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Identification and characterization of a proteasome deubiquitinase inhibitor

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"Toliko je bilo u životu stvari kojih smo se bojali,

a nije trebalo. Trebalo je živjeti."

Ivo Andrić

To my parents

ABSTRACT

The 26S proteasome has emerged as an attractive therapeutic target in the treatment of cancers. Proteasome inhibitors have been shown to selectively kill cancer cells. The FDA approval of bortezomib for the treatment of multiple myeloma and mantle cell lymphoma has clinically validated the 26S proteasome as a therapeutic target in oncology. Despite the acceptable therapeutic index, patients treated with this drug manifest several toxic side effects and moreover, the poor outcome of bortezomib treatment has been associated with acquired resistance to the drug. Currently, many efforts are made to develop new proteasome inhibitors that act through mechanisms distinct from that of bortezomib.

In this theses we uncover a novel proteasome inhibitor of the 19S deubiquitinase activity, b-AP15. The inhibition of the proteasome function by this small molecule inhibitor is achieved through the inhibition of deubiquitinating enzymes UCHL5 and USP14 resulting in the induction of poly-ubiquitinated proteins in cells. b-AP15 elicits anti-tumor effects both *in vitro* and *in vivo* that is associated with the inhibition of proteasome function. In syngenic and xenograft mouse models, representing both solid and leukemic malignancies, b-AP15 exhibited potent antitumor activity causing tumor regression, reduced tumor growth and delayed tumor onset.

b-AP15 elicits similar, but yet distinct, cellular response as bortezomib. b-AP15 induces rapid apoptotic response which is associated with strong increases of chaperone expression and strong induction of oxidative stress. Notably, these responses are stronger in cells exposed to b-AP15 than bortezomib. Similarly to bortezomib, b-AP15 induced ER stress being involved in the induction of apoptosis. b-AP15 is a reversible inhibitor of deubiquitinase activity and proteasome function. Despite being reversible inhibitor, b-AP15 treated cells rapidly commit to apoptosis/cell death which we here report is due to rapid uptake and enrichment of the drug into cells.

Bortezomib-mediated resistance has been associated with anti-apoptotic Bcl-2 overexpression and induction of aggresome formation in cancer cells. Here we report that b-AP15 induced apoptosis is insensitive to the overexpression of Bcl-2 and that b-AP15 blocks aggresome formation suggesting that bortezomib-mediated resistance could be overcome by b-AP15 treatment. Furthermore, we found that b-AP15 is more toxic to cancer cells than immortalized normal cells with differences larger than those observed for bortezomib, suggesting more favorable therapeutic window of b-AP15 compared to bortezomib. The findings in this thesis strongly suggest that b-AP15 is a good candidate for clinical drug development.

LIST OF PUBLICATIONS

I. D'Arcy P*, Brnjic S*, Olofsson H M, Fryknäs M, Lindsten K, De Cesare M, Perego P, Sadeghi B, Hassan M, Larsson R, Linder S.
Inhibition of proteasome deubiquitinating activity as a new cancer therapy.
Nature Medicine, 2011, 17(12), 1636-40
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II. **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.
Antioxidants & Redox Signaling, 2013, doi: 10.1089/ars.2013.5322

III. Wang X, Mazurkiewicz M, **Brnjic S**, Zhang X, Fryknäs M, Gullbo J, Larsson R, Stafford W, Arnér E, D'Arcy P, Linder S.
The 19S deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death.
Manuscript

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

CONTENTS

LIST OF ABBREVIATIONS

AAA+ ATPases	ATPases associated with diverse cellular activities
Admr1	Adhesion regulating molecule 1
AMP	Adenosine monophosphate
AP-1	Activating protein 1
Ar-Φ	Aromatic-aliphatic
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Bad	Bcl-2 associated death promoter
Bag-1	Bcl-2 associated athanogene
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
Bik	Bcl-2 interacting killer
Bim	B-cell lymphoma 2 interacting mediator of cell death
Bip	Binding immunoglobulin protein
Bcl-2	B-cell lymphoma 2
BL	Binding loop
CDK	Cyclin dependent kinase
CHIP	Carboxyl terminus of Hsc70 Interacting Protein
CHOP	C/EBP-homologous protein
CKI	Cyclin kinase inhibitor
CP	Core particle
DMB	Dynein motor binding
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FDA	Food and Drug Administration
GFP	Green fluorescent protein
Grp78	78 kDa glucose-regulated protein
HCC	Hepatocellular carcinoma
HDAC6	Histone deacetylase 6
HECT	Homologous to E6-AP carboxy-terminus
Hsp	Heat shock protein
Hul	HECT ubiquitin ligase
IF	Intermediate filament
IκB	Inhibitor of kappa-light-chain-enhancer of activated B cells
IRE1	Inositol requiring protein 1
IU1	USP14 inhibitor 1
JAMM	JAB1/MPN/Mov34 metalloenzyme
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
LC3	Microtubule-associated protein 1A/1B-light chain 3
Lys	Lysine
MCPIP	Monocyte chemotactic protein-induced protein
MDa	Mega Dalton

Mdm2	Mouse double minute 2 homolog
MHC	Major histocompatibility complex
MPN	Most probable number
MTOC	Microtubule organizing center
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	Nanometer
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
ODC	Ornithine decarboxylase
OTU	Ovarian tumor protease
PERK	Double stranded RNA-activated protein kinase-like ER kinase
PGPH	Peptidylglutamyl-peptide hydrolyzing
POH1	Pad one homolog-1
Pru	Pleckstrin-like receptor for the ubiquitin
RDPs	Rapidly degraded proteins
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RP	Regulatory particle
Rpn	Regulatory particle non-ATPase subunit
Rpt	Regulatory particle triple-A protein
SDPs	Slowly degraded proteins
shRNA	Small hairpin ribonucleic acid
TGF-β	Transforming growth factor-β
Ub	Ubiquitin
UBA	Ubiquitin-associated
Ubal	Ubiquitin aldehyde
UBL	Ubiquitin-like
Ubp	Ubiquitin specific protease
UbVS	Ubiquitin vinyl sulfone
Uch	Ubiquitin carboxyl-terminal hydrolase
UCHL5	Ubiquitin carboxyl-terminal hydrolase L5
UIM	Ubiquitin interacting motif
UPP	Ubiquitin proteasome pathway
UPR	Ubiquitin proteasome response
USP	Ubiquitin specific protease
Xbp1	X box–binding protein-1
Å	Ångström

INTRODUCTION

CANCER AND CANCER TREATMENT

Cancer consists of more than hundred different types of diseases that can affect any organ of the body. The different types of cancerous diseases share many features, like their origin from normal cells, limitless proliferation, loss of differentiation, and invasion of surrounding tissues leading to metastasis to other sites in the body. Cancer development is a multistep process involving a high number of mutations and other genetic and epigenetic alterations which drive the transformation of normal human cell to the malignant phenotype. Moreover, cancer cells interact with the microenvironment (i.e. fibroblasts, vessels, macrophages, etc.) to fully display their phenotype [1].

Cancer is a leading cause of death worldwide. In 2008, 7.6 million people died from cancer which accounts for 13% of all deaths. Deaths from cancer are expected to rise to over 13.1 million in 2030 [2].

Significant improvement of cancer survival has been reported in economically developed countries due to early diagnosis, universal access to health care and improvements in cancer therapy. The combined use of surgery, radiation therapy (RT) and chemotherapy accounts for most of the cured cases. RT accounts for 40% of cancer cure and utilization of RT in new cancer cases is increasing [3]. The common use of RT is in combination with surgery and/or chemotherapy. Most of the cytotoxic chemotherapeutic agents used to treat cancer today are DNA-damaging agents. The increasing knowledge in aberrant signaling pathways involved in cancer cell proliferation and survival has opened the door to new therapeutic strategies. At the present, there is an emphasis on designing new small molecules to repair these molecular defects in cancer cells in an attempt to eradicate the tumor. Some of these aberrations are appearing in a broad range of cancers making them attractive targets for drug development. However, combination therapy will still be essential for tumor eradication. The notion that cancer cells bear a high number of genetic aberrations, the blockade of a single pathway would not in many cases be enough to eradicate the tumor and furthermore, probable resistance mechanisms will defeat single agents. The huge challenge in the future is to identify patients who will respond to certain drugs which will be combined in a most effective way.

THE UBIQUITIN PROTEASOME PATHWAY IN CANCER

Background

The degradation of cellular proteins is a highly complex and tightly regulated process that regulates many processes important for cell growth and survival. The concept of protein turnover is almost 60 years old. Before the discovery of the ubiquitin proteasome pathway (UPP), 20 years ago, it was known that some intracellular protein degradation occurred via the lysosome, in a process termed autophagy. This provided a mechanism to explain how intracellular proteins existed in a dynamic balance between synthesis and degradation but it did not explain all aspects of the regulation of intracellular proteins. With the discovery of the ubiquitin system in the late 1970s and early 1980s a new era in

the protein degradation field occurred [4]. Aaron Ciechanover, Avram Hershko, and Irwin Rose were awarded the Nobel Prize in Chemistry in 2004 for the discovery of ubiquitin-mediated protein degradation.

Estimates suggest that more than 80% of cellular proteins are degraded by the UPP, indicating the importance of this pathway in the regulation of many of the cellular processes [5]. The UPP is implicated in the degradation of abnormal and damaged proteins, of cell cycle regulators, of proteins encoded by oncogenes and tumor suppressor genes, in the processing of antigens, and the activation or degradation of transcription factors [6, 7].

As such this pathways has been implicated in the pathogenesis of many human diseases, including neurodegenerative disorders, viral diseases and cancer [8]. In this thesis, the main focus will be on UPP and its role in cancer.

Overview of the UPP

The proteasome has been highly conserved during eukaryotic evolution, with simpler forms found even in archaebacteria and eubacteria [9, 10]. The proteasome is a large multisubunit protease (2.5 MDa) that is found in the cytosol and in the nucleus of eukaryotic cells [11]. It is an ATP-dependent protease [12, 13]. This large structure consists of approximately 50 different subunits and is arranged into two subcomplexes: catalytic 20S core particle (CP) capped by the 19S regulatory particle (RP), which together form the 26S proteasome. The CP is a barrel-shaped structure of ~730 kDa consisting of four heptameric rings, whereas the 19S RP is a ~930 kDa complex constituting at least 19 different subunits. The 19S RP binds to one or both ends of the CP to form an enzymatically active proteasome. The most abundant proteasome species at least in yeast cells is composed of one CP capped by two RP. It is still unclear whether there are functional differences between different species, although the high degree of conservation does suggest a common function [7, 14, 15].

Proteins destined to be degraded by the proteasome are initially tagged by a covalently linked poly-ubiquitin chain that is recognized as a degradation signal by the 19S RP. Once recognized, the ubiquitin chain is removed and the protein is unwound in an ATP dependent manner and fed into the center of the 20S complex, where it is degraded into small peptides (Figure 1) [16].

