From Oncology and Pathology
Karolinska Institutet, Stockholm, Sweden

STUDIES OF B-AP15 – A NOVEL INHIBITOR OF PROTEASOME DEUBIQUITINASE ACTIVITY

Chao Sun

孙超

Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by Eprint AB 2017

ISBN 978-91-7676-662-0

Studies of b-AP15 – a novel inhibitor of proteasome deubiquitinase activity
THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i CMM lecture Hall L8:00 Karolinska Universitetssjukhuset, Solna.

Fredagen den 28 april, 2017, kl 9:00

av

Chao Sun
M.Sc.

Principal Supervisor:
Professor Robert Harris
Karolinska Institutet
Department of Clinical Neuroscience
Division of Neuroimmunology

Opponent:
Professor Petter Höglund
Karolinska Institutet
Department of Medicine, Huddinge
Division of Hematology

Examination Board:
Professor Antonio Barragan
Stockholm Universitet
Department of Molecular Biosciences

Professor Tomas Ekström
Karolinska Institutet
Department of Clinical Neuroscience

Associate Professor Anna-Lena Ström
Stockholm University
Department of Neurochemistry

Dedicated to my family

ABSTRACT

Bortezomib was the first FDA approved proteasome inhibitor that was initially very successful in treatment of multiple myeloma patients but acquired resistance and adverse side-effect highly decreased patients' quality of life. Development of 2nd generation proteasome inhibitors that could overcome these shortcomings is thus of prime medical importance.

Our group has developed b-AP15 as such a candidate, which targets a different subunit of the proteasome than does bortezomib. In study I, we determined that use of CpdA as a co-translational translocation inhibitor in a co-treatment protocol greatly enhanced proteasome inhibition by b-AP15.

Aggresome formation is a resistance mechanism evident after bortezomib treatment. In study II, we demonstrated that b-AP15 did not induce aggresome formation under the same conditions and interestingly we observed less aggresome formation with co-treatment of b-AP15 and bortezomib compared to single treatment with bortezomib.

In study III, we demonstrated that lymphoma cell lines were as sensitive to b-AP15 as other cancer cell lines previously reported. The apoptosis induced by b-AP15 correlated to accumulation of polyubiquitination and the ER stress response.

In study IV, we observed that the gene expression patterns and apoptosis induction mechanisms of b-AP15 and bortezomib were similar, but not identical. Both induced the expression of Hmox-1 but only b-AP15 could induce ER stress. This study also revealed that ROS scavengers could reduce the apoptosis induced by b-AP15, which was due to activation of AP-1

In study V, the gene expression pattern following Piperlongumine treatment was similar to that of other proteasome inhibitors and the drug could block the ubiquitin–proteasome system in cancer cells. However, Piperlongumine was determined not to be a classic but instead interfered upstream of UPS system.

The overall conclusion is that further development of proteasome inhibitors such as b-AP15 should be continued, as increased efficacy is expected following clinical translation.

LIST OF SCIENTIFIC PAPERS

I. **Sun C**, Roboti P, Puumalainen M, Fryknäs M, Wang X, D'Arcy P, Hult M, High S, Linder S and Swanton E. Elevation of proteasomal substrate levels sensitizes cells to apoptosis induced by inhibition of preteasomal deubiquitinatases. PLoS One. 2014 Oct 6

II. Brnjic S, **Sun C**,Mazurkiewicz M, Larsson R, Fryknä s M, Swanton L, High S, Linder S, D'Arcy P. Inhibitor of proteasome deubiquitinase activity inhibits cytoprotective- aggresome formation in cancer cells. (Manuscript)

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. Brnjic S, Mazurkiewicz M, Fryknäs M, **Sun C**, Zhang X, Larsson R, D'Arcy P, Linder S. Induction of Tumor Cell Apoptosis by a Proteasome Deubiquitinase Inhibitor Is Associated with Oxidative Stress. Antioxid Redox Signal. 2014 Dec 10;21(17):2271-85.

V. Jarvius M*, Fryknäs M*, D'Arcy P*, **Sun C**, Rickardson L, Gullbo J, Haglund C, Nygren P, Linder S, Larsson R. Piperlongumine induces inhibition of the ubiquitin-proteasome system in cancer cells. BiochemBiophys Res Commun.2013 Feb 8;431(2):117-23.

* Equal Contribution

CONTENTS

LIST OF ABBREVIATIONS

3D-CRT	3-dimensional conformal radiation therapy
53BP1	p53 binding protein 1
5-FU	5-fluorouracil
AML	Acute myeloid leukemia
ASCT	Autologous stem cell transplantation
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2
BCRP	Breast cancer resistance protein
Bik	Bcl-2 interacting killer
Bim	B-cell lymphoma 2 interacting mediator of cell death
Bmf	Bcl-2 modifying factor
BMSCs	Bone marrow stromal cells
Bok	Bcl-2 related ovarian killer
CAM	Cell-adhesion molecular
CDK	Cycline–dependent kinases
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DDR	DNA damage response
DUBs	Deubiquitinating enzymes
EGFR	Epidermal growth factor receptor
EGFR-TKIs	EGFR tyrosine kinase inhibitors
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
GITR	Glucocorticoid-induced TNFR-related protein
HECT	Homologous to E6-AP C terminus
HIF	Hypoxia-inducible transcription factor family
Hmox-1	Heme oxygenase (decycling) 1
HR	Homologous recombination
HRK	Harakiri
HSCT	Hematopoietic stem cell transplantation
IAPs	Inhibitor of apoptosis proteins
IGF	Insulin-like growth factor
IGRT	Image-guided radiation therapy
IMRT	Intensity-modulated radiation therapy
MCL	Mantle-cell lymphoma
MCL	Mantle cell lymphoma
MDM2	Mouse double minute 2-homolog
MDR1	Multi-drug resistance protein 1
MJD	Machado–Jakob-disease proteases
MOMP	Mitochondrial outer membrane permeabilization

MRP1	MDR-associated protein 1
NCCN	National Comprehensive Cancer Network
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NSCLC	Non-small-cell lung carcinoma
OS	Overall survival
OTU	Ovarian tumour-like proteases
PD	Parkinson's disease
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
PSMB5	Proteasome subunit β5
PTEN	Phosphatase and tensin homolog
PUMA	p53 up-regulated modulator of apoptosis
RING	Really interesting newly discovered gene
Rpn	Regulatory particle non-ATPase subunit
SCF	SKP1–CUL1–F‑box protein
STAT1	Signal transducer and activator of transcription 1
TNFR	Tumor necrosis factor receptor
TRF1	TBP (TATA box-binding protein)-related factor 1
UCH	Ubiquitin carboxy-terminal hydrolases
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific proteases
VEGF-A	Vascular endothelial growth factor-A
VHL	Von Hippel-Lindau
XIAP	X chromosome-linked IAP protein

1 INTRODUCTION

1.1 CANCER AND CANCER TREATMENT

1.1.1 What is Cancer

As a top deadly disease, cancer is a serious threat to public health across the world. It is estimated by International Agency for Research on Cancer GLOBOCAN that a total of 8.2 million deaths and 14.1 million newly diagnosed cases were recorded from 184 countries worldwide in 2012[1]. According to the definition by NIH (National Cancer Institute), cancer is "a collection of diseases in which some of the body's cells continue to divide and spread into surrounding tissues". Tumorigenesis is considered as a multiple-step progress in which normal cells develop into highly malignant cells through genetic alteration.

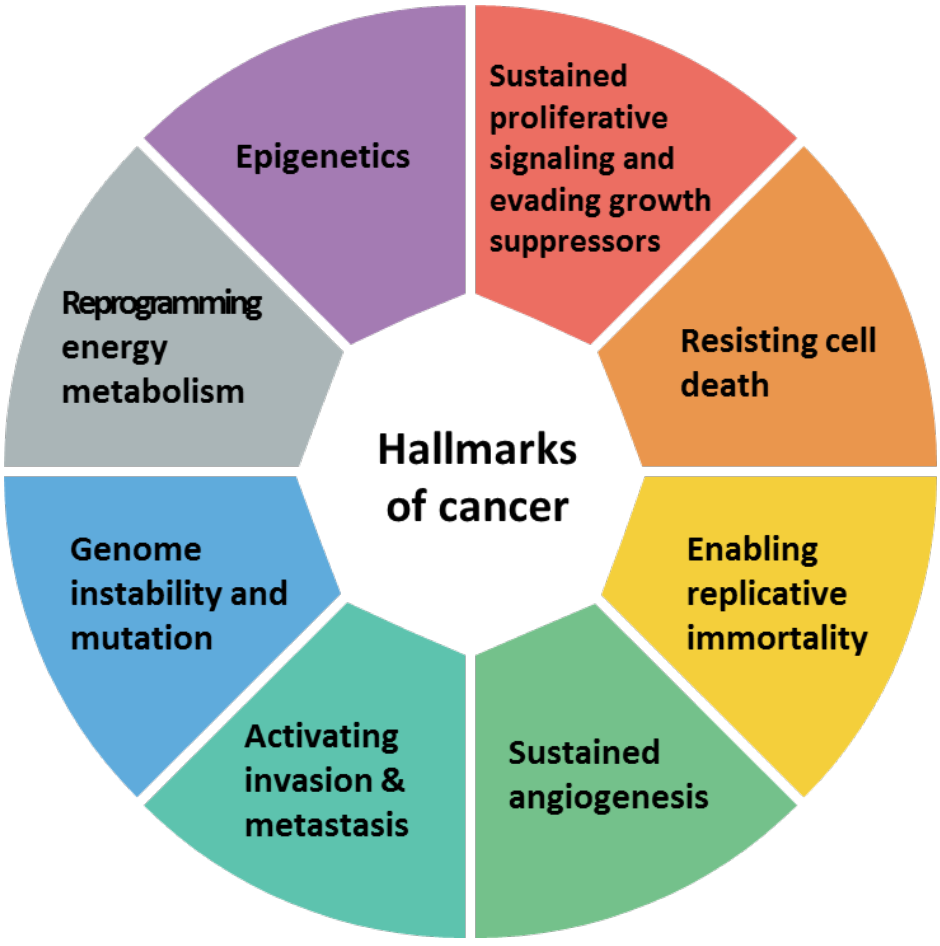

Figure 1: 8 hallmarks of cancer.

In general, cancer is characterized by the following eight hallmarks (Figure 1): 1) sustained proliferative signaling and evading growth suppressors, which is defined by the healthy cell number and cell proliferation in the analyzed samples; 2) resisting cell death, which is

investigated by detecting and evaluating the programmed cell death process, apoptosis; 3) enabling replicative immortality, which is identified by the up-regulated level of telomerase; 4) sustained angiogenesis, which is identified by the up-regulated level of vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (FGF); 5) activating invasion and metastasis, which exhibits incomplete or absent epithelial-mesenchymal transition (EMT); 6) genome instability and mutation, which presents altered chromosomal stability; 7) reprogramming energy metabolism, which shows increased glycolysis level; and 8) epigenetics[2].

1.1.2 Cancer treatment

Cancer is usually classified into different categories based on organ or tissue-of-origin affected. Common treatments of cancer include surgery, chemotherapy, radiation, immunotherapy and targeted therapies. In addition, there are other newly developed treatments such as stem cell transplantation, hormone therapy and precision medicine. Some patients may have good responses to mono-treatments, but most patients need to receive different combinations of treatments. A combination of cancer therapies has been used to overcome the disadvantages and limitations of mono-treatments, and to produce long-lasting anti-cancer responses for patients not benefiting from mono-treatments. With the development of systemic therapeutics, patients' life span can be expanded, and some tumor types can be completely cured.

1.1.2.1 Surgery

As a local treatment surgery can be used to cure the cancer constrained in one area if it has not spread. Surgery can be performed in a traditional way using surgical instruments to remove the cancerous tissue from the body. There are also several other types of surgeries without the use of scalpels, including cryosurgery, lasers, hyperthermia and photodynamic therapy. Cryosurgery destroys cancer tissue by extreme cold in the form of liquid nitrogen or argon gas that can be applied to internal tumors, such as prostate and liver tumors, and external tumors like skin squamous cell carcinoma [3]. Laser therapy is usually used to treat cancer cells in superficial areas such as the cervix, penis and vagina using high-intensity light, like CO_2 lasers and argon lasers. Hyperthermia treatment destroys cancer cells through high temperature, combined with radiation or chemotherapy for a synergistic effect [4]. Photodynamic therapy uses a photosensitizer that stays shorter in healthy cells than in cancer

cells, and when the photosensitizer is exposed to visible light it triggers photochemical reactions and produces singlet oxygen to destroy cancer cells [5].

1.1.2.2 Radiation therapy

High-energy radiation is used in radiation therapy to suppress tumor growth and to destroy cancer cells through DNA damage. According to NIH, this procedure is extensively used in almost 50% of cancer patients. Radiotherapy can be given as an externally-delivered photobeam or as internally-placed radiation material. External delivery radiation therapy methods include three-dimensional conformal radiation therapy (3D-CRT), intensity-modulated radiation therapy (IMRT), image-guided radiation therapy (IGRT) and stereotactic radiosurgery. Internal radiation material can be applied in tumor tissue or the body cavity. In systemic radiation therapy a radioactive substance is administered by oral or intravenous injection to reach tissues throughout the body. The treatment planning of radiation therapy involves precision location using CT scan or MRI, PET and ultrasound scans. Radiation therapy has some side-effects, including normal cell damage, which occurs during the treatment or many years after the treatment.

1.1.2.3 Chemotherapy

Chemotherapy refers to the use of drugs to suppress cancer cell growth and to kill cancer cells, which is widely used in almost half of cancer patients. More than 100 drugs have been used for cancer chemotherapy. Chemotherapeutic drugs can be classified by their chemical structures, derivation and mechanisms of action. Chemotherapeutic drugs are normally grouped as alkylating agents, mitotic inhibitors, anti-tumor antibiotics, anti-metabolites, topoisomerase inhibitors, corticosteroids, miscellaneous chemotherapeutic agents and alkyl-lysophospholipids. Some agents work through more than one mechanism, thus belonging to various groups. Chemotherapeutic drugs can be administered in different ways, such as orally, intravenous injection, intramuscular injection, intrathecal injection and intra-arterial injection. Chemotherapeutic drugs may induce side-effects such as damage to normal body cells. It is therefore necessary to use optimal drug doses for individualized treatment and to minimize unwanted systemic effects. The leading cause for chemotherapy failure is acquired drug resistance. During recent years' research on cancer therapy has focused on the development of specific inhibitors targeting the oncogenic mutations that hyperactivate growth regulatory pathways.

1.1.2.4 Immunotherapy

Immunotherapy is based on the concept that cancer development is naturally monitored by the immune system, which has the potential to suppress malignant cells [6]. Cancer immunotherapies include various strategies to stimulate immune effector mechanisms or that neutralize suppressive or inhibitory immune responses. There are various approaches used to induce immune effector cell activation, including vaccines specific for antigens expressed by tumors; treatment with cytokines such as IL-2 and IFN-α to stimulate the host's immune system; adoptive cellular therapy with tumor-infiltrating lymphocytes; administration of oncolytic viruses; and antibodies to enhance the co-stimulatory signals such as targeting the tumor necrosis factor receptor (TNFR) superfamily members 4‑1BB, OX40, glucocorticoid-induced TNFR-related protein (GITR), CD27, CD40 and TNFRSF14. As for the approaches to eliminate immune suppressive mechanisms, there are antibodies targeting surface markers of regulatory T cells such as CD25, LAG3 and T cell-immunoglobulin-mucin domain protein TIM3, and antibodies against immune-checkpoint molecules, such as programmed cell death protein 1(PD-1) and cytotoxic T lymphocyte associated protein 4(CTLA-4) [6, 7].

There are limitations in the exploration of successful immunotherapies for cancer, such as screening of suitable and effective antigens, low response rates, restriction to specific tumor types, and immune-related adverse events. After years of disappointing failures, cancer immunotherapy has achieved some success clinically. The Sipuleucel-T vaccine was approved to treat asymptomatic and metastatic hormone-refractory prostate cancer by the FDA in 2010[8]. The anti-CTLA-4 antibody ipilimumab was approved to treat melanoma by the FDA in 2011. The PD1 antibody nivolumab was approved to treat metastatic melanoma and advanced and metastatic non-small-cell lung cancer in 2014 and 2015 respectively [9].

1.1.3 Clinical successes and failures in cancer treatment

1.1.3.1 Multiple Myeloma

Over the past 15 years the diagnosis and therapy of multiple myeloma have achieved great advances, and patients' survival rates have significantly increased with the increasing number of new drugs [10]. Patients are usually treated with high-dose chemotherapy and transplantation of peripheral blood or bone marrow stem cells. According to the 2016 guidelines of the National Comprehensive Cancer Network (NCCN) for multiple myeloma, newly diagnosed non-transplantation patients are treated with a triplet regimen consisting of bortezomib and dexamethasone combined with either cyclophosphamide or lenalidomide; or

melphalan and prednisone combined with bortezomib, lenalidomide or thalidomide. Transplantation patients are treated with a triplet regimen consisting of bortezomib and dexamethasone, combined with lenalidomide, thalidomide, or cyclophosphamide for induction therapy, and autologous stem cell transplantation (ASCT). These treatments are followed by maintenance therapy. After initial treatment of dexamethasone/lenalidomide/ bortezomib with or without ASCT, the 18-month progression-free survival (PFS) rate was 75% and the overall survival (OS) rate was 97%. After initial therapy with cyclophosphamide/bortezomib/dexamethasone, 5-year PFS and OS rates were 42% and 70%, respectively. For the patients who experienced relapsing multiple myeloma, numerous regimens such as carfilzomib/dexamethasone, elotuzumab/lenalidomide/ dexamethasone, and Ixazomib/lenalidomide/dexamethasone have been demonstrated to improve the median PFS [11].

