Potential New Drugs in Lymphoma

MARYAM DELFOROUSH
Abstract


Lymphomas are malignant tumours arising from cells in the lymphatic system. They are classified as B-cell lymphomas, T-cell lymphomas and Hodgkin lymphoma (HL). Of the B-cell lymphomas, one of the most common is diffuse large B-cell lymphoma (DLBCL). Many patients with lymphomas can be successfully treated however patients who relapse or are refractory have a poor prognosis, warranting further investigations to identify potential targets and develop novel drugs.

Picropodophyllin (PPP), a potent and selective inhibitor of IGF-1R, inhibits malignant cell growth with low or no toxicity on normal cells in preclinical models. In paper I, we investigated the potential benefits of using PPP against DLBCL and found that the anti-tumor effects of PPP might possibly be explained by IGF-1R-unrelated mechanism(s). However, the inhibitory effects of PPP on lymphoma cells together with its low toxicity in vivo makes it a promising drug candidate for treatment. Melflufen, a derivative of melphalan, is currently being evaluated in a clinical phase I/II trial in relapsed or refractory multiple myeloma. In paper II, we confirmed previous reports of superior potency of melflufen over melphalan. Being active in cell lines and primary cultures of lymphoma cells as well as in a xenograft model in mice, melflufen considered being a candidate for further evaluation in treatment. bAP-15, a novel inhibitor of proteasome activity, inhibits ubiquitin specific peptidase 14 (USP14) and ubiquitin carboxyl-terminal hydrolase L5 (UCHL5). In paper III, we investigated the activity of b-AP15 in DLBCL and HL cell lines and compared the results to standard drugs used in treatment. Results showed inhibition of the proteasome and growth inhibition/cytotoxicity with IC50-values in the micromolar range. Treatment failure and lack of clinical benefit of proteasome inhibitors like bortezomib in DLBCL patients inspired us investigating for possible new targets, with major focus on proteasome inhibitors in DLBCL. In paper IV, we suggested that UCHL5 and/or USP14, as new targets for proteasome inhibitors in DLBCL, be further evaluated.

The findings in this thesis suggest that PPP, Melflufen and b-AP15 are potential candidates for clinical drug development and UCHL5 and/or USP14 are new potential targets for proteasome inhibitors in DLBCL.

Keywords: lymphoma, diffuse large B-cell lymphoma, DLBCL, picropodophyllin, PPP, J1, melflufen, prodrug, cancer therapeutics, alkylating agents, b-AP15, proteasome inhibitors, DUB inhibition, UCHL5, USP14

Maryam Delforoush, Department of Immunology, Genetics and Pathology, Rudbecklaboratorium, Uppsala University, SE-751 85 Uppsala, Sweden.

© Maryam Delforoush 2016

ISSN 1651-6206
ISBN 978-91-554-9524-4
urn:nbn:se:uu:diva-280546 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-280546)
“Find your voice & when you do, fill the damn silence.”
Meredith Grey
To My Beloved Family ♥

تقدّيم به خانواده نازنينم ♥
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III Delforoush M, Sun C, Strömberg T, Strese S, Enblad G & Linder S, Gullbo J. Inhibition of the 19S proteasome by bAP-15 in lymphoma cell lines. *Manuscript*

IV Delforoush M, Berglund M, Edqvist P-H, Sundström C, Gullbo J, Enblad G. Expression of possible targets for new proteasome inhibitors in diffuse large B-cell lymphoma. *Submitted for publication*

Reprints were made with permission from the respective publishers.
Contents

Introduction ............................................................................................. 15
Normal lymphatic system ........................................................................ 15
Functions of the lymphatic system .......................................................... 16
Bone Marrow ........................................................................................ 17
Lymph nodes ......................................................................................... 17
B-Lymphocytes ...................................................................................... 17
Normal lymphocyte development ......................................................... 17
Cellular origin of B-cell lymphomas ...................................................... 19
Malignant lymphomas ........................................................................... 20
Diffuse large B-cell lymphoma .............................................................. 20
Treatment of DLBCL in an international perspective ............................ 21
Chemotherapy ....................................................................................... 21
Immunotherapy ..................................................................................... 22
Radiotherapy ......................................................................................... 22
CNS-prophylaxis .................................................................................. 22
Salvage therapy .................................................................................... 23
Oncogenic mechanisms and potential targets in DLBCL ....................... 23
New therapeutic approaches ................................................................. 26
Hodgkin lymphoma ............................................................................. 28
Treatment of Hodgkin lymphoma in an international perspective ......... 28
Radiation therapy .................................................................................. 28
Chemotherapy ....................................................................................... 29
Other treatment ................................................................................... 30
Drug resistance in cancers .................................................................... 30

New alternative approaches for lymphoma treatment ......................... 31
IGF-1R Inhibitors ................................................................................ 31
Picropodophyllin (PPP) ........................................................................ 31
Alkylating agents and cancer therapy .................................................. 32
Melflufen ............................................................................................. 34
Proteasome structure and function ....................................................... 35
Potential of 26S proteasome inhibition in cancer therapy ...................... 37
b-AP15 .............................................................................................. 38
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Activated B-Cell - like</td>
</tr>
<tr>
<td>ADRM-1</td>
<td>Adhesion Regulating Molecule 1</td>
</tr>
<tr>
<td>ASCT</td>
<td>Autologous Stem Cell Transplantation</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-Cell Lymphoma 2</td>
</tr>
<tr>
<td>BCL6</td>
<td>B-Cell Lymphoma 6</td>
</tr>
<tr>
<td>BCR</td>
<td>B-Cell Receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt's Lymphoma</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton’s Tyrosine Kinase</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>CP</td>
<td>Core Particle</td>
</tr>
<tr>
<td>CSR</td>
<td>Class-Switch Recombination</td>
</tr>
<tr>
<td>DHL</td>
<td>Double-Hit Lymphoma</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse Large B-Cell Lymphoma</td>
</tr>
<tr>
<td>DPL</td>
<td>Double-Protein-expression Lymphomas</td>
</tr>
<tr>
<td>DUBs</td>
<td>Deubiquitinating enzymes</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>FL</td>
<td>Follicular Lymphoma</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric Microculture Cytotoxicity Assay</td>
</tr>
<tr>
<td>FOXP1</td>
<td>Forkhead box-P1</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal Center</td>
</tr>
<tr>
<td>GCET1</td>
<td>Germinal Center B-Cell Expressed Transcript 1</td>
</tr>
<tr>
<td>HDC</td>
<td>High Dose Chemotherapy</td>
</tr>
</tbody>
</table>
HL Hodgkin Lymphoma
HSC Hematopoietic Stem Cells
Hsp70B’ Heat shock protein 70B’
Ig Immunoglobulin
IGF Insulin-Like Growth Factor
IPI International Prognostic Index
LDH Lactico Dehydrogenase
LMO2 LIM domain only 2
LPL Lymphoplasmacytic Lymphoma
LT-HSC Long-Term Haematopoietic Stem Cell
MCL Mantle Cell Lymphoma
MDM2 Murine Double Minute 2
MM Multiple Myeloma
MPP Multipotent Progenitor cells
MUM1 Multiple Myeloma oncogene 1
MZBCL Marginal Zone B-Cell Lymphoma
NF-κB Nuclear Factor Kappa B
PARP Poly ADP-Ribose Polymerase
PET FDG-Positron Emission Tomography
PD-1 Programmed cell Death 1
PD-L1 Programmed cell Death Ligand 1
PMBL Primary Mediastinal B-cell Lymphoma
PPP Picropodophyllin
PSMB5 Proteasome Subunit β5
PTC Peptichemio
RBC Red Blood Cell
RT Radiotherapy
SHM Somatic Hypermutation
S-LDH Serum Lactate Dehydrogenase
SLL Small Lymphocytic Lymphoma
ST-HSC Short-Term Haematopoietic Stem Cell
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD</td>
<td>T-cell Dependent</td>
</tr>
<tr>
<td>TI</td>
<td>T-cell Independent</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine Kinase Inhibitor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>UB</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UCHL5</td>
<td>Ubiquitin C-terminal Hydrolase L5</td>
</tr>
<tr>
<td>USP14</td>
<td>Ubiquitin Specific Peptidase 14</td>
</tr>
</tbody>
</table>
Introduction

Lymphomas are a group of malignant tumours in which cells of the lymphatic system grow uncontrollably and become abnormal. Lymphomas can start in almost any organ of the body since there is lymph tissue all over the body [1]. In 2014, 1871 new lymphoma cases were registered in Sweden, giving an incidence rate of 38.7 cases per 100 000 and it is the 8th most common cancer (both in men and women) in Sweden [2].