The rates of protein synthesis, folding and degradation in cells have been estimated in mouse L929 cells. A typical protein was defined to be 450 residues, leading to estimation that the protein content of these cells was 2.6×10^9 proteins per cell. Proteasomes are fed with both slowly degraded proteins (SDPs) and rapidly degraded proteins (RDPs). Approximately 30% of newly synthesized proteins in mammalian cells are degraded with a half-life of <10 min. In actively dividing cells (1 day doubling time), RDPs provide 1.3×10^6 substrates per min per cell. Slowly degraded proteins have a half-life average of ~2 days. The contribution of SDPs to proteasomal degradation amounts to ~5×10^5 substrates per min per cell. The total amount of proteasomal substrates is therefore ~2×10^6 proteins degraded per min per cell, generating ~10^8 oligopeptides per min per cell [17-19].
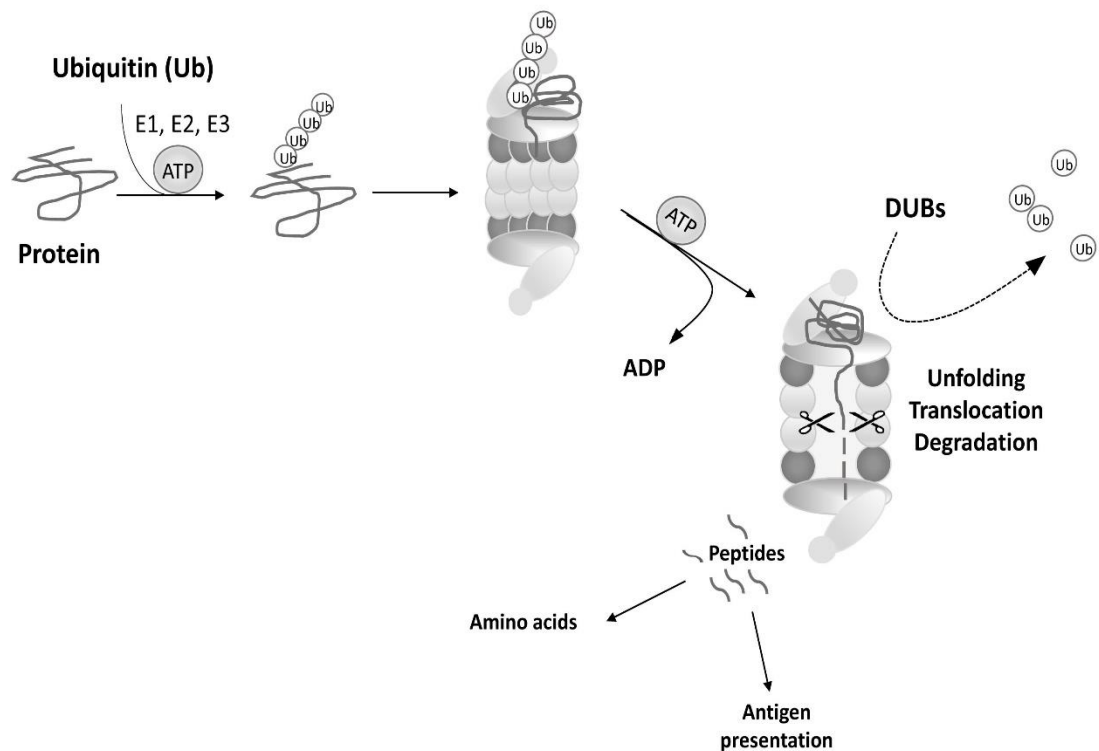
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Figure 1. Ubiquitin-dependent protein degradation.

Ubiquitination

Ubiquitination is a multi-step process leading to labeling of a substrate for degradation by the proteasome. A key protein in this process is ubiquitin (Ub), which is a highly conserved small (8.5kDa) regulatory protein consisting of 76 amino acids.

Ubiquitination is dependent on three distinct enzymes, Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3). These enzymes activate free ubiquitin and catalyze its covalent addition to substrate protein.

Ubiquitin is first activated by the E1 enzyme in the presence of ATP, forming a high-energy thiolester bond between the carboxy-terminal glycine residue of ubiquitin and the active site cysteine of E1. In mammalian organisms, a single, functional E1 enzyme has been found. Once activated, the ubiquitin is transferred from E1 to a cysteine residue of one of the 30 to 40 ubiquitin carrier proteins or E2s, thereby generating yet another thiolester intermediate. Specific E2s function in the degradation of various types of substrates by conjugating with various E3 ligases. E3 ligases bind target substrates and attach ubiquitin from E2 to a lysine residue of a substrate, resulting in the formation of ubiquitin chain covalently linked to the substrate (Figure 2). There are >1000 different types of E3 ligases in cells making them the main specificity factor in the UPP [20-22]. There are three different classes of ubiquitination: i) mono-ubiquitination: a single ubiquitin is bound to the substrate, ii) multiubiquitination or poly-monoubiquitination: several single ubiquitin moieties are bound to the substrate, iii) poly-ubiquitination: substrates are tagged with poly-ubiquitin chains. Different cellular processes are regulated depending on the type of the modification of the substrate [23, 24]. For example, mono-ubiquitination has been shown to control numerous cellular processes such as receptor transport, viral budding and DNA repair [25]. Most substrates require poly-ubiquitination to be delivered to the proteasome. The attachment of a chain of four or more ubiquitin molecules to a protein is required for efficient degradation [26, 27].

There are several types of ubiquitin linkages depending on the lysine residue involved in the formation of the poly-ubiquitin chain involving one or more of the seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) of the ubiquitin moiety. Ubiquitin chains can vary in their length and linkage type. Depending on the type of poly-ubiquitin chain, modified proteins are destined to different cellular fates. The best known is the Lys48-linked poly-ubiquitin chain being involved in the proteasomal degradation of proteins [6], whereas the Lys63-linked poly-ubiquitin chain has been shown to be involved in DNA repair, DNA replication and signal transduction processes [23]. This has been the picture for many years but the recent studies have added another level of complexity. Linkages other than Lys48 appear to be involved in targeting proteins to the proteasome for degradation with poly-ubiquitin chains linked through Lys6, Lys11, Lys27, Lys29, or Lys33 [28]. Even Lys63 chains, which have been assumed to provide a signaling rather than degradation function, can target for proteasome degradation [29].
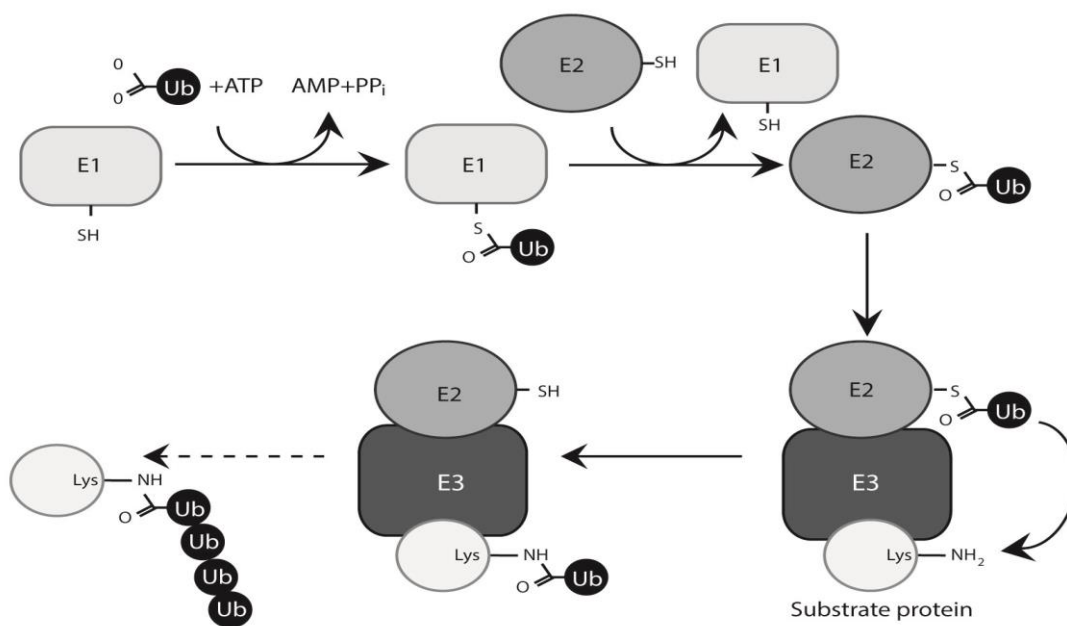

Figure 2. The ubiquitination system

The process of ubiquitination can be reversed by specific deubiquitinating enzymes (DUBs), thereby controlling the balance between E3 ligases and DUBs. The balance of chain-extending and chain-trimming activities is required for proper proteasome function. Through opposing activities of HECT ubiquitin ligase 5 (Hul5) and the proteasome DUB, Ubp6/USP14, ubiquitin chains on bound substrates are extensively remodeled by the 26S proteasome [30].

Most of the proteasomal substrates are recognized by poly-ubiquitin chains [6]. In contrast, the enzyme ornithine decarboxylase (ODC) is recognized by the proteasome through a mechanism independent of ubiquitination. The enzyme plays a key role in the polyamine biosynthesis. Instead of binding poly-ubiquitin chains, ODC degradation by the proteasome involves binding of polyamine-induced protein (antizyme) [31, 32].

19S regulatory particle

The ubiquitinated substrate is recognized by the 19S RP followed by the removal of the ubiquitin chain by the deubiqitinating ezymes (DUBs), unfolding and translocation of

the unfolded substrate protein to the 20S CP for degradation. The 19S RP consists of at least 19 different subunits with molecular masses ranging from 10 to 110 kDa and can be divided into two subcomplexes: the lid and the base [26].

The lid of the 19S RP is composed of nine non-ATPase subunits Rpn3, Rpn5–Rpn9, Rpn11, Rpn12, and Rpn15 (Figure 3). Overall, Rpn3, Rpn7, Rpn6, Rpn5 and Rpn9 form the fingers of the hand-shaped lid structure. Rpn11/POH1, the only essential DUB of the proteasome, lies in the palm of the hand and makes extensive contacts with Rpn8, Rpn9 and Rpn5 [26, 33-35].

The base is composed of ten subunits and six are related AAA⁺ ATPase (Rpt1-Rpt6) that form a hetero-hexameric ring with the specific order of Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 [36]. A loop, called Ar-Φ (aromatic-aliphatic) loop, protrudes from every subunit into the center of the ATPase ring [37]. The AAA⁺ domains of the ATPase subunits are responsible for the contact with the folded protein substrate through the loops. The loops undergo conformational changes by utilizing the energy from ATP hydrolysis resulting in the production of pulling pulses that unravel the tertiary and secondary structures of protein substrates. The unfolded substrate is then translocated through a narrow central pore into the 20S CP peptidase chamber for degradation [38-40]. The ATPase ring plays an important role in the opening of the gated channel for substrate degradation, primarily involving two of the six ATPases (Rpt2 and Rpt5) [41, 42].

Rpn1 and Rpn2 are the largest subunits of the proteasome which are associated with the ATPase ring within the base (Figure 3). Rpn1 and Rpn2 are important subunits regarding ubiquitin chain dynamics by binding enzymes that disassemble or extend ubiquitin chains. For example, DUB Ubp6/USP14 binds proteasomes via Rpn1 and the ubiquitin ligase Hul5 binds proteasomes through Rpn2 [30, 43, 44].
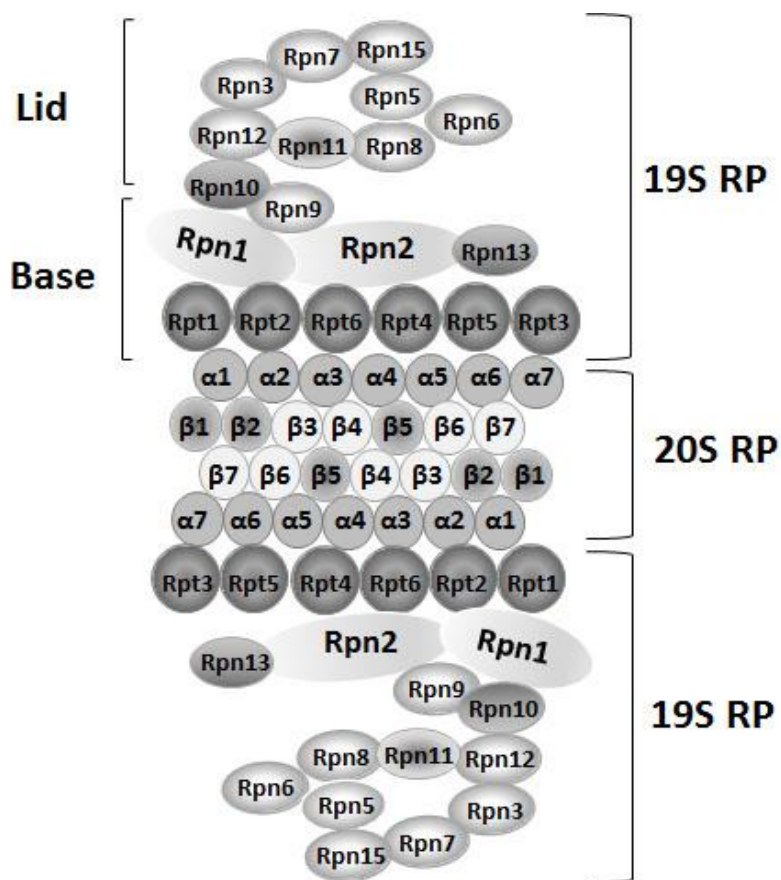

Figure 3. Schematic diagram of the 26S proteasome complex. Yeast nomenclature for the different subunits.