1.1.3.2 Acute Myeloid Leukemia

Of all acute myeloid leukemia (AML) patients, about 40–45% of younger (18-60 years old) and 10–20% of older patients will be cured using current standard chemotherapy [12]. The "7 + 3" induction regimen (7-day continuous intravenous cytarabine infusion and 3 daily doses of anthracycline, typically daunorubicin or idarubicin) is considered as the standard treatment for AML; and the higher dose cytarabine and nucleoside analogue doublets are the most modern approaches [13]. Only a small number of patients can receive allogeneic hematopoietic stem cell transplantation (HSCT) to prevent from recurrence, but for older patients the transplantation may induce higher morbidity and mortality [14]. In recent years, a better understanding of AML genomic and epigenomic changes has facilitated targeted treatments for specific subgroups. However, the outcomes in older patients (>60 years old) remain highly unsatisfactory and effects in relapsed or refractory patients are still poor [15].

1.1.3.3 Malignant Glioma

In comparison with great improvement in the therapeutic outcomes of other types of cancer, the clinical outcomes of malignant glioma are disappointing. World Health Organization defined the anaplastic high-grade gliomas and glioblastomas as malignant gliomas [16]. Generally, diagnosed glioblastoma patients without any treatment will die in a few months. At present, glioblastoma is treated with gross total resection, radiotherapy and combined with concomitant DNA alkylating agent temozolomide, showing a limited efficacy. The median survival is extended to 14.6 months by combining temozolomide to surgery and

radiotherapy, only one year longer than natural course, with 2-year survival rate less than 25% [17]. Almost all malignant glioma patients will recur, and salvage therapy has no effect.

1.1.3.4 Metastasis

Metastasis is a multi-step process in which cancerous cells spread from the original location to distant organs through the circulatory system before establishing a secondary tumor at the new areas [18]. According to the statistics, metastasis causes about 90% of cancer deaths [19]. The process of metastasis includes invasion, survival, entering into the blood stream and colonization. In terms of metastatic cancers, the treatment should be directed to both the primary site and the secondary tumors. Moreover, the diagnosis of metastatic cancers is often a sign of more widely metastatic sites that have not been detected. These factors result in extreme difficulty for the treatment of metastatic cancer [20].

1.2 THE UBIQUITIN-PROTEASOME SYSTEM AND CANCER

1.2.1 The ubiquitin network

The synthesis and degradation balance is responsible for the maintenance of steady-state levels of cellular proteins. The two predominant cellular systems that regulate intracellular protein degradation are the cytoplasmic ubiquitin-mediated and the vacuolar pathways [21]. The ubiquitin-mediated pathway is responsible for about 80% protein turnover in cells [22]. As a conserved protein in eukaryotic cells, ubiquitin is coupled to lysine residues on target proteins through enzymatic reaction cascades [23]. Many components of the ubiquitin-proteasome (UPS) system are considered as potential targets for cancer therapy, due to its involvement in the pathogenesis of cancer.

There are 3 keys enzymes (E1, E2 and E3) involved in the multiple-step ubiquitination process (Figure 2). In eukaryotic cells, ubiquitin is an 8kDa conserved protein in its free form or conjugated to protein substrates. In the first step of the ubiquitination process, ubiquitin is activated by E1 enzyme. The high energy-consuming activity requires ATP for a thio-ester bond formation between E1 enzyme and ubiquitin. Then the activated ubiquitin is transferred from E1 to E2 conjugating enzyme by trans-thiolation. Finally, ubiquitin from the E2-linked complex is attached to the lysine residue of the substrate protein by E3 ubiquitin ligases [23-25]. The human genome contains around 40 E2 enzymes and more than 600 E3 ligases [26]. The process of ubiquitination is reversible. Ubiquitination is controlled by deubiquitinating enzymes (DUBs), a divergent family of isopeptidases that reverse ubiquitination by

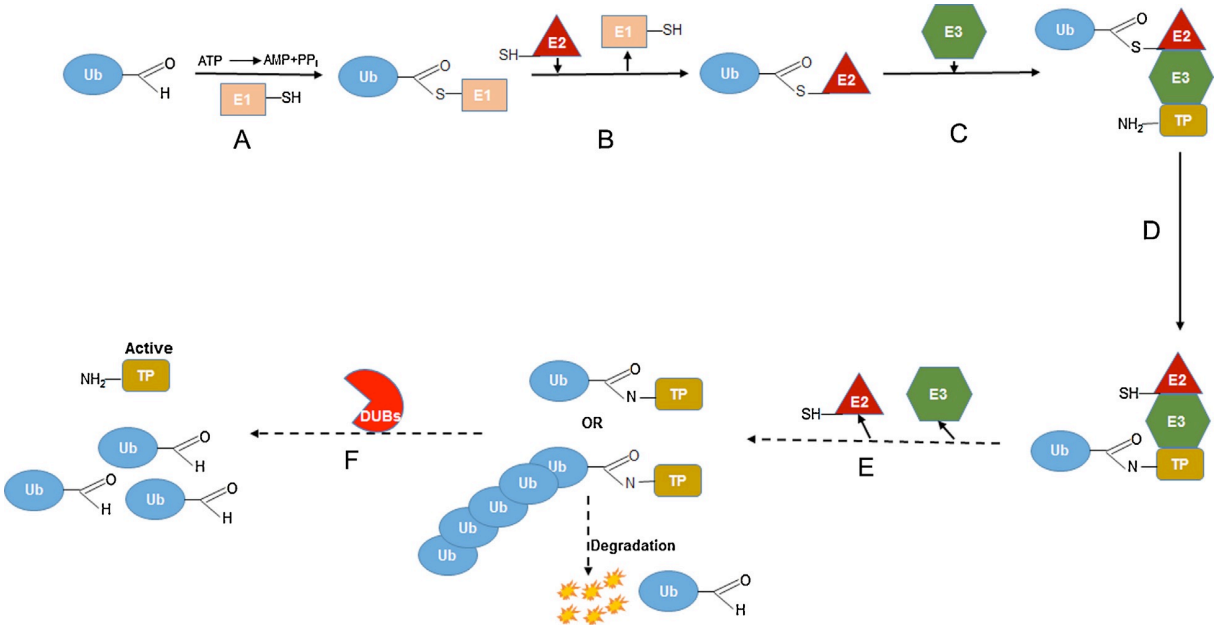

Figure 2. The ubiquitination and deubiquitination
(From: Deubiquitinases and cancer: A snapshot)

removing conjugated ubiquitin tags [27, 28].

Ubiquitinated proteins are degraded in proteasome or lysosome. The proteasome is a 26S complex comprising of several multi-catalytic proteases. These proteases will turn the polyubiquitinated proteins into short peptides. The proteasome consists of two 19S regulatory domains that recognize ubiquitinated proteins and a 20S core domain in charge of the protein degradation [29, 30]. Deubiquitination is one of the most important bases of the ubiquitin proteasome system (UPS), in which signal ubiquitin or multiple ubiquitin chains are removed from a target. More than 100 kinds of DUBs have been identified and characterized in human cells.

Ubiquitination is a major post-translational modification affecting protein stability, localization or interaction pattern. It has been discerned that several tumor suppressors or oncogenes involved in the ubiquitin conjugation and deconjugation pathways are altered in cancerous states. The ubiquitin-proteasome system therefore becomes a potential target for anti-cancer drug development.

1.2.2 E3 ubiquitin ligases in cancer

Oncoprotein stabilization and tumor suppressor gene destabilization are the main inducers of cancer. Cancer cells carry genetic mutations, resulting in unlimited growth and survival in adverse conditions. Dysregulation of ubiquitin pathways contributes to a protein turnover

defect, which is associated with the clearing of oncoproteins and the stabilization of tumor suppressor proteins [31, 32]. Many components of the ubiquitin system have been reported to be correlated with tumors and are thus considered as potential candidates for cancer therapy, (ubiquitin ligase system, dysregulation of deubiquitinating enzymes and the 26S proteasome [32, 33]).

E3 ligases are the most essential components of the ubiquitin conjugation procedure due to their involvement in the binding of both the specific target proteins and substrates. E3 ligases are a diverse group of proteins, including HECT (homologous to E6-AP C terminus)-type, single RING (Really interesting newly discovered gene)-type, Cul4 (Cullin 4)-base-type, SCF (SKP1–CUL1–F box protein)-type, and ECV (Skp1/Cdc53 or Cul1/F-box protein)–type. E3 ligases selectively target tumor suppressive proteins or oncogenic proteins and have a strong relation with the development of cancer [34]. The SCF-type E3 ligase contains 4 components, including Skp1, Cul1, and Rbx1/Roc1, functioning as the invariable subunits, and a target protein receptor subunit F-box protein [35].

F-box protein Fbw7 mutations and its target gene mutations are reported in a variety of human cancers [36]. The E3 SCF^{Fbw7} complex ubiquitinately phosphorylates oncoproteins, such as Notch, c- Jun, cyclin E and c-Myc. As the first reported E3 ligase for the G1/S phase progression negative regulator p27, SCF^{Skp2} can indirectly regulate cycline–dependent kinases (CDK) [37]. Increased levels of F-box protein SKP2 and reduced levels of p27 are observed in many types of cancer [38]. The tumor suppressor Von Hippel-Lindau (VHL) is an ECV-type E3 ligase component, including elongin C, elongin B, Cullin 2 and bx1/ROC1. HIF-1α is an unstable subunit of the hypoxia-inducible transcription factor family (HIF). VHL E3 ligase regulates HIF-1α activity and induces oxygen-dependent ubiquitin-mediated destruction. Unhydroxylated HIF-1α cannot bind VHL protein and therefore accumulates in cells during hypoxia. VHL mutations or hypoxia can prevent ubiquitination of HIF-1α and increase HIF expression, thus activating the pathways to promote angiogenesis and cell survival, which are the advantages utilized by cancer cells [39, 40].

Tumor suppressor p53 acts as a sequence-specific transcription factor in apoptosis, DNA repair and the cell cycle. Numerous studies have shown that the prominent function of p53 is to regulate the process of apoptosis and pathways of cell cycle arrest. Many genes involved in the signaling transduction of apoptosis processes are induced by p53 to activate both the intrinsic and extrinsic apoptosis pathways [41]. Mitochondrial outer membrane

permeabilization (MOMP) plays an important role in the multiple-step signaling transduction of intrinsic apoptosis [42]. p53 regulates MOMP by transcriptionally inducing the expression of a variety of BH3-only pro-apoptotic proteins (Puma, Noxa, Bad, Bax,). It is evidenced by the experiments of gene knockout mice that these pro-apoptotic proteins work together to mediate this p53-induced apoptosis mechanism [43, 44]. p53 transcription enhances the cell surface death receptor levels, such as Killer/Dr5 and Fas to activate the extrinsic apoptosis pathways [45, 46]. Transcriptional activation of p21/WAF1, 14-3-3σ and cdc25C by p53 arrests cells at G1 phase and G2/M phases [47].

The activity and stability of p53 is regulated by ubiquitination. A series of RING or HECT subgroup E3 ligases have been identified to target multiple lysines on p53 for ubiquitination [48]. More than 10 RING-type E3 ligases are recognized to be involved with 26S proteasome-mediated p53 degradation. As a RING-containing E3 ligase, mouse double minute 2-homolog (MDM2) functions as an E3 ligase to ubiquitinated p53, which has a highly-conserved N-terminus. MDM2 acts on the p53 transactivation domain to consistently suppress p53 function. Wild-type p53 can also be downregulated by over-expression or amplification of MDM2, which leads to the proteasome-mediated degradation of p53 and promotes carcinogenesis in many human cancers [49]. As with the HECT-type E3 ligases, ARF-BP1 directly acts on p53 for ubiquitination, and WWP1 mediates the poly-ubiquitination of p53 to regulate different cell functions [50].

1.2.3 Deubiquitination and DUBs

Deubiquitination serves as the reverse process of protein ubiquitination. Ubiquitin is recycled in the ubiquitin-proteasome system (UPS), and the ubiquitin tag can be removed from the target complex by deubiquitinating enzymes (DUBs). DUBs release ubiquitin tags to rescue the marked protein from degradation, which is composed of several intracellular peptidases involved in ubiquitin maturation, recycling and editing, to remove ubiquitin chains from target complexes. Human DUBs can be classified into 5 groups, including ubiquitin-specific proteases (USP), ovarian tumor-like proteases (OTU), ubiquitin carboxy-terminal hydrolases (UCH), JAMM/MPN metalloproteases and Machado–Jakob-disease proteases (MJD)[51]. USP, the largest DUB family, is observed to be associated with most cancers and is involved in regulating multiple signaling pathways.

1.2.4 DUBs in cancer

DUBs are implicated to control processes relevant to tumorigenesis, including DNA damage response pathways, regulation of cell-cycle, regulation of histones and signaling pathways associated with cancer [52].

1.2.4.1 DNA damage response pathways

The different damages to or lesions in DNA and deficient DNA repair mechanisms are associated with genetic alterations and tumorigenesis. USP1 plays an important role in regulating DNA damage responses. The deubiquitination of two important proteins in Fanconi's anemia pathway, FANCD2 and PCNA, is mediated by USP1. Human USP2a, USP4, USP7, USP10, USP29 and USP42 are involved in the regulation of the p53 tumor suppressor protein, exerting broad effects on DNA damage repair [53]. USP28 is required to stabilize checkpoint kinases Chk2 and 53BP1 and regulate the apoptosis upon to DNA damage [54].

1.2.4.2 Regulation of the cell cycle

DUBs can exert influences on cell cycle regulation to induce cellular transformation and malignancy. USP13, USP37, USP39, USP44 and CYLD serve as key regulators in mitosis, thus contributing to the cell-cycle regulation [52]. USP 50 and UbpM (USP16) regulate cell cycle progression by controlling the G2/M checkpoint [55]. USP7 plays an important role in cell differentiation and proliferation, which is shown in its regulation of phosphatase and tensin homolog (PTEN), and FOXO localization [56, 57]. Usp22 regulate shelterin protein TRF1 (TBP (TATA box-binding protein)-related factor 1) level through deubiquitination, which affects cell cycle and apoptosis genes [58].

1.2.4.3 Regulation of histones

Histone ubiquitination affects DNA damage responses and cellular homeostasis maintenance. In response to DNA damage, up-regulated mono-ubiquitination was observed in H2A, H2B, H3 and H4. Mono-ubiquitination of histone H2A is related to transcriptional inhibition and maintenance of genome integrity. USP10 can deubiqutinate the mono-ubiquitinated H2A to induce androgen receptor-mediated gene activation [59]. USP16 is a specific DUB for histone H2A, while USP49 and USP42 are specific DUBs for histone H2B. USP1, USP3, USP7, USP12, USP 22 and USP46 can deubiqutinate both histones H2A and H2B to regulate the cellular DNA repair and transcription and gene expression [55].

1.2.4.4 Signaling pathways associated with cancer

There is an increasing focus on DUB-involved cancer pathways in various tumor types, representing a potential drug target. The NF-κB pathway is constitutively activated and frequently deregulated in different kinds of cancer [60]. USP4, USP14, USP15 and USP31 have been reported as negative regulators of NF-κB signaling through deubiquitination of multiple NF-κB pathway-associated molecules [61]. Epithelial-mesenchymal transition (EMT) is an epithelial plasticity process, involved in cancer metastasis. TGF-β signaling pathways, as one of the best-characterized promoters of EMT, can be stimulated by a number of cytokines and influenced by DUBs to promote tumor metastasis and progression [62]. USP4, USP11 and USP15 deubiquitinate and stabilize the TGF-β receptor I (ALK5), permitting sustained Smad activation and resulting in the enhancement of TGF-β signaling [63-65].