The WHO classifies Lymphomas as to B-cell lymphoma, T-cell lymphoma and Hodgkin lymphoma (HL) [3]. Approximately 85% of all lymphomas occurring in the Western world are B-cell lymphoma with the incidence of T-cell lymphoma and Hodgkin lymphoma less common. Of the B-cell lymphomas, most common are chronic lymphatic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). The current study focuses primarily on DLBCL and HL where chemotherapy is intensively employed as a treatment modality.

Normal lymphatic system

The spleen, thymus, lymph nodes, bone marrow and the lymphatics (small connecting vessels linking the lymph nodes and connecting with the circulatory system to return excess fluid into the circulation) comprise the lymphatic system, crucial in the body’s defense against infection and cancer. Figure 1 shows the distribution of lymph nodes throughout the body with clustering about the groin, neck and armpits. The nodes contain specific white blood cells known as lymphocytes. In a widened portion of the thoracic duct known as the cisterna chyli, fluid from several lymph-collecting vessels is received. Two main ducts, the right and thoracic, drain lymph fluid into the common iliac veins that merge to form the inferior vena cava and thus return lymph fluid to the central circulation. Crucial in the fight against infection, the spleen also removes and destroys worn out red blood cells while haematopoiesis takes place in the bone marrow of the central skeleton [4].
Functions of the lymphatic system

Clonal production of immunocompetent lymphocytes and macrophages, specific to the immune response, takes place in the bone marrow, lymph nodes and other lymphoid tissues. Moreover, maintenance of extracellular...
pressure and fluid volume is via the lymph system as it returns excess water and dissolved substances from the interstitial fluid to the circulation [4].

Bone Marrow

Via a process known as haematopoises, the cells of the immune system are initially derived from the bone marrow. It is during this process that stem cells derived in the bone marrow will differentiate into mature stem cells of the immune system or will migrate out of the bone marrow and continue to mature elsewhere. Granulocytes, immature thymocytes, B cells, natural killer cells, red blood cells and platelets all are produced in the bone marrow. The formation of tumors may often originate from the transformation of normal stem cells. The regulation of self-renewal in both stem cells and cancer cells may occur via similar signaling pathways and cancer cells may include cancer stem cells [4].

Lymph nodes

Located along the lymphatic vessels throughout the body are small organs known as lymph nodes. Varying in size (~0.1 - 2.0 cm) they cluster in areas of converge-lymph vessels. These nodes are the filters through which lymphocytes percolate into the blood system [4].

B-Lymphocytes

Being partially mature in the bone marrow, B cells complete their maturation in secondary lymphoid organs and differentiate and undergo rearrangement of the immunoglobulin genes to be able to produce antibodies [5].

Normal lymphocyte development

In bone marrow, long-term haematopoietic stem cells (LT-HSC) reconstruct the entire haematocyte system. Having the ability of self-renewal, LT-HSC differentiate into short term HSC (ST-HSC) and further into multipotent progenitor cells (MPP). Through a series of intermediate steps, MPP differentiate down to different paths [6]. One of these paths will end up to pro-B cells as the first irrevocably committed B-cell precursor. During these early stages of B-cell development, rearrangement of the B-cell receptor (BCR) starts. The rearrangement is a complex process. To achieve the heavy and light chains of a functional BCR, DNA double strand breaks are induced and
variable (V), diversity (D) and joining (J) gene segments are unify together [5]. Once the B-cell undergoes functional rearrangement the pre-B-cell receptor (pre-BCR) is expressed on the surface [7].

The first requirement of a functional BCR in a B-cell is the expression of a pre-B-cell receptor for further development towards a mature B-lymphocyte. Appropriate pre-BCR signaling leads the cells to the small-pre-B stage, and the rearrangement of the Ig light chain locus begins. An antigen-specific cell surface receptor is formed together with Igα and Igβ, the light and heavy chains respectively. Due to auto-reactivity, about 80% of immature B-cells succumb. Surviving B-cells leave the bone marrow and migrate to the spleen and transformed into transitional B-cells [8, 9]. Functional B-cells develop further into either marginal zone B cells (upon a TNF-α family B-cell activator receptor signaling) or mature naïve B-cells in the spleen [6, 10].

Naïve B-cells have the ability to interact with foreign antigen in either a T-cell dependent (TD) or T-cell independent (TI) manner. B-cells usually enter the follicle of secondary lymphoid organs (including lymph nodes) following TD activation. There, undergoing heavy clonal expansion, B-cells form structures called germinal centers (GCs).

In the next step of B-cell maturation, B-cells undergo somatic hypermutation (SHM) and class-switch recombination (CSR) in the GCs [11]. SHM produces point mutations in the Ig V gene to bring about antibodies with elevated affinities for the matching antigen while CSR is a process in which the constant region of the antibody is replaced to a secondary one, producing different antibody classes (IgA, IgG, IgE respectively). These processes finalize the production of high-affinity antigen-specific antibodies [12]. In the end, there are three possible fates for GC B-cells:

- apoptosis,
- going through further rounds of SHM and CSR
- differentiation into memory B-cells or long-lived plasma cells [11, 13] (Figure 2).
Cellular origin of B-cell lymphomas

Most of the recognized entities of B-cell lymphomas can be traced to particular stage(s) of B-cell differentiation according to the current model of lymphomagenesis. However further investigations to clarify the extent to which these malignancies maintain the molecular and physiological property of certain normal B-cell subsets is needed. Undergoing VDJ recombination, B-cells convey specific heavy and light chain IG gene rearrangement with or
without the presence of SHM. Each daughter cell will carry identical IG gene rearrangements whenever a B-cell goes through malignant transformation and clonal proliferation. The information on the origin and clonal history of the B-cell tumors is provided by IG gene rearrangements [15, 16]. Whether the cell of origin has undergone SHM and affinity maturation under the influence of antigens will be indicated by the presence of somatic mutations in the V region of the IG genes [15].

DLBCL and HL are examples of GC or post-GC derived B cell malignancies with somatically hypermutated IG genes [17-19].

Malignant lymphomas

Comprising a heterogeneous group of tumors, malignant lymphomas are tumors originating from transformed lymphoid cells. The WHO-classification defines these malignances according to postulated and known lineage and places them into three main categories: B-cell neoplasms, T and NK cell neoplasms and HL. Comprising some 85% of all malignant lymphomas are the B-cell neoplasms according to the American Cancer Society [20]. The major B-cell lymphoma types are as follows:

- Small lymphocytic lymphoma (SLL)
- Chronic Lymphotic Leukemia (CLL)
- Lymphoplasmacytic lymphoma (LPL)
- Follicular lymphoma (FL)
- Diffuse Large B-cell lymphoma (DLBCL)
- Burkitt’s lymphoma (BL)
- Mantle cell lymphoma (MCL)
- Marginal zone B-cell lymphoma (MZBCL) [20]

Diffuse large B-cell lymphoma

Worldwide, approximately 30% to 40% of all newly diagnosed malignant lymphoma cases are DLBCL [21]. There are two principally different ways in which DLBCL’s can arise; de novo, without a former history of an indolent lymphoma, or by transformation from an indolent lymphoma, most often FL [22, 23]. Common symptoms are enlarged lymph nodes, pruritus, night sweats and weight loss [15] and a significant risk factor for developing DLBCL is immunodeficiency which is commonly associated with EBV infection [24] and autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus) [25].
Based on recent studies DLBCL, with respect to the B-cell differentiation gene expression profile, can be divided into three subgroups; one with features of germinal center cells (GC-DLBCL), another which resembles activated B-cells (ABC-DLBCL) [26] and the third Primary Mediastinal B-cell Lymphoma (PMBL) [27]. Not only different with respect to their gene expression profiles, these subtypes depend on different oncogenic pathways and are distinguished by significant differences in overall survival following standard treatment [28]. To subdivide DLBCL into GC or non GC subtypes, Hans et al., described an algorithm which is identified by immunohistochemical stainings directed against CD10, BCL6 and MUM1 [29]. Berglund et al. has re-confirmed the possibility of using immunohistochemical stainings for DLBCL subdivision and prognostication [30]. However, this algorithm has later been shown to be of less value for patients treated with R-CHOP or similar [31, 32]. Furthermore, a new and improved algorithm has been demonstrated including immunohistochemical stainings against GCET1, CD10, IRF-4, BCL-6, MUM1 and FOXP1 [33]. Another algorithm with a slightly different approach is based on CD10, GCET1, MUM1, LMO2 and FOXP1 [34]. However a more simplified algorithm based only on MUM1 and FOXP1 has also been proposed [35].