Most substrates dock at the proteasome via specific ubiquitin receptors. There are five ubiquitin receptors known to associate with the proteasome: two 19S RP base subunits, Rpn10 and Rpn13, and three extrinsic ubiquitin receptors Rad23, Dsk2, and Ddi1. Yeast cells with mutations in all five ubiquitin receptors remain viable, indicating that other ubiquitin receptors may exist [45-48]. Rpn10 was the first receptor found to bind the poly-ubiquitin chain [45]. It contains two C-terminal ubiquitin-interacting motif (UIM) for ubiquitin binding. These two domains cooperate when there is more than one ubiquitin present in a conjugate [46, 49]. The other integral ubiquitin receptor, Rpn13, binds ubiquitin via pleckstrin-like receptor for the ubiquitin (Pru) domain. Rpn13 bind Lys48-linked di-ubiquitin with high affinity. Rpn13 serves also as a receptor for Uch37/UCHL5 deubiquitinating enzyme, linking chain recognition and disassembly together [48]. The extrinsic ubiquitin receptors (Rad23, Dsk2, and Ddi1) contain ubiquitin-like (UBL) domain which bind to the 19S RP subunits Rpn1, Rpn10 and Rpn13 and ubiquitin-associated (UBA) domain binding poly-ubiquitinated substrates [47]. There is a theory that conjugates arrive at the proteasome on these extrinsic receptors and are handed off to intrinsic receptors, Rpn10 and Rpn13. The UBA domains in these ubiquitin receptors may have preferential affinities for different ubiquitin linkages and different chain lengths [50, 51].

The lid complex is connected to the base complex by interactions between Rpn12 and Rpn2, and between Rpn11 and Rpn1. Rpn10 might be involved in stabilizing these interactions [33]. The major activity of the lid and the base is proposed to be deubiquitination [34, 35, 52, 53].

20S core particle

The basic understanding of the structure of the 20S CP came from the studies of the archaeon *Thermoplasma acidophilum*. The prokaryotic 20S CP consist of homo-oligomers of the same α and β subunits, i.e., the αββα structure. The eukaryotic 20S CP is a cylinder-shaped multimeric complex which is formed by axial stacking of four heteroheptameric rings composed of two outer α-rings and two inner β-rings. Each ring contains seven homologous members resulting in the general structure of the complex $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ [54, 55].

In yeast and in higher eukaryotes the 20S CP contains three proteolytically active sites β1, β2 and β5, while the other β-subunits, β3, β4, β6 and β7 are catalytically inactive. The catalytically active β-subunits contain catalytically active threonine residues at their N-termini. The β1, β2, and β5 subunits are associated with caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing), trypsin-like, and chymotrypsin-like activities, respectively, which confer the ability to cleave peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino-acid residues, respectively. The active sites face the interior space of the 20S CP [56, 57]. The three peptidase activities of the 20S proteasome can be easily probed with specific fluorogenic peptides and are very often monitored as a measure of proteasomal activity *in vitro*. Many of the proteasome inhibitors have these activities as targets [58].

While β rings possess proteolytically active sites, the outer α rings provide attachment sites for the 19 S RP and control the access of substrates to the catalytic chamber by serving as a gated channel. The channel of the α-ring is very narrow (~13 Å in diameter) and is mainly composed of α2, α3 and α4 subunits. Thus, substrates destined to be degraded must pass this narrow channel to access the active sites of catalytic enzymes at the center of the α-ring. Substrate proteins are degraded into oligopeptides ranging in length from 3 to 15 amino-acid residues. The resulting peptide products are hydrolyzed

to amino acids by different peptidases [54, 59-61]. If not hydrolyzed to amino acids, oligopeptides can be used by histocompatibility complex (MHC) class I molecules for presenting intracellular/endogenous antigens to the immune system [62].

Deubiquitinating enzymes of the 26S proteasome

Why do proteasomes deubiquitinate substrates? Substrate deubiquitination is required for efficient proteolysis. Access to the opened channel into the 20S CP is sterically impeded by covalently linked poly-ubiquitin chains, thus cleavage and removal of the ubiquitin chain is required for substrate translocation and degradation [34, 35]. Deubiquitinating enzymes are also responsible for the recycling of free ubiquitin in the cell. This process is vital for keeping sufficient levels of free ubiquitin that can be used for chain assembly. If not cleaved from the substrate, some ubiquitin can be translocated into the CP along with the substrate and be degraded [63]. DUB activity is also required for clearing substrate-free poly-ubiquitin chains stuck to the proteasome so that new poly-ubiquitinated substrates can bind to these sites. Yet, another function of deubiquitinating enzymes is to rescue poorly ubiquitinated substrates from degradation by trimming the poly-ubiquitin chain from the distal end [64].

There are at least 98 DUBs encoded by the human genome which are divided into six classes: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian-tumor proteases (OTUs), Machado–Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases (JAMMs) and monocyte chemotactic protein-induced protein (MCPIP) [65].

Three DUBs are associated with the proteasome: Rpn11/POH1, Ubp6/USP14 and Uch37/UCHL5 (yeast/human nomenclature). Rpn11/POH1 is a metalloprotease which belongs to the JAMM domain family and is an integral part of the lid of the 19S RP. Ubp6/USP14 and Uch37/UCHL5 are cysteine proteases and members of the ubiquitin C-terminal hydrolases (UCH) and ubiquitin specific proteases (USP) families, respectively. They are physically associated with the base complex of the 19S RP. Deubiquitination is carried out by the hydrolysis of the isopeptide bond in ubiquitin–protein conjugates [34, 35, 43, 52, 53].

Rpn11/POH1

Rpn11/POH1 is essential for viability in yeast and cancer cells [66, 67]. In addition to its function as a DUB, Rpn11/POH1 is essential for 26S proteasome structure and activity. Knock-down experiments of Rpn11/POH1 disrupted the assembly of the 26S proteasome leading to the inhibition of the activity of the proteasome [68]. Rpn11/POH1 contains a JAMM/MPN$^+$ motif sequence containing two histidine residues and an aspartic residue coordinating a zinc ion, which is important for the proteolytic activity of this deubiquitinating enzyme [69, 70]. Indeed, JAMM/MPN$^+$ motif was required for cell viability of cancer cells mentioned before [67]. Hydrolysis of ATP is required for Rpn11/POH1-dependent deubiquitination, suggesting that deubiquitination is coupled to protein unfolding by the ATPases. Rpn11/POH1 is localized directly above the entrance of the ATPase ring by being cross-linked to the N-terminal coiled-coil domain of Rpt3. This position is ideal for removal of ubiquitin from substrates immediately before the translocation of the substrate into the CP. The activity of Rpn11/POH1 is thought to be delayed until the proteasome is committed to degrade the substrate [34, 35, 71]. Rpn11/POH1-mediated deubiquitination promotes substrate degradation by cutting at the base of the chain resulting in the release of entire poly-ubiquitin chain. To allow cleavage without disengaging from the receptor, an ubiquitin chain must be long enough to span the distance between receptor and DUB. At least four ubiquitin moieties are necessary to span the distance between receptors Rpn10 or Rpn13

and Rpn11/POH1 [26, 34, 35]. Lys63-linked poly-ubiquitin chains are the preferable substrates for Rpn11/POH1 [72].

Ubp6/USP14

Unlike Rpn11/POH1, *Saccharomyces cerevisiae* orthologue of USP14, Ubp6, is nonessential for cell viability [73]. Knock-down of Ubp6/USP14 had no detectable effect on proteasome structure indicating that it is not an integral subunit of the proteasome [64]. The full-length human Ubp6/USP14 contains 494 amino acids, with a 9-kDa UBL domain at its N-terminus followed by a 45-kDa catalytic domain. The catalytic domain of Ubp6/USP14 resembles an extended right hand comprised of three domains: Fingers, Palm, and Thumb. The binding pocket of ubiquitin is located between the Fingers and the Palm–Thumb scaffold and contains two surface loops (BL1 and BL2) that are located above, which block the binding of ubiquitin. Thus, the blockade of the ubiquitin binding groove by loops BL2 and BL1 must be removed in order for Ubp6/USP14 to catalyze deubiquitination [53, 74]. Ubp6/USP14 has been shown to reversibly associate with the Rpn1 subunit of the base in the 19S RP via its UBL domain. It has been proposed that the binding of Ubp6/USP14 to the base of the 19S RP induce conformational changes in the two loops to make the active site for ubiquitin accessible [74]. Association with the proteasome increases Ubp6/USP14 catalytic activity several hundredfold. Ubp6/USP14 can be dissociated from the proteasome under high-salt conditions [43, 64]. Ubp6/USP14 is located at the largest distance from the entrance of the pore of all the ubiquitin-interacting subunits in the RP, which may allow it to clip extended or unnecessary ubiquitin chains from substrates [26]. Lys48-linked poly-ubiquitin chains are the preferable substrates for Ubp6/USP14 and are cleaved from their distal end or within the chain [74, 75].

Ubp6/USP14 has several functions at the proteasome, which are dependent on the deubiquitinating activity of this enzyme but also non-catalytic effects of Ubp6/USP14 have been reported. Ubp6/USP14 is important for ubiquitin recycling. Loss of ubiquitin pools severely impair the ability of the proteasome to clear unwanted proteins from the cell, being dependent on ubiquitin for tagging, and result in protein accumulation. Depletion of free ubiquitin up-regulates proteasome bound Ubp6/USP14 and loss of Ubp6/USP14 results in increased degradation of ubiquitin and decreased levels of monomeric ubiquitin [75-77].

Ubp6/USP14 was shown to inhibit proteasome activity independently of its deubiquitinating function by delaying the breakdown of proteins by the proteasome. It is suggested that Ubp6/USP14 prevents deubiquitination of the substrate by Rpn11/POH1. This allows the substrate to be docked at the proteasome for a longer time, thus resulting in more extensive trimming of ubiquitin chains, which reduces substrate binding affinity to the proteasome and favors its release back to the cytosol [75, 78]. Conversely, the small molecule USP14 inhibitor IU1 was shown to reduce chain trimming and stimulate proteasome degradation, indicating the ability of USP14 to inhibit the proteasome through its deubiquitinating activity [79]. Ubp6/USP14 is also involved in the regulation of gate opening of the 20S core particle. Binding of ubiquitin conjugates to the 26S proteasome increases peptide hydrolysis by increasing 20S gate opening. Ubiquitin conjugates interact with Ubp6/USP14 and in this way stimulate gate opening, enabling the substrate to be degraded [80]. It has been found that most of the cellular Ubp6/USP14 is not associated with the proteasome, indicating that it may be involved in other cellular processes [64].