1.2.5 Proteasome-associated DUBs in cancer

1.2.5.1 26S proteasome-associated DUBs

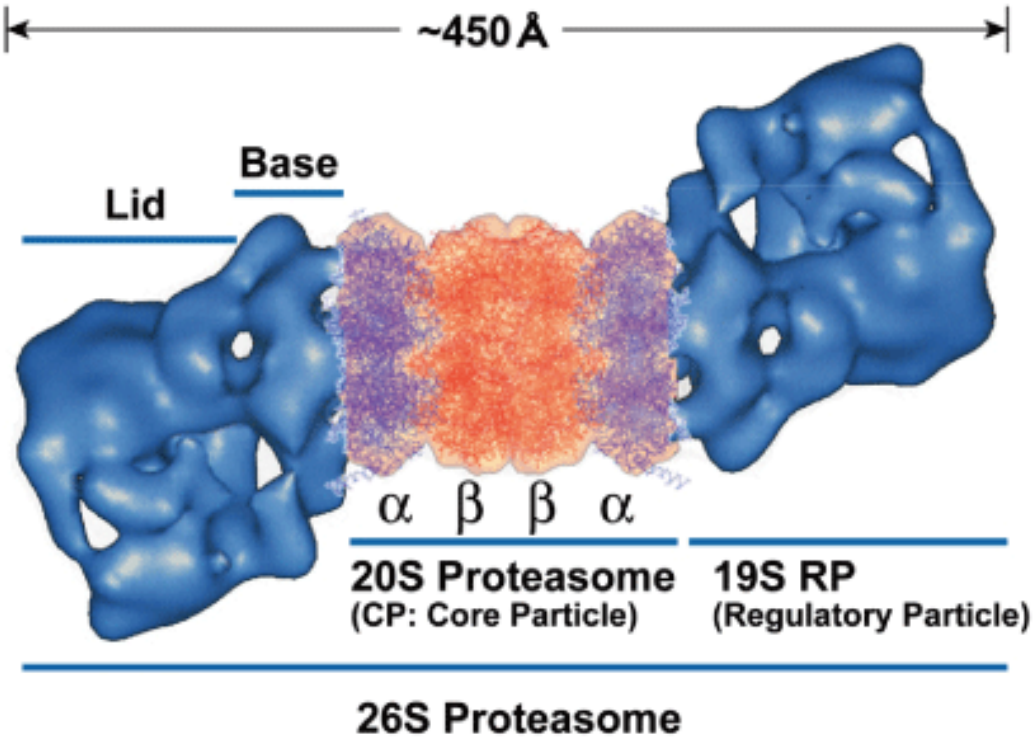

Figure 3.3D structure of 26S Proteasome.
(http://www.tanpaku.org/e_icsg2008/07_01.php)

The human 26S proteasome contains approximately 50 subunits, which can be classified as 3 sub-complexes based on their structures and functions (Figure 3): one 20S proteolytic core

particle is in charge of degrading proteins into amino acids and two cap 19S regulatory particles play a recognition and deubiquitination role in the UPS. The 20S core particle is formed by 28 protein subunits, which have two stacked β-rings in the central chamber and two α-rings in the outside chamber. Proteolytic active parts are placed within the internal cavity and determine the cleavage of different types of peptides [66]. To ensure the specificity of proteasome-associated degradation of ubiquitinated substrates, the 19S regulatory particles function as the recognition of substrate and control the access of proteins into the proteasome. The 19S regulatory particles comprise one lid with nine non-ATP Rpn subunits (Rpns 3, 5, 6, 7, 8, 9, 11, 12, and 15), one base containing six ATPases (Rpt1, 2, 6, 3, 4, 5) and four non-ATP Rpn subunits (Rpns 1, 2,10, and 13) [67].

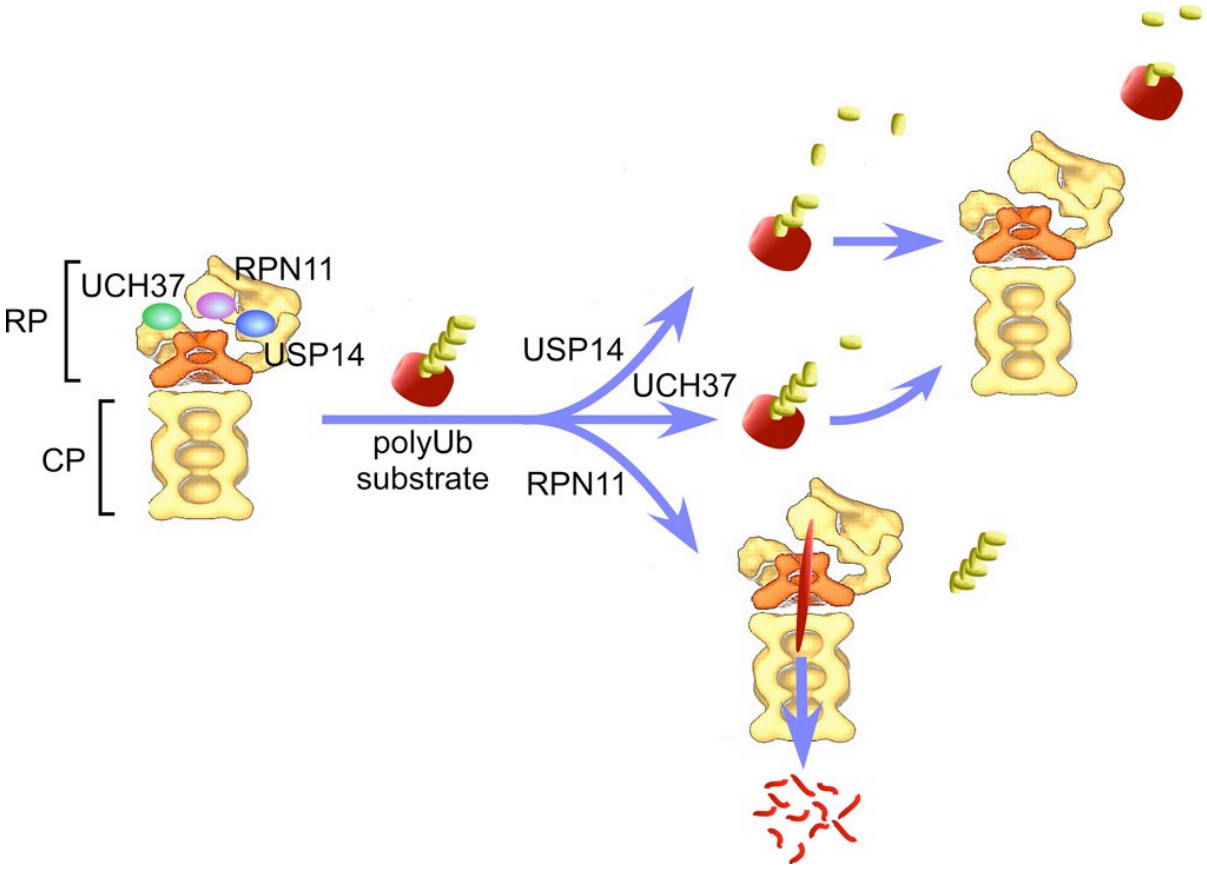

Figure 4. Three different DUBs of proteasome
(From: Trimming of Ubiquitin Chains by Proteasome associated Deubiquitinating Enzymes)

Three different DUBs, including UCHL5 (also known as UCH37), USP14 and Rpn11, are located in the human proteasome 19S subunit, belonging to the USP, UCH and JAMM families, respectively (Figure 4). Of these, Rpn11 can remove the proximal end of the ubiquitin chain from the substrate to the poly-ubiquitin direction, and UCHL5 and USP14 can remove ubiquitin from the top of the ubiquitin chain (distal tip) to the substrate direction.

Because of the directional difference, Rpn11 can induce substrate degradation by liberating ubiquitin from the complex, and conversely UCHL5 and USP14 can suppress substrate degradation by removing ubiquitin from the complex. By cleaving the iso-peptide bond and releasing the poly-Ub chains, USP14 can rescue the incorrectly poly-Ub- labeled substrate proteins [68]. Inhibition of USP14 by small molecules can enhance substrate degradation through proteasome [69]. These 3 DUBs have differential preferences for the position of deubiquitination, with Rpn11 only cutting at K63 and UCHL5 preferably cutting at K48[70].

1.2.5.2 The role of 26S proteasome-associated DUBs in cancer

Rpn11 is required for cancer cell viability and also confers multidrug resistance to a spectrum of anti-cancer drugs [71, 72]. Screening for genetic abnormalities shows the involvement of USP14 in ovarian carcinogenesis [73]. USP14 is associated with lymph node and liver metastases, which is highly evident in colorectal cancer [74]. UCLH5 acts as an important regulator in oncogenic signaling. UCHL5 regulates TGF-β/Smad signaling, a critical regulator of cell proliferation, differentiation, and tumor pathogens, thereby allowing deubiquitination and stabilization of the TGF receptor [75]. Elevated level of UCHL5 is associated with esophageal squamous cell carcinoma outcome and recurrence [76]. POH1 was identified as an important DUB to regulate ErbB2 levels, while the over-expression of ErbB2 is contributable to malignancy and poor prognosis of breast cancer [77].

1.3 CHEMOTHERAPY AND CANCER DRUG RESISTANCE

1.3.1 Chemotherapy of cancer

The treatment with cytotoxic drugs, termed chemotherapy, is mainly used as a combination treatment strategy with surgery and radiotherapy, sometimes including immunotherapy. Chemotherapeutic reagents can be divided into different types according to the mechanisms of action, molecular structure and the relationship with other drugs. Based on the mechanisms of action, chemotherapeutic drugs can be classified into different groups: anti-metabolites, which damage the cell by acting as a natural substitute molecule for metabolites of DNA and RNA syntheses; alkylating agents, which act on all cell cycle phases to damage the DNA; anti-tumor antibiotics, which interfere with the intracellular DNA to suppress cell growth; mitotic inhibitors, which target tubulins to destroy the normal mitotic spindles function; topoisomerase inhibitors, which interrupt DNA replication in cancer cells; corticosteroids, which refers to natural hormones and hormone-like drugs that may induce apoptosis and be useful in some specific types of cancer; and alkyl-lysophospholipids, which

disturb the membrane phospholipid metabolism to destroy cancer cell[78, 79]. The major limitation for chemotherapy is intrinsic (innate) and acquired (adaptive) resistance.

1.3.2 Cancer drug resistance

Drug resistance affects the effectiveness of cancer chemotherapy, which is evident for most drugs during the treatment, with patients potentially ending up in an uncontrollable situation. Cancer drug resistance can be recognized as intrinsic resistance and acquired resistance. Tumors showing intrinsic resistance may present this character before encountering any chemotherapeutic drugs. Acquired resistance can be observed in tumors, which are sensitive in the early stage and later become insensitive to similar drugs [80]. New drug development should therefore focus more on drug resistance and its related signaling pathways, in addition to extension of patients' life span and improvement of their life quality. Several mechanisms have been suggested to contribute to the cancer drug resistance (Figure 5).

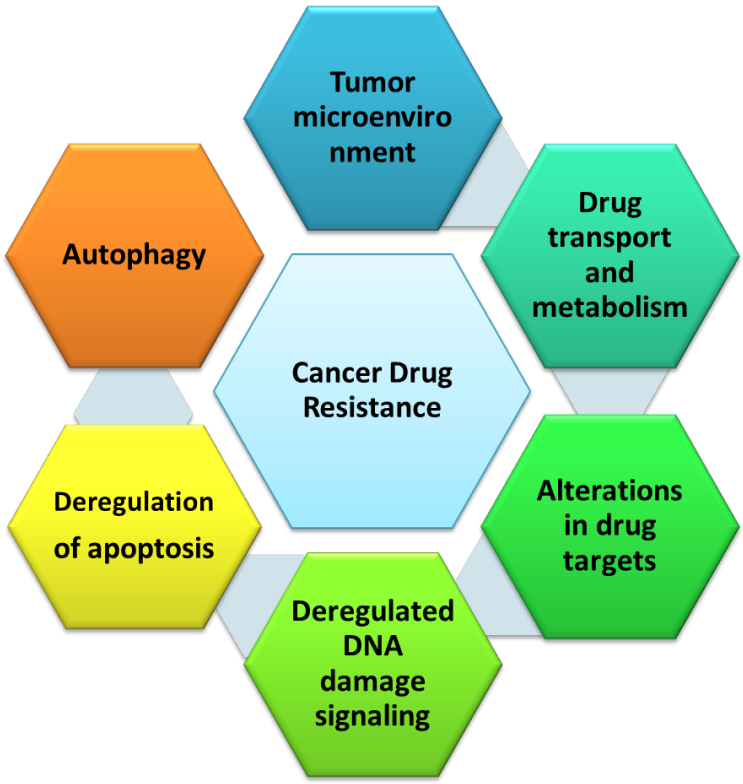

Figure 5. Mechanisms of cancer drug resistance.

1.3.2.1 Drug transport and metabolism

A number of cell membrane transporter proteins are responsible for the resistance to many commonly used chemotherapeutics. For example, multi-drug resistance protein 1 (MDR1), breast cancer resistance protein (BCRP) and MDR-associated protein 1 (MRP1) have been

indicated in multi-drug resistance [81]. As the first identified ATP-binding cassette (ABC) transporter, MDR1 is up-regulated in many tumors and can be induced by chemotherapy [82]. Expression of MRP1 in prostate, lung and breast is consistently associated with drug resistance in those cancer types [83]. Cytotoxic vincristine and etoposide are excellent substrates of MRP1, and the presence of GSH in living cells at mill molar concentrations significantly broadens the spectrum of solutes transported by MRP1[84]. BCRP possesses a very broad substrate and inhibitor specificity, which is different from MDR1 or MRP1. BCRP is associated with chemo-resistance in solid tumors, such as breast cancer, and acute myeloid leukemia [85].

1.3.2.2 Alterations in drug targets

Mutations or decreased expression of drug targets affect drug response and resistance. The development of drugs targeting specific mutations in cancer cells has achieved clinical success in cancer therapy, but the acquired drug resistances limit the efficacy of these drugs. An important anti-cancer drug target is epidermal growth factor receptor (EGFR) and its related signaling pathways. Non-small-cell lung carcinoma (NSCLC) patients with mutations in exons 19 and 21 show good response to the EGFR- tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib. The most common mechanism resulting in disease relapse and acquired drug resistance for EGFR-TKIs in lung cancer patients is the T790M gatekeeper mutation that affectes ATP binding in these kinases and reduces sensitivity to EGFR-TKIs. As a 1st generation inhibitor of the break point cluster-Abelson (BCR-ABL) tyrosine kinase, Imatinib is efficient for the treatment of chronic myelogenous leukemia patients. However, patients will relapse after receiving Imatinib treatment, due to ABL gene mutations and increased levels of BCR-ABL transcripts. NSCLC patients carry anaplastic lymphoma kinase (ALK) rearrangements that affect response to the ALK inhibitor crizotinib, and resistance to crizotinib, due to ALK fusion protein mutations and (insulin-like growth factor 1 receptor) IGF-1R pathway activation [86-88].

1.3.2.3 Deregulated DNA damage signaling

In normal cells DNA damage response (DDR) factors promote DNA damage repair immediately when recognizing DNA lesions. DDR gene inactivation can induce the loss of genomic integrity and increase the risks of cancer development. In order to have anti-cancer effects, most chemotherapeutic drugs induce damage in tumor cells DNA with defected DNA repair mechanisms. Chemotherapy resistance in DDR-dysregulated tumors can be

induced by compensation of deficient DNA damage signaling. Patients with inactivating mutations in tumor suppressor genes BRCA1/2 show defective DNA repair responses, and are sensitive to DNA damage reagents such as the inter-strand DNA crosslinking drugs cisplatin and carboplatin, and poly (ADP-ribose) polymerase inhibitors [89]. Investigation of cell lines carrying a germ-line BRCA2 mutation from an ovarian carcinoma patient before platinum treatment and after drug resistance revealed that the resistant cells carry a secondary BRCA2 mutation acquired by the tumor cells in patients. This leads to the restoration of BRCA2 function and the DNA damage repair signaling homologous recombination (HR), which compensates for the DDR defection and develop resistance [90]. Loss of p53 binding protein 1 (53BP1) can restore the end resection in BRCA1-deficient cells, which leads to partial restoration of HR, thereby decreasing the efficacy of chemotherapy for HR deficiency [91]. TP53 is a well-known DDR gene with different mutations, responsible for differential effects on cancer cells responding to chemotherapy. In advanced germ cell cancer patients, especially those with primary mediastinal nonseminomas, the genetic studies of cisplatin resistance reveal that TP53 mutations are detected dominantly in cisplatin-resistant cancer cells [92].

1.3.2.4 Deregulation of apoptosis

Cancer therapeutic strategies mainly target anti-apoptotic proteins or stimulate pro-apoptotic molecules expression [93]. Up-regulation of pro-survival factors, such as inhibitor of apoptosis proteins (IAPs) and anti-apoptotic B cell lymphoma-2 (Bcl-2) family members, is observed to be associated with acquired chemotherapy resistance. IAPs can suppress apoptosis against apoptotic stimulations from chemotherapeutic agents in cancer cells. The human IAP member X chromosome-linked IAP protein (XIAP) binds and inhibits the key apoptotic effector proteases caspases 3, 7, and 9, while cellular IAP1 (cIAP-1) and cellular IAP2 (cIAP-2) negatively regulate caspase 8 activation. XIAP and cIAPs are highly correlated with the sensitivity of chemotherapy responses in various cancers such as primary nodal diffuse large B-cell lymphomas, colorectal cancer, advanced head and neck cancer, pancreatic cancer and acute myelogenous leukemia [94-98]. Bcl-2 protein families take an important part in intrinsic apoptotic pathways regulation. Bcl-2 family members are classified into 3 groups: one anti-apoptotic subfamily (e.g. Bcl-2, Bcl-xL, Bcl-w and Mcl-1) avoid cells from apoptosis; two pro-apoptotic subfamilies: BH3-only proteins (e.g. Bik, Bad, Bid, Bim, Bmf, Noxa, Hrk, and Puma) and Bax-like multi-domain proteins (e.g. Bax, Bak and Bok) [99, 100]. Up-regulation of anti-apoptotic protein Bcl-2 and Bcl-xL is associated

with chemoresistance in CD34+ acute myeloid leukaemia cells [101], and Bcl-2 level is correlated with chemotherapy sensitivity in breast cancer patients [102]. Deletion or blocking of pro-apoptotic protein Bax promotes chemoresistance in human colorectal cancer and ovarian cancer cells [103, 104].

1.3.2.5 Autophagy

Autophagy plays a dual role in tumorigenesis, contributing to both cell death and cell survival. The induction of autophagy in response to cancer therapy can inhibit tumor initiation and facilitate anti-cancer drug-related cancer cell survival during metabolic stress. There is significant evidence for upregulation of autophagy in tumor cells, with the autophagic cytoprotective response driving acquired resistance to chemotherapy. The EGFR-TKIs gefitinib or erlotinib up-regulate the autophagy level through inhibition the PI3K/Akt/mTOR signaling pathway in human lung cancer cell lines, and gefitinib- or erlotinib-induced cytotoxicity was increased by pharmacological inhibition of autophagy [105]. Imatinib-induced autophagy may 'antagonize' TKI-induced cell death and be responsible for intrinsic resistance in chronic myeloid leukemia stem cells. As autophagy inhibitors, anti-malarial chloroquine and hydroxychloroquine derivatives have been tested in serval clinical trials with cytotoxic autophagy inducers in combination chemotherapy [106]. Autophagy inhibition and combination chemotherapy may overcome resistance or restore sensitivity in temozolomide-treated glioblastoma multiforme, 5-FU-treated colorectal cancer and tamoxifen and trastuzumab-treated breast cancer [107].