Treatment of DLBCL in an international perspective

Continuous improvement in the treatment armamentarium, particularly the introduction of immunotherapy (anti-CD20, rituximab) and haemopoietic growth factors, still leaves differentiation and individualization of treatment based on a few rough clinical factors. Already described in 1993, the International Prognostic Index (IPI) is still forming the basis for the selection of treatment. Five factors comprise the IPI; stage III-IV disease, S-LDH (Serum Lactate Dehydrogenase) above the upper normal limit, age over 60 years, ECOG (Eastern Cooperative Oncology Group) performance status of 2 or above and more than one extranodal site. However, one of the most important factors is still age when it comes to choosing appropriate treatment regimen [36].

Chemotherapy

Since the first description of a combined chemotherapy regimen (Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone) (CHOP; Table 1) in 1978, several other regiments have been investigated [37, 38]. Despite the multitude of regimens tested, CHOP remains in the arsenal and is often used with generally tolerable side effects even in the elderly population. Slightly less than half of all patients are cured with the CHOP treatment [39]. More
intense chemotherapy treatment regimens have been developed recently which improve outcomes in younger patients and have thus been incorporated into the new treatment strategies [39, 40].

Table 1. CHOP chemotherapy. Reprinted by permission from [14].

<table>
<thead>
<tr>
<th>Drug Group</th>
<th>Drug</th>
<th>Major side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Cyclophosphamide (C)</td>
<td>Alkylating</td>
</tr>
<tr>
<td>H</td>
<td>Doxorubicin (H)</td>
<td>Anthracycline</td>
</tr>
<tr>
<td>O</td>
<td>Vincristine (O)</td>
<td>Vinca-alkaloid</td>
</tr>
<tr>
<td>P</td>
<td>Prednisone (P)</td>
<td>Cortico-steroid</td>
</tr>
</tbody>
</table>

**Immunotherapy**

Adding rituximab to the chemotherapy regime (R-CHOP) has significantly improved overall survival as well as progression free survival in DLBCL patients [41, 42]. Rituximab’s effect is mainly through chemo-/immune-sensitisation and cell death triggered by antibody dependent cytotoxicity, complement dependent cytotoxicity and apoptosis [43, 44]. The interaction of rituximab with several regulatory cell signaling pathways results in a synergistic action with chemotherapeutic drugs [44]. This effect is dependent upon tumor cells expressing CD20 which is necessary for the rituximab anti-CD20 antibody to express its effect [43]. However because of mechanisms not yet fully elucidated, not all patients respond [43].

**Radiotherapy**

The treatment of DLBCL has long included radiotherapy (RT) and while there is some evidence in the literature to support its use, it is not sufficient for a general recommendation. Yet there is debate regarding its (RT) significance [40, 45, 46]. Notable exceptions are primary DLBCL in the testis and central nervous system (CNS) where RT could play a role [40].

**CNS-prophylaxis**

The involvement of more than one extranodal site places DLBCL patients at a higher risk of relapse in the CNS [47]. Administration of CNS prophylaxis should be considered in this group [48] but it may not be optimal. Intrave-
Salvage therapy

After relapse following chemotherapy or in primary refractory patients, salvage chemotherapy followed by high dose chemotherapy with autologous stem cell transplantation (ASCT) is standard therapy. The choice of salvage chemotherapy differs. DHAP (dexamethasone, ara-C and cisplatin) has been the most frequently used regimen however phase II studies suggest ICE regimen (ifosfamide, carboplatin, etoposide) induces a higher rate of remissions [50]. However, in a randomized study no difference between these regimens could be shown but patients in the GC group had a better survival rate when treated with R-DHAP [51, 52].

For patients responding to salvage therapy, high-dose chemotherapy (HDC) followed by ASCT [53] is performed. Two regimens which are considered prior to ASCT are BEAC (BCNU, etoposide, cytarabine, and cyclophosphamide) and BEAM (BCNU, etoposide, cytarabine, and melphalan) [54].

Even though complete remission and an increase in overall survival are achieved, the prognosis for patients who relapse after R-CHOP based therapy is poor [55]. Further development of new approaches in the management of DLBCL and improvement in the outcome is of importance [56, 57].

Oncogenic mechanisms and potential targets in DLBCL

Various oncogenic pathways in DLBCL have recently been identified and could be possible targets for future therapy (Table 2) [58].

In recent years several studies have shown that the presence of rearrangement of the MYC gene (MYC-R), an oncogene involved in the pathogenesis of BL and DLBCL, results in poor prognosis following treatment with R-CHOP [59-61]. Forming a heterodimer with MYC-associated factor X, the transcribed MYC protein binds to the promoter region of its target genes. It regulates the transcription of 10 - 15% of genes, involved in diverse functions, such as cell proliferation, cell growth and DNA replication. Also apoptosis and micro-RNAs are regulated by MYC [62-64]. Not binding to promoters of silent genes, MYC activation only stimulates the already active program in a cell, explaining why the effects of high MYC expression are diverse in some cell types [64]. According to published studies in 7% to 21% of DLBCL cases, MYC-R is observed. A negative predictor of survival in DLBCL patients is the MYC-IG translocation partner gene and thus a po-
tential targeted therapy acting on the MYC oncogenesis pathway should be further investigated [65].

As therapeutic targets in GC DLBCL, the apoptosis regulator B-cell lymphoma 2 protein (BCL-2) and the zinc finger transcription factor-BCL6 are often upregulated in DLBCL. There are ongoing investigations on inhibitors targeting these proteins [66]. Earlier studies of BCL2 inhibitors have shown toxicity due to BCL-XL blockade, however newly developed agents show more promising results with fewer side effects [67-69]. In investigations on BCL6 inhibitors, reduction in the size of the tumors was also observed in mice models [70, 71].

A subgroup of aggressive lymphomas with both MYC and BCL2 gene rearrangements called Double-hit lymphoma (DHL) is characterized by a rapidly progressing clinical course which is refractory to aggressive treatment and short survival. It includes diffuse large B-cell lymphoma with MYC translocation combined with another translocation involving BCL2 or BCL6. Some cases were recently characterized as immunohistochemical double-hit lymphomas (ie, double-protein-expression lymphomas [DPLs]) which have a similar clinical course with further overexpression of MYC or BCL2 proteins. Patients with genetic or immunohistochemical double-hit lymphomas have a poor prognosis with the present treatment [72].

Protein 53 (p53), a protein named after its molecular weight of ~53 kDa, is a transcription factor regulating the expression of a large group of genes. Ubiquitylation and proteasomal degradation are involved in production of p53 protein under normal conditions at low levels. Several E3 ubiquitin ligases are mediating ubiquitylation but the main one is the Murine Double Minute 2 (MDM2) ligase. DNA damage, nucleotide depletion and hypoxia are some of the cellular stress signals to which p53 is very sensitive. However, p53 is stabilized and undergoes post-translational changes and exerts its multi-faceted effect while not responding to stress signals, including regulation of autophagy, senescence or apoptosis, glucose metabolism and mitochondrial respiration. Cell cycle arrest in G1 and/or G2 phase can be induced by high levels of accumulated p53. TP53 (the p53 gene) is mutated or deleted in about 50% of all human cancers, while in many tumors with wild type TP53 other regulatory mechanisms of the p53 pathway are corrupted. Multiple studies have demonstrated that poor prognosis in lymphomas are associated with mutations in the TP53, especially in the GC subtype, whereas some studies have failed to demonstrate this. However in haematological malignancies compared to solid tumours, mutations in the p53 gene are less common and in only 10-20% of all tumours in B-cell lymphomas, TP53-mutations occur. Here, other disruptions of the p53 pathway, such as the key regulatory factor MDM2, may influence p53 function [14].

