performed on many aspects of apoptosis by different research groups. General proposed mechanisms of chemo- and immunotherapy-induced apoptosis have been composed from the separate results of these experiments, leading to the simplified model presented in Figure 7.1. One major problem of this model is that it is composed of results of so many different experiments, and therefore a “general” mechanism actually does not exist. In addition, not all mechanisms of apoptosis are completely unraveled and still many controversies exist, which are highlighted in this thesis.

The paradoxic mechanism of action of cytostatic agents like Ara-C
One of the inconsistencies of chemotherapy treatment is the mechanism of action of cytostatic drugs like Ara-C. Like other chemotherapeutic agents Ara-C is supposed to exert its action preferably in cycling cells. 30-32 Incorporation of Ara-C into DNA, a process specifically occurring during S-phase of the cell cycle, and leading to termination of DNA synthesis, is assumed to be a key event in the mechanism of killing of proliferating leukemic cells. 19 In addition, Ara-C inhibits DNA repair by blocking topoisomerase I-mediated DNA religation. 33 The unexpected killing by Ara-C of cells not in proliferation or active phase of the cell cycle, as demonstrated in chapter 2 of this thesis for G0-B-CLL cells, revealed that Ara-C also induces apoptosis via other mechanisms of action. Although this observation was not expected, a similar phenomenon is seen in patients with acute leukemia treated with chemotherapy, since not only the rapidly dividing leukemic blasts, but also normal resting B and T lymphocytes are frequently killed by the cytostatic drugs. It is intriguing why these G0 cells are killed, while in former studies specific deletion of cycling cells was demonstrated. 10,33 In chapter 2, we additionally studied B-CLL cells that were stimulated with CD40L. Culturing of B-CLL cells for 7 days on irradiated mouse fibroblasts expressing human CD40L induced proliferation in these B-CLL cells resulting in the following cell cycle distribution: 15% S/G2M, 69% G1, and 16% G0. These cells were less responsive to Ara-C than primary B-CLL cells, but after 48 to 72 hrs of incubation specific deletion of cells from the S-phase of the cell cycle was observed, suggesting that these CD40 stimulated B-CLL cells behaved more or less like normal proliferating cells. The G0 cells that were left in this CD40L-stimulated population were unresponsive to Ara-C like other G0 cells in proliferating cell lines. Apparently, these CD40L-stimulated “G0” cells are not identical to primary B-CLL cells.
It was demonstrated in chapter 2 that both downregulation of RNA synthesis and Mcl-1 expression appear to contribute to the mechanism of action of Ara-C in G\textsubscript{o}-B-CLL cells. Enhanced expression levels of Bcl-2 but perhaps also of Mcl-1 cause the prolonged survival observed in B-CLL cells.\textsuperscript{20} Nowadays, clinical studies are ongoing to investigate the potential of Bcl-2 antisense oligonucleotides (Oblimersen) in the treatment of B-CLL.\textsuperscript{34} Based on the results of chapter 2, Mcl-1 may be another interesting target to downregulate. This hypothesis has to be evaluated in \textit{in vitro} studies aimed at specific downmodulation of this gene in B-CLL cells, for instance by siRNA or antisense RNA strategies.

The findings described in chapter 2 will probably not directly imply a clinical application for Ara-C in the treatment of B-CLL, especially since combination therapy using a combination of fludarabine and Ara-C was not more effective than treatment with fludarabine alone.\textsuperscript{35} However, the unexpected potential of Ara-C, which has been considered to be S-phase specific, to kill G\textsubscript{o}-B-CLL cells makes it likely that other nucleotide analogues such as gemcitabine or clofarabine may be effective in killing B-CLL cells as well.