1.3.2.6 Tumor microenvironment

The cellular interactions and extracellular matrix, tumor blood flow and vasculature, and tumor hypoxia and acidity are important aspects of the microenvironment in solid tumors [108]. Cells in the tumor microenvironment can induce acquired chemo resistance through such mechanisms as cellular interactions to desensitize cancer cells to apoptosis, soluble factors to promote survival and tumor growth, direct physical cell in contact with tumor cells and improved tumor microenvironment hypoxia after the initial chemotherapy exposure to promote the survive of tumor cells [108, 109]. An example for the interactions between tumor cells and stromata is that myofibroblasts decrease the apoptosis and promote resistance to chemotherapy in a pancreatic ductal adenocarinoma model by reducing the expression of STAT1 and caspases [110]. In addition to solid tumors, the soluble factors in bone marrow microenvironments of multiple myeloma such as IL-6, fibroblast growth factor

(FGF)-3, insulin-like growth factor (IGF)-1 and IFN-α are associated with resistance to cytotoxic therapy through signaling pathways including Ras/Raf/MEK-ERK1/2 pathway, PI3K/AKT pathway, JAK-STAT pathway and Src-family tyrosine kinase pathways. Physical cell contact through integrin-mediated adhesion in multiple myeloma microenvironments takes an essential role in resistance to chemotherapy- induced apoptosis through the CAM super family [111].

1.4 THE PROTEASOME AS A CANCER THERAPY TARGET

1.4.1 Unfolded protein in neurological disorders

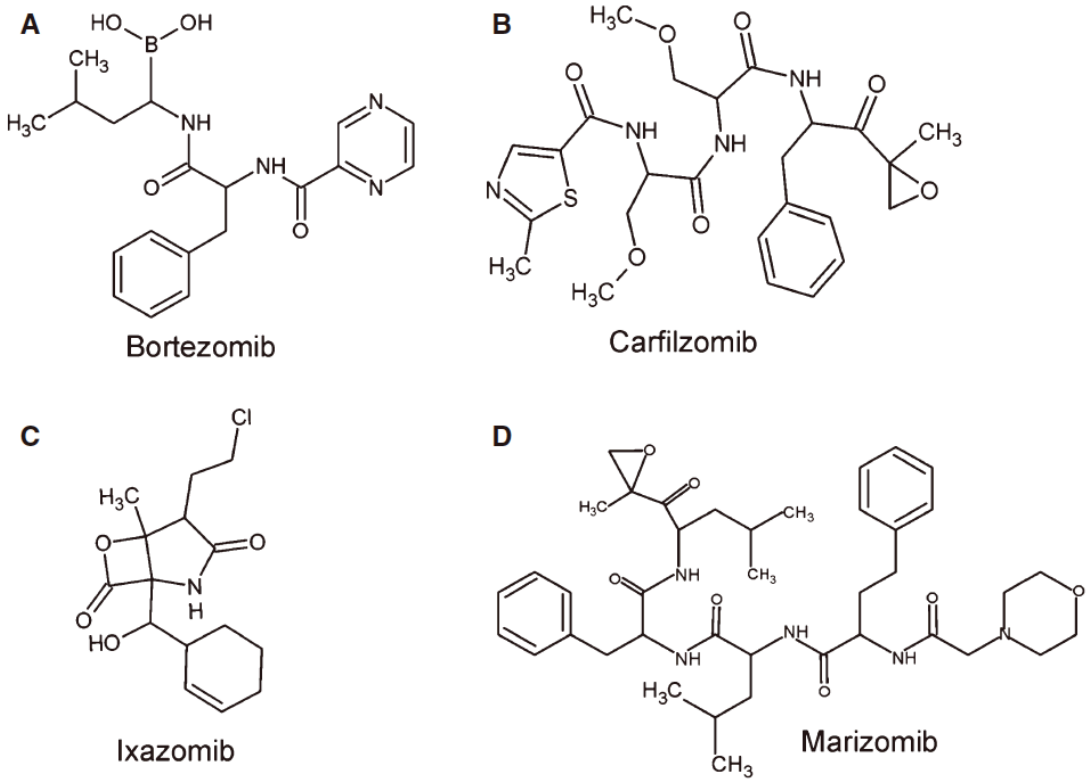

Figure 6. Chemical structures of 20S proteasome inhibitors. (A) Bortezomib, (B) Carfilzomib, (C) Ixazomib, (D) Marizomib.
(From: Proteasome inhibitors –molecular basis and current perspectives in multiple myeloma)

The ubiquitin-proteasome system degrades misfolded and aggregated proteins in mammalian cells in order to maintain normal cell functions. The endoplasmic reticulum (ER) stresses from unfolded proteins accumulation can trigger a series of defense mechanisms, which is known as the unfolded protein response (UPR) [112]. A variety of diseases have been associated with unfolded protein response pathways, such as cancer, neurodegenerative disorders, inflammation and metabolic diseases. The importance of UPR in tissue

homeostasis is mostly studied in neurodegenerative disorders [113]. For example, Parkinson's disease (PD) is characterized as the loss of dopaminergic neurons from the substantia nigra, which is associated with the formation of intraneuronal protein aggregates, termed Lewy bodies [114]. α-synuclein is a small presynaptic protein that tends to misfold and aggregate. It is a main component of Lewy bodies and the post-translational modifications of α-synuclein, such as phosphorylation, ubiquitination and oxidative nitration. In Lewy bodies, it is strongly associated with the pathogenesis of PD [115]. Parkin functions as an ubiquitin E3 ligase, and loss function of parkin in PD, which results in disrupted protein ubiquitination, α-synuclein accumulation and the formation of Lewy bodies, also promotes oxidative and nitrated proteins formation in affected brain regions. Elevated levels of oxidative or nitrative stresses trigger the UPR and ER-associated degradation, with subsequent increase in apoptosis, which directly leads to the degeneration of dopaminergic cells [116, 117].

1.4.2 Proteasome inhibitors in cancer therapy

Deregulation of the ubiquitin proteasome pathway can result in increased or reduced degradation of targets proteins, thus contributing to oncopathogenesis, such as down-regulation of tumor-suppressor proteins p53 and cyclin-dependent kinase (CDK)-interacting proteins $p27^{Kip1}$, or activation of oncogenic proteins NF-κb [118-120]. The proteasome is considered as a promising target for cancer therapy due to the abundance of proteasome in proliferating cells and the important role of the 26S proteasome in regulating diverse biological process of cell functions. A number of 20S proteasome inhibitors (e.g. botezomib, carfilzomib, oprozomib and marizomib) (Figure 6) and 19S proteasome inhibitors (e.g. b-AP15, WP1130 and Azepan-4-ones) have been developed and characterized [66, 121]. Of the commonly studied proteasome inhibitors, bortezomib, ixazomib and carfilzomib have been approved for treatment of multiple myeloma or mantle-cell lymphoma (MCL). Of the first-generation proteasome inhibitors, bortezomib was the first FDA-approved proteasome inhibitor and has been commonly used in first-line and relapsed and/or refractory settings in patients with multiple myeloma or MCL. The second-generation proteasome inhibitors include carfilzomib, ixazomib and oprozomib. In 2012, carfilzomib was approved by the FDA as a single agent for the treatment of multiple myeloma in patients who had received at least two prior lines of therapy, or with disease refractory to the most recent line of treatment. Other new proteasome inhibitors have also been evaluated in clinical trials, including the orally bioavailable reversible peptide boronate ixazomib, the irreversible epoxyketone

oprozomib, the intravenous β-lactone marizomib, and the boronate delanzomib. In 2015, ixazomib was granted by FDA combined with lenalidomide and dexamethasone for the second-line treatment of multiple myeloma [122]. Several mechanisms have been proposed to be involved in the proteasome inhibitor-induced apoptotic effect in tumor cells, such as interference with NF-κB activity, the change of the balance between pro-apoptotic and anti-apoptotic proteins, the interference with cell cycle proteins degradation, the inhibitory effects on angiogenesis and DNA repair, and the potent induction of endoplasmic reticulum stress [123].

1.4.3 Bortezomib

As the proteasome inhibitor first approved by FDA, bortezomib (also known as Velcade®) reversely interacts with N‑terminal threonine of catalytic β subunits resident in the proteasome to inhibit the chymotrypsin-like, the trypsin-like and post-glutamyl peptide hydrolysing activities [124]. The targets signal pathways and action mechanism of bortezomib have been well studied. NF-κB and its inhibitor IκB are present as the inactive complex in cytoplasm, which can be activated by the proteasomal degradation of I-κB. Bortezomib protects IκB from degradation, binding to the promoters of target genes and subsequent translocation of NF-κB to the nucleus. The cell adhesion of multiple myelomas to bone marrow stromal cells (BMSCs) results in the NF-κB-dependent over-expression of IL-6. Inhibition of NF-κB can thereby suppress IL-6-dependent growth of multiple myeloma cells [125]. Bortezomib activates the intrinsic, extrinsic and the ER stress response apoptotic pathways in cancer cells. To activate the intrinsic apoptotic pathways, bortezomib induces pro-apoptotic protein Bax accumulation, resulting in the inhibition of caspase-9 activation and anti-apoptotic Bcl-2[126], promotes down-regulation of apoptosis inhibitors, and up-regulation of pro-apoptotic protein Noxa [127], like XIAP and Bcl-2 through blocking NF-κB expression [128], and also induces p53-dependent apoptosis [129]. To activate the extrinsic apoptotic pathways, bortezomib increases death-inducing receptors, Fas and DR5, and caspase-8 activation and Fas-mediated signaling. ER stress-induced apoptosis will be initiated by bortezomib via caspase-2 activation [130].

In spite of the significant efficacy of monotherapy and combination therapy of bortezomib, patients with multiple myeloma still show primary or secondary resistance to bortezomib in their therapy process. Resistance to bortezomib treatment can be attributed to several mechanisms, including alterations of proteasome subunit compartment, impaired pro-

apoptotic protein accumulation, over-expression of the endoplasmic reticulum chaperone protein, and tumor microenvironments. Over-expression of proteasome subunit β5 (PSMB5) protein results in the upregulation of various proteolytic activities of proteasome and contributes to bortezomib resistance. Mutations (such as Ala49Thr, Met45Val and Cys52Phe) located in the S1 specificity pocket of the proteasome β5-subunit (PSMB5) protein decrease the binding affinity of the PSMB5 to bortezomib. Over-expression of anti-apoptotic protein Bcl-2, which interact with the proapoptotic Bcl-2 family member Noxa, and may inhibits Noxa-induced apoptosis and protect the cancer cells from bortezomib treatment [131].

The aggregation and accumulation of misfolded proteins can be promoted by proteasome inhibitors. Endoplasmic reticular chaperones Grp78/BiP and heat shock proteins can be bound to misfolded proteins, thus preventing them from aggregation and promoting degradation. Therefore, upregulation of protein chaperones Grp78, Hsp27, Hsp70 and Hsp90 have been demonstrated to increase cellular resistance to bortezomib [132-136]. IL-6 and IGF-1 in the microenvironment could affect the growth of tumor cells, thus inducing resistance to bortezomib by activating NF-κB through PI3-K/Akt and Raf/MEKK1pathways [137, 138]. There are other mechanisms involved in the resistance to bortezomib, such as upregulation of reducing equivalents like NADPH [139].

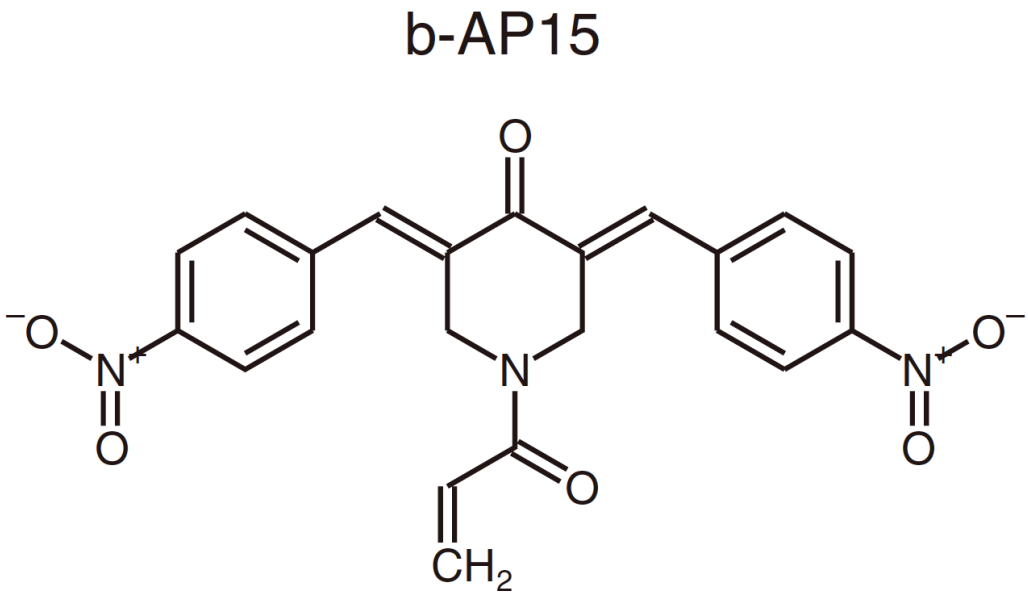

Figure 7. Chemical structures of b-AP15.
(Inhibition of proteasome deubiquitinating activity as a new cancer therapy)

1.4.4 b-AP15/VLX1500

b-AP15 (3,5-bis [(4-nitrophenyl) methylidene]-1-prop-2-enoylpiperidin-4-one, NSC687852) (Figure 7) was originally identified as a small molecule that induces p53-independent apoptosis pathways in a HCT116 colon carcinoma cell line-based screen of synthetic small molecules compounds [140]. Later studies characterized that b-AP15 is an inhibitor of the UPS and induces cathepsin-D- dependent lysosomal apoptosis [141]. Additionally, it elicits a similar gene expression profile with several known proteasome inhibitors. b-AP15 reversely inhibits the activities of two 19S proteasome-associated DUBs USP14 and UCHL5. Treatment with b-AP15 induces the accumulation of ubiquitin conjugates and inhibitors of cell cycle-dependent kinases, such as CDKN1A, CDKN1B and tumor suppressor p53, thus resulting in inhibition of the UPS. b-AP15 induces apoptosis of colon cancer cells, which is not responsive to TP53, BBC3 and Bax disruption and Bcl-2 over-expression [142]. b-AP15 induces both intrinsic and extrinsic apoptosis in multiple myeloma cell lines by activating caspase 3, caspase 8 and caspase 9, and also gives rise to the mitochondrial apoptosis pathway through activation of proapoptotic Bcl-2 family members Bax [143]. In addition, animal studies have demonstrated the efficacy of b-AP15 in squamous carcinoma model, colon carcinoma model, lung carcinoma model, breast carcinoma model, acute myeloid leukemia model and multiple myeloma models. Bortezomib-resistant multiple myeloma cells can be sensitized by b-AP15. Synergistic anti-multiple myeloma activity is observed in the combined treatment of b-AP15 with lenalidomide, suberoylanilide hydroxamic acid and dexamethasone [144]. A recent study shows that b-AP15 can exert equivalent inhibitory potency in both 20S proteasome and 19S regulatory domains [145].

2 HYPOTHESIS AND AIM

Scientific Hypothesis of this thesis

Compared to bortezomib, b-AP15 is a more effective proteasome inhibitor in malignant cells.

Scientific Aim of this thesis

My PhD thesis aimed to explore different resistance mechanisms of b-AP15, thus improving the therapeutic efficiency of b-AP15.

Specific scientific goals for each study were as follows.

Study **I**: to investigate whether the increase of misfolded proteasome substrates could sensitize the therapeutic functions of ubiquitin–proteasome system (UPS) inhibitors in cancer cells.

Study **II**: to assess if b-AP15, a novel small molecule inhibitor of proteasome deubiquitinase activity, could induce the accumulation of poly-ubiquitin with the absence of aggresome formation.

Study **III**: to study the therapeutic effects of b-AP15 in lymphoma cell models, and confirm b-AP15 could induce apoptosis in lymphoma cell models, as a proteasome inhibitor.

Study **IV**: to characterize the cellular response to proteasome DUB inhibition of bAP-15 treatment.

Study **V**: to examine whether piperlongumine was an inhibitor of the ubiquitin–proteasome system (UPS) in the induction of apoptosis in cancer cell models.

3 METHODS

Materials and methods used in different studies are detailed in each article. In the following section, some of the important methods are discussed.

3.1 CELL LINES AND CELL CULTURE

As the major malignant cell model, HCT116 cells were involved in studies I, II, IV and V. hTERT-RPE1 cells functioned as a non-malignant cell model in studies I, II, IV and V. HeLa cells were used in immunofluorescence experiments to demonstrate aggresome formation using different proteasome inhibitors. MelJuSo-UbG76V-YFP cells functioned as an ubiquitination reporter cell line. To confirm the therapeutic mechanism of bAP15 in lymphoma, 8 DLBCL lymphoma cell lines and 4 Hodgkins lymphoma cell lines were used in study III.