A major characteristic of ABC DLBCL is the constitutive activation of the NF-κB pathway in which targeting shows therapeutic potential [73, 74]. An important set of molecular factors are involved in NF-κB pathway, lead-
ing to proliferation, invasion and metastasis [74]. In the ABC subtype of DLBCL, loss of A20 and mutations in CD79A/B, MYD88 and CARD11 are often associated with a constitutively active NF-κB [75]. Targeting of the Bruton’s tyrosine kinase (BTK) by Ibrutinib is not toxic in patients with CARD11 or MYD88 mutations alone, since they are pathway members downstream of BTK, but is highly toxic in ABC DLBCL patients with mutations in CD79A/B upstream of BTK [76, 77]. Other patients that could also potentially benefit from Ibrutinib treatment are those who have mutations in other upstream pathway members of the BCR/NF-κB, such as SYK and LYN [69].

Being important for different processes relevant to cancer, PI3K/AKT/mTOR signaling is linked to the BCR/NF-κB pathway through SYK and CD79 [78, 79]. Using SYK inhibitors seems promising and it is under investigation [80]. Another potent mechanism for the constitutive activation of the PI3K/AKT/mTOR pathway in DLBCL is mutations of the PTEN tumor suppressor gene which are one of the hallmarks of GC DLBCL [81, 82]. Additional classes of drugs targeting this pathway (molecules that block PI3K signaling, Akt inhibitors and mTOR inhibitors) have been developed and are under clinical investigation [83-86].

One of the immune checkpoint pathways with a great potential, having a key role in the maintenance of self-tolerance and control of excessive immune responses, is the programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway. These pathways are utilized by cancer cells to suppress anti-tumour immune responses and to evade immune surveillance [87]. Activated T cells, B cells and natural killer cells express PD-1, and immune cells (including macrophages, dendritic cells and lymphocytes) and non-immune cells (including tumour cells) express PD-L1 [88, 89]. Beneficial therapeutic effects in solid tumours have been recently demonstrated by PD-1 and PD-L1 blockade and appear to be a highly promising anti-cancer strategy. Therapeutic activity of PD-1 blockade has also been shown in haematolymphoid malignancies, including HL and FL, as well as potential utility in DLBCL in early clinical trials [90].
Table 2. Oncogenic mechanisms and potential targets in DLBCL subtypes. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology [58], copyright 2014.

<table>
<thead>
<tr>
<th>DLBCL subtype</th>
<th>Cell of origin</th>
<th>Oncogenic mechanisms</th>
<th>Potential targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCB</td>
<td>Germinal centre B-cell</td>
<td>BCL2 translocation, EZH2 mutations, PTEN deletions</td>
<td>BCL6, EZH2, PI3K/Akt</td>
</tr>
<tr>
<td>ABC</td>
<td>Post-germinal centre B-cell</td>
<td>NF-κB activation, CARD11 mutations, MYD88 mutations, CD79B mutations, A20 deletions</td>
<td>BCR, CBM complex, IRAK-4, JAK–STAT</td>
</tr>
<tr>
<td>PMBL</td>
<td>Post-thymic B-cell</td>
<td>NF-κB activation, 9p24 amplification, REL amplification, JAK2 mutations, CIITA translocations</td>
<td>JAK–STAT, PD-1</td>
</tr>
</tbody>
</table>

New therapeutic approaches

Rituximab has a crucial role on the treatment and the outcome of DLBCL. It bonds traditional chemotherapy to advanced therapies aiming at particular targets on tumor cells [91]. Although the majority of patients with DLBCL can be cured with R-CHOP, a growing concern is there for those whom fail R-CHOP or become rituximab resistant. Therefore, identifying unique molecular targets is of importance [21]. A number of molecules currently under evaluation are listed in Table 3.
Table 3. List of targeted therapies for DLBCL. Information derived from www.clinicaltrials.gov. Reprinted by permission from [69].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molecule class</th>
<th>Targeting feature</th>
<th>Status (FDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7438</td>
<td>EZH2 inhibitors</td>
<td>$EZH2$ activating mutations</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>ABT-199</td>
<td>BCL2 inhibitors</td>
<td>BCL2 overexpression</td>
<td>Phase I trial</td>
</tr>
<tr>
<td>Ibrutinib</td>
<td>BTK inhibitors</td>
<td>Constitutively active NF-κB pathway</td>
<td>Phase III trial</td>
</tr>
<tr>
<td>Entospletinib</td>
<td>SYK inhibitors</td>
<td>Constitutively active NF-κB pathway</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>BKM120</td>
<td>PI3K blockade</td>
<td>PI3K/Akt/mTOR activation</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>MK2206</td>
<td>Akt inhibitors</td>
<td>PI3K/Akt/mTOR activation</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>Everolimus</td>
<td>mTOR inhibitors</td>
<td>PI3K/Akt/mTOR activation</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>Veliparib</td>
<td>PARP1 inhibitors</td>
<td>PARP1 in tumors with HR defects</td>
<td>Phase II trial</td>
</tr>
<tr>
<td>SGN-CD70A</td>
<td>CD70 blockade</td>
<td>CD70 – CD27 signaling</td>
<td>Phase I trial</td>
</tr>
<tr>
<td>-</td>
<td>CD27 agonists</td>
<td>CD70 – CD27 signaling</td>
<td>Not investigated</td>
</tr>
<tr>
<td>Pembrolizumab, Nivolumab, Pidilizumab Atezolizumab</td>
<td>PD-1 blockade</td>
<td>PD-1 – PD-L1/2 signaling</td>
<td>Phase II trial</td>
</tr>
<tr>
<td></td>
<td>PD-L1 blockade</td>
<td>PD-1 – PD-L1 signaling</td>
<td>Not investigated</td>
</tr>
<tr>
<td>RO4929097</td>
<td>γ-secretase inhibitor</td>
<td>Notch signaling</td>
<td>Phase I trial</td>
</tr>
</tbody>
</table>
Hodgkin lymphoma

The malignant disorder Hodgkin lymphoma evolves from the lymphoid tissue. Approximately 200 people are diagnosed with HL in Sweden every year with many young adults among them [2]. In the early stages short time prognosis is excellent. However with a primary progressive or relapsed disease the prognosis is poorer and many young patients will succumb [92]. Many patients suffer from severe secondary complications of intensive chemotherapy and/or RT [93, 94]. Thus the search for new prognostic markers continues in an effort to further optimize treatment. The goal being to strike a balance between enough treatment to effect a cure while minimizing complications and unnecessary suffering. There is also an effort to develop less toxic drugs that act synergistically with conventional treatment modalities and to avoid the possible evolution of drug resistant tumor cells. Most patients with limited or untreated HL possess a defect in cell mediated immunity which leaves them susceptible to infections. Whether the observed impaired immunity results from HL or constitutes a predisposition to it, is unclear [95].

Treatment of Hodgkin lymphoma in an international perspective

Throughout the world treatment varies but generally speaking, patients in low stages (I and II) receive a combination of a few regiments of chemotherapy combined with RT. On the other hand patients in advanced stages (III and IV) are treated with several courses of CT. Recently the trend in treatment has been toward a more individualized approach guided by FDG-PET [96] with the aim of providing sufficient cure while avoiding many of the possible complications [97].

Radiotherapy

The first curative treatment, RT, was developed by Peters in 1958 and Kaplan in 1966 [98, 99]. Under their protocols RT was given to large fields covering both tumors and nearby unaffected tissue. In later years the protocol called for smaller fields only covering the involved lymph node region in order to reduce side effects. Today RT directed only at the involved lymph node itself is being tested [100].
Chemotherapy

While single cytostatic drugs are known to have an effect on HL, it was not until the mid-1960s that the combination 4 drugs came into use. Mechloretamine, vincristine, procarbazine and prednisone (the MOPP regime) revolutionized the treatment of advanced HL [101]. An alternative regime, ABVD, was introduced in 1975 (Table 4) which contains “non-cross resistant” drugs yet shows effects similar to MOPP [102]. Continued research revealed that ABVD had fewer secondary complications than MOPP or the combination MOPP/ABVD [102]. Since then alternative regimes have been developed such as BEACOPP (Table 5) [103]. Today the chemotherapies most widely used are ABVD and BEACOPP. In 2003 an increased survival benefit for BEACOPP over COPP/ABVD in advanced stages of HL was demonstrated [104, 105].