Uch37/UCHL5

The Uch37/UCHL5 deubiquitinase is well conserved from fungi to humans [81]. An orthologue of human Uch37 has not been found in *Saccharomyces cerevisiae*. However, the orthologue in *Saccharomyces pombe*, Uch2, is nonessential for viability [82]. When identified, Uch37/UCHL5 was first shown to be a stoichiometric component of the 26S proteasome [83], but has later been shown, in several reports, to reversibly associate with the proteasome [81, 84-86]. In contrast to Rpn11/POH1, Uch37/UCHL5 is not important for the activity or the structure of the 26S proteasome. Uch37/UCHL5 consists of 329 amino acids (molecular mass 37K) and has two functional domains, a catalytic domain (UCH-domain) and a C-terminal domain. The deubiquitinase activity of the Uch37/UCHL5 is enhanced when bound to 26S proteasome [64] via the Rpn13/Admr1 receptor in the 19S RP base complex. The N-terminal Pru domain of Rpn13/Admr1 binds to the proteasome (Rpn2 subunit of the 19S RP), whereas it's C-terminal domain binds to the C-terminus of Uch37/UCLH5. The UCH-domain contains an active-site crossover loop, and unless displaced, blocks substrate entry. This auto-inhibitory function is reversed by binding of Uch37/UCHL5 to Rpn13/Admr1, resulting in the release of the loop and activation of the enzyme. Incorporation of the Uch37/UCHL5 to 26S proteasome is required for efficient deubiquitinating activity [81, 84, 86]. Similarly to Ubp6/USP14, Uch37/UCHL5 removes ubiquitin from the distal end of the chain. Thus, it does not remove chains en as a single unit but progressively shortens them. While Ubp6/USP14 is able to release di- and tri-ubiquitin from substrates, Uch37/UCHL5 releases only mono-ubiquitin from chains [52, 75]. Uch37/UCHL5 cleaves both Lys48- and Lys63-linked poly-ubiquitin chains [87]. It is believed that Uch37/UCHL5 suppresses protein degradation by shortening the chain of inappropriately or poorly modified substrates [52, 64]. In contrast, a recent study has suggested that Uch37/UCHL5 promotes the degradation of specific proteasome substrates, nitric oxide synthase and IκB-α [88]. In conclusion, Uch37/UCHL5 can suppress the degradation of some substrates while promoting the degradation of others.

Cooperative functions of proteasomal DUBs

It is unclear why so many activities are required for deubiquitination at the proteasome and how they are related to one another, but there are some suggestions that Rpn11/POH1, Ubp6/USP14, and Uch37/UCHL5 cooperate to provide effective processing of ubiquitin chains. As mentioned above, the substrate must be committed to degradation before the poly-ubiquitin chain is removed by Rpn11/POH1. Both Usp6/USP14 and Uch37/UCHL5 trim poly-ubiquitin chains from the distal end before the poly-ubiquitin chain is removed by Rpn11/POH1 [49]. It has been proposed that Ubp6/USP14 and Uch37/UCHL5 complement the function of Rpn11/POH1. Rpn11/POH1 cleaves the entire ubiquitin chains resulting in the release of the substrate to be degraded by the proteasome. Substrate-free poly-ubiquitin chains remain associated with the proteasome and must be removed so that new substrate-bound poly-ubiquitin chains can bind. Ubp6/USP14 and Uch37/UCHL5 accomplish this function by hydrolyzing these poly-ubiquitin chains. The exact difference in catalytic function between Ubp6/USP14 and Uch37/UCHL5 are not clear, but the activities appear to operate in similar ways. Double knockdown of Ubp6/USP14 and Uch37/UCHL5 results in inhibition of cell growth, decreased protein degradation, and accumulation of poly-ubiquitinated proteins, a cellular phenotype similar to that observed after knock-down of Rpn11/POH1 [68]. RNAi-mediated down-regulation of either DUB alone creates a complete opposite phenotype where the cell growth was not affected and reduced levels of poly-ubiquitinated proteins was observed, indicating that each enzyme could compensate for loss of function of the other [64].

The role of proteasomal deubiquitinases in cancer

As mentioned previously, the important role of Rpn11/POH1 for cancer cell viability makes it an obvious target for cancer therapy [66, 67]. Over-expression of Rpn11/POH1 has been shown to promote resistance to several chemotherapeutic drug being used in the clinic today, e.g. vinblastine, doxorubicin, cisplatin. Rpn11/POH1-induced drug resistance is suggested to be mediated through AP-1 transcription factors [89]. Rpn11/POH1 is also involved in the regulation of the ErbB2 receptor, which has been associated with poor prognosis and malignancy of breast cancer when highly expressed. It is believed that Rpn11/POH1 deubiquitinates ErbB2 and instead of destruction, ErbB2 is rescued from proteasomal degradation [90]. Thus, inhibiting Rpn11/POH1 would be beneficial for breast cancer patients with ErbB2 positive tumors.

To date, there are only a few studies on the potential role of Ubp6/USP14 in cancer. Thus, the understanding of the role of Ubp6/USP14 in cancer biology is very limited. Levels of Ubp6/USP14 were first found to be elevated in leukemic and colon cancer cell lines [91, 92]. The levels of Ubp6/USP14 have also been associated with overall survival rate in colorectal cancer patients, being worse in patients with high expression of the enzyme than in those with low expression levels. This high expression correlated also with histological stage, lymph node and liver metastases [93]. Recently, a study on lung adenocarcinoma revealed a high expression of Ubp6/USP14 in both cell lines and tumor tissue. The overexpression of Ubp6/USP14 promoted cell proliferation by induction of β-catenin and was significantly correlated with overall survival of lung adenocarcinoma patients [94].

There are several studies on Uch37/UCHL5 and its role in oncogenesis. Deregulation in the transforming growth factor-β (TGF-β) signaling cascade is a common occurrence in human cancers. In the late stage cancers, TGF-β switches from being a tumor suppressor to a tumor promoter. Cancer cells use TGF-β to initiate cell migration and metastases [95]. Uch37/UCHL5 has been shown to interact with Smad7 involved in TGF-β signaling. Thus, up-regulation of Uch37/UCHL5 plays an important role in the late stage of tumor development. Smad7 has been shown to function as an adaptor that recruits the Smurf E3 ubiquitin ligase to the TGF-β receptor complex to promote its ubiquitination and proteasomal degradation. Uch37/UCHL5 interaction leads to deubiquitination of the TGF-β receptor thereby rescuing it from proteasomal degradation and promotion of TGF-β signaling [96]. Taken together aberrant Uch37/UCHL5 levels play important role in cell migration and metastases which has been shown by Cutts and colleagues [97].

Another way of Uch37/UCHL5 to promote cell survival is by altering the expression of apoptosis mediators. Silencing of Uch37/UCHL5 in lung adenocarcinoma epithelial cell line A549 was shown to induce apoptosis through activation of caspase-3 and caspase-9. In these cells anti-apoptotic Bcl-2 protein was down-regulated while pro-apoptotic Bax protein was up-regulated. Conversely, the overexpression of Uch37/UCHL5 had the opposite effect [98].

Uch37/UCHL5 has been shown to be up-regulated in several carcinoma tissues compared to the adjacent normal tissues [99-101]. In esophageal squamous cell carcinoma the expression of Uch37/UCHL5 was closely related with lymph node metastases and TNM (Classification of Malignant Tumors) stage and was also significantly correlated with patients' overall survival and disease free survival [100]. Uch37/UCHL5 was also found to be highly expressed in hepatocellular carcinoma (HCC) tissues. In HCC cell lines, Uch37/UCHL5 promoted cell migration and invasion by deubiquitinating PRP19, an essential RNA splicing factor [101].

The ubiquitin proteasome pathway in cell physiology

The Role of UPP in cell proliferation and cell death

Many important targets of the 26S proteasome that have been identified are important players in cell proliferation and cell death including cyclins [102-104], tumor suppressor protein p53 [105], pro-apoptotic protein Bax [106], cyclin dependent kinase inhibitor (CKI) p27 [107] and the nuclear factor kappaB (NF-κB) inhibitor, IκB [108]. The degradation of specific cellular proteins by the 26S proteasome determines whether a cell proliferates or dies [109]. The UPP is involved in the regulation of many of the apoptosis pathway molecules and generally contributes to apoptosis resistance in cancer cells. Thus, the induction of apoptosis by proteasome inhibitors is out of key importance [110]. The UPP regulates levels of both pro- and anti-apoptotic proteins and inhibition of the proteasome activity up-regulates pro-apoptotic factors such as p53 and Bax while reducing levels of anti-apoptotic proteins such as Bcl-2 [111]. The proteasome activity has been shown to be elevated in many kinds of cancer contributing to tumorigenesis by providing cancer cells with anti-apoptotic protection and uncontrolled cell division [106, 112-114].

Cell cycle

In normal cells, the cell cycle is tightly controlled by a number of signaling pathways leading to cell growth, DNA replication and cell division. The UPP is involved in the degradation of many of the proteins that regulate the cell cycle leading to cell growth. Uncontrolled cell proliferation is a hallmark of cancer [1]. There are three key classes of regulatory molecules involved in the control of the cell cycle: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs), which require programmed and periodic expression, and degradation for cell cycle progression. The most important mechanism of cell cycle control is the activation of CDKs, which are regulated by the ubiquitin-dependent proteolysis of cyclins (D, E, A, B) and CKIs (p21 and p27) [115]. There are several ubiquitin ligases involved in the regulation of cell cycle, which are responsible for the ubiquitination of the CKIs or cyclins and their degradation by the 26S proteasome [116]. Their primary targets are tumor suppressor proteins p53, p21 and p27 [117-119]. Cyclin D and E have been shown to be up-regulated in different malignancies while CKIs (p21 and p27) are suppressed in several cancer types to promote tumor progression [120-122]. Inhibition of proteasome function leads to accumulation of p27 and p21 followed by the induction of cell cycle arrest and apoptosis [109, 123, 124].

p53

The tumor suppressor p53 is an important regulator of apoptosis. p53 levels are tightly regulated through interaction with its negative regulator Mdm-2, which is an E3 ubiquitin ligase. Under normal conditions it is held inactivated and ubiquitinated by Mdm2 leading to degradation by the 26S proteasome [105, 119, 125]. In response to DNA damage, hypoxia and inappropriate oncogene signaling, wild-type p53 is induced resulting in activation of transcription of genes that induce growth arrest and apoptosis [126]. Mutations in the p53 gene are common in many types of cancer leading to tumor progression [127]. Induction of p53-dependent apoptosis by proteasome inhibitors has been demonstrated in different malignancies [128-130]. In contrast, proteasome

inhibitors are also able to induce apoptosis independently of the p53 status in cancer cells [131, 132].

NF-κB

Nuclear factor kappaB (NF-κB) is a transcription factor that is also involved in the regulation of apoptosis pathways and its activation is regulated by the UPP. When activated, NF-κB suppresses apoptosis by initiating the transcription of genes encoding anti-apoptotic proteins such as Bcl-2 and IAPs (inhibitors of apoptosis) [133-135]. Under normal circumstances, NF-κB is sequestered in the cytoplasm through association with its endogenous inhibitor IκB [136]. Upon stimulation, IκB is phosphorylated, poly-ubiquitinated by an E3 ubiquitin ligase and degraded by the 26S proteasome, resulting in the translocation of NF-κB into the nucleus and thereby initiating the transcription of anti-apoptotic genes [108]. It has been shown that NF-κB is constitutively active in certain malignancies, promoting tumor cell survival [137, 138]. By using proteasome inhibitors, the degradation of IκB by the proteasome is prevented, resulting in the inhibition of NF-κB transcriptional activity and induction of tumor cell apoptosis [139, 140]. However, there are several studies showing that NF-κB activity enhances tumor cell sensitivity to apoptosis. NF-κB has been shown to contribute to cell death by transcriptional up-regulation of its pro-apoptotic target genes i.e. p53, Bax [141-143]. A study on the mouse model of hepatocellular carcinoma (HCC) shows that NF-κB acts rather as a tumor suppressor than a tumor promoter [144]. In this case, treatment with proteasome inhibitors would perturb NF-κB function as a tumor suppressor.

Bcl-2 family

A number of members of the Bcl-2 family of anti-apoptotic and pro-apoptotic proteins are regulated by proteasomal degradation. The ratio of pro-apoptotic versus anti-apoptotic Bcl-2 members plays an important role in determination of cellular fate. The anti-apoptotic Bcl-2 protein is an integral mitochondrial protein, which upon apoptotic stimuli blocks apoptosis and preserves mitochondrial integrity [145]. Reduced levels of Bcl-2 protein through proteasomal degradation results in induction of apoptosis by release of pro-apoptotic signals [146]. Similarly, pro-apoptotic members of the Bcl-2 family (Bax, Bak, Bad, Bim, Bik, and Bid) also undergo proteasomal degradation, favoring cell survival [147]. It has been demonstrated that Bax is a direct target of the proteasome and its degradation is a survival mechanism in human cancer cells. Under normal conditions, Bax is localized in the cytosol as a monomer. During apoptosis, Bax translocates to mitochondria and undergoes conformational changes to form a functional dimer resulting in the loss of mitochondrial membrane potential and release of cytochrome c. In turn cytochrome c activates the caspase cascade resulting in cellular apoptosis [106]. Increased proteasomal degradation of Bax has been associated with poor prognosis in chronic lymphocytic leukemia [148]. Inhibition of proteasomal degradation of Bax, in Jurkat T-cell leukemia cells overexpressing Bcl-2, was sufficient to overcome protective Bcl-2 effects on apoptosis. Thus, blocking proteasomal degradation may enhance the activities of pro-apoptotic proteins rather than those of anti-apoptotic proteins [106].