All the cells were cultured in humidified 5% CO_2 atmosphere at 37°C. HCT116 cells[142] were cultured in McCoy's 5A modified medium with 10% serum. RPE1 cells[142] were cultured in DMEM:F12 medium with 10% serum. HeLa cells were cultured in DMEM medium with 10% serum. MelJuSo cells[146] were cultured in DMEM medium with 10% serum. The 12 different lymphoma cell lines were cultured in RPMI medium with 10% serum, 2nM glutamine, 100U/mL penicillin, and 100mg/mL streptomycin.

3.2 APOPTOSIS ASSAY

Apoptosis between different proteasome inhibitors, combinations, doses or treatment times were compared using an ELISA kit, M30-Apoptosense ELISA[147]. The antibody of the M30 kit is specific for a neoepitope of cytokeratin 18, which is cut by caspase 3, 7 or 9 during the occurrence of apoptosis.

Aliquots of 104 target cells were seeded in 96-well plates and cultured for 16 hours with different treatment protocols. NP40 lysis buffer was added at the end of the treatment protocol to terminate the culturing, after which the apoptosis assay was conducted using the M30-Apoptosense ELISA kit.

3.3 WESTERN BLOTTING

Different target cells were lysed in NP40 lysis buffer with freshly added proteasome inhibitor cocktails on ice. Lysates were cleared by 13000g centrifugation for 10 mins. The Bradford assay was conducted to measure protein concentration in each sample. Samples

were adjusted to equivalent concentrations with 5X western blot loading buffer. 3-8% gradient gels were used to separate ubiquitin-conjugated proteins. Proteins were transferred to PVDF membranes, which were blocked with 5% dry milk in PBST buffer. Finally, interested proteins could be detected using primary and secondary antibodies; an ECL kit was used for to enable visualization by chemiluminescence.

3.4 IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

Microscope coverslips were placed in 6-well plates and then were sterilized with 70% ethanol and 15 mins UV exposure. Target cells were seeded and incubated overnight. Cells were treated with different proteasome inhibitors, combinations, doses or periods.

After treatment medium was removed from the plate, and following washing with PBS, 4% formaldehyde was used to fix cells for 20 mins. 1% Triton X-100 in PBS was used for permeablization for 15 mins. Plates were then blocked with 5% dry milk in PBS for 30 mins. Coverslips were removed from the plate and then incubated with different specific primary antibodies for 16 hours at 4^{o}C. The 2nd antibody was incubated at 20^{o}C for 1 hour. Finally, coverslips were transferred to microscope slides and mounted with DAPI mounting media.

4 SUMMARY AND DISCUSSION

The PhD project aimed to understand therapeutic actions and different resistance mechanisms of b-AP15. I explored how ROS, the chaperone and aggresome systems worked during b-AP15 treatment; tested the action of b-AP15 resistance; and explored the possibility of using b-AP15 to overcome bortezomib resistance. I also tested b-AP15 application in lymphoma cell models. To the end, I was involved in the project of verifying the role of piperlongumine as a proteasome inhibitor.

4.1 STUDY I: SENSITIZING CELLS TO APOPTOSIS BY INCREASING MISFOLDED PROTEINS

Background:

As a 19S proteasome subunit inhibitor, b-AP15 can inhibit proteasome function in cells. After the proteasome is blocked by b-AP15, a huge quantity of polyubiquitinated proteins was accumulated in cells. According to previous study results, b-AP15 exhibits promising anti-cancer effects in different solid tumor models. As a potential chemotherapy candidate, the compound was very toxic in cancer cells by inducing apoptosis, but not toxic in non-malignant cells.

Hypothesis:

Cells might be more sensitive to b-AP15 treatment, with increase of the total number of polyubiquitinated proteasome substrates.

Methods:

Sec61-mediated protein translocation inhibitor (CpdA) was used to increase the cellular levels of proteasome substrates in tumor cells and non-malignant cells. Later, cells were exposed to b-AP15 or bortezomib for certain times or concentrations. Western blotting was used to determine the accumulation of misfolded proteins, ER stress markers and apoptosis response markers. Cell survival was assessed under the same treatment conditions, with confirmed results using the MelJuSo-UbG76V-YFP reporter cell line by FACS.

Results:

By comparing the treatment outcome between b-AP15 and bortezomib, it was shown that b-AP15 could induce the accumulation of high molecular weight polyubiquitinated conjugates, thus resulting in higher apoptosis in cancer cells. CpdA was a co-translational translocation inhibitor, which could affect protein translocation between endogenous prosaposin and the ER. The co-treatment of CpdA and b-AP15 could consequently enhance proteasome

inhibition. Therefore, CpdA pre-treatment was conducted in order to increase polyubiquitinated proteins in different cell models in order to make them sensitive to b-AP15 treatment. Adding extra cysteine into HCT116 cells did not rescue the apoptosis mediated by b-AP15. Furthermore, there was no significant decrease of cellular cysteine levels by the co-treatment of CpdA and b-AP15.

REFLECTIONS

This study introduced me to the field of proteasome inhibitors and multiple myeloma. I learned some basic knowledge of misfolded protein response elements, such as ER stress, chaperones and apoptosis and drug resistance. I also learned strategies about how to increase the therapeutic window in the development of cancer and drugs, and even about the whole drug discovery and development process.

There were several technical challenges for this project: i) developing a heat shock method to increase the level of chaperone expression in cancer cells; ii) human and mouse primary cell culture; iii) quantitative analysis of cell death in multiple cell lines, and different treatment protocols.

4.2 STUDY II: B-AP15 INHIBITS CYTOPROTECTIVE AGGRESOME FORMATION IN CANCER CELLS.

Background:

Proteasome inhibitors can kill cancer cells by inducing an acute proteotoxic stress response characterized by the accumulation of poly-ubiquitinated proteins, ER stress and the production of reactive oxygen species (ROS). The aggresome pathway is described as an escape mechanism from proteasome inhibitor-induced cytotoxicity. Previously, we have determined in Study I that b-AP15 and bortezomib were both proteasome inhibitors, although b-AP15 treatment resulted in the accumulation of higher molecular weight polyubiquitinated proteins and higher apoptosis, compared with bortezomib.

Hypothesis:

b-AP15 will induce the accumulation of poly-ubiquitin with the absence of aggresome formation.

Methods:

Hela and HCT-116 cells were treated with different concentrations of b-AP15 or bortezomib +/- CpdA at different time points. Ubiquitin and aggresome in cells were determined by immunofluorescence staining and visualized with confocal microscopy. Solubility of aggresome or aggregates was confirmed by the exposure of cells to saponin, which also

released the cytoplasm. Proteins involved in HDAC6 ubiquitination pathways were demonstrated using immunoprecipitation and immunofluorescence confocal microscopy

Results:

In Hela cells, after an overnight treatment, bortezomib induced aggresome formation. However, b-AP15 did not induce aggresome formation under the same conditions. We showed that b-AP15 could induce vimentin perinuclear clustering. Interestingly, we observed less aggresome formation in the co-treatment of b-AP15 and bortezomib compared with the single treatment of bortezomib. It is suggested that b-AP15 could disrupt aggresome formation. In this study, we also determined that aggresome formation was independent of ubiquitin, which was not inhibited by b-AP15. Chaperone binding to polyubiquitin not inhibited by b-AP15. In conclusion, it was shown that HDAC6 ubiquitination may be mediated by b-AP15 treatment.

REFLECTIONS

During this study, I gained knowledge about aggresome and cyto-protective mechanisms in cells were treated with proteasome inhibitors. Similar to study I, this is another resistance mechanism, and the study of which further enriched my knowledge about tumor resistance. I also learned some knowledge about neurological disorders such as Parkinson's disease due to the similarity of aggresomes to Lewy bodies and the role of UPS inhibition in neurodegenerative diseases. The knowledge of HDAC6 ubiquitination was another learning outcome from this study. This study enhanced my practical skills of immunofluorescence confocal microscopy.

4.3 STUDY III: INHIBITION OF PROTEASOME BY B-AP15 IN LYMPHOMA CELL LINES.

Background:

Bortezomib has been well studied as a proteasome inhibitor, exhibiting significant anti-cancer activity in different cancer cells. Bortezomib is the first FDA approved proteasome inhibitor drug for multiple myeloma and later also approved for lymphoma treatment. Although bortezomib had very good performance in the beginning of treatment, numbers of patients acquired resistance to the compound. New chemotherapy drugs are needed to overcome the resistant of bortezomib in multiple myeloma and lymphoma treatments.

Hypothesis:

b-AP15 can inhibit the 19S proteasome in lymphoma cell lines.

Methods:

Cell viability of 12 different lymphoma cell lines was evaluated using a fluorometric microculture cytotoxicity assay in 96-well microtiter plates. The ER stress response and apoptosis markers were determined by western blotting.

Results:

A significant concentration-dependent decrease in viability of all lymphoma cell lines (both DLBCL and Hodgkins lymphoma) was observed in response to b-AP15 treatment. We demonstrated that lymphoma cell lines were as sensitive to b-AP15 as other cancer cell lines previously reported.

The treatment of all cell lines with b-AP15 promoted the accumulation of high-molecular weight polyubiquitin conjugates, which were correlated to the expression of heat shock protein 70 (Hsp70B´). Interestingly, all markers, such as polyubiquitin conjugates, Hsp70B´, caspase-3 expression and PARP cleavage showed significant concentration-dependent responses to b-AP15 treatment.

REFLECTIONS

In this study, I learned clinical and epidemical knowledge about lymphomas. This study gave me a good chance to practice floating cell culturing and to learn the fluorometric microculture cytotoxicity assay.

4.4 STUDY IV: PROTEASOME INHIBITOR INDUCED CANCER CELL APOPTOSIS BY OXIDATIVE STRESS

Background:

b-AP15 is well studied as a 19S proteasome inhibitor, which interferes with deubiquitinase (DUBs): USP14/UCHL5 on the 19S proteasome subunit. b-AP15 induces accumulation of polyubiquitinated proteasome substrates by blocking the cellular protein turnover and inhibiting proteasome.

Hypothesis:

Compared with clinically approved 20S proteasome inhibitors, b-AP15 targets the 19S proteasome subunit. b-AP15 will be an efficient chemotherapy drug candidate, especially for current proteasome inhibitor-resistant tumors.

Methods:

To compare the cellular stress responses between b-AP15 and bortezomib, a q-PCR assay was used to examine the pattern of transcripts for 84 genes related to stress responses. Microarray analysis was also conducted with b-AP15- or bortezomib-treated cells.

Live-cell analysis of UPS activity was conducted in ubiquitin reporter cell lines MelJuSo UbG76V-YFP cells following either b-AP15 or bortezomib treatment. The generated fluorescence was continuously detected using an IncuCyte FLR instrument.

Results:

The gene expression patterns of b-AP15- or bortezomib-treated cells were similar but not identical, which is consistent with the previous study that although both compounds are proteasome inhibitors they function via different mechanisms. This study showed the apoptosis induced by b-AP15 was also different to bortezomib-mediated apoptosis. Both b-AP15 and bortezomib could induce the expression of Hmox-1 [heme oxygenase (decycling) 1], but only b-AP15 could induce ER stress. The expression of Hmox-1 was correlated with the accumulation of high molecular weight polyubiquited proteasome subtracts during b-AP15 treatment. The study also showed that ROS scavengers could reduce the apoptosis induced by b-AP15, with activation of AP-1 to mediate b-AP15-derived apoptosis.

REFLECTIONS

This study gave me the opportunity to deepen my knowledge of drug discovery and development, especially how to pattern candidate drugs according to the existing drugs and how to perform subsequent studies with candidate drugs. I also learned basic knowledge of ROS signaling in proteasome inhibitor drug applications.

4.5 STUDY V: CONFIGURATION OF PIPERLONGUMINE AS A PROTEASOME INHIBITOR

Background:

Piperlongumine is a natural product from the plant Piper longum. In previous studies, it was demonstrated that the compound is cytotoxic to tumor cell lines and has anti-cancer effects in different solid tumor models.

Hypothesis:

As an inhibitor of the ubiquitin proteasome system, piperlongumine will induce apoptosis in cancer cells.

Methods:

A fluorometric microculture cytotoxicity assay (FMCA) was performed to measure the cytotoxicity of piperlongumine. *In vitro*, a proteasome activity assay was introduced to examine the protein turnover activities in different proteasome subunits following piperlongumine treatment. A Cellomics Oxidative Stress I Kit was used to evaluate oxidative stress induced by piperlongumine.

Results:

In this study, it could be confirmed that the gene expression pattern following piperlongumine treatment was similar to that of other proteasome inhibitors. Piperlongumine treatment could block the ubiquitin–proteasome system in cancer cells. However, the accumulation of polyubiquitinated proteasome substrates was induced by direct proteasome inhibitor, data suggest piperlongumine was not a proteasome inhibitor, but interfere the upstream of UPS system. In MelJuSo cells, we confirmed that the cytotoxic effects of piperlongumine treatment were correlated with oxidative stress.

REFLECTIONS

In this study, I had the opportunity to obtain some basic knowledge about how to discover and develop drugs from natural compounds. It also allowed me to work with a compound that was neither a 19S-proteasome inhibitor nor a 20S-proteasome inhibitor, but still performed the activity of proteasome inhibition. This study also enriched my understanding of the UPS system and ROS signaling.

4.6 DISCUSSION

4.6.1 Accumulation of different molecular weight polyubiquitinated proteins by different proteasome inhibitors

A previous study indicated that b-AP15 could induce higher molecular weight polyubiquitinated proteins compared to those induced with bortezomib [142]. In my first study, we confirmed this difference following treatment of HCT116 cells, and during my PhD studies, I also studied these effects using several other cell models (including Hela, MelJuSo and RPE1 cells). We know that the proteasome includes 3 subunits, and that during protein turnover the 19S subunit is first linked to the polyubiquitinated proteins, after which the protein will insert into the 20S subunit [148]. Bortezomib is an inhibitor of the 20S subunit, which means even we inhibit the function of 20S subunit, polyubiquitinated proteins will still recognize and be linked to the 19S subunit, and that DUBs on the 19S subunit will still remove some ubiquitin from the conjugates. However, b-AP15 is a direct inhibitor of the 19S subunit, which will totally block the function of the proteasome. No DUBs on the proteasome will thus be active, so the molecular weight of polyubiquitinated proteins will be higher. In study V, the action of piperlongumine treatment led to accumulation of the highest molecular weight polyubiquitinated conjugates. Considering that b-AP15 is a direct inhibitor of the DUDs on the 19S subunit, this must thus indicate that piperlongumine acts upstream in the UPS systems and does not function as a proteasome inhibitor.

4.6.2 Increasing the therapeutic window of proteasome inhibitors

During the first day of my PhD I remember there was an interesting cartoon posted in the lab, which said "Remember both salt and a gun can kill cancer cells." This is a good example of how we should consider drug development for cancer treatment. The strategy for cancer drug development must not only identify compounds which can kill cancer cells, but that also do not have too many side-effects. According to previous studies, proteasome inhibitors distinguish cancer and non-malignant cells by their protein turnover rates [122]. Cancer cells synthesize huge amounts of proteins compared to non-malignant cells; and at the same time cancer cells always carry a lot of mutations. For cancer drug development, we thus need to identify compounds that can make this distinction. In our studies, we could demonstrate that b-AP15 was very sensitive in inducing apoptosis in cancer cell lines (HCT116 and RPE1), but did not induce too much apoptosis in non-transformed and primary cells (human fibroblast cells). b-AP15 thus fulfills the requirement for a cancer drug that primarily targets malignant cells.

We introduced another small molecule CpdA that is also known as CAM741 [149] into our study. CpdA was originally discovered as a vascular cell adhesion molecule 1 inhibitor [150], a low dose application of CpdA inhibiting protein translational translocation in the ER [149]. Exposure of cells to low concentrations of CpdA will only slightly reduce the growth rate of malignant cells, and it will not induce apoptosis or any other cytotoxic effect in either cancer or normal cells. The apoptosis induced by b-AP15 correlated to the accumulation of polyubiquitinated conjugates assembled from misfolded proteins and ubiquitin. We pretreated cells with CpdA to achieve a high amount of misfolded proteins, and then blocked the proteasome using b-AP15. To scientific rationale of this co-treatment was that by accumulating sufficient amounts of polyubiquitinated proteins through the action of the non-toxic compound CpdA we could lower the dose of b-AP15 required for its toxic treatment effects. We thus expected to increase the therapeutic window of b-AP15 by using CpdA.

While the rationale was sound, the reality proved different. In HCT116 cells we did observe that CpdA sensitized the apoptosis induced by b-AP15. However, the non-transformed RPE1 cells and human fibroblast cells were also sensitized by CpdA, which means that instead of increasing the therapeutic window we decreased the therapeutic efficacy of b-AP15. A similar phenomenon following co-treatment with CpdA and bortezomib has been reported by another group [151].

An important perspective is that cell lines are not a good model to mimic normal cells in the human body. Furthermore, proteasome inhibitors do have side effect on stem cells, germ cells and pluripotent cells as these also have a high amount of protein synthesis. Nonetheless, considering that CpdA is not toxic to cells it is still a promising compound to investigate in its capacity to increase the therapeutic efficacy of proteasome inhibitors.

4.6.3 Dominant cytotoxicity mechanisms of proteasome inhibitors

In the academic world, there is a continuous debate about the underlying cytotoxic mechanisms of action of proteasome inhibitors. One part of the academic society believes the dominant cytotoxicity to be induced by increased levels of ROS and ER stress and focus on polyubiquitinated conjugate accumulation. However, the other part considers that it is the stabilization of anti-tumor proteins that is critical for this anti-cancer activity.