Table 4. ABVD chemotherapy. Reprinted by permission from [106].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group of drug</th>
<th>Major side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
<td>Hematologic, cardiac</td>
</tr>
<tr>
<td>Bleomycin (B)</td>
<td>Other</td>
<td>Pulmonary</td>
</tr>
<tr>
<td>Vinblastine (V)</td>
<td>Vinca-alkaloid</td>
<td>Hematologic</td>
</tr>
<tr>
<td>Dacarbazine (D)</td>
<td>Alkylating</td>
<td>Hematologic</td>
</tr>
</tbody>
</table>

Table 5. BEACOPP chemotherapy. Reprinted by permission from [106].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Group of drug</th>
<th>Major side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin (B)</td>
<td>Other</td>
<td>Pulmonary</td>
</tr>
<tr>
<td>Etoposide (E)</td>
<td>Topoisomeras II inhibitor</td>
<td>Haematologic</td>
</tr>
<tr>
<td>Doxorubicin (A)</td>
<td>Anthracycline</td>
<td>Cardiac, haematologic</td>
</tr>
<tr>
<td>Cyclophosphamide (C)</td>
<td>Alkylating</td>
<td>Haematologic, nausea</td>
</tr>
<tr>
<td>Vincristine (O)</td>
<td>Vinca-alkaloid</td>
<td>Nausea, neuropathy</td>
</tr>
<tr>
<td>Procarbazine (P)</td>
<td>Alkylating</td>
<td>Haematologic</td>
</tr>
<tr>
<td>Prednisone (P)</td>
<td>Cortico-steroid</td>
<td>Endocrine-diabetes etc</td>
</tr>
</tbody>
</table>
Other treatment

For primary refractory patients or patients experiencing relapse, the same second-line salvage regimens as in DLBCL are used followed by HDC and ASCT. For patients who relapse after ASCT, allogeneic SCT is the only strategy with a curative potential but still it’s role in HL remains controversial [107]. For this group of patients, new therapeutic strategies are being tested. One recent development is the chemotherapy-antibody conjugate brentuxumab-vedotin which has shown very promising results [108]. Another treatment approach which seems very promising is targeting the immune checkpoint pathways and checkpoint blockade in HL treatment [109].

Drug resistance in cancers

The increasingly sophisticated design of cancer chemotherapy has yet to yield a treatment regime that is 100% effective against disseminated cancer. Individual resistance to anticancer drugs or to the treatment with them may result from a variety of factors including variations in patients or in the somatic cell genetic differences in the tumors themselves. Even in those of the same tissue origin there may exist variation [110]. There are many reasons for acquired resistance to a broad range of anticancer drugs but probably most common is the expression of one or more energy-dependent transporters that detect and eject the drugs from the cells. Other mechanisms exist such as insensitivity to drug induced apoptosis and/or drug-detoxifying mechanisms. Research on acquired drug resistance continues to yield important information on methods to circumvent the problem in order to improve cancer chemotherapy [111]. Despite significant recent advancements made, the major cause of treatment failure of DLBCL remains drug resistance [112]. R-CHOP has resulted in a dramatic improvement in outcome with most patients responding to it [113, 114]. However, with regard to DLBCL, the search for potential mechanisms of drug resistance is of particular importance [114].
New alternative approaches for lymphoma treatment

IGF-1R Inhibitors
Due to its central role in cancer cell signaling and overexpression in many cancers, IGF-1R has become an attractive clinical target over the last decade [115, 116]. Various monoclonal antibodies and tyrosine kinase inhibitors (TKIs) against IGF-1R are currently under investigation in the treatment of solid tumors [117]. Most antibody-based IGF-1R inhibitors function by blocking IGF-ligand binding to the receptor, blocking intracellular signaling primarily through the PI3K/AKT pathway and by decreasing expression of IGF-1R at the cell surface by mediating receptor internalization. Positive responses to IGF-1R therapies have encouraged further research into biomarkers that will identify the optimal target population [118].

Picropodophyllin (PPP)
A member of the cyclolignan family, Picropodophyllin (PPP), has been described as an inhibitor of IGF-1R. PPP can apparently discriminate between the IGF-1R and the insulin receptor [119]. It has been thought that PPP inhibits IGF-1R by utilizing the MDM2 E2 ligase, causing its down regulation [120] and in several human cancer models inducing tumor regression and inhibition of metastasis [121]. In this observation and also in another study it has been shown that the anti-tumor effects of PPP could possibly be governed via inhibition of microtubules (during assembly of the mitotic spindle) and do not result from targeting the IGF-1R. However, further investigations are needed to understand more in detail such an IGF-1R-independent mechanism(s) [122, 123].
Alkylating agents and cancer therapy

Alkylating agents as one of the earliest and most commonly used anticancer drugs have been used for the treatment of cancer for over six decades. During the early 1940s their documented use in cancer therapy began. Having dual natures, most of these agents are capable of great destruction and even greater healing. A remarkable example of these double edged swords that lead to the discovery of its potential for cancer treatment by Dr Cornelius Packard Rhoads, is the discovery of nitrogen mustard used during World War II for military purposes [124]. The mechanism of action is similar in most alkylating agents but differs in their clinical efficacy. Alkylating anticancer drugs are effective during all phases of cell cycle and a wide variety of cancers are treated by using them [125]. Acting directly on DNA results in crosslinking and DNA strand breaks and abnormal base pairing which inhibits cell division and eventually result in cell death [124, 126].

The limiting factors for alkylating agents’ clinical efficacy are systemic toxicity and drug resistance. Several unfavorable side effects do occur in long-term use including permanent infertility; decreasing sperm production in males and causing menstruation cessation in females. Some alkylating agents can also lead to secondary cancers years after the therapy, such as acute myeloid leukaemia. Therefore, intense efforts is being placed on the development of combinations of alkylating agents with other anticancer agents or inhibitors of DNA repair enzymes, topoisomerases, COX-2, p34 cdc2 kinase, phosphatases, multi-drug resistance proteins, anti-vascular agents or natural products such as aqueous extract of walnut bark (Juglans
regia L.), to improve their clinical efficacy and minimizing the toxic side effects [127-132].

Alkylating agents are reacting with only one DNA strand (monofunctional) or reacting with an atom on each of the two DNA strands to crosslink the strands covalently (bifunctional) [133]. Alkylating agents have three different mechanism of action which leads to disruption of DNA function and ultimately cell death (Figure 4):

- In the first mechanism of action, alkylating agents cause DNA damage by involvement in formation of cross-bridge bonds between atoms in the DNA. In this process, bifunctional alkylating agents link together two bases and the crosslinking makes DNA strands inseparable for synthesis or transcription (Figure 4A).
- A second mechanism by which alkylation can cause mutations due to mispairing of the nucleotides leading to mutations (Figure 4B).
- The third mechanism of action of alkylating agents is by attaching alkyl groups to DNA bases, resulting in fragmentation of DNA during the DNA repair enzymes attempts to replace the alkylated bases (Figure 4C).

Figure 4. Mechanism of action of alkylating agents. Modified from Expert Opinion on Therapeutic Patents [124], copyright 2007.
Alkylating agents are generally separated into six classes:

- alkyl sulfonates: busulfan
- ethyleneimines and methylmelamines: hexamethylmelamine or altretamine
- nitrogen mustards: mechlorethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil
- nitrosoureas: carmustine, lomustine
- triazenes: dacarbazine, procarbazine, temozolomide
- the platinum-containing antineoplastic agents: cisplatin, carboplatin, oxaliplatin [124].

Melflufen

In the end of the seventies, Peptichemio (PTC) was synthesized as a cocktail of six interesting sarcolysine (a stereoisomer of melphalan) containing peptides and it was studied in several preclinical and clinical trials, before the production stopped in the mid-eighties [134]. Peptides of PTC were individually studied and it was discovered that the most effective of the six and even more effective than melphalan was the tripeptide L-prolyl-m-L-sarcolysin-p-L-fluorophenylalanine ethyl ester (P2) [135-137]. As an intermediate of melphalan and P2, the dipeptide melflufen (L-melphalanyl-p-L-fluorophenylalanine ethyl ester, previously called J1) was synthesized, and soon chosen to be the leading compound [138]. The mechanism of action is targeted alkylation of tumor cell DNA. Melflufen is highly lipophilic and rapidly enters into the cells. Hydrophilic melphalan is released when peptidases that are overexpressed in the cytoplasm of most malignant cells, cleave melflufen. Melflufen showed 50- to 100-fold higher cytotoxicity compared with that of melphalan (measured by IC50) in studies of 20 different human cancers in vitro [139].
Proteasome structure and function