**The controversial role of the death receptor pathway in chemotherapy-induced apoptosis**

Cytostatic agents have been reported to induce apoptosis via the mitochondrial pathway.\textsuperscript{23,24} However, involvement of the death receptor pathway in chemotherapy-induced apoptosis has also been shown by some investigators who reported that activation of the death receptor pathway by chemotherapy occurred via extracellular triggering of death receptors due to drug-induced upregulation of Fas receptor and ligand leading to autocrine or paracrine induction of Fas-mediated apoptosis.\textsuperscript{36-40} In the study described in chapter 3, chemotherapy-induced apoptosis in AML and ALL leukemic cell lines was shown to be completely mediated via the mitochondrial pathway. Although AML and ALL cell lines were used in this study instead of primary leukemic cells, we suppose that especially the ALL cell lines resemble the primary ALL cells, and that the results can be extrapolated to the clinical situation. The death receptor pathway was not involved, although one of the key regulators of this pathway, namely caspase-8, likely played an important role in the execution of the leukemic cells by chemotherapy as well. This unexpected involvement of caspase-8 in mitochondria-mediated apoptosis makes it likely that in some former studies in which chemotherapy-induced caspase-8 activation was found incorrect involvement of the death receptor pathway has been concluded.

The group of Fulda postulated that the pathway of chemotherapy-induced apoptosis is cell-type dependent.\textsuperscript{41} In so-called type I cells both the death receptor and the mitochondrial pathway were activated upon drug treatment, whereas in type II cells selective induction of the mitochondrial pathway was demonstrated. According to
this theory, AML and ALL cells are categorized as type II cells. Fulda et al. also proposed that type II cells are characterized by the fact that Fas-induced apoptosis is enhanced via a mitochondrial bypass (see Figure 7.1). In our studies, however, apoptosis induced by Fas agonistic antibodies or TRAIL was completely mediated by the death receptor pathway, since complete inhibition of Fas-induced apoptosis was found in cells overexpressing FLIP while no inhibition was observed in cells with Bcl-2 overexpression. Apparently, the classification of cells is more complicated than a distinction into type I and II cells as suggested by the group of Fulda.

The impact of common elements involved in different apoptotic cascades
An important finding of this study was that, apparently, caspase-8 plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia. To investigate this theory in more detail, additional studies should be performed in which caspase-8 expression is downregulated in leukemic cells, for instance via a siRNA-based protocol, and subsequently the effect of this knockdown on sensitivity to chemotherapy is analyzed. If these data are in line with our theory this may have implications for therapy. It is known that caspase-8 is downregulated in certain tumors. Therefore, therapeutic strategies focusing at modulation and activation of caspase-8 may sensitize drug-resistant malignancies to chemotherapy. However in practice this will be complicated, since caspase-8 is also present in normal healthy cells, which will also be destroyed when caspase-8 is not specifically targeted in malignant cells. On the other hand, regular chemotherapy also destroys normal hematopoiesis which may be restored by allogeneic SCT.

The existence of common important elements in the mitochondrial and the death receptor pathway may also explain the synergistic effect observed using combination therapies. Various studies have reported on increased sensitivity of cancer cells to Fas or TRAIL when patients were simultaneously treated with low dose chemotherapy. This cotreatment with chemotherapy may cause upregulation of death receptors, but additionally it may activate common apoptotic elements such as caspase-3 or caspase-8, which later on facilitates TRAIL-induced apoptosis. Cross resistance of Fas/TRAIL resistant cells to anticancer agents which is commonly observed in leukemia patients, can also partly be explained by defects in a common element of both pathways, such as caspase-8. When defects in common elements of the two pathways are detected in a patient with leukemia this may be important in further treatment stratification. Moreover, if CTL-induced target cell death is also largely mediated via the death receptor pathway, defects in for instance caspase-8 may also cause decreased responsiveness to cellular immunotherapy in these patients.
The influence of effector-target interactions on the kinetics and the execution pathway of CTL-induced target cell death

In response to recognition of target antigen by their membrane TCRs, CTLs potentially use two pathways for lysis of target cells. 49:50 Cell lysis occurs either via a rapid PFN/GrB-mediated mechanism of cell death (<90 min) 51, or alternatively, via activation of the death receptor pathway in the target cell 50;52;53 causing relatively slow elimination of this target cell (>4 hrs) 54 as also demonstrated in chapters 4 and 6 of this thesis. It is largely unknown how these pathways are activated. Since cytotoxic T lymphocytes can use secretion of perforin (PFN) and granzyme B (GrB) to kill their target cells, why would they in addition have the potential to induce death receptor-mediated apoptosis? Virus-infected and tumorigenic cells have indeed been reported to be predominantly eliminated via rapid granule-mediated cell death. 55:56 Death receptor-mediated cell death, in contrast, primarily has been described to be involved in eliminating autoreactive T cells and downsize immune responses after infection. 57-60 In chapter 4 another role of the death receptor pathway is described, namely, execution of human target cells by cytotoxic T cells if not all target cells have been destroyed by release of PFN/GrB. In addition the death receptor pathway has also been shown to be involved in CTL-induced non MHC-restricted target cell death (Chapter 6).