By introducing co-treatment of CpdA and proteasome inhibitors we not only induce apoptosis in cancer cells but also kill non-malignant cells. Considering the function of CpdA is only to create more misfolded protein in the cell (but not additional functional proteins) we assume that the amount of anti-tumor proteins is maintained at the same level. However, by introduce the substrates for UPS we do increase the apoptosis. According to this finding, we can thus conclude that although the evidence could not exclude the possibility of stabilization of anti-tumor proteins, there is quite solid evidence to support the polyubiquitinated protein hypothesis.

Through inhibition of the proteasome, protein degradation in the cell is blocked. As we know, tumor suppression proteins are rapidly turned over by the UPS system, and a blocked UPS will lead to accumulation of significant amounts of tumor suppressors. The high level of tumor suppressors will be cytotoxic to cancer cells, Myc and p53 upregulation by bortezomib inhibition inducing apoptosis in different cancer models [152-154]. A previous study of our group demonstrated Bcl-2 over-expression in bortezomib resistant cells. p53 and p21 accumulation through the action of b-AP15 treatment has also been reported before [142]. Taken together this suggests that tumor suppressor stabilization also plays an important role in proteasome inhibitor application, and that gene expression in apoptosis pathways can be also interfered by long-term expose to these compounds. Further investigation in this field is warranted to provide unequivocal evidence for this effect in to avoid any uncertain risk of clinical applications.

Some other people claim that the major mechanism of proteasome inhibitor is inefficient amino acid turnover. Previous studies have reported amino acid depletion to be important in yeast and murine cells [155]. Amino acids are reused in protein turnover. When old or misfolded proteins are recognized and linked with ubiquitin chains, the protein will be degraded by the proteasome and the amino acid and short peptides are released back into the cytosol, which will rebuild into new peptide or proteins. By blocking the proteasome, protein synthesis will eventually be compromised as there will be no available amino acids to build up. As a result, there will be no new proteins made. However, our studies suggest that block of the proteasome does not result in amino acid depletion, as using cysteine as a marker of intracellular amino acids we did not observe significant amino acid down-regulation.

4.6.4 Acquired resistant to bortezomib and b-AP15

Bortezomib is the most successful clinically approved proteasome inhibitor used in the treatment of multiple myeloma [156]. However, bortezomib is also known to be easy to develop acquired resistance to [156]. Although the mechanism of this resistance is yet unclear, several papers have reported that aggresome formation and cellular chaperone responses are correlated with resistance [132, 157].

Our next approach was thus to try to set up cell lines that were resistant to both bortezomib and b-AP15 in order to have a test system that mimics the clinical situation. To this end, HCT116 cells were first exposed to low concentrations of bortezomib and b-AP15, and then the concentrations were increased every month. After 14 months, we had successfully achieved bortezomib resistant cell lines. However, we could not increase the tolerance of HCT116 cells to b-AP15. These results were thus of great significance, as they indicate that acquired resistance to b-AP15 is less likely than that known to develop to bortezomib, showcasing the potential high value of b-AP15 as a novel candidate drug.

The cytotoxicity of proteasome inhibitors is correlated with the accumulation of polyubiquitinated proteins [142]. The underlying mechanism of aggresome protection is not clear either. However, we know that polyubiquitinated chains conjugated to misfolded proteins could upregulate ROS induction and ER stress [122]. Our major hypothesis for aggresome protection is that the cellular aggresome is insoluble in the cytoplasm. We show (the data was not included in the thesis or appendix paper) that, using saponin to release the cytoplasm of the cell which treated with bortezomib. As we expected aggresomes were not eliminated from the cells. Which suggested aggresome is an insoluble structure in the cell,

and cell by assemble aggresome will high reduce the polyubiquitin chains which are distributed in the cytoplasm. Only the polyubiquitin chain on the surface of the aggresome are still active to induce cytotoxicity.

Data presented in my second study demonstrated that following treatment of Hela cells with bortezomib or b-AP15 aggresomes only formed in bortezomib treated cells (but not in the b-AP15 treatment group). To confirm this result, I tested more treatment conditions (data was not included in this thesis or the appendix papers). We confirmed with 3, 6 or 12 hours of treatment that aggresomes only formed in the bortezomib treatment group. However, after 18 and 24 hours of treatment b-AP15 also induced aggregates and aggresomes. Both bortezomib and b-AP15 induce cytotoxicity through apoptosis. Compared to aggresomes that are evident after 6 hours of bortezomib treatment, it takes at least 18 hours for b-AP15 to assemble aggresomes. Since the apoptosis program has already started by this time point, the aggresome formation is too late to rescue cells from apoptosis, and this explains the lack of development of cell resistance to b-AP15. Interestingly, we had also observed co-treatment with b-AP15 could overcome the early aggresome formation associated with bortezomib treatment. This suggests that b-AP15 should have another target that is outside of UPS but involved in the aggresome formation pathway.

The major candidate for this alternative target is HDAC6, which is known as an atypical deacetylase [158]. Within the aggresome pathway HDAC6 plays an important role in stepwise development of the aggresome, from polyubiquitinated conjugates into aggregates and finally into aggresomes [159]. In our study, we showed that b-AP15 treatment led to significantly increased polyubiquitination of HDAC6. Considering that b-AP15 is a DUB inhibitor, and DUBs are not only localized on the proteasome but are also distributed in the cytoplasm, we assume that b-AP15 treatment frees up DUBs which inhibit de-ubiquitination of HDAC6. The recruiting and transporting functions of HDAC6 are mediated by the polyubiquitination, HDAC6 is only active when the polyubiquitin chains are partially removed by DUBs. Similar published results also support our hypothesis, such as that using HDAC6 inhibitors during bortezomib treatment reduces aggresome formation [160].

Further studies will be needed to determine: which DUBs are involved in de-ubiquitination of HDAC6; is it directly inhibited by b-AP15; or just due to the DUBs on the 19S proteasome being inhibited by b-AP15, and the free DUBs in the cytoplasm binding to the substrates which are accumulated by UPS malfunction. Our study also suggests that the

proteasome pathway presents new potential for drug development, such as using HDAC6 and tubulin inhibitors to overcome proteasome inhibitor resistance, or co-treatment to reduce the concentration of proteasome inhibitors and increase their therapeutic efficacy.

Chaperone overexpression is another phenomenon that is associated with bortezomib resistant [161]. According to a previous study in the group, HSPA6 is the chaperone that is highly induced by b-AP15 treatment [142]. It is quite important to know the role of chaperones in b-AP15 treatment. In study I, II, III and IV we observed that HSPA6 expression was highly drug concentration dependent on b-AP15, and this also correlated with the accumulation of polyubiquitinated proteins. In study I, II and III our results indicate that HSPA6 expression is also correlated to bortezomib treatment. Especially in study I, we demonstrated that HSPA6 expression can also be up-regulated by CpdA co-treatment. In study V we detected a drug concentration-dependent relationship between HSPA6 and piperlongumine. Taken together these data suggest that HSPA6 is the chaperone involved in cellular response mechanisms against UPS inhibition, polyubiquitination being the trigger of HSPA6 expression. Although the HSPA6 expression level is correlated with the accumulation of polyubiquitinated proteins, apoptosis is still most highly induced in the high polyubiquitinated group. We assume the HSPA6 has a cellular defense activity, but that this could not fully reverse the cytotoxicity induced by UPS inhibitors.

In order to address whether HSPA6 has a cellular protection function or not, we designed as experiment to mimic the high level of HSPA6 observed in clinical cancer patients. We pre-treated HCT116 cells for a short time to mildly heat shock the cells, which were then left to recover. This mild heat shock did not induce much cellular apoptosis. The results showed that HSPA6 expression can detected by western blotting from 3 hours, with a peak at about 6-8 hours, and then the expression decreases. We thus compared b-AP15 treatment with or without pre-heat shock and 6 hours' recovery. Interestingly, there was significant inhibition of b-AP15-induced apoptosis in the pre-heat shock group. This suggests that HSPA6 can only play a cellular defense role if the cell already has a high HSPA6 expression level. Although HSPA6 expression may act in concert with UPS inhibition, the cell seemingly cannot express enough chaperone to reverse the apoptosis already induced by UPS inhibition.

We also test the HSPA6 levels in our 14-month-cultured bortezomib or b-AP15 resistant cells, but there was no significant upregulation of HSPA6 expression. Although our cells are super resistant to bortezomib, there is thus no obvious evidence that HSPA6 over-expression

explains the bortezomib super-resistance. Despite that our *in vitro* simulation of bortezomib resistance and heat shock responses are not fully represented of the clinical *in vivo* situation experienced by patients, our results still confirmed the importance of aggresome formation and chaperone responses. Further investigation should focus on determining the key mechanisms underlying the bortezomib resistant.

In summary, we can conclude that both aggresome formation and HSPA6 expression can downregulate the apoptosis mediated by bortezomib, and that it is relatively easy to induce bortezomib resistance in cells. Luckily, our research candidate b-AP15 is harder to induce resistance to, and could also inhibit aggresome formation. It is thus a promising candidate for further clinical development.

Some other interesting data generated during the aggresome project in the cell line models we tested (Hela, HCT116 and MelJuSo cells), was that aggresome formation had similar kinetics with the same proteasome inhibitors. Aggresomes formation always started as random small aggregates inside the cytosol, then small aggregates started to get together organized along cytosolic microtubules, culminating in a dominate aggresome close to the nuclei. Through pre-treatment with CpdA we achieved a significant increase in size of aggresome formation. By using anti-ubiquitin K48 and p62 specific antibodies we could detect the co-localization of aggresomes, polyubiquitin and p62. We determined that b-AP15 did not interfere with function of the microtubule system.

Taken together we thus confirmed that bortezomib-mediated aggresome formation is p62 chaperone-dependent, that polyubiquitin chains are a major component of aggresomes, and that b-AP15 inhibition of aggresome is microtubule-independent.

4.6.5 Application of b-AP15 in lymphoma

Bortezomib is not only approved for clinical use of treatment in multiple myeloma, but is also FDA approved for the treatment of mantle cell lymphoma (MCL) [162]. In my third study, we introduced multiple lymphoma cell lines to test general sensitivity to b-AP15.As expected, lymphoma cells exhibited clear concentration-dependent reduction in viability, and in the western blot assay we observed a correlation between polyubiquitinated protein accumulation, ER stress marker expression and apoptosis marker expression. We included both HL and DLBCL cell lines in this study and compared with adherent tumor cells (HCT116, Hela and MelJuSo cells). The lymphomas cells were very sensitive to b-AP15

treatment, if we compare with HCT116 cells there was about a 5-to-10-fold sensitivity difference to lymphoma cells. We assume that this may be due to the lymphoma cells being non-adherent cells, which might increase their chance of exposure (due to their relatively larger surface area). There was no significant difference between HL and DLBCL cells. Our result suggests further consideration of b-AP15 development in lymphoma treatment.

One systematic limitation of b-AP15 and some of its structural analogues is its very low solubility. In all the *in vitro* studies we used DMSO to dissolve the compound, which was further diluted in PBS. For *in vivo* studies we tried to use Tween20 (detergent) to dissolve the compound. The solubility problem restricts the research of b-AP15. However, the non-polarity structure of b-AP15 makes it very easy to pass through cell membranes.

As the lymphoma cells were very sensitive to b-AP15, we employed concentrations of b-AP15 much higher than the EC50 concentration. Using the cell survival and western blot assays, we could then observe that the induced cytotoxic effects switched from cell apoptosis to cell necrosis. As for u2940 and su-DHL-6, we could detect decreases in apoptosis, polyubiquitination and ER stress markers at the highest concentrations of b-AP15. Interestingly, in WSU-NHL cells, all the markers cycled between increased and decreased expression, before increasing again, which we assume is an off-target effect of b-AP15 treatment together with interference of HDAC6 de-ubiquitination. This suggests that there is more than one target in the b-AP15 treatment profile. Further investigation will be necessary to discover and understand the other target or targets of b-AP15 treatment prior to clinical application.

There was an unpredictable incident during this project in that 4 of the 12 lymphoma cell lines which we received from our collaborator did not pass the cell identification check. Although in the end this did not affect the conclusion of this study, it still taught me an important lesson. Similar systematic confounding events that occurred in my studies was contamination with mycoplasma. Another systematic issue concerned the appropriateness of our model. Bortezomib is a successful drug in multiple myeloma treatment, and which was also reported as good choice for non-solid tumor treatment. However, the major cancer model we were working with is HCT116 cells which is a colon cancer model. Most of the animal studies involved solid tumor models. Although you could argue as HCT116 cells is a good model for study general cancer mechanisms there were several gene mutation models available in HCT116. But if we consider the off-target effects and that a cytotoxic switch

between apoptosis to necrosis was only observed with non-adherent cells, this suggests that multiple cell models should be employed.

4.6.6 Comparison of therapeutic mechanisms in bortezomib and b-AP15 treatments

In our study, we clearly demonstrate that b-AP15 induced apoptosis much more rapidly than did bortezomib (Fig. 2A). Bax and Bak are not required for the apoptosis pathway induced by b-AP15, but they do play dominate roles in bortezomib-induced apoptosis (Fig 2B). b-AP15 is a 19S proteasome inhibitor and bortezomib is a 20S proteasome inhibitor, and the 19S proteasome inhibitor always accumulated higher molecular weight proteasome substrates compared with 20S proteasome inhibitors. This indicates that the polyubiquitin chains on the b-AP15-induced conjugates are longer than the bortezomib-induced conjugates. We thus suggest that b-AP15-induced rapid apoptosis is due to the longer polyubiquitin chain and does not require Bak and Bax.

Oxidative stress has been reported as one of the major s for proteasome inhibitor- induced apoptosis [163, 164] and Hmox-1 is induced in response to oxidative stress [165]. In our study, we show that b-AP15 highly upregulated Hmox-1 gene expression induced ROS much more so than did bortezomib. Subsequent experimentation comparing employing a ROS scavenger (Trolox and NAC) suggested that ROS induction is the key mechanism underlying b-AP15-induced apoptosis.

Proteasome inhibitors could induce oxidative stress through ASK1following activation of p38-MAPK and JNK [166], as reported for bortezomib-treated cells [167]. Our results confirmed b-AP15 could induce the activation of p38-MAPK and JNK, but that only JNK is a direct downstream mediator of the oxidative stress induced by b-AP15.

ER stress was also reported as a conservative phenomenon occurring during proteasome inhibitor treatment, as has previously been reported for bortezomib [168]. In our study we demonstrate that b-AP15 treatment could upregulate CHOP expression, which is considered as the marker of ER stress. However, the ER stress responses induced by b-AP15 and bortezomib are different, as only b-AP15 could induce the phosphorylation of Eif2- α.

So while bortezomib and b-AP15 are both proteasome inhibitors, both can induce apoptosis, and both can induce of oxidative stress and ER stress, the underlying mechanisms for these effects differ between the two drugs. Furthermore b-AP15 induces a more rapid apoptosis

than does bortezomib. We consider that this indicates that b-AP15 has high potential as a candidate in anti-cancer applications, especially in settings of overcoming bortezomib resistance.

4.6.7 Proteasome inhibitors and neuronal disorders

Similar phenomena such as aggresome formation, oxidative stress and ER stress induced by proteasome inhibitors is also characteristic of human neurodegeneration disease, such as Parkinson's Disease, which is characterized by the formation of protein aggregates and Lewy bodies. There is currently no clinical, in *vivo* or *in vitro* data to suggest that proteasome inhibitor treatment will lead to neurodegeneration disorders, but peripheral neuropathy is reported as a major side-effect of bortezomib in the clinical applications [169]. Peripheral neuropathy is commonly described as damage, inflammation or desecration to peripheral neurves [170]. During bortezomib treatment, peripheral neuropathy has reported as symptoms including neuropathic pain, hypotension, sexual dysfunction and constipation [171, 172].

Cancer cells exposed to bortezomib are killed within the first days of treatment but non-malignant cells are not seemingly affected in this time frame. The binding of bortezomib to proteasomes has been demonstrated to be reversible. Although in the first days after treatment the accumulation of polyubiquitinated proteins is not problematic, after prolonged periods (weeks and months), cell types such as peripheral neurons that accumulate excessive levels of polyubiquitinated proteins and form aggresomes might have reduced viability day by day. To avoid such long time peripheral neuropathy is another motivation for further development of 2nd or 3rd generation proteasome inhibitors, which is focusing on irreversible inhibitors. Previous studies of our group [142] tested if b-AP15 is a reversible proteasome inhibitor, and our findings were that DUBs will release bound b-AP15 within hours. We thus hypothesis that b-AP15 is also a reversible proteasome inhibitor, which likely to cause peripheral neuropathy in clinical application.

4.6.8 Solubility of b-AP15

b-AP15 was well studied in our group for many years. This compound has very promising *in vivo*, *in vitro* and even clinical trial data that surpass the effects of bortezomib. However, the poor solubility of b-AP15, requiring DMSO for dissolution, is problematic. Local precipitation in the injection area will lead to local tissue damage near the injection area. More than 10 structural analogues were thus synthesized as new b-AP15 analogues and

tested. Some of these exhibit similar cellular responses and therapeutic effects, and have improved solubility, such as VLX1570, which has about 70% of EC50 capability as b-AP15 and can be dissolved in PBS. VLX1561-1567 are also structural analogues of b-AP15, which are similar to VLX1570 are currently in continued clinical development.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis focused on the development of a 2nd generation proteasome inhibitor. Our major candidate b-AP15 was investigated in studies I-IV and the natural product piperlongumine was tested in study V. The findings of this thesis contributed a tiny brick to the Great Wall, understanding the ubiquitin proteasome system. It may inspire other researchers for future determinations in multiple myeloma & lymphoma, chemotherapy reagents or other emerging treatment.