Proteasomes are large non-lysosomal, multienzyme complexes (ca. 2.4 MDa). They are found in all eukaryotes, archaea and in some bacteria. They are constitutively present in the nucleus and cytoplasm of all eukaryotic cells and form the principal pathway of degradation of intracellular proteins, unfolded and misfolded proteins. Recent research has demonstrated that proteasomes enter the nuclei through nuclear pores. The 26S proteasome complex contains two 19S regulatory subunits and a single 20S core catalytic subunit [140]. This 20S catalytic subunit consists of a 28 subunit core and two outer and inner heptameric rings arranged axially to form a hollow center core [141]. The two outer rings are known as the α subunit and consist of seven polypeptides which are a part of the structural makeup of the 20S proteasome. Also made up of seven polypeptides, the two inner rings are known as β subunits (figure 6). The central core chamber, consisting of three catalytic subunits, is formed by the β subunits:

- β1 has chymotrypsin like activity i.e., specific for peptides with tyrosine and phenylalanine at the carboxyl position.
- β2 has trypsin like activity i.e., specificity for peptides having arginine or lysine at the carboxyl position.
- β5 has post-glutamyl peptidyl hydrolytic-like activity since it cleaves the peptides after acidic residues like aspartate or glutamate [140].
Seven different genes encode the seven subunits of the $\alpha$ and $\beta$ subunits. These two subunits are assembled in a specific manner so as to impart selectivity in function to the 20S proteasome [141]. The 19S regulatory subunit is a 700 kDa complex consisting of 20 different polypeptides. Present at both ends of the 20S proteasome, the 19S complex serves two main functions: it acts as a lid that must be opened to allow entry of proteins into the catalytic core (20S) for subsequent degradation, and it regulates the unfolding of polyubiquitinated proteins [140]. Along with other polypeptides, the 19S regulatory unit consists of six different ATPases. Conformational change in the 19S subunit that opens the lid that covers the 20S requires ATP hydrolysis thus facilitating entry of intracellular proteins into catalytic core [141].

The first step in the degradation pathway of unwanted proteins is polyubiquitination, i.e., tagging of proteins with the protein molecule ubiquitin (Ub). Consisting of 76 amino acids, ubiquitin, a small protein, is highly conserved in the eukaryotes. Synthesized as a precursor form it requires modification by deubiquitinating enzymes (DUBs) like Ubiquitin Specific Peptidase 14 (USP14) and Ubiquitin C-terminal Hydrolase L5 (UCHL5), at the C-terminal end in order to expose the substrate conjugation site i.e., glycine carboxylate [142]. Ubiquitin has seven lysine residues that are involved in polyubiquitination and it is covalently conjugated to protein substrates. This process involves three different ubiquitin associated enzymes, E1, E2 and E3. In the presence of ATP, E1 (ubiquitin activating enzyme) binds with ubiquitin to activate it. E2 (ubiquitin carrier enzyme) transfers the Ub molecule from E1 to E3, an ubiquitin ligase enzyme [142]. Imparting specificity to the whole process are the many different subtypes of E3 that are available to bind to specific substrates targeted for degradation. Coordination between E2 and E3 allows for the transfer of the ubiquitin molecule to the lysine residue of the substrate via an isopeptide bond. The three ubiquitin-associated enzymes repeat this process several times leading to poly-ubiquitination of the target protein. The 19S subunit recognizes these polyubiquitinated proteins and they are processed for degradation [140]. It can be stated that the 26S proteasome functions are regulated by the energy and ubiquitination state of proteins in the cell. If the above would be stated as a rule, then some exceptions exist. In mammalian cells it has been noted that different regulatory subunit, 11S bind to the 20S subunit to form a 26S complex (figure 6). This complex also has protein degradation activity though in an energy and ubiquitination independent manner [141].

36
Potential of 26S proteasome inhibition in cancer therapy

The proteasomes, known to regulate the endoplasmic reticulum and cell homeostasis, are considered to be an effective target for cancer therapy. Their importance is much more pronounced in immunoglobulin producing multiple myeloma (MM), Waldenstrom macroglobulinemia cells or other similarly highly secretory cells. In such cells the disruption of normal proteasome function leads to accumulation and aggregation of large amounts of misfolded and/or unfolded proteins which can prove detrimental to the cell by causing proteotoxicity [143]. Several different proteasome inhibitors have been tested but the first to reach clinical trials was the dipeptidyl boronic acid small molecule bortezomib (Velcade®). Boretzomib is now an established therapy for multiple myeloma however concerns have been raised regarding the drug’s toxicity presenting as thrombocytopenia and as periph-

Figure 6. Composition and organization of the proteasome and their intracellular activators. Modified from Nature Reviews Neuroscience [141], copyright 2001.
eral neuropathy (incidence ≥ 30%). Inhibitor specificity and drug resistance in cases of prolonged treatment were also of concern [144].

Both the success and associated concerns with bortezomib use have lead many researchers to look for novel proteasome inhibitors. An entirely new class of proteasome inhibitors was identified in 2010 termed the syrbactins. Structurally distinct from known proteasome inhibitors, this class was shown to bind and inhibit the eukaryotic proteasome in a novel way [145]. Later on it was reported that TIR-199, a natural product-derived syrbactin structural analog, should be further evaluated for development into a clinical drug for treatment of multiple myeloma and other cancers [146].

For nine years bortezomib continued to be the only drug approved by the FDA for the treatment of multiple myeloma. Recently a second-generation proteasome inhibitor, carfilzomib, was approved by the FDA. Carfilzomib has a superior toxicity profile and demonstrated activity in the relapsed and refractory settings [147].

In 2013 a novel 19S regulatory particle inhibitor, b-AP15, was investigated which was shown to selectively block deubiquitylating activity of USP14 and UCHL5 without inhibiting proteasome activity. In both MM cell lines and patient MM cells, b-AP15 decreases viability. Even in the presence of bone marrow stromal cells, it has been demonstrated that b-AP15 inhibits proliferation of MM cells and overcomes bortezomib-resistance [147].

b-AP15

b-AP15 induces anti-tumor effects both in vitro and in vivo. It is a novel small molecule inhibitor of the proteasome, which blocks proteasome function via inhibition of deubiquitinating enzymes, UCHL5 and USP14. In syngeneic and xenograft mouse models, b-AP15 exhibited potent antitumor activity in both solid and leukemic malignancies. Tumor regression, reduced tumor growth and delayed tumor onset were demonstrated [148]. b-AP15 also appears to increase chaperone expression and induces rapid apoptotic response which is associated with strong induction of oxidative stress with rapid uptake and enrichment of the drug into the cells [149].

b-AP14 blocks aggresome formation and induces apoptosis insensitive to the over expression of Bcl-2. It has therefore been suggested that bortezomib-mediated resistance could be overcome by b-AP15 treatment. It is also more toxic to cancer cells than to immortalized normal cells with larger differences than those observed with bortezomib. In comparison, this suggests a more favorable therapeutic window. These findings bode well for b-AP15 as a good drug for clinical development [149].
Figure 7. The chemical structure of bAP-15.
Present investigation

Aims
The overall aim of the studies presented in this thesis was to investigate possible new drugs for the treatment of lymphomas and diffuse large B-cell lymphoma in particular and to investigate specifically:

- The activity of PPP (Picropodopyllin), Melflufen (J1) and b-AP15 to determine if they should be further evaluated as a drug in lymphoma treatment.
- The expression of possible targets for proteasome inhibitors in patients with DLBCL and to correlate the findings to clinical parameters and outcome.
Summary of papers

Paper I

*Picropodophyllin (PPP) inhibits proliferation and survival of diffuse large B-cell lymphoma (DLBCL) cells*

Background

Picropodophyllin (PPP) was originally presented as a selective inhibitor of the insulin-like growth factor-1 receptor (IGF-1R). Due to its overexpression in many cancers, the IGF-1R has been suggested as a promising molecular target in treatment of cancer [115]. However, the results from clinical trials using different modes of IGF-1R-targeted therapy have so far been disappointing [150]. In contrast, PPP has been well tolerated and showed clinical effect in a phase I/II study in lung cancer patients. Although PPP has been suggested as a potential therapy for the B-cell neoplasms, there were no reports investigating the potential benefits of using PPP against DLBCL [122]

Results & discussion

Four cell lines of diffuse large B-cell lymphoma together with primary tumor cells derived from lymph nodes in four DLBCL patients were used. PPP dose-dependently inhibited proliferation/survival in all cell lines and primary cell preparations. The observed, early effects of PPP on cell cycle phase distribution together with the lack of decreased phosphorylation of IGF-1R suggested that the anti-tumour effects of PPP did not primarily result from targeting the IGF-1R but could possibly be governed via inhibition of microtubules, in particular, during assembly of the mitotic spindle.

