Summary

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 40% of adult Non-Hodgkin lymphoma and is characterized by large B-cells. Although this lymphoma is classified as one disease, DLBCL is clinically, morphologically and genetically a heterogeneous group of tumors that is treated with chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone, CHOP) in combination with the CD20 chimeric monoclonal antibody rituximab (R). Despite this aggressive therapy, 30-40% of the patients will eventually die due to the disease. Fatal outcome usually results from failure to achieve complete remission or the occurrence of an relapse. Many studies have shown that inhibition of the apoptosis signaling pathways is strongly related to response to chemotherapy and eventual clinical outcome. Apoptosis is an ATP dependent, physiological form of cell death which can be triggered by a variety of stimuli. Upon induction of apoptosis, cystein aspartic acid-containing proteases (caspases) are proteolytically cleaved and activated. Caspases can be divided into effector caspases (caspase 3, 6 and 7) which carry out cell death, and initiator caspases (caspase 2, 8, 9, and 10) that mainly activate effector caspases. Once activated, effector caspases execute cell death through cleavage of key cellular proteins, including substrates involved in cell structure, signaling, cell cycle control and DNA repair. Two major apoptosis pathways have been elucidated; an intrinsic stress-induced pathway and an extrinsic death receptor-induced pathway.

Intrinsic apoptosis pathway

The intrinsic or stress-induced pathway can be triggered by many stimuli, including growth factor deprivation, oxidants, Ca^{2+} overload, oncogene activation, DNA-damaging agents, and microtubule-attacking drugs. These death stimuli activate BH3-only (Bcl-2-homology domain 3) proteins, a pro-apoptotic subgroup of the Bcl-2 family, that initiate apoptosis via direct or indirect activation of Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist/killer). Once activated, Bax and Bak insert in the mitochondrial outer membrane and form oligomers, resulting in mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c in the cytosol. In the cytosol, cytochrome c binds Apaf-1 (apoptotic protease activating factor-1) and procaspase-9, resulting in a large protein complex, also designated as the apoptosome. Pro-caspase 9 becomes activated and active caspase 9 in turn cleaves and activates downstream effector caspases such as pro-caspase 3.

Extrinsic apoptosis pathway
The extrinsic or death receptor-induced pathway is triggered by binding of a ligand to death receptors (DR) of the TNF (tumor necrosis factor) receptor super family. These death receptors contain a cytosolic death domain (DD) and include TNFR1 (binding with TNF-α), Fas/CD95 (binding with FasL), DR3 (binding with APO3L), TRAIL receptor R1/DR4 and R2/DR5 (both binding with TRAIL/Apo2L). Upon ligand binding, death receptors recruit FADD (Fas Associated Death Domain) protein, that binds directly or indirectly via TRADD pro-caspase 8 and/or 10, forming a death inducing signaling complex (DISC), resulting in activation of caspase 8 and 10. Once caspase 8 and/or 10 are activated, cells can undergo apoptosis by signaling through two different pathways. 1) Caspase 8 and/or 10 can cleave and activate pro-caspase 3 directly. 2) When the amount of caspase 3 initially activated by caspase 8 and/or 10 is insufficient to trigger the apoptotic process, caspase 3 can be activated indirectly through caspase 8 mediated cleavage of Bid, a pro-apoptotic member of the Bcl-2 family. Cleaved Bid interacts with Bax or Bak resulting in disruption of the mitochondrial membrane and induction of the intrinsic apoptosis pathway.

This thesis summarizes our studies on mechanisms of apoptosis resistance in DLBCL and alternative targeted therapies that could restore apoptosis sensitivity.

In chapter 2, we demonstrated that a caspase 8 inhibition only profile, illustrated by low percentages of active caspase 3 positive DLBCL cells and expression of c-Flip was related to a favorable outcome, whereas a caspase 9 inhibition only profile, characterized by expression of Bcl-2 and XIAP was strongly predictive for a poor response to chemotherapy and overall survival time. Most of the chemotherapeutic drugs used in the treatment of DLBCL induce apoptosis primarily via the intrinsic caspase 9 mediated apoptosis pathway, further indicating that only inhibition of this pathway is seriously involved in resistance to chemotherapy induced apoptosis. To investigate possible mechanisms that underlie disruption of chemotherapy-induced caspase 9 mediated apoptosis, functional analysis of apoptosis pathways in DLBCL cells was necessary.