In study I, there is no clear evidence to distinguish whether UPS inhibition or stabilization of tumor suppressors is the dominant mechanism underlying the proteasome inhibitor application. Future experiments will be needed to test if the stability of tumor suppressors also plays an important or even dominant role in the cytotoxicity induced by those 2 compounds. However, we also sensitized non-malignant cells to b-AP15. Due to the limited number of non-malignant cell models tested in this project we cannot draw any significant conclusion about increasing the therapeutic window of b-AP15 through co-treatment with the non-toxic drug CpdA. Future studies are needed to employ more primary or non-transformed cell models in this investigation, and it will be of primary importance to also test the co-treatment in animal models or in clinical trials.

In study II we report that the chaperone response and aggresome formation are two major resistance phenomena that occur with long-term bortezomib treatment. We showed that chaperone HSPA6 expression was highly upregulated following b-AP15 treatment, and if we over-expressed HSPA6 by pre-heat shocking the HCT-116 cells we could also inhibit the apoptosis induced by b-AP15. Taken together this suggests that the chaperone response plays a general role in UPS inhibition, no matter what compound interferes with DUBs on whichever proteasome subunit. The general applicability of this observation in different tumor and inhibitor systems would thus be an interesting future research focus. With respect to aggresome formation, we noted that co-treatment of b-AP15 and bortezomib decrease their formation, suggesting that b-AP15 inhibits a target such as HDAC6. In study IV we also recorded differences in ER stress, oxidative stress, ROS induction and metabolism interfere in mitochondria by b-AP15 and bortezomib treatments. While our knowledge is certainly increasing, future investigation should more precisely determine the differences in mechanisms of action between different classes of proteasome inhibitors.

We tested the action of b-AP15 in lymphoma cell models in study III, and demonstrated that they were as sensitive to b-AP15 as other cancer cell lines previously reported. In some of these lymphoma models we observed off-target effect are very interesting and important for further studies. The efficacy of b-AP15 in additional types of cancer is warranted.

In study V our data suggested that piperlongumine was not a proteasome inhibitor, but instead interferes upstream of UPS. Further study will be needed to determine exactly how piperlongumine mediates its effects, but these observations are significant as they open for the possibility of upstream targeting as a therapeutic modality, which might have fewer adverse side-effects than those experienced with current proteasome inhibitors. Importantly, this study also indicates that an alternative drug development program is possible using ancient knowledge of the natural world. Asian medicine is the oldest recorded in the world, and the medicine men of bygone eras certainly seem to have had more awareness of what mother nature can provide than our current high-technology approaches.

As I mentioned in the beginning of discussion in this thesis, anti-cancer drug development is like dancing on the tip of a knife. In order to eliminate more cancer cells we want to have the as powerful a therapy as possible, However, to avoid side-effects of the therapy we want to lower the concentration and does as much as possible. It is just like a loop and paradox. Theoretically we have a very low chance to discover a compound which is very effective on tumors but that induces only mild side-effects. Years of study in chemotherapy reagent development still relies on the shotgun method, a huge amount of money, time and expectation, but the improvement in therapy is limited.

Immunotherapy is an emerging technique focused on targeting treatments and has very promising prospects. We could use immunotherapy to first treat the tumor, making it less proliferative and stable, greatly reducing the chance of tumor metastasis. As a second measure we could expose the unhealthy cancer cells to proteasome inhibitors, with a short treatment time, and lower concentrations with limited side-effects, I order to further weaken or eradicate the tumor and prevent both resistance and relapse. Another aspect of reducing unwanted side-effects with proteasome inhibitors (e.g. death of normal cells) would be to localize treatment to the solid tumor site. A recently developed medical device at Karolinska Institutet, the Extroducer^{TM} is such a device that opens new possibilities for targeted delivery within tissues, including the CNS. By using this minimally invasive access catheter access is afforded to most organs, and injection of all kinds of reagents or cells is possible, as well as

biopsy of the solid tumor [173]. We can thus imagine use of the Extroducer^{TM} to deliver proteasome inhibitors into the solid tumor. Instead of continuing the search for next-generation chemotherapy reagents we are maybe already at a stage when co-treatments with existing compounds and targeted delivery could be in focus instead.

ACKNOWLEDGEMENT

The years I spent at Karolinska Institutet have been an unforgettable adventure that increased my knowledge in life science and greatly improved my soft skills. During this time I have learned a lot how to communicate with people and society. I made a lot of friends at KI and matured during my studies.

First, I want to use this chance to give my most sincerely appreciations to my main supervisor, Prof. Robert Harris. Bob, thanks you so much for helping me to finish my Ph.D. It was impossible for me to reach this moment without your great support and trust. Your inspired me in a lot in scientific thinking. You gave me millions of good ideas on how to write my thesis. I learned a lot on constructive thinking, emotional control and communication skills from you.

I also want to say thank you to my other supervisors, Prof. Stig Linder, you gave me the chance to perform my master thesis work in your group and kindly offered the financial support in this project. After the project, you give me the chance to start my Ph.D at KI with Dr. Pädraig Darcy, who supported me a lot in my daily work and was always helpful in any situation. I also want to thank all the colleges that I interacted with in Linder's group and all other collaborators in all my projects. Thank you so much for all the help and support you offered to me in my study.

My mentor, Prof. Roman Zubarev. It was my great honor to work in your group during my master's project and I appreciated you accepting to be my mentor. You are a very smart, knowledgeable and humored person. You join everyone working in your lab, like in a family. I still remember the beer and talks we had during the lab meetings.

I also want to give my deep appreciation to Assoc. Prof. Ingeborg Van der Ploeg, Prof. Karin Dahlman-Wright, Prof. Anders Gustafsson, Prof. Marianne Schultzberg, Prof. Gert Helgesson, Dr. Yujuan Zheng, John Steen and Sara Elg. Thank you so much for all the support and trust you gave to me during my Ph.D.

Finally and mostly, I want to give my deepest appreciation to my families: 爸爸妈妈感谢你们给我生命，给我一个家。尤其是对于我妈妈，感谢你把这个世界上最真挚的，纯洁的，无私的爱，毫无保留的给予我。你对我的肯定，赞许和信任，是我一次一次

的战胜生命中的困难并勇敢的走下去的原动力。我的妻子雷莹，感谢你我最艰难的时刻无条件的对我的妥协、信任、支持和帮助。感谢你们，我爱你们。

REFERENCES

1. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. **136**(5): p. E359-86.
2. Menyhart, O., et al., *Guidelines for the selection of functional assays to evaluate the hallmarks of cancer.* Biochim Biophys Acta, 2016. **1866**(2): p. 300-319.
3. Luo, X.M., et al., *Advances in cryoablation for pancreatic cancer.* World J Gastroenterol, 2016. **22**(2): p. 790-800.
4. Wust, P., et al., *Hyperthermia in combined treatment of cancer.* Lancet Oncol, 2002. **3**(8): p. 487-97.
5. Abrahamse, H. and M.R. Hamblin, *New photosensitizers for photodynamic therapy.* Biochem J, 2016. **473**(4): p. 347-64.
6. Sharma, P., et al., *Novel cancer immunotherapy agents with survival benefit: recent successes and next steps.* Nat Rev Cancer, 2011. **11**(11): p. 805-12.
7. Farkona, S., E.P. Diamandis, and I.M. Blasutig, *Cancer immunotherapy: the beginning of the end of cancer?* BMC Med, 2016. **14**: p. 73.
8. Gardner, T.A., B.D. Elzey, and N.M. Hahn, *Sipuleucel-T (Provenge) autologous vaccine approved for treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer.* Hum Vaccin Immunother, 2012. **8**(4): p. 534-9.
9. Sharma, P. and J.P. Allison, *Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential.* Cell, 2015. **161**(2): p. 205-14.
10. Rajkumar, S.V. and J.L. Harousseau, *Next-generation multiple myeloma treatment: a pharmacoeconomic perspective.* Blood, 2016. **128**(24): p. 2757-2764.
11. Anderson, K.C., et al., *NCCN Guidelines Insights: Multiple Myeloma, Version 3.2016.* J Natl Compr Canc Netw, 2016. **14**(4): p. 389-400.
12. Bose, P., P. Vachhani, and J.E. Cortes, *Treatment of Relapsed/Refractory Acute Myeloid Leukemia.* Curr Treat Options Oncol, 2017. **18**(3): p. 17.
13. Tamamyan, G., et al., *Frontline treatment of acute myeloid leukemia in adults.* Crit Rev Oncol Hematol, 2017. **110**: p. 20-34.
14. Dombret, H. and C. Gardin, *An update of current treatments for adult acute myeloid leukemia.* Blood, 2016. **127**(1): p. 53-61.
15. Dohner, H., D.J. Weisdorf, and C.D. Bloomfield, *Acute Myeloid Leukemia.* N Engl J Med, 2015. **373**(12): p. 1136-52.
16. Price, R.L. and E.A. Chiocca, *Evolution of malignant glioma treatment: from chemotherapy to vaccines to viruses.* Neurosurgery, 2014. **61 Suppl 1**: p. 74-83.
17. Wick, W., et al., *MGMT testing--the challenges for biomarker-based glioma treatment.* Nat Rev Neurol, 2014. **10**(7): p. 372-85.
18. Jafri, M.A., M.H. Al-Qahtani, and J.W. Shay, *Role of miRNAs in human cancer metastasis: Implications for therapeutic intervention.* Semin Cancer Biol, 2017.
19. Monteiro, J. and R. Fodde, *Cancer stemness and metastasis: therapeutic consequences and perspectives.* Eur J Cancer, 2010. **46**(7): p. 1198-203.
20. Chakraborty, S. and T. Rahman, *The difficulties in cancer treatment.* Ecancermedicalscience, 2012. **6**: p. ed16.
21. Schwartz, A.L. and A. Ciechanover, *The ubiquitin-proteasome pathway and pathogenesis of human diseases.* Annu Rev Med, 1999. **50**: p. 57-74.
22. D'Arcy, P., X. Wang, and S. Linder, *Deubiquitinase inhibition as a cancer therapeutic strategy.* Pharmacol Ther, 2015. **147C**: p. 32-54.
23. Popovic, D., D. Vucic, and I. Dikic, *Ubiquitination in disease pathogenesis and treatment.* Nat Med, 2014. **20**(11): p. 1242-53.
24. Fulda, S., K. Rajalingam, and I. Dikic, *Ubiquitylation in immune disorders and cancer: from molecular mechanisms to therapeutic implications.* EMBO Mol Med, 2012. **4**(7): p. 545-56.

25. Ciechanover, A. and K. Iwai, *The ubiquitin system: from basic mechanisms to the patient bed.* IUBMB Life, 2004. **56**(4): p. 193-201.
26. Grabbe, C., K. Husnjak, and I. Dikic, *The spatial and temporal organization of ubiquitin networks.* Nat Rev Mol Cell Biol, 2011. **12**(5): p. 295-307.
27. Wilkinson, K.D., *DUBs at a glance.* J Cell Sci, 2009. **122**(Pt 14): p. 2325-9.
28. Komander, D., M.J. Clague, and S. Urbe, *Breaking the chains: structure and function of the deubiquitinases.* Nat Rev Mol Cell Biol, 2009. **10**(8): p. 550-63.
29. Baumeister, W. and A. Lupas, *The proteasome.* Curr Opin Struct Biol, 1997. **7**(2): p. 273-8.
30. Gu, Z.C. and C. Enenkel, *Proteasome assembly.* Cell Mol Life Sci, 2014. **71**(24): p. 4729-45.
31. Kirkin, V. and I. Dikic, *Ubiquitin networks in cancer.* Curr Opin Genet Dev, 2011. **21**(1): p. 21-8.
32. Hoeller, D. and I. Dikic, *Targeting the ubiquitin system in cancer therapy.* Nature, 2009. **458**(7237): p. 438-44.
33. Yang, Y., et al., *Targeting the ubiquitin-proteasome system for cancer therapy.* Cancer Sci, 2009. **100**(1): p. 24-8.
34. Kitagawa, K., Y. Kotake, and M. Kitagawa, *Ubiquitin-mediated control of oncogene and tumor suppressor gene products.* Cancer Sci, 2009. **100**(8): p. 1374-81.
35. Cardozo, T. and M. Pagano, *The SCF ubiquitin ligase: insights into a molecular machine.* Nat Rev Mol Cell Biol, 2004. **5**(9): p. 739-51.
36. Minella, A.C., M. Welcker, and B.E. Clurman, *Ras activity regulates cyclin E degradation by the Fbw7 pathway.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9649-54.
37. Carrano, A.C., et al., *SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27.* Nat Cell Biol, 1999. **1**(4): p. 193-9.
38. Frescas, D. and M. Pagano, *Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer.* Nat Rev Cancer, 2008. **8**(6): p. 438-49.
39. Baldewijns, M.M., et al., *VHL and HIF signalling in renal cell carcinogenesis.* J Pathol, 2010. **221**(2): p. 125-38.
40. Robinson, C.M. and M. Ohh, *The multifaceted von Hippel-Lindau tumour suppressor protein.* FEBS Lett, 2014. **588**(16): p. 2704-11.
41. Riley, T., et al., *Transcriptional control of human p53-regulated genes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 402-12.
42. Tait, S.W. and D.R. Green, *Mitochondria and cell death: outer membrane permeabilization and beyond.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 621-32.
43. Jeffers, J.R., et al., *Puma is an essential mediator of p53-dependent and -independent apoptotic pathways.* Cancer Cell, 2003. **4**(4): p. 321-8.
44. Michalak, E.M., et al., *In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute.* Cell Death Differ, 2008. **15**(6): p. 1019-29.
45. Wu, G.S., et al., *KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene.* Nat Genet, 1997. **17**(2): p. 141-3.
46. Muller, M., et al., *p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs.* J Exp Med, 1998. **188**(11): p. 2033-45.
47. Chen, J., *The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression.* Cold Spring Harb Perspect Med, 2016. **6**(3): p. a026104.
48. Pant, V. and G. Lozano, *Limiting the power of p53 through the ubiquitin proteasome pathway.* Genes Dev, 2014. **28**(16): p. 1739-51.
49. Devine, T. and M.S. Dai, *Targeting the ubiquitin-mediated proteasome degradation of p53 for cancer therapy.* Curr Pharm Des, 2013. **19**(18): p. 3248-62.
50. Chao, C.C., *Mechanisms of p53 degradation.* Clin Chim Acta, 2015. **438**: p. 139-47.
51. Lim, K.H. and K.H. Baek, *Deubiquitinating enzymes as therapeutic targets in cancer.* Curr Pharm Des, 2013. **19**(22): p. 4039-52.
52. Fraile, J.M., et al., *Deubiquitinases in cancer: new functions and therapeutic options.* Oncogene, 2012. **31**(19): p. 2373-88.

53. Jacq, X., et al., *Deubiquitylating enzymes and DNA damage response pathways.* Cell Biochem Biophys, 2013. **67**(1): p. 25-43.
54. Zhang, D., et al., *A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response.* Cell, 2006. **126**(3): p. 529-42.
55. McClurg, U.L. and C.N. Robson, *Deubiquitinating enzymes as oncotargets.* Oncotarget, 2015. **6**(12): p. 9657-68.
56. Song, M.S., et al., *The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network.* Nature, 2008. **455**(7214): p. 813-7.
57. van der Horst, A., et al., *FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP.* Nat Cell Biol, 2006. **8**(10): p. 1064-73.
58. Atanassov, B.S., et al., *Gcn5 and SAGA regulate shelterin protein turnover and telomere maintenance.* Mol Cell, 2009. **35**(3): p. 352-64.
59. Draker, R., E. Sarcinella, and P. Cheung, *USP10 deubiquitylates the histone variant H2A.Z and both are required for androgen receptor-mediated gene activation.* Nucleic Acids Res, 2011. **39**(9): p. 3529-42.
60. Jing, H. and S. Lee, *NF-kappaB in cellular senescence and cancer treatment.* Mol Cells, 2014. **37**(3): p. 189-95.
61. Pal, A., M.A. Young, and N.J. Donato, *Emerging potential of therapeutic targeting of ubiquitin-specific proteases in the treatment of cancer.* Cancer Res, 2014. **74**(18): p. 4955-66.
62. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
63. Zhang, L., et al., *USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor.* Nat Cell Biol, 2012. **14**(7): p. 717-26.
64. Al-Salihi, M.A., et al., *USP11 augments TGFbeta signalling by deubiquitylating ALK5.* Open Biol, 2012. **2**(6): p. 120063.
65. Inui, M., et al., *USP15 is a deubiquitylating enzyme for receptor-activated SMADs.* Nat Cell Biol, 2011. **13**(11): p. 1368-75.
66. Borissenko, L. and M. Groll, *20S proteasome and its inhibitors: crystallographic knowledge for drug development.* Chem Rev, 2007. **107**(3): p. 687-717.
67. Lander, G.C., et al., *Complete subunit architecture of the proteasome regulatory particle.* Nature, 2012. **482**(7384): p. 186-91.
68. Lee, B.H., et al., *USP14 deubiquitinates proteasome-bound substrates that are ubiquitinated at multiple sites.* Nature, 2016. **532**(7599): p. 398-401.
69. Lee, B.H., et al., *Enhancement of proteasome activity by a small-molecule inhibitor of USP14.* Nature, 2010. **467**(7312): p. 179-84.
70. D'Arcy, P. and S. Linder, *Proteasome deubiquitinases as novel targets for cancer therapy.* Int J Biochem Cell Biol, 2012. **44**(11): p. 1729-38.
71. Spataro, V., K. Simmen, and C.A. Realini, *The essential 26S proteasome subunit Rpn11 confers multidrug resistance to mammalian cells.* Anticancer Res, 2002. **22**(6C): p. 3905-9.
72. Gallery, M., et al., *The JAMM motif of human deubiquitinase Poh1 is essential for cell viability.* Mol Cancer Ther, 2007. **6**(1): p. 262-8.
73. Wada, T., et al., *Screening for genetic abnormalities involved in ovarian carcinogenesis using retroviral expression libraries.* Int J Oncol, 2009. **35**(5): p. 973-6.
74. Shinji, S., et al., *Ubiquitin-specific protease 14 expression in colorectal cancer is associated with liver and lymph node metastases.* Oncol Rep, 2006. **15**(3): p. 539-43.
75. Wicks, S.J., et al., *The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling.* Oncogene, 2005. **24**(54): p. 8080-4.
76. Chen, Y., et al., *Expression and clinical significance of UCH37 in human esophageal squamous cell carcinoma.* Dig Dis Sci, 2012. **57**(9): p. 2310-7.
77. Liu, H., et al., *Regulation of ErbB2 receptor status by the proteasomal DUB POH1.* PLoS One, 2009. **4**(5): p. e5544.