Taken together, the inhibitory effects of PPP in DLBCL cells with its low toxicity *in vivo*, makes it a promising drug candidate in the treatment of this disease. However, the anti-tumour effects of PPP might here be explained by other, possibly IGF-1R-unrelated mechanism(s). The novelty of the paper is the description of another alternative mechanism of action for PPP as probably not being an IGF-1R inhibitor.
Paper II

*In vitro and in vivo activity of melflufen (J1) in lymphoma*

**Background**

For almost 60 years, melphalan has been used in the treatment of various hematologic malignancies and today it is part of standard therapy for multiple myeloma but also as part of myeloablative regimens in association with stem cell transplantation in lymphomas. An optimized derivative of melphalan, providing targeted delivery of active metabolites to cells expressing aminopeptidases is called Melflufen (melphalan flufenamide ethyl ester, previously called J1) [151]. In a series of *in vitro* and *in vivo* experiments, performed preferably on different solid tumor models and multiple myeloma, the activity of melflufen was compared with melphalan. Melflufen is currently being evaluated in a clinical phase I/II trial in relapsed or relapsed and refractory multiple myeloma [152].

**Results & discussion**

Cytotoxicity of melflufen was assayed in twelve lymphoma cell lines and in primary tumor cells by Fluorometric Microculture Cytotoxicity Assay and in two of the cell lines, cell cycle analyses were performed. Melflufen was also investigated in a xenograft model with subcutaneous lymphoma cells inoculated in mice. Melflufen showed activity with cytotoxic IC50-values in the submicromolar range (0.011-0.92 µM) in the cell lines, corresponding to a mean of 49-fold superiority (p<0.001) in potency vs. melphalan. In the primary cultures melflufen yielded slightly lower IC50-values (2.7 nM to 0.55 µM) and an increased ratio vs. melphalan (range 13-455, average 108, p<0.001). A clear accumulation in the G2/M-phase of the cell cycle was exhibited in treated cell lines. Melflufen also showed significant activity and no or minimal side effects in the xenografted animals.

Our observation confirms previous reports of a superiority of melflufen compared to that of melphalan. Melflufen was active in cell lines and primary cultures of lymphoma cells, as well as in a xenograft model in mice and turns out to be a candidate for further evaluation in the treatment of this group of patients.
Paper III

_Inhibition of the 19 S proteasome by bAP-15 in lymphoma cell lines_

Background

Inhibition of the 26S proteasome, an attractive therapeutic target in the treatment of cancers, has been shown to selectively kill cancer cells [147]. Bortezomib, a proteasome inhibitor, is approved by FDA for the treatment of multiple myeloma and mantle cell lymphoma. However, patients treated with bortezomib show toxic side effects and moreover, eventually acquire resistance to the drug. Many efforts are currently been made to elaborate new proteasome inhibitors that behave through mechanisms different from that of bortezomib [153].

Results & discussion

We have studied the effects of b-AP15, a novel proteasome inhibitor of the 19S deubiquitinase activity, on a panel of nine cell lines from DLBCL and three cell lines from HL and compared the results to standard drugs used in treatment of DLBCL and HL. All cell lines showed a dose dependent reduction of viability. The inhibition of the proteasome function by b-AP15 was achieved through inhibition of the proteasome by accumulation of ubiquitinated proteins. Moreover, we have also shown that proteotoxic stress is the underlying mechanism of sensitivity of lymphoma cells to the deubiquitinase inhibitor b-AP15.

In conclusion this study show solid _in vitro_ effects of a new drug, bAP-15, a novel small molecule inhibitor of DUBs of the proteasome USP14 and UCHL5 in the 19S regulatory particle of the proteasome, on both HL and DLBCL cell lines. The activity is associated with accumulation of high-molecular weight polyubiquitin conjugates, increases in inducible form of heat shock protein 70B´ (Hsp70B´), and induction and cleavage of PARP and caspase-3. Further investigation of b-AP15 seems warranted based on these differences, while bortezomib has failed to demonstrate clinical activity among these patients. bAP-15 is currently used in Phase I/II studies on myeloma and if those turn out promising, we plan to perform studies on lymphoma patients.
Paper IV

*Expression of possible targets for new proteasome inhibitors in diffuse large B-cell lymphoma*

**Background**

Catalytic proteasome subunit β5 (PSMB5), within the 20S core particle (20S CP) of the proteasome, is selectively inhibited by the dipeptidyl boronic acid bortezomib. Changes in the structure and expression of PSMB5 are mechanisms involved in bortezomib resistance [154]. However, there are a few biomarkers predicting bortezomib resistance, and most of these markers include differentiation of the plasma cells [155]. New inhibitors of PSMB5 (e.g. carfilzomib), which are under development, overcome bortezomib resistance in multiple myeloma by binding irreversibly to PSMB [156]. However, studies on mechanisms of bortezomib resistance on different cancers are currently being performed on cell lines. To our knowledge, there is no study on the relation between PSMB5 expression and effect of bortezomib in vivo [157] and despite promising preclinical data, proteasome inhibitors like bortezomib have failed to demonstrate significant clinical benefit in DLBCL [153]. Furthermore, a receptor of the 26S proteasome subunit, adhesion regulating molecule 1 (ADRM-1), has been reported to be upregulated in various solid cancers and in acute leukaemia but its expression in lymphoma has so far not been investigated.

**Results & discussion**

Tumor material from ninety-two patients with DLBCL treated with either R-CHOP like (n=69) or CHOP like (n=23) regimens were stained for possible targets of proteasome inhibitors. The primary target molecule of bortezomib, PSMB5 was not detected in the tumor cells in any of the cases but showed an abundant expression in cells in the microenvironment (e.g. small lymphocytes). However, the DUBs of the proteasome, UCHL5 and USP14 were detected in the cytoplasm of the tumor cells in 77% and 74% of the cases, respectively. ADRM1 was detected in 98% of the cases. There was no correlation between the expression of any of the studied markers and clinical outcome or GC/non-GC phenotype.

The findings in this study suggest that UCHL5 and/or USP14 should be further evaluated as new targets for proteasome inhibitors in DLBCL. The lack of expression of PSMB5 on the tumor cells might provide an explanation of the relatively poor results of bortezomib in DLBCL. The novelty of the paper is that it is the first time that potential targets for proteasomes have been studied in lymphomas.
Conclusion & future perspectives

This thesis aimed to investigate possible new drugs in the treatment of lymphomas and in particular, diffuse large B-cell lymphoma. Furthermore, this thesis attempted to explore: the activity of PPP (Picropodopyllin), Melflufen (J1) and b-AP15 as new candidates for cancer treatment; the expression of possible targets for proteasome inhibitors in patients with DLBCL; to correlate the findings to clinical parameters and outcomes and to contribute to new approaches in the treatment of DLBCL.

PPP has been suggested as a potential therapy for the B-cell neoplasm multiple myeloma [121, 158] and for different classes of lymphoma [159-162]. However, there were no reports available on the investigation of the potential benefits of using PPP against DLBCL. IGF-1R has been suggested as a promising molecular target in treatment of cancer due to its overexpression in many different cancers [115, 116]. However, disappointing results from clinical trials using different modes of IGF-1R-targeted therapy and unacceptable toxicity when combined with traditional cytotoxic drugs [150], put PPP in the spotlight. It has been well tolerated in a phase I/II clinical study and possibly contributed to disease stabilization in lung cancer patients [163]. In paper I, we have shown that PPP could be considered as a new potential drug for DLBCL treatment due to the inhibitory effects in DLBCL cells together with its low toxicity in vivo.

The findings of Wickstrom et al [164], has promoted us to further investigate the activity of melflufen in lymphoma and the results presented in paper II, have shown that melflufen is indeed an active drug both in Hodgkin and diffuse large B-cell lymphoma cell lines in vitro. A similar pattern in patient cells clearly suggesting that melflufen appears to be a candidate for further evaluation in the treatment of this group of malignant diseases.