JNK pathway

The inhibition of the proteasome leads to the induction of apoptosis by activating the stress kinase JNK (c-Jun-N-terminal Kinase). JNK belongs to the mitogen-activated

protein kinase (MAPK) family. Different mechanisms of JNK induced apoptosis by proteasome inhibitors have been proposed. Yang and colleagues show that bortezomib induces JNK pathway in non-small cell lung cancer cells probably by induction of p21, resulting in growth arrest and apoptosis [149]. In another study, bortezomib-induced apoptosis in multiple myeloma cells is associated with translocation of JNK from cytosol to mitochondria and release of cytochrome c [150].

The Role of UPP in protein quality control

Organisms have evolved protein quality control systems to avoid disruption of cellular function by accumulation of abnormal proteins. Heat shock proteins (Hsps) or molecular chaperones and UPP are central players in the protein quality control processes. The quality control system must be able to distinguish between native (properly folded, and assembled) proteins and non-native or abnormal proteins, which include partially unfolded, misfolded, or incorrectly modified proteins (i.e. unassembled subunits of complexes). Molecular chaperones are induced upon accumulation of non-native proteins in the cytosol in an attempt to repair them. Chaperones bind to and stabilize exposed hydrophobic residues through ATP-dependent interactions, allowing the protein to achieve proper folding. If this fails, the non-native proteins are ubiquitinated and targeted for degradation by the 26S proteasome. Thus, the UPP and and molecular chaperones are closely connected [151, 152]. Interaction of chaperones and the proteasome is not only limited to cytosolic proteins and chaperones, but also rather important for the folding of newly synthesized proteins in the endoplasmic reticulum (ER) [153].

The quality control in the cytosol and ER

The close relationship between UPP and molecular chaperones is strengthened by the evidence that inhibition of UPP results in up-regulation of Hsps and that Hsps (Hsp70 and Hsp90) are required for ubiquitination and degradation of some substrates [154, 155]. Hsp70 is recruited to the 26S proteasome by binding to a co-chaperone protein Bag-1, which contains an UBL domain for the proteasomal binding [156]. Binding of Hsp70 and Hsp90 to different co-chaperones defines whether a protein becomes repaired or degraded by the 26S proteasome. CHIP (C-terminus of Hsp70-Interacting Protein) is one of the co-chaperones that is also an ubiquitin E3 ligase. CHIP contains domains that interact with both Hsps and ubiquitin conjugating enzymes (E2s), thereby having important role in determining the fate of damaged proteins by modulating both the chaperone activity and the ubiquitin conjugating activity [157, 158]. Damaged proteins are recognized by Hsp70 or Hsp90 and with the help of other co-chaperones they are repaired and returned to cellular function. If this fails, CHIP triggers the ubiquitination of the damaged protein for the degradation by the 26S proteasome. The activity of DUBs is also involved in the decision of repair or degradation. As already mentioned above, Ubp6/USP14 and Uch37/UCHL5 rescue substrates from proteasomal degradation by removing the ubiquitin from chains attached to damaged proteins, thereby allowing the substrate to associate longer with the proteasome, giving a chance to molecular chaperones to repair the damage and rescue the protein from degradation [151]. Proteasome inhibitors induce the expression of various molecular chaperones or heat shock proteins [159]. These are recruited by high molecular weight poly-ubiquitin conjugates which are induced upon proteasome inhibition [160].

The UPP plays an important role in the quality control of newly synthesized proteins. After being synthesized, proteins enter the ER in their unfolded state and are then folded to reach their functional three dimensional structure [161]. The ER is responsible for folding about 7500 different proteins and 30-80% of all newly synthesized proteins end

up misfolded, indicating an important role of the UPP to degrade and prevent accumulation of non-functionally proteins [162]. Because the UPP is located in the cytosol, misfolded proteins have to be transported from ER back to the cytosol for degradation (retro-translocation), a process known as ER-associated degradation (ERAD) [163]. Transport across ER membrane in both directions is mediated through different channels. Proteasomes have also been shown to associate with ER membrane through binding of 19S RP base to these channels and a model has been proposed in which ATPases of the 19S RP are responsible for extracting proteins from ER through the channel for degradation by the proteasome [164]. A proper folding is mediated by chaperones residing in the lumen of the ER and the most abundant one is the heat shock protein BiP/Grp78 (an ER homologue of Hsp70). Misfolded or unfolded proteins are prone to aggregation and BiP/Grp78 is responsible to prevent aggregation of proteins bound to it and in this way keep them soluble which is important for proper retro-translocation through the channels. To promote folding and prevent aggregation of proteins BiP/GRP78 uses the energy from ATP hydrolysis within the ER [162, 165].

Inhibition of the 26S proteasome results in the accumulation of unfolded proteins in the ER lumen and induction of ER stress, which in turn elicits the unfolded protein response (UPR) [166]. UPR is primarily pro-survival mechanism which functions to increase expression of BiP/Grp78 to limit protein aggregation, to increase biosynthesis of structural components of the ER and to inhibit protein synthesis in an attempt to reduce the load on the ER. If the cyto-protective mechanisms fail, UPR ultimately triggers apoptosis [167]. There are three major UPR pathways mediated by the activation of three stress sensor proteins: transcription factor-6 (ATF6), inositol-requiring 1α (IRE1α), and protein kinase RNA-like ER kinase (PERK). In resting cells, these sensor proteins are associated with BiP/Grp78 and are inactive. Induction of ER stress leads to dissociation of BiP/Grp78 and activation of the sensor proteins [168, 169]. Upon activation, ATF6 initiates the transcription of genes encoding ER chaperones to promote protein folding and genes promoting ERAD. The second sensor IRE1α activates transcription factor X box–binding protein-1 (Xbp1) which is also involved in the activation of genes encoding chaperones and ERAD proteins. Activated PERK phosphorylates the translation initiation factor-2α subunit (eIF2α) to reduce the rate of protein translation in order to decrease the load of misfolded proteins on the ER. Prolonged, intense ER stress is cytotoxic. PERK is involved in the induction of apoptosis by activating pro-apoptotic transcription factor C/EBP-homologous protein (CHOP) (reviewed by Claudio Hetz) [170]. IRE1α also sensitizes cells to apoptosis through activation of c-Jun N-terminal kinase (JNK) and an ER resident caspase, caspase 12 in mice or caspase 4 in humans [171-173]. The three UPR pathways are often activated together, but selective activation of some pathways together with suppression of others can occur [170]. Prolonged UPR activation has been shown to induce reactive oxygen species (ROS) and cell death [174].

The role of UPP in response to oxidative stress

One of the major consequences of aerobic life is the formation of reactive oxygen species (ROS) that results in severe damage to DNA, protein, and lipids. In contrast, ROS production is beneficial for the destruction of pathogenic micro-organisms [175]. ROS constitutes of a variety of partially reduced metabolites of oxygen (e.g., superoxide anions, hydrogen peroxide, and hydroxyl radicals) which have higher reactivity than molecular oxygen [176]. The main source of ROS are the mitochondria. The mitochondrial electron transport chain is the main source of ATP in mammalian cells and during energy transduction ROS are generated from electron leakage from the respiratory chain. It has been estimated that 1-2% of the total oxygen consumption of mitochondria generates ROS [177]. Another source of ROS are the cellular oxidases,

e.g. NADPH oxidase [178]. Organisms have developed a series of defense mechanisms to fight ROS, which include both enzymatic antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase) and non-enzymatic antioxidants (Vitamin C, Vitamin E and others) [179, 180]. If the cellular antioxidant capacity is overwhelmed by ROS, this results in the induction of oxidative stress [181]. The outcome of oxidative stress depends on the dose and duration of the exposure to ROS, as well as the cell type. Typically, low levels of ROS promote cell proliferation while intermediate doses result in growth arrest and senescence. Very severe oxidative stress induces cell death via either apoptosis or necrosis. Necrotic cell death is thought to result from a higher amount and exposure to ROS than the amount necessary to elicit apoptotic cell death [182].

ROS cause modifications to the amino acids of proteins that generally result in loss of protein function and/or enzymatic activity. Oxidized proteins may become misfolded leading to aggregate formation [183]. The UPP is responsible for the removal of the oxidized proteins [184]. About 70%–80% of turn-over of oxidized protein has been attributed to the UPP [185]. Moreover, oxidized proteins can be degraded by the free 20S CP in an ATP-independent manner and without the presence of ubiquitin [186]. In the case of low levels of oxidative stress, the cellular repair mechanisms consisting of antioxidants and chaperones can reduce and refold the oxidatively damaged proteins and if this fails damaged proteins are either degraded by the proteasomes or they form aggregates which are cleared by autophagy [180].

Paradoxically, ROS can promote normal cellular proliferation and can also induce apoptosis in tumor cells. Elevated ROS levels have been implicated in the cell survival and tumor progression by the induction of transcription factors NF-κB and activator protein 1 (AP1) [187]. On the other hand, enhanced levels of ROS increase p53 expression and stress kinase JNK pathway that results in the induction of apoptosis [188]. Cancer cells have higher levels of ROS than normal cells in order to promote cell survival and tumor progression and for this modest levels of ROS are required [189]. However, increased ROS levels in cancer cells by using proteasome inhibitors leads to apoptosis induction and suppression of tumor growth. Proteasome inhibitors have been shown to induce ROS in different cancer cells. In human leukemia cells, the inhibition of the proteasome activity resulted in the induction of apoptosis by triggering the pro-apoptotic stress kinase JNK [190, 191]. Moreover, proteasome inhibitors have been shown to induce loss of mitochondrial membrane potential resulting in the induction of ROS and apoptosis [192, 193].

Protein aggregation and aggresome pathway

As mentioned above, some misfolded proteins are beyond salvation by chaperones or are not degraded by the 26S proteasome. Instead, these are more prone to aggregation through association of the hydrophobic domains with one another. Normally, hydrophobic surfaces of proteins are buried in the protein´s interior. Partially folded proteins expose hydrophobic domains which are prone to aggregation through association with each other. Aggregated proteins show poor solubility in water or detergents and by non-native secondary structure. Aggregates are defined by poor solubility in water or detergents and by non-native secondary structure [194]. Aggregates are sequestered into intracellular foci named inclusion bodies which are usually present in low copy numbers, often one per cell. Very quickly after their formation, inclusion bodies are transported on microtubules to the microtubule organizing center (MTOC) where they are localized to form the aggresome (Figure 4) [195]. Most aggresomes are enriched in poly-ubiquitin but there are some exceptions like those formed by the expression of misfolded GFP-250 (GFP fused to a 250–amino

acid fragment of the cytosolic protein, p115) which do not contain appreciable poly-ubiquitin [196]. Most aggresomes are pericentrosomal structures of 60-80 nm, containing misfolded, aggregated, ubiquitinated proteins. Depolymerization of the microtubules with nocodazole prevents formation of the perinuclear aggresomes and induces the production of small protein aggregates that are dispersed throughout the cytoplasm. Thus, the movement of inclusion bodies requires intact microtubules and motor dynein complex is responsible for the transport of inclusion bodies along microtubules [183, 195, 197]. Moreover, aggresome formation requires the microtubule-associated deacetylase HDAC6 which binds both poly-ubiquitinated proteins and dynein proteins, through its ubiquitin binding domain (BUZ finger) and a dynein motor binding (DMB) domain, respectively, thereby acting to recruit inclusion bodies to dynein motors for transport to aggresomes. It has been shown that aggresomes do not form in HDAC6-deficient cells. Instead, dispersed micro-aggregates are observed throughout the cytoplasm [198]. HDAC6 can also modulate aggresome formation through its regulation of ubiquitin-dependent protein degradation. HDAC6 binds ubiquitin with high affinity which promotes poly-ubiquitin chain stability, leading to the escape of the ubiquitinated protein from proteasomal degradation and instead allowing the ubiquitinated substrate to accumulate in the aggresomes [199, 200]. The DUB Ataxin-3 has been implicated in aggresome formation, although the prices role of the enzyme is still unknown. Ataxin-3 has been shown to interact with HDAC-6 and dynein motor, raising the possibility that Ataxin-3 could serve as an adaptor linking poly-ubiquitinated proteins to the dynein motor for the transport to the aggresomes [201].