78. Estanqueiro, M., et al., *Nanotechnological carriers for cancer chemotherapy: the state of the art.* Colloids Surf B Biointerfaces, 2015. **126**: p. 631-48.
79. Baskar, R., et al., *Cancer and radiation therapy: current advances and future directions.* Int J Med Sci, 2012. **9**(3): p. 193-9.
80. Liu, F.S., *Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review.* Taiwan J Obstet Gynecol, 2009. **48**(3): p. 239-44.
81. Gottesman, M.M., T. Fojo, and S.E. Bates, *Multidrug resistance in cancer: role of ATP-dependent transporters.* Nat Rev Cancer, 2002. **2**(1): p. 48-58.
82. Sarkadi, B., et al., *Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system.* Physiol Rev, 2006. **86**(4): p. 1179-236.
83. Deeley, R.G., C. Westlake, and S.P. Cole, *Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins.* Physiol Rev, 2006. **86**(3): p. 849-99.
84. Cole, S.P., *Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future.* Annu Rev Pharmacol Toxicol, 2014. **54**: p. 95-117.
85. Mao, Q. and J.D. Unadkat, *Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update.* AAPS J, 2015. **17**(1): p. 65-82.
86. Xu, Y., et al., *Acquired resistance of lung adenocarcinoma to EGFR-tyrosine kinase inhibitors gefitinib and erlotinib.* Cancer Biol Ther, 2010. **9**(8): p. 572-82.
87. Camidge, D.R., et al., *Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study.* Lancet Oncol, 2012. **13**(10): p. 1011-9.
88. Gorre, M.E., et al., *Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification.* Science, 2001. **293**(5531): p. 876-80.
89. Dhillon, K.K., E.M. Swisher, and T. Taniguchi, *Secondary mutations of BRCA1/2 and drug resistance.* Cancer Sci, 2011. **102**(4): p. 663-9.
90. Sakai, W., et al., *Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma.* Cancer Res, 2009. **69**(16): p. 6381-6.
91. Bouwman, P., et al., *53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers.* Nat Struct Mol Biol, 2010. **17**(6): p. 688-95.
92. Bagrodia, A., et al., *Genetic Determinants of Cisplatin Resistance in Patients With Advanced Germ Cell Tumors.* J Clin Oncol, 2016.
93. Hassan, M., et al., *Apoptosis and molecular targeting therapy in cancer.* Biomed Res Int, 2014. **2014**: p. 150845.
94. Muris, J.J., et al., *Immunohistochemical profiling of caspase signaling pathways predicts clinical response to chemotherapy in primary nodal diffuse large B-cell lymphomas.* Blood, 2005. **105**(7): p. 2916-23.
95. Yang, X.H., et al., *XIAP is a predictor of cisplatin-based chemotherapy response and prognosis for patients with advanced head and neck cancer.* PLoS One, 2012. **7**(3): p. e31601.
96. Hector, S., et al., *Clinical application of a systems model of apoptosis execution for the prediction of colorectal cancer therapy responses and personalisation of therapy.* Gut, 2012. **61**(5): p. 725-33.
97. Tamm, I., et al., *Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias.* Clin Cancer Res, 2000. **6**(5): p. 1796-803.
98. Lopes, R.B., et al., *Expression of the IAP protein family is dysregulated in pancreatic cancer cells and is important for resistance to chemotherapy.* Int J Cancer, 2007. **120**(11): p. 2344-52.
99. Adams, J.M. and S. Cory, *The Bcl-2 apoptotic switch in cancer development and therapy.* Oncogene, 2007. **26**(9): p. 1324-37.
100. Martinou, J.C. and R.J. Youle, *Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics.* Dev Cell, 2011. **21**(1): p. 92-101.

101. Konopleva, M., et al., *The anti-apoptotic genes Bcl-X(L) and Bcl-2 are over-expressed and contribute to chemoresistance of non-proliferating leukaemic CD34+ cells.* Br J Haematol, 2002. **118**(2): p. 521-34.
102. Yang, D., et al., *Bcl-2 expression predicts sensitivity to chemotherapy in breast cancer: a systematic review and meta-analysis.* J Exp Clin Cancer Res, 2013. **32**: p. 105.
103. Zhang, L., et al., *Role of BAX in the apoptotic response to anticancer agents.* Science, 2000. **290**(5493): p. 989-92.
104. Yang, X., et al., *Hsp70 promotes chemoresistance by blocking Bax mitochondrial translocation in ovarian cancer cells.* Cancer Lett, 2012. **321**(2): p. 137-43.
105. Han, W., et al., *EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells.* PLoS One, 2011. **6**(6): p. e18691.
106. Amaravadi, R.K. and C.B. Thompson, *The roles of therapy-induced autophagy and necrosis in cancer treatment.* Clin Cancer Res, 2007. **13**(24): p. 7271-9.
107. Swampillai, A.L., P. Salomoni, and S.C. Short, *The role of autophagy in clinical practice.* Clin Oncol (R Coll Radiol), 2012. **24**(6): p. 387-95.
108. Tredan, O., et al., *Drug resistance and the solid tumor microenvironment.* J Natl Cancer Inst, 2007. **99**(19): p. 1441-54.
109. Castells, M., et al., *Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death.* Int J Mol Sci, 2012. **13**(8): p. 9545-71.
110. Muerkoster, S.S., et al., *Role of myofibroblasts in innate chemoresistance of pancreatic carcinoma--epigenetic downregulation of caspases.* Int J Cancer, 2008. **123**(8): p. 1751-60.
111. Shain, K.H. and W.S. Dalton, *Environmental-mediated drug resistance: a target for multiple myeloma therapy.* Expert Rev Hematol, 2009. **2**(6): p. 649-62.
112. McConkey, D.J. and K. Zhu, *Mechanisms of proteasome inhibitor action and resistance in cancer.* Drug Resist Updat, 2008. **11**(4-5): p. 164-79.
113. Hetz, C., E. Chevet, and H.P. Harding, *Targeting the unfolded protein response in disease.* Nat Rev Drug Discov, 2013. **12**(9): p. 703-19.
114. Harris, R.A. and S. Amor, *Sweet and sour--oxidative and carbonyl stress in neurological disorders.* CNS Neurol Disord Drug Targets, 2011. **10**(1): p. 82-107.
115. Beyer, K. and A. Ariza, *alpha-Synuclein posttranslational modification and alternative splicing as a trigger for neurodegeneration.* Mol Neurobiol, 2013. **47**(2): p. 509-24.
116. Shimura, H., et al., *Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease.* Science, 2001. **293**(5528): p. 263-9.
117. Buhlman, L.M., *Parkin loss-of-function pathology: Premature neuronal senescence induced by high levels of reactive oxygen species?* Mech Ageing Dev, 2017. **161**(Pt A): p. 112-120.
118. Love, I.M., D. Shi, and S.R. Grossman, *p53 Ubiquitination and proteasomal degradation.* Methods Mol Biol, 2013. **962**: p. 63-73.
119. Lu, Z. and T. Hunter, *Ubiquitylation and proteasomal degradation of the p21(Cip1), p27(Kip1) and p57(Kip2) CDK inhibitors.* Cell Cycle, 2010. **9**(12): p. 2342-52.
120. Amit, S. and Y. Ben-Neriah, *NF-kappaB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach.* Semin Cancer Biol, 2003. **13**(1): p. 15-28.
121. Liu, N., et al., *Inhibition of 19S proteasome-associated deubiquitinases by metal-containing compounds.* Oncoscience, 2015. **2**(5): p. 457-66.
122. Manasanch, E.E. and R.Z. Orlowski, *Proteasome inhibitors in cancer therapy.* Nat Rev Clin Oncol, 2017.
123. Crawford, L.J., B. Walker, and A.E. Irvine, *Proteasome inhibitors in cancer therapy.* J Cell Commun Signal, 2011. **5**(2): p. 101-10.
124. Orlowski, M. and S. Wilk, *Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex.* Arch Biochem Biophys, 2000. **383**(1): p. 1-16.

125. Chauhan, D., et al., *Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B.* Blood, 1996. **87**(3): p. 1104-12.
126. Fennell, D.A., A. Chacko, and L. Mutti, *BCL-2 family regulation by the 20S proteasome inhibitor bortezomib.* Oncogene, 2008. **27**(9): p. 1189-97.
127. Qin, J.Z., et al., *Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells.* Cancer Res, 2005. **65**(14): p. 6282-93.
128. Mitsiades, N., et al., *Molecular sequelae of proteasome inhibition in human multiple myeloma cells.* Proc Natl Acad Sci U S A, 2002. **99**(22): p. 14374-9.
129. Pandit, B. and A.L. Gartel, *Proteasome inhibitors induce p53-independent apoptosis in human cancer cells.* Am J Pathol, 2011. **178**(1): p. 355-60.
130. Gu, H., et al., *Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells.* Mol Cancer Ther, 2008. **7**(8): p. 2298-307.
131. Smith, A.J., et al., *Noxa/Bcl-2 protein interactions contribute to bortezomib resistance in human lymphoid cells.* J Biol Chem, 2011. **286**(20): p. 17682-92.
132. Oerlemans, R., et al., *Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein.* Blood, 2008. **112**(6): p. 2489-99.
133. Ruckrich, T., et al., *Characterization of the ubiquitin-proteasome system in bortezomib-adapted cells.* Leukemia, 2009. **23**(6): p. 1098-105.
134. Kern, J., et al., *GRP-78 secreted by tumor cells blocks the antiangiogenic activity of bortezomib.* Blood, 2009. **114**(18): p. 3960-7.
135. Gutman, D., A.A. Morales, and L.H. Boise, *Acquisition of a multidrug-resistant phenotype with a proteasome inhibitor in multiple myeloma.* Leukemia, 2009. **23**(11): p. 2181-3.
136. Shringarpure, R., et al., *Gene expression analysis of B-lymphoma cells resistant and sensitive to bortezomib.* Br J Haematol, 2006. **134**(2): p. 145-56.
137. Kuhn, D.J., et al., *Targeting the insulin-like growth factor-1 receptor to overcome bortezomib resistance in preclinical models of multiple myeloma.* Blood, 2012. **120**(16): p. 3260-70.
138. Voorhees, P.M., et al., *Inhibition of interleukin-6 signaling with CNTO 328 enhances the activity of bortezomib in preclinical models of multiple myeloma.* Clin Cancer Res, 2007. **13**(21): p. 6469-78.
139. Soriano, G.P., et al., *Proteasome inhibitor-adapted myeloma cells are largely independent from proteasome activity and show complex proteomic changes, in particular in redox and energy metabolism.* Leukemia, 2016. **30**(11): p. 2198-2207.
140. Erdal, H., et al., *Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 192-7.
141. Berndtsson, M., et al., *Induction of the lysosomal apoptosis pathway by inhibitors of the ubiquitin-proteasome system.* Int J Cancer, 2009. **124**(6): p. 1463-9.
142. D'Arcy, P., et al., *Inhibition of proteasome deubiquitinating activity as a new cancer therapy.* Nat Med, 2011. **17**(12): p. 1636-40.
143. Feng, X., et al., *Proapoptotic effects of the novel proteasome inhibitor b-AP15 on multiple myeloma cells and natural killer cells.* Exp Hematol, 2014. **42**(3): p. 172-82.
144. Tian, Z., et al., *A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5 induces apoptosis in multiple myeloma and overcomes bortezomib resistance.* Blood, 2014. **123**(5): p. 706-16.
145. Huang, L., K. Jung, and C.H. Chen, *Inhibitory effect of b-AP15 on the 20S proteasome.* Biomolecules, 2014. **4**(4): p. 931-9.
146. Menendez-Benito, V., et al., *Endoplasmic reticulum stress compromises the ubiquitin-proteasome system.* Hum Mol Genet, 2005. **14**(19): p. 2787-99.

147. Hagg, M., et al., *A novel high-through-put assay for screening of pro-apoptotic drugs.* Invest New Drugs, 2002. **20**(3): p. 253-9.
148. Collins, I., et al., *Chemical approaches to targeted protein degradation through modulation of the ubiquitin-proteasome pathway.* Biochem J, 2017. **474**(7): p. 1127-1147.
149. Harant, H., et al., *Inhibition of vascular endothelial growth factor cotranslational translocation by the cyclopeptolide CAM741.* Mol Pharmacol, 2007. **71**(6): p. 1657-65.
150. Garrison, J.L., et al., *A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum.* Nature, 2005. **436**(7048): p. 285-9.
151. Mitsiades, C.S., et al., *Antimyeloma activity of heat shock protein-90 inhibition.* Blood, 2006. **107**(3): p. 1092-100.
152. Sterz, J., et al., *The potential of proteasome inhibitors in cancer therapy.* Expert Opin Investig Drugs, 2008. **17**(6): p. 879-95.
153. Orlowski, R.Z. and D.J. Kuhn, *Proteasome inhibitors in cancer therapy: lessons from the first decade.* Clin Cancer Res, 2008. **14**(6): p. 1649-57.
154. Williams, S.A. and D.J. McConkey, *The proteasome inhibitor bortezomib stabilizes a novel active form of p53 in human LNCaP-Pro5 prostate cancer cells.* Cancer Res, 2003. **63**(21): p. 7338-44.
155. Suraweera, A., et al., *Failure of amino acid homeostasis causes cell death following proteasome inhibition.* Mol Cell, 2012. **48**(2): p. 242-53.
156. Richardson, P.G., et al., *A phase 2 study of bortezomib in relapsed, refractory myeloma.* N Engl J Med, 2003. **348**(26): p. 2609-17.
157. Franke, N.E., et al., *Impaired bortezomib binding to mutant beta5 subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells.* Leukemia, 2012. **26**(4): p. 757-68.
158. Li, L. and X.J. Yang, *Tubulin acetylation: responsible enzymes, biological functions and human diseases.* Cell Mol Life Sci, 2015. **72**(22): p. 4237-55.
159. Kawaguchi, Y., et al., *The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress.* Cell, 2003. **115**(6): p. 727-38.
160. Catley, L., et al., *Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells.* Blood, 2006. **108**(10): p. 3441-9.
161. Shah, S.P., S. Lonial, and L.H. Boise, *When Cancer Fights Back: Multiple Myeloma, Proteasome Inhibition, and the Heat-Shock Response.* Mol Cancer Res, 2015. **13**(8): p. 1163-73.
162. Hambley, B., P.F. Caimi, and B.M. William, *Bortezomib for the treatment of mantle cell lymphoma: an update.* Ther Adv Hematol, 2016. **7**(4): p. 196-208.
163. Perez-Galan, P., et al., *The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status.* Blood, 2006. **107**(1): p. 257-64.
164. Sun, L., et al., *Combination of atiprimod and the proteasome inhibitor bortezomib induces apoptosis of mantle cell lymphoma in vitro and in vivo.* Leuk Res, 2012. **36**(3): p. 363-8.
165. Morse, D. and A.M. Choi, *Heme oxygenase-1: from bench to bedside.* Am J Respir Crit Care Med, 2005. **172**(6): p. 660-70.
166. Fujino, G., et al., *Thioredoxin and TRAF family proteins regulate reactive oxygen species-dependent activation of ASK1 through reciprocal modulation of the N-terminal homophilic interaction of ASK1.* Mol Cell Biol, 2007. **27**(23): p. 8152-63.
167. Hideshima, T., et al., *Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341.* Blood, 2003. **101**(4): p. 1530-4.
168. Fribley, A., Q. Zeng, and C.Y. Wang, *Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells.* Mol Cell Biol, 2004. **24**(22): p. 9695-704.

169. Kaplan, G.S., et al., *Proteasome inhibitors in cancer therapy: Treatment regimen and peripheral neuropathy as a side effect.* Free Radic Biol Med, 2017. **103**: p. 1-13.
170. Visovsky, C., *Chemotherapy-induced peripheral neuropathy.* Cancer Invest, 2003. **21**(3): p. 439-51.
171. Ravaglia, S., et al., *Immune-mediated neuropathies in myeloma patients treated with bortezomib.* Clin Neurophysiol, 2008. **119**(11): p. 2507-12.
172. Delforge, M., et al., *Treatment-related peripheral neuropathy in multiple myeloma: the challenge continues.* Lancet Oncol, 2010. **11**(11): p. 1086-95173.
173. Lundberg, J., S. Jonsson, and S. Holmin, *New endovascular method for transvascular exit of arteries and veins: developed in simulator, in rat and in rabbit with full clinical integration.* PLoS One, 2010. **5**(5): p. e10449.