Based on the results described in chapters 4 and 6, we hypothesize that the strength of interaction between target and effector cell determines the kinetics of target cell death and perhaps also the pathway of cell death. More specifically, we assume that weak effector-target interactions may be sufficient to induce the death receptor pathway of apoptosis, but cannot or only marginally lead to the release of PFN/GrB. In Chapter 4 was shown that the T cell clone C6-2 slowly killed EBV-LCL derived from a patient with leukemia. The same CTL was previously shown to be capable of rapidly killing monocytes of the same patient, 61 indicating that the execution machinery of the T cell is intact. Since the EBV-LCL could be killed within 4 hrs by another CTL clone (HY-A1), the observed differences in kinetics of killing are also not caused by any defects in one of the apoptotic mechanisms of the target cell. Therefore, more likely they are caused by differences in effector-target interaction, for instance due to variant levels of peptide presentation by the HLA molecules on the different target cells. Probably, the Cw6-restricted CTL clones recognize minor antigens highly expressed on monocytes but poorly expressed on EBV-LCL from the patient resulting in a low avidity interaction between TCR and MHC/peptide complex. The low amounts of PFN that were released are assumed to be a consequence of this weak interaction, as well as the fact that target cell death was at least partially executed via the death receptor pathway (Chapter 4).

The results shown in Chapter 6 also support the hypothesis that low avidity effector-target interactions only lead to death-receptor-induced apoptosis of target cells, while
strong effector-target interactions mainly induce PFN/GrB-induced target cell death. In addition to MHC-restricted target cell death, CD8+ T cells were demonstrated to be capable of killing target cells in a non MHC-restricted manner. For the latter mechanism of lysis the TCR was not involved, implying a low avidity effector-target interaction. This non MHC-restricted target cell death was demonstrated to be completely mediated via activation of the death receptor pathway in the target cell. In contrast, in case of a strong effector-target cell interaction, as involved in TCR-dependent MHC-restricted recognition of target cells, the same cytotoxic T cells used PFN/GrB release as the main execution mechanism to induce rapid target cell death.

Why weak effector-target interactions can lead to activation of the death receptor pathway, but not to the release of PFN and GrB remains largely unknown. A model has been proposed assuming that after ligand binding the TCR complex has to undergo a number of modifications, including clustering of receptors, tyrosine phosphorylation steps and SH2 interactions, before a signal can be transmitted. The requirement for these modifications results in a temporal lag between ligand binding and receptor signaling. At each step of this cascade the MHC/peptide-TCR complex may dissociate making it very unlikely that low-avidity MHC/peptide-TCR complexes can ever assemble the final signaling complex resulting in release of PFN and granzymes. However, a sufficiently high density of low-affinity MHC/peptide complexes can support the early steps of the process and lead to a distinct (partial) signaling pathway resulting in death-receptor-mediated apoptosis. This model is supported by some murine studies demonstrating that interaction of CTLs with target cells exogenously loaded with single-amino-acid variants of the optimal MHC-binding peptide resulted in Fas-mediated target cell death while PFN/GrB-mediated cell lysis was absent. When the same less-than-optimal peptides were endogenously processed, using a virus-based infection model, CTL-mediated cell death was completely absent. In Chapter 6 similar results were described showing that the HLA-A*0205 expressing cell line ALL-HP was not killed within 4 hrs by the HLA-A*0201-restricted T cell clone HA2.27 (Figure 6.1). Unpublished results of our group revealed that exogenous loading of this HLA-A*0205 positive cell line with high concentrations of HA-2 peptide resulted in rapid PFN/GrB-mediated cell death by CTL HA2.27. By artificially offering more specific MHC/peptide complexes to the TCR, the avidity of the MHC/peptide-TCR complex is apparently sufficiently enhanced to overcome a certain threshold of TCR triggering and making PFN/GrB release possible. In conclusion, small differences in avidity of MHC/peptide-TCR complexes can highly affect recognition by that specific TCR and determine which effector mechanisms can be used by the T cell.