In addition to misfolded and aggregated proteins, molecular chaperones and proteasome components (19S and 20S proteasome subunits) are recruited to the aggresomes, presumably to aid in the clearance of the aggregated proteins [197, 202]. The most consistent component of the aggresome is the intermediate filament protein vimentin. It forms a cage-like structure wrapped around the aggresome. The function of the vimentin cage is unclear but has been proposed to contribute to the stability of the aggresome [183, 195].
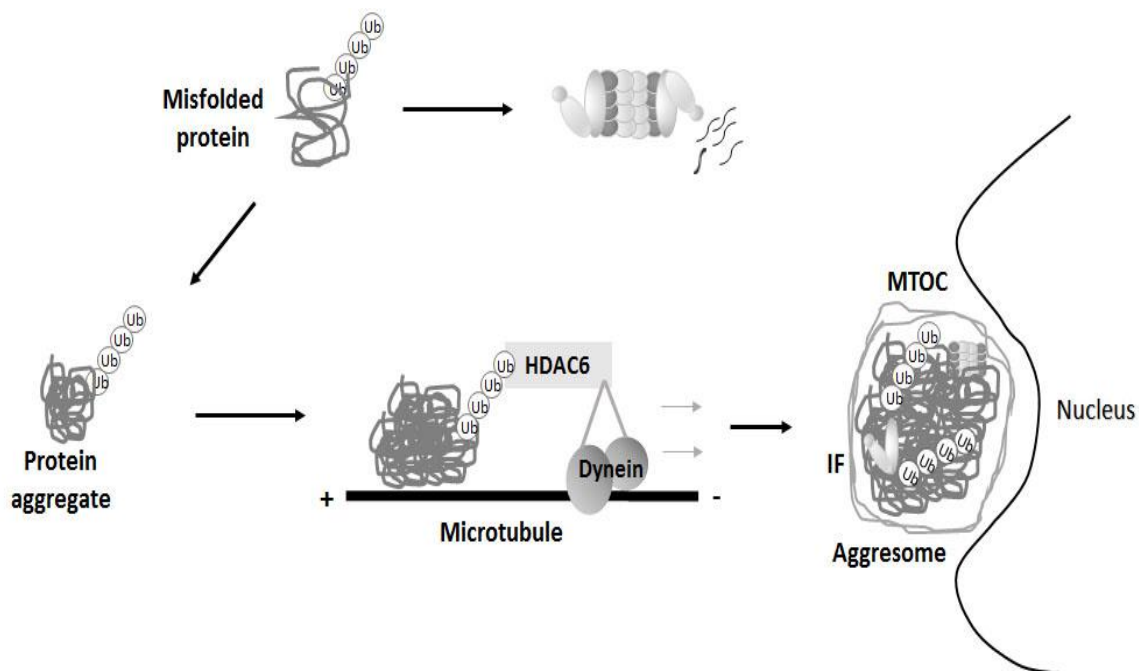

Figure 4. Aggresome pathway. IF-intermediate filament. MTOC- microtubule organizing center.

The association of proteasomes with aggresomes has led to the hypothesis that aggresomes might be cleared via the UPP [203]. However, it has been shown that aggregated proteins are poor substrates of the proteasome and can actually inhibit proteasome activity [204, 205]. Autophagosomes and lysosomes accumulate around the periphery of the aggresome, suggesting a role for autophagy in the clearance of aggresomes [206]. There are studies showing that aggresome clearance can be facilitated by the induction of autophagy. Autophagy is a degradation pathway that mediates bulk clearance of cytosolic proteins and organelles by the lysosome. The content to be degraded by the autophagy is sequestered in autophagy vesicles with double membranes and are called autophagosomes. Autophagosomes subsequently fuse to lysosomes where the autophagic contents are released and degraded by lysosomal enzymes [206-208]. A protein, p62, is responsible for recruiting aggresomes to autophagosomes by binding ubiquitins associated with aggresomes and a component of the autophagic membrane LC3, thereby acting as a bridge that connects ubiquitinated aggresomes to autophagosomes [209]. The fusion of autophagosomes to lysosomes is controlled by HDAC6, which recruits an actin-remodelling machinery responsible for the assembly of actin network that stimulates autophagosome–lysosome fusion and substrate degradation [210]. Recently, a mechanism for clearance of aggresomes was proposed where both the ubiquitin-proteasome and autophagy pathways are involved. Hao and colleagues show that aggresomes are first broken into several large fragments and then smaller microaggregates, suggesting a "de-aggregation" step that precedes final clearance by autophagy. In their model, deubiquitinating enzyme Rpn11/Poh1, plays an important role by producing unanchored free ubiquitin chains which bind and activate HDAC6. In turn, HDAC6 induces an actinomyosin system that promotes the de-aggregation and autophagic clearance of the aggresome [211].

The formation of aggresomes followed by proteasome inhibition in cancer cells is thought to be cytoprotective. Bortezomib has been shown to induce formation of aggresomes in human pancreatic cell lines leading to cytoprotective response to proteasome inhibition, probably by shuttling poly-ubiquitinated proteins to lysosomes for degradation. Inhibition of aggresome formation with HDAC6 inhibitors or RNAi resulted in the sensitization of these cells to bortezomib treatment indicating cytoprotective role of aggresomes [212]. Inhibiting both the proteasomal degradation of proteins and the aggresome formation results in accumulation of poly-ubiquitinated proteins and significant cell stress, followed by the induction of apoptosis [213]. Malignant cells use the aggresome pathway as an alternative mechanism for ubiquitinated protein degradation and disposal, which can potentially compensate for proteasome pathway inhibition and contribute to drug resistance [214].

INHIBITORS OF THE UBIQUITIN PROTEASOME PATHWAY

Selectivity of the proteasome inhibitors

Protein homeostasis plays an essential role in processes of cancer cell growth, development and survival [215]. As described above, the 26S proteasome is crucial for the execution of many cellular functions and moreover, increased proteasome activity has been associated with malignant disease. Thus, tumorigenic cells are expected to be more dependent upon proteasomal activity thereby being more sensitive to its blockade [106, 216]. Many types of actively proliferating malignant cells have been shown to be more sensitive to proteasome inhibitors than normal cells [217-219]. A concrete mechanistic explanation for this selectivity is not yet available but many have suggested

that this selectivity is linked to proliferation, deregulation of the cell cycle and induction of apoptosis.

As described earlier, cancer cells use excessive proteasomal degradation to degrade proteins that inhibit cell growth e.g. p53, p27 and Bax [105, 106, 220]. Proteasome inhibitors elevate the levels of these short-lived proteins and might selectively restore apoptosis in these cells [106, 123, 128]. Alternatively, the selectivity of proteasome inhibitors for tumor cells has been suggested to depend on the expression of oncogenes, such as c-Myc, that deregulate cell proliferation and also induce apoptosis. The induction of pro-apoptotic protein Noxa by proteasome inhibitor has been shown to depend on the basal levels of c-Myc. Thus, proteasome inhibition selectively induces apoptosis in tumor cells compared to normal cells by being dependent on the expression of the oncogene for the induction of pro-apoptotic signals [221].

The cell cycle check point and DNA repair systems are defective in cancer cells and when treated with proteasome inhibitors cancer cells are unable to correct the cell cycle transition blockade and are driven into apoptosis while normal cells retain checkpoints that allow cells to recover from proteasome inhibition. Moreover, one could speculate that cancer cells are more vulnerable to proteasome inhibition by being dependent on increased protein synthesis to be able to proliferate. These cells might accumulate defective proteins at a much higher rate than normal cells, which increases their dependency on the proteasome as a disposal mechanism [111]. Indeed, it has been reported that B-cell chronic lymphocytic leukemia cells have higher levels of ubiquitin-conjugated proteins and higher levels of chymotrypsin-like activity compared with normal lymphocytes when treated with proteasome inhibitor lactacystin [222]. But the picture is more complicated, Guzman and colleagues report that quiescent malignant cells are more susceptible to proteasome inhibitors than their proliferating normal counterparts [223]. Further understanding of the molecular basis for the selectivity of proteasome inhibitors is needed for their development as anticancer agents.

Bortezomib

Bortezomib (PS-341, Velcade ®; Millennium Pharmaceuticals) is the first proteasome inhibitor to gain approval by the US Food and Drug Administration (FDA). The approval of bortezomib for the treatment of multiple myeloma and mantle cell lymphoma has validated the UPP as a suitable target for the development of novel therapies for the treatment of cancer [224, 225].

Bortezomib is a synthetic dipeptide boronic acid that acts as reversible inhibitor of the proteolytic activities of the 20S CP, namely the chymotryptic-like, caspase-like and to a lesser extent trypsin-like activities [226-228]. The inhibition of chymotryptic-like and caspase-like activities is achieved through interactions of bortezomib with the active N-terminus threonine of the $\beta5$ or $\beta1$ subunit, respectively [140].

Preclinical studies of bortezomib have demonstrated the ability of this proteasome inhibitor to induce growth arrest and apoptosis in a wide range of tumor cell lines and animal models of hematologic and solid tumor malignancies [139, 229-231]. Multiple mechanisms of bortezomib induced growth arrest and apoptosis have been suggested including the inhibition of the NF-κB activation [230], induction of p53 [128], induction of CKIs p27 and p21 [232], activation of the stress kinase JNK [149] and induction of pro-apoptotic protein Noxa [221]. Many groups have reported the importance of bortezomib-mediated inhibition of the pro-survival NF-κB in cancer cells by preventing IκB degradation resulting in the induction of cell death [139, 230, 233, 234]. However, there are conflicting reports that NF-κB inhibition may not be the key mechanism of bortezomib anti-cancer activity and conversely bortezomib treatment may lead to

activation of NF-κB [235]. Thus the apoptotic activity of bortezomib may be dependent on cell type and the presence of alternative signaling pathways.

It has been shown that multiple myeloma cells derived from relapsed patients have elevated NF-κB levels and that aberrant activation of NF-κB contributes to drug-resistance [236, 237]. With this in mind, a phase I clinical trial using bortezomib as a single agent was conducted on patients with refractory multiple myeloma. One of the patients had complete response with improvements in eight other patients observed indicated by the reduction of plasma cells in the bone marrow. From this phase I study, it was concluded that bortezomib was well tolerated [238]. This study was followed by several other phase I, II and III studies where bortezomib was used as a single agent or in combination with other conventional anti-cancer drugs like doxorubicin, dexamethasone and several others [233, 239]. Furthermore combination studies showed that bortezomib could sensitize cancer cells to conventional chemotherapy treatments and overcome drug resistance in several hematological malignancies. [229, 240]. However; combination strategies on solid tumor types have been less successful, for example bortezomib and docetaxel did not show any significant anti-tumor effect in hormone refractory prostate cancer [241]. Thus, although bortezomib showed promising results in patients with hematologic malignancies, less efficacy was observed in patients with solid tumors.

In addition, the use of bortezomib in clinics has been hampered by the development of acquired drug resistance and the occurrence of serious side effects [242]. Many multiple myeloma patients that show initial responses later relapse, and a significant sub set of patients do not respond to the treatment at all [243]. Moreover, despite being well tolerated, bortezomib has been associated with many toxic side effects including peripheral neuropathy, low platelet and erythrocyte counts and joint pain, among many others [244].