We were inspired by the D'Arcy et al [148] investigation that identified a novel small molecule inhibitor of USP14 and UCHL5, b-AP15, demonstrating a unique mechanism of proteasome inhibition. We examined the anti-lymphoma activity of b-AP15 using our in vitro model of lymphoma and the results in paper III, have suggested that bortezomib-mediated resistance could be overcome by b-AP15. While bortezomib has failed to demonstrate clinical activity among these patients, further investigation of b-AP15 seems warranted based on the differences we have investigated in paper III.
The major obstacle for treating DLBCL patients is still drug resistance though it's significantly improved during the last decades [165], warranting further investigations to identify potential targets. As described in paper III, proteasome inhibitors like bortezomib, have failed to demonstrate significant clinical benefit in DLBCL. In paper IV, the expression of potential targets for proteasome inhibitors was studied in ninety-two DLBCL patients treated with curative intention with either R-CHOP like or CHOP like regimens. Based on the findings in this study, further evaluation of UCHL5 and/or USP14 as new targets for proteasome inhibitors in DLBCL seems warranted.

Clinical development of PPP (Picropodopyllin), Melflufen (J1) and b-AP15 as new candidate drugs and further evaluation of UCHL5 and/or USP14 as new targets for proteasome inhibitors in DLBCL patients, seems warranted in the light of the results presented in this thesis and hopefully lead us towards better treatment of lymphoma patients.
Acknowledgements

I would like to thank everyone whom has directly or indirectly contributed to this thesis and to my PhD journey. I could never have done it by myself!

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Gunilla Enblad, not only for her tremendous academic support but for believing in me and always being supportive, understanding and patient. Thanks for your never ending care and for giving me the opportunity and the means to develop as a scientist. You have been a great supervisor, with your ability to always see positive aspects even when things seem not to be going well!

I would also like to thank my co-supervisor, Mattias Berglund, for sharing your scientific knowledge and experience, coming up with ideas for my projects in the beginning of my postgraduate studies and also giving me excellent hints in writing my thesis.

To Joachim Gullbo: Thank you for introducing me to your lab and making me feel welcome and well taken care of when I needed it the most and also, for the productive scientific discussions that helped me in writing papers.

To Thomas Strömberg: How you manage to be completely devoted to science, yet still know everything about everything and remain sane at the same time is definitely a mystery to me! You have guided me through paths of frustration and helped me to develop my scientific thinking skills more than I think you realize, for this I am truly grateful.

To Rose-Marie Amini: Thanks for your tremendous knowledge in haemathopathology and for showing me what malignant B-cells look like.

To Jenny Rubin: Thanks for sharing your knowledge about the world of SELDI-TOF, and for our great talks about anything and everything.

To past and present members of the Oncology group: Thank you; Nongnit, for your sincere happiness for me in my victories and the shoulder you always have ready when things get a bit too much for my heart to bear; Linda,
for nice conference trips together. I enjoyed all the interesting, funny, weird and crazy chats with you! Ulf, for your contagious laughter and for the cheerful atmosphere at work and also Erika, Gustaf, Patrik, Norafiza, Ingrid, Daniel, Fredrik, Martin and Xuping for all the nice chats during coffee and lunch breaks.

To all members of GI G group: Thanks for adopting me and making me feel like a part of your group. A very special thanks to: Emma for sharing all the adventures of being a mom and the passionate football chats. Too bad we don’t cheer for the same team ☺ Lotta, for being such a fantastically nice, caring, girl and for all the not science-related talks during lunch time; Hannah, for being always available to listen no matter what. I ‘m so glad we got the opportunity to get closer. Your warmth, frankness and never ending solutions to my never ending problems is amazing! I feel blessed having you girls as my friends.

To all members of KlinFarm group: Thanks for being super friendly. Special thanks to: Lenna Lenhammar for your great help and your skillful technical assistance and Sara Stresse for answering all my questions regarding FMCA and also the great collaboration in writing articles.

I would like to thank all my fantastic co-authors and associates, specially: Per-Henrik Edqvist, Christer Sundström, Patrik Georgii-Hemming, Johan Lennartsson, Malin Wickström, Rolf Larsson and Chao Sun.

To Ammar: Thank you so much for helping me during the hard times of my research life, giving me advice on how to cope with it and especially for listening to me complain about research every time we met in the corridors and elevators. I wish the best for both you and Hanan in the future.

I owe great gratitude to the “Girls Club”: Ruta, Fernanda, Tonge and Victoria. Thank you for your true friendship and the way you all made life easier for me and of course gossip shared! I am a better person when I am with you girls! ☺ Tonge and Victoria thanks for your unlimited support at all times and for always being there for me. Fernanda, thanks for sharing the joy and frustration of dissertation process. We made it girl! Ruta, you are truly more like a sister to me! You have helped me cope with so much and have been there for me through the good and the bad times. You have a very special place in my heart lady!

To the “one and only” Eric: Thanks for letting your charming wife Ruta spend a lot of time with me and enlighten my life! ☺ Thanks a million for proofreading my papers and my thesis.
A big “thank you!” to all amazing family friends or better to say “my family in Sweden”: Ellahe, Maria, Reza, Farzaneh, Shahin, Farnaz, Behdad, Karin, Shahriyar, Faranak and Saeed, thanks for your friendship and always welcoming me and my family to your homes and all the wonderful times we spend together and for always asking how my research life goes!

To Tabassom, Azita and Shokooh: Thanks for making my life much easier by taking care of my boys when I needed it most and giving me the time to work on my papers and thesis. Your friendship means the world to me ladies!

To Millaray: Thanks for everything! Your love, support, knowledge, encouragement, ideas, positivity, laughers and friendship. You are an angle and it wouldn’t be the same without you!

To Sonja: My German partner in crime! It’s crazy to think that I have only known you for 5 years! Thanks for absolutely everything. For all the WhatsApp messages, sharing memorable moments, crazy laughs and introducing German culture to me. You have no idea how much you mean to me, you are a very special person in my life… you are my sister by choice!

To my “Forever Friends”, Saba, Elham and Mahsa: “Sometimes in life, you find special friends who change your life just by being part of it. When you're down and the world seems dark and empty, they lift you up in spirit and make that dark and empty world suddenly seem bright and full. They make you laugh until you can't stop. These friends are “Forever Friends”... You have forever friends, and forever have no end.” Thanks for being my forever friends girls!

No words can express the extent of gratitude I feel towards my family, still, I will try to do so. With all my love to:

My aunt, Sima: Simply thanks for being YOU! Since the day I could remember, you were such a kind caring aunt for me. You taught me how to deal with the ups and downs in life. You are a super strong woman and you are definitely my role model. Thanks for always being concerned about my research life and sending me all your positive energy each and every time we talk. I miss you every day!

My in-laws, Fattaneh, Katy, Keyvan and Ghazaleh: Thanks for all heart-warming and encouraging words especially during past few months and thanks for always being there for me no matter what.
My second mom and dad, Lila and Abbas and my two lovely cousins Sam and Tina: Thank you so much for taking very good care of me since the day I have moved to Sweden! You made me feel so welcome as if I am in my own home though I was away from home. Lila, thanks for introducing me to the world of research in Sweden and also thanks for your all time support and love. You'll always have my heart! Abbas, thanks for loving me like your own daughter and for your life-long support. Sam and Sofie, thanks for sharing all the baby drama and your unconditional love. Tina, you are the sweetest cousin one could ever have! Thanks for always being a shoulder to lean on and also thanks for your hard work to teach me Swedish. You are truly my safe haven “kusinvitamin”!

My brother, Milad: Thanks for your endless love and your hard efforts to understand all the aspects of my projects! You are the best sibling I could ask for and you deserve the best of the best. We are going to be inseparable throughout life just as we have been in the past. I am very proud of the person you are and the strength you show! ♥

The two heroes of my life, my mom and my dad: I owe you every second of my life! You taught me never stop trying, never stop believing in me and never give up. The strength both of you has shown in life is the strength that has brought me to this day. From the bottom of my heart, I thank you for your unlimited love, sacrifices and efforts. I’ll love you till the day I die! ♥

My princes Radin and Arvin: I am the luckiest mom in the whole world for having you. Since the day both were born, your kind beautiful faces have brought a smile to my face and given me the strength not to give up and be a fighter! My greatest accomplishment in life is being your mom and it always will be! ♥

My soul mate, my best friend and my forever love, Kian: Thanks for your endless support and always being there for me. Thank you darling, for making all my dreams come true. Thanks for helping me a lot with the images in my thesis and the graphical support and most important of all designing the cover of my thesis. I love it! I'll never love you any less than I do, right this second and can’t wait for the fabulous days ahead with you and our children. You will forever be my “always”! ♥
References


69. Georgiou, K., Genetics of diffuse large B-cell lymphoma, in Department of Laboratory Medicine. 2015, Karolinska Institutet: Stockholm. p. 78.


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)