In chapter 3, we showed that apoptotic cells can be detected with an easy and highly sensitive method using 7AAD staining in combination with fluorescent beads and the pancaspase inhibitor zVAD-FMK. This method required few cells and it was possible to detect apoptosis reproducibly in isolated lymphoma cells of DLBCL biopsies and in hematopoietic cell lines.

In a previous micro array study we had demonstrated that a subset of DLBCL with poor clinical outcome was characterized by a gene expression profile reflecting constitutive activation with concomitant inhibition of the intrinsic apoptosis pathway. It seemed that
intrinsic resistance to chemotherapy and clinical outcome depended on the balance between pro- and anti-apoptotic genes. We hypothesized that chemotherapy resistance was caused by inhibition of the intrinsic apoptosis pathway. In chapter 4, we found that expression profiles of apoptosis related genes in isolated lymphoma cells divide DLBCL cases into one group with low expression levels of both pro- and anti-apoptotic genes and one group with high expression levels of these genes. DLBCL with high expression levels of pro- and anti-apoptotic genes frequently appeared to be chemotherapy-refractory and were characterized by high levels of constitutive caspase 9 activity and mitochondrial membrane depolarization without induction of apoptosis, indicating that there is a disruption of the apoptosis pathway downstream of caspase 9 activation. In chapters 5, 6 and 7 we tested alternative therapies that could circumvent downstream disruption of the intrinsic apoptosis pathway and restore sensitivity to cell death.

We hypothesized that inhibition of the downstream anti-apoptotic protein XIAP by a XIAP antagonist would restore apoptosis sensitivity. In chapter 5, we showed that the small-molecule XIAP antagonist induced apoptosis in isolated DLBCL cells, including chemotherapy-refractory cases. XIAP antagonist-sensitive DLBCL cases were characterized by high expression levels of XIAP, relatively low expression levels of Bcl-2, and by constitutive caspase-9 activation. Furthermore, the XIAP antagonist was not toxic for peripheral blood mononuclear cells and tonsil germinal center B-cells from healthy donors.

An other pathway to induce apoptosis is the extrinsic apoptosis pathway that can be triggered by hsTRAIL/Apo2L. In chapter 6, we demonstrated that a subset of DLBCL cases including chemotherapy-refractory lymphomas was sensitive to hsTRAIL/Apo2L. In addition, hsTRAIL/Apo2L induced apoptosis in DLBCL cells and in B-cell lines demonstrated high expression levels of the apoptosis inhibitors Bcl-2 and/or XIAP, suggesting that these anti-apoptotic proteins did not contribute to resistance to hsTRAIL/Apo2L-induced apoptosis in DLBCL.

An alternative way to circumvent disruption of the apoptosis pathways is to induce cell death independent of apoptosis in DLBCL. In chapter 7, we found that the novel human type I CD20 mAb ofatumumab induced CDC in DLBCL cell lines and all chemotherapy-refractory DLBCL cases tested. Ofatumumab was more effective in inducing CDC of DLBCL cells compared to rituximab. Sensitivity of DLBCL to ofatumumab- and rituximab-induced CDC was dependent of CD55 and CD59 expression, although this inhibitory effect was relatively limited for ofatumumab-induced CDC.

Finally, in the concluding chapter 8, we describe possible mechanisms responsible for intrinsic resistance to apoptosis in DLBCL and how these mechanisms can be used in
development and application of targeted therapies that can improve clinical outcome in DLBCL patients. We showed that upstream activation of the intrinsic apoptosis pathway with concomitant downstream inhibition of this pathway is a key-event in chemotherapy refractory DLBCL. Downstream inhibition of caspase 3 activity could be restored by targeting XIAP with small-molecule XIAP antagonists, either or not in combination with a Bcl-2 antagonist. In addition, \textit{in vitro} sensitivity to the XIAP antagonist could be predicted based on biological markers suggesting the possibility of pre-defining patients most likely to benefit from XIAP antagonist therapy. We expect that especially R-CHOP refractory DLBCL patients will benefit from monotherapy with the small-molecule XIAP antagonist and that adverse side effects will be very limited.