Resistance to bortezomib can occur either at the level of the proteasome or downstream of the enzymatic activity. In different bortezomib-resistant cell lines, mutations or over-expression of the β5-subunit encoding gene *PSMB5* has been shown to contribute to resistance [245, 246]. Resistance mechanisms downstream of the proteasome include overexpression of molecular chaperones, alterations to regulators of the apoptotic machinery, induction of aggresome formation.

A correlation between loss of sensitivity to bortezomib and up-regulation of the pro-survival chaperone BiP/Grp78 controlling ER homeostasis has been observed. Knockdown of BiP/Grp78 or pretreatment with inhibitors down-regulating BiP/Grp78, in cells resistant to bortezomib, has been shown to restore cells sensitivity [247].

Over-expression of the anti-apoptotic mediator Bcl-2 has been implicated in mediating bortezomib resistance. Down-regulation of Bcl-2 with shRNA sensitized Jurkat cells to bortezomib treatment, suggesting that endogenous levels of Bcl-2 are sufficient to protect against bortezomib treatment. Furthermore, synergistic effects were observed in cells treated with an inhibitor of the Bcl-2 protein (ABT-737) and bortezomib [248].

Aggresome formation induced by bortezomib has also been reported to be resistance mechanism. Ubiquitin conjugates induced by bortezomib treatment are sequestered into aggresomes, which is the way of cancer cells to dispose proteotoxic proteins in order to promote survival. Disrupting aggresome formation with histone deacetylase inhibitors has been shown to overcome bortezomib resistance [212].

A new generation of proteasome inhibitors

The proteasome is an attractive target for cancer therapy and there is a need for new proteasome inhibitors which will overcome drug-resistance and have lower toxic side effects to be able to improve the outcome of patients.

A new generation of proteasome inhibitors with irreversible modes of action and which are orally bioavailable is currently being evaluated. These new proteasome inhibitors are being developed with the aims of reducing toxicity, overcoming bortezomib resistance, enhancing anti-tumor activity and improving effectiveness of proteasome inhibition. Carfilzomib a peptide-epoxyketone that irreversibly inhibits the chymotrypsin-like activity of the proteasome has recently been approved by the FDA for the treatment of multiple myeloma patients who have received at least two prior therapies. A major advantage of using carfilzomib as a therapeutic agent is its ability to overcome bortezomib resistance. Similarly to bortezomib, carfilzomib is, administered intravenously. However, several orally bioavailable proteasome inhibitors are currently in clinical trials [249, 250].

Marizomib is an orally available irreversible proteasome inhibitor that inhibits all three (chymotrypsin-like, caspase-like and trypsin-like) activities of the 20S CP [251, 252]. The advantage of inhibiting all three activities is thought to be due to a more extensive inhibition of proteasome, thereby inducing pro-apoptotic pathways to a greater extent than inhibiting the chymotrypsin-like activity alone [253]. Marizomib shows clinical activity on bortezomib-refractory multiple myeloma patients and is well tolerated with no peripheral neuropathy side effects [252]. In conclusion, the new generation of proteasome inhibitors are creating opportunities to overcome resistance and enhance clinical outcomes. However, one of the biggest challenges that has yet to be overcome is to identify proteasome inhibitors that have better effects on advanced solid tumors. Targeting other components of ubiquitin proteasome pathway is an intriguing possibility and it has been suggested that targeting the UPP upstream of the 20S CP may lead to improved therapy options for cancer patients [254].

Targeting deubiquitinating enzymes

As mentioned above, 98 DUBs have been identified in the human genome and recent evidence suggests that many of these are overexpressed or show altered activity in tumor cells [255]. Therefore, finding inhibitors that target DUB activity may therapeutically be beneficial. Several pan-DUB inhibitors, which target both proteasomal and non-proteasomal DUBs have recently been described.

The C-terminally modified vinyl sulfone derivative of ubiquitin, UbVS, is an irreversible DUB inhibitor that is used as an active site directed probe to detect enzymatically active DUBs [53]. Ubiquitin aldehyde (Ubal) is reversible inhibitor of ubiquitin C-terminal hydrolases (UCHs) and ubiquitin specific proteases (USPs) that prevents the hydrolysis of poly-ubiquitin chains on substrate proteins *in vitro* [256]. Due to their high molecular mass and lack of specificity these inhibitors have not been considered as therapeutic agents and instead are primarily used as research tools. However, as research tools they may be fundamental in aiding the design and identification of small molecule DUB inhibitors to be used as therapeutic agents.

WP1130 (degrasyn) is a small-molecule compound that inhibits several DUBs, both proteasomal and non-proteasomal, including USP9$_X$, USP5, USP14 and UCHL5. In cells, WP1130 inhibition of DUB activity resulted in the accumulation of poly-ubiquitinated conjugates, down regulation of anti-apoptotic mediators such as MCL-1 and up-regulation of pro-apoptotic proteins, such as p53 [257]. Furthermore, Pham and

colleagues showed that WP1130 in combination with bortezomib had antitumor activity in lymphoma animal model [258].

A more specific small molecule inhibitor of DUB activity is IU1. IU1 is a reversible inhibitor of USP14 and binds specifically to the proteasomal-associated form of USP14 inhibiting its DUB activity. IU1 has been shown to enhance the degradation of proteasome substrates and has been suggested as a treatment option for neurodegenerative diseases that are associated with the accumulation of misfolded and aggregated proteins. Due to its capacity to increase proteasome activity this inhibitor may not be a suitable therapeutic agent for cancer treatment [78].

Recently, a curcumin analog (AC17) has been shown to irreversibly inhibit the deubiquitinase activity of the 19S RP. AC17 was shown to inhibit NF-κB activity and to reactivate p53 in human lung cancer cells and was also shown to have anti-tumor activity in an *in vivo* model of human lung cancer. This anti-tumor activity was associated with proteasome inhibition, NF-κB blockade and p53 reactivation. However, it is still unclear which deubiquitinating enzymes are inhibited with AC17 [259].

Although many DUB inhibitors have been identified and characterized in recent years, their broad range of activity limits their clinical use, thus the identification of inhibitors with more specific targets is required.

AIMS OF THESIS

The general aim of the thesis was to investigate the potential of a deubiquitinase inhibitor, b-AP15, as a therapeutic agent for cancer treatment.

The specific aims of these studies were;

- **Paper I:** To elucidate the mechanism of action of b-AP15.

- **Paper II:** To characterize the response of tumor cells to b-AP15 and to compare this response to the elicited by the clinically used proteasome inhibitor bortezomib.

- **Paper III:** To examine cellular uptake and metabolism of b-AP15 and to examine reversibility of target binding and commitment to cell death.

- **Paper IV:** To examine the faith of misfolded proteins accumulating in b-AP15-exposed cells.

RESULTS & DISCUSSION

PAPER I

Inhibition of proteasome deubiquitinating activity as a new cancer therapy.

A screen conducted to identify compounds that induce p53 independent apoptosis led to identification of a number of compounds, one of which was NSC687852 (b-AP15). This compound was later identified in a screen of compound inducing the lysosomal apoptosis pathway [260, 261]. In this study we show that b-AP15 is a potent proteasome inhibitor that functions via a novel mechanism, namely the inhibition of two of the 19S RP associated deubiquitinating enzyme, UCHL5 and USP14.

Firstly we sought out to elucidate the effect of b-AP15 on the cellular proteasome function. We used a reporter cell line expressing a proteasome targeted fluorescent substrate: ubiquitin tagged yellow fluorescent protein (Ub^{G76V}-YFP). Upon proteasome inhibition, the Ub^{G76V}-YFP reporter protein accumulated in cells, indicating impaired proteasome degradation. b-AP15 induced a dose-dependent accumulation of the Ub^{G76V}-YFP. Proteasome inhibition is associated with the induction of poly-ubiquitin conjugates, which were indeed observed in b-AP15 treated cells. Importantly, compared to the proteasome inhibitor, bortezomib, b-AP15 induced poly-ubiquitin conjugates of a higher molecular weight, indicating different mechanism of action.

Many proteasome inhibitors inhibit the proteolytic activities of the 20S CP. Thus, we next investigated if b-AP15 inhibited this activity by using fluorogenic substrates for chymotrypsin-like, caspase-like and trypsin-like activities, respectively. b-AP15 was unable to perturb these activities, thus we hypothesized that b-AP15 might act upstream of the 20S CP. Chemical structure of b-AP15 contains an α-β dienone with two sterically accessible β carbons. A similar pharmacophore has been described in a variety of deubiquitinase inhibitors, thus we hypothesized that b-AP15 may inhibit DUB activity [262]. Firstly, we showed using a combination of tagged DUB active site probes (HA-UbVS) and the flurogenic DUB substrate (Ub-AMC) that b-AP15 was not a general inhibitor of total deubiquitinase activity in cells. To further investigate the inhibitory effects of b-AP15 on DUB activity we used biochemical assays with purified 19S RP and 26S proteasomes on different proteasome substrates and K48- and K63- linked ubiquitin tetramer chains. b-AP15 inhibited deubiquitination of the proteasome substrates and disassembly of K48 and K63 chains, indicating the possibility of b-AP15 being a proteasomal DUB inhibitor. Three DUBs are linked to the proteasome POH1, UCHL5 and USP14. UCHL5 and USP14 are cysteine proteases, while POH1 is a metalloprotease. We ruled out the possibility that b-AP15 inhibited POH1 by using a general cysteine protease inhibitor for cysteine DUBs (UCHL5 and USP14) and a metal chelator for the metalloprotease POH1. After co-treating purified 19S regulatory particles with b-AP15 and cysteine protease inhibitor some deubiquitinase activity was still observed while co-treatment with b-AP15 and metal chelator resulted in abolished DUB activity, suggesting that b-AP15 inhibited one or both of the cysteine proteases. The hypothesis was further supported by using hemagglutinin-tagged ubiquitin vinylsulphonone (HA-UbVS) as a probe, which is utilized for detecting active DUBs. Treatment with b-AP15 in biochemical assays and in cells resulted in a loss of Ub-VS labeling of two DUBs with molecular weights corresponding to UCHL5 and USP14. From these experiments we concluded that b-AP15 inhibited the activities of the cysteine proteases UCHL5 and USP14.

In cells, b-AP15 induced cell cycle arrest and apoptosis when used at concentrations required for the accumulation of poly-ubiquitin conjugates. Moreover, decreased cell viability was observed at similar concentrations thereby coupling proteasome inhibition to the cytotoxic effects of the drug. Importantly, b-AP15 induced apoptosis was insensitive to disruption of p53 or Bcl-2 overexpression. We further investigated the selectivity of b-AP15 and showed that it was more toxic to colon carcinoma cells than to immortalized epithelial cells or peripheral blood mononuclear cells. Notably, differences observed with b-AP15 were larger compared to bortezomib, indicating that b-AP15 might have a larger therapeutic window compared to bortezomib.

Next we evaluated the anti-tumor activity of b-AP15 in different animal models representing both solid tumors and hematological malignancies. Daily administration of b-AP15 to mice with FaDu squamous carcinoma xenografts resulted in significant antitumor activity and induction of caspase cleaved keratin 18 in mouse plasma, an indicator of drug-induced apoptosis in human tumor xenografts [263]. Bcl-2 overexpression has been reported to mediate resistance to proteasome inhibitors [248]. So we examined disease-free survival in mice with HCT-116 colon carcinoma xenografts overexpressing Bcl-2. The onset of tumor growth was delayed in the treated group with two out of six mice disease free at the termination of the study, suggesting that b-AP15 may be used to overcome Bcl-2-mediated drug resistance in tumors.
In previous models b-AP15 was administered daily so we investigated the anti-tumor effect of b-AP15 using a less frequent schedule. Treatment with b-AP15 every fourth day significantly reduced tumor growth in mice with orthotopic breast carcinoma with decreased numbers of pulmonary metastases detected in b-AP15 treated mice. We also observed increased accumulation of K48-linked poly-ubiquitin chains and activated caspase 3 in tumor section staining, coupling anti-tumor activity of b-AP15 *in vivo* to the inhibition of proteasome function.
Lastly, the effect of b-AP15 on tumor cell invasion was investigated in an acute myeloid leukemia model. Eight out of ten b-AP15 treated mice had regressed leukemia. Animals in the control group had massive invasion of myeloid leukemic cells into the liver tissue and invasion of leukemic blasts in the ovary tissue which was absent in biopsies from the b-AP15 treated groups.