From a physiological point of view it is not surprising that weak effector-target interactions could not lead to the release of PFN/GrB. Misdirected effector molecules
would cause a lot of damage to neighboring tissues. In the complex selection mechanism taking place in the thymus to delete potentially self-reactive T cells, it is important, however, that weak interactions can lead to death-receptor-mediated apoptosis.

**Overexpression of anti-apoptotic genes as a method to study the role of different apoptotic pathways**

Enhancing the expression of FLIP has been demonstrated to be an effective method to block the death receptor pathway in different leukemic cell lines (Chapters 3 and 4 of this thesis). PI-9 is considered to be an efficient and selective inhibitor of Granzyme B. Retroviral introduction of PI-9, FLIP, or both constructs into a target cell would therefore provide an elegant approach to study all effector mechanisms of a CTL. However, in chapter 5 it was demonstrated that overexpression of PI-9 inhibited Fas-induced apoptosis. Apparently, proteins that are supposed to act specifically in a certain pathway may interfere in other pathways, which may lead to incorrect conclusions.

Retroviral transfer of PI-9 can thus not be used to study CTL-induced PFN/GrB-mediated target cell death. Alternative strategies to evaluate the role of PFN and GrB in CTL-mediated cytotoxicity may be measuring PFN/GrB release using flow cytometry (as shown in chapter 4) or performing inhibition experiments using EGTA (chapter 6) or concanamycin A (CMA). The Ca\(^{2+}\)-chelator EGTA efficiently inhibits all (Ca\(^{2+}\)-dependent) secretion pathways in the T cell,\(^{66}\) while CMA is an inhibitor of vacuolar type H\(^{+}\)-ATPase that inhibits PFN-based cytotoxicity, mostly by accelerated degradation of PFN caused by an increase in the pH of lytic granules.\(^{67}\)

The inhibitory effect of PI-9 on Fas-induced cell death was much more pronounced in one of the two cell lines studied. We speculate that this may be caused by differential usage of Fas-cell death pathways by the two cell lines studied. In the earlier mentioned type I cells, Fas-induced apoptosis is completely dependent on the death-receptor pathway. In these cells the highest inhibition by PI-9 was observed. In type II cells in contrast, Fas-induced apoptosis is partly mediated via the mitochondrial pathway of apoptosis. Dependent on the site of interference of PI-9 in the apoptotic cascade, the inhibitory effect may differ between type I and II cell lines. This may also explain why this inhibitory effect was not observed by other groups that only studied one or two (type II) cell lines.

**Conclusion**

In conclusion, the results described in this thesis demonstrate that apoptosis induction in leukemic cells after treatment with chemotherapy or (cellular) immunotherapy is very complex and frequently dependent on the target cell studied, and on the interaction
between target and effector cell. It was shown that G₀ cells derived from patients with B-CLL compared to G₀ cells from patients with acute leukemia responded differently to Ara-C treatment, which may be partially explained by the different mechanisms of action exerted by Ara-C. In this thesis the role of the death receptor pathway in both chemotherapy-induced and CTL-induced apoptosis of leukemic cells was unraveled in more detail, at least in the cell lines that were studied. Considering future strategies in the treatment of leukemia, it will be worthwhile to focus on the investigation and development of agents that restore normal or therapy-induced apoptosis in resistant leukemic cells. These agents should be combined with conventional treatment modalities. For B-CLL, already a number of agents are under investigation which target not only Bcl-2, but also other anti-apoptotic proteins in CLL cells. Other attractive targets are proteins that are common in multiple apoptotic pathways, for instance caspase-8. Defects in caspase-8 or other common caspases like caspase-3 may cause multiple drug resistance and also cross resistance to immunotherapeutic interventions. Modulation or activation of these proteins will sensitize the leukemic cell simultaneously to all these forms of therapy.

REFERENCES