In conclusion, we identified b-AP15 as a novel proteasome inhibitor that functions by inhibiting the DUB activity of the 19S RP and which displays anti-tumor activity both *in vitro* and *in vivo*.

PAPER II

Induction of tumor cell apoptosis by a proteasome deubiquitinase inhibitor is associated with oxidative stress.

Proteasome inhibition leads to the induction of a wide range of cellular responses. In this study we try to delineate the different cellular responses induced by b-AP15 compared to the conventional proteasome inhibitor bortezomib.

To identify which molecular pathways were affected by b-AP15 treatment, we performed transcriptional profiling on 84 genes whose expression is known to be altered by chemotherapeutical reagents, including proteasome inhibitors. Analysis of the gene expression profiles showed that both b-AP15 and bortezomib induced the up-regulation of a common subset of genes involved in oxidative stress, ER stress, heat shock and the immediate early response. Notably, the HSPA6 gene which encodes HSP70B´protein was induced >1000 fold following b-AP15 treatment compared to 60 fold induction by bortezomib. Hsp70B´has been shown to be induced by proteasome inhibitors and is induced under conditions of severe proteotoxic stress [264]. One reason for this disparity could be the accumulation of higher molecular weight ubiquitin conjugates as a consequence of UCHL5 and USP14 inhibition observed in b-AP15 treated cells compared to bortezomib. Moreover, the induction of genes associated with oxidative stress (HMOX-1, PHOX/p67) and immediate early response (JUN, GADD34, GADD45A, and GADD45B) were overall strongly induced by b-AP15 compared to bortezomib. Lastly, the expression of genes associated with ER stress were similarly induced by both drugs.

Both b-AP15 and bortezomib activated caspase-4, an ER resident caspase that is activated upon ER stress induction [172]. The role of ER stress in b-AP15- and bortezomib-induced apoptosis was assessed by chemical inhibitors or siRNA against caspase-4, either of which resulted in the inhibition of apoptosis induced by both drugs. The data suggest contribution of ER stress in induced apoptosis. In contrast to bortezomib, b-AP15 induced phosphorylation of eIF2α in several different cell lines. Activated eIF2α has been suggested to reduce the load of misfolded proteins on the ER promoting cell survival [265]. Bortezomib has been shown previously to inhibit ER kinase (PERK) leading to the inhibition of phosphorylation of eIF2α and has been proposed to be important for the induction of apoptosis by this agent [266]. Despite the phosphorylation of eIF2α, b-AP15 induced a more rapid apoptosis compared to bortezomib, evident from our studies on the kinetics of apoptosis induction. Thus, suggesting that translational suppression might not be important for the induction of apoptosis by agents that impair proteasome function.

We further investigated the role of ROS production in b-AP15 induced apoptosis. To assess the levels of ROS production by b-AP15 and bortezomib we utilized fluorescent ROS probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) and determined that both drugs induced ROS production, although levels of ROS were higher in b-AP15 treated cells. Consistent with these results the expression of HMOX-1 protein, which is considered to be one of the most sensitive and reliable indicators of cellular oxidative stress [267], was higher following b-AP15 treatment. Others have reported the important role of ROS production in mediating bortezomib-apoptosis, so we sought to

investigate if the ROS production generated by b-AP15 was involved in the induction of apoptosis. Using different anti-oxidants to scavenge ROS resulted in inhibition of apoptosis by both b-AP15 and bortezomib, confirming previous findings. The role of ROS in apoptotic signaling induced by bortezomib and other proteasome inhibitors has been controversial, due to ability of anti-oxidants, such as NAC and Vitamin C, to form complexes with these drugs [268, 269]. Anti-oxidants used in this study did not interact with b-AP15, suggesting that b-AP15 induced apoptosis could indeed be mediated by oxidative stress.

Prolonged oxidative stress has been reported to inactivate the proteasome function, which raised a question if b-AP15 induced ROS production contributed to the same phenomenon. To investigate this further we utilized a reporter cell line expressing a proteasome targeted fluorogenic substrate, Ub^{G76V}-YFP. Treatment of these cells with b-AP15 in the presence of a scavenger during drug treatment did not affect the accumulation of the Ub^{G76V}-YFP signal, indicating that the inhibition of the proteasome function was due to the direct action of b-AP15 and not due to increased ROS levels. Additional experiments with different b-AP15 analogues confirmed that proteasomal blocking was associated with induction of oxidative stress. b-AP15 analogues that generated poly-ubiquitin conjugates similarly to b-AP15 also induced high levels of HMOX-1 protein, whereas analogues that failed to induce poly-ubiquitin conjugates were unable to induce HMOX-1 expression. Furthermore, we show that stress kinase JNK is activated upon b-AP15 treatment and is involved in the induction of apoptosis by b-AP15. The activation of JNK was further shown to be mediated by b-AP15 induced oxidative stress.

In this paper we conclude that b-AP15 is a more potent inducer of apoptosis compared to bortezomib and that oxidative stress is a key mediator of the strong pro-apoptotic potential of b-AP15.

PAPER III

The 19S deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death.

In this study we address several questions with regard to the mechanism of action and pharmacology of the deubiquitinase inhibitor b-AP15.

As already mentioned in papper I, b-AP15 contains a pharmacophore with an α-β dienone with two sterically accessible β carbons that has been described to inhibit deubiquitinase activity. The α, β-unsaturated dienone serve as a Michael acceptor that interacts with thiol groups of cysteines in deubiquitinating enzymes. b-AP15 also contains another Michael acceptor (an acrylamide residue) that potentially could be reactive. We sought out to investigate which of these Michael acceptors mediated the biological activity of b-AP15. New compounds were synthesized in which the acrylamide residue and the α, β-unsaturated carbonyls were substituted. Compared to b-AP15, compounds with the substituted acrylamide induced cell death to a similar degree as b-AP15. In contrast, substitution of the unsaturated carbonyls lead to reduced cytotoxic effects. Thus, the biological activity of b-AP15 is governed primarily by the reactivity of α, β-unsaturated carbonyl pharmacophore.

A DUB inhibitor, AC17, with similar chemical properties to b-AP15 have been reported to be irreversible inhibitor [259]. However, in paper I we report that b-AP15 is reversible inhibitor. The reversibility of b-AP15 was further investigated in this study. We used Ub-VS to detect active proteasomal USP14. Dilutions of b-AP15 treatment of cell extracts lead to reappearance of the active USP14, indicating b-AP15 to be a reversible inhibitor of deubiquitinase activity. We also investigated if the inhibition of proteasome function and induction of apoptosis by b-AP15 was reversible in cell lines. We utilized a reporter cell line expressing a proteasome targeted fluorescent substrate, ubiquitin tagged yellow fluorescent protein (Ub^{G76V}-YFP). Treatment of cells with low b-AP15 concentrations for one hour resulted in the induction of poly-ubiquitin conjugates, which started to decline four hours after drug removal. Similar pattern was observed for the reporter protein Ub^{G76V}-YFP and proteasome substrate $p21^{Cip1}$, indicating that b-AP15 was reversible inhibitor of the proteasome function. However, the cleavage of caspase 3 and PARP was observed twenty four hours after wash-out, suggesting that despite reversible inhibition of the proteasome function cells still committed to apoptosis after one hour of b-AP15 exposure.

In study I, we also observed that higher concentrations of b-AP15 were required to inhibit deubiquitinase activity in biochemical assays than in assays of proteasomal function on cells. The reason for this difference was further investigated in this study. When using Ub-AMC as a substrate for DUBs, an $IC_{50} \sim 17\mu M$ of b-AP15 was observed while $1\mu M$ of b-AP15 was sufficient to inhibit the activity of USP14 in colon carcinoma cells. We hypothesized that the reason for this discrepancy could be due to effective uptake of b-AP15 from the medium and enrichment of this compound in cells, which also could explain the fast commitment of cells to apoptosis. Distribution from medium to cells was examined by the incubation of b-AP15 in the presence or the absence of cells. Only one hour of incubation with b-AP15 resulted in strong uptake of the drug by cells from

the medium. Using radiolabeled b-AP15 we observed rapid uptake of the drug into cells after thirty minutes incubation. Drug washout resulted in a ~40% decrease of radiolabeled b-AP15 over two hours with a ~50% decrease observed up to ten hours. These results lead us to the assumption that a sufficient amount of drug is available for proteasome inhibition even after washout, when using high drug concentrations. Consistent with our hypothesis, treatment of reporter Ub^{G76V}-YFP expressing cells with higher b-AP15 concentrations resulted in the accumulation of poly-ubiquitin conjugates that were still abundant eight hours after drug removal. Moreover, we observed low concentrations of free intracellular b-AP15, suggesting that majority of the drug molecules may bind to cellular macromolecules. As described earlier Michel acceptors react with thiol groups in cysteines of proteins, suggesting that b-AP15 was binding to intracellular thiols. Pretreatment of cells with N-ethylmaleimide, a covalent inhibitor of DUB activity and a Michael acceptor, prior the treatment of cells with radiolabeled b-AP15 resulted in the inhibition of the cellular uptake of b-AP15, indicating that the uptake of b-AP15 was via a thiol-dependent mechanism.

Since it is unlikely that b-AP15 binds solely to the thiols present in the proteasomal deubiquitinases, we also report an off-target activity of b-AP15, namely irreversible inhibition of thioredoxin reductase. The thioredoxin enzymatic system is important for redox regulation of cellular function. We investigated the importance of the thioredoxin reductase inhibition by b-AP15 on the cellular response. The inhibition of thioredoxin reductase on its own could not explain the strong induction of oxidative stress and apoptotic activity of b-AP15 observed in paper II, suggesting that it is of limited importance for b-AP15-mediated cytotoxicity. Another important enzymatic system in regulating cellular redox-homeostasis is the glutathione system. In contrast to thioredoxin, glutathione reductase was not inhibited by b-AP15, possibly because of the selectivity of b-AP15 for some cellular cysteines present in thioredoxin reductase but not in glutathione reductase.

From this study we conclude that b-AP15 is a reversible inhibitor of deubiquitinating activity and proteasome function. Although b-AP15 is reversible inhibitor, cells treated with b-AP15 rapidly become committed to cell death within one hour of drug treatment. This commitment to cell death may be explained by the rapid uptake and enrichment in cells and by the harmfulness of misfolded proteins not deposited in aggresomes (see paper IV). These findings are encouraging for the development of b-AP15 for clinical use.

PAPER IV

Inhibitor of proteasome deubiquitinase activity inhibits cytoprotective-aggresome formation in cancer cells.

Blockade of the proteasome results in the accumulation of poly-ubiquitinated conjugates that are prone to aggregation. Such aggregates are sequestered at the peri-nuclear region in complexes termed aggresomes. Bortezomib has been shown to induce the aggresome pathway, which may be used by malignant cells as an alternative mechanism for sequestering and degrading poly-ubiquitinated proteins, potentially resulting in drug resistance [212-214]. In this study, we investigate if blocking the deubiquitinating activity of the proteasome by b-AP15 is involved in the induction of aggresome pathway.

The p62 protein is an ubiquitin-binding scaffold protein that has been shown to co-localize with ubiquitinated protein aggregates and is a key mediator of aggresome formation [209]. Treatment of cells with either b-AP15 or bortezomib for shorter time points resulted in the formation of dispersed aggregates throughout the cytosol, evident from the co-localization of p62 and K48-polyubiquitin in the formed aggregates. Longer exposure to bortezomib, resulted in the formation of a single juxta-nuclear poly-ubiquitin aggregate that co-localized with p62 in >60% of the cells when treated with bortezomib. In contrast, prolonged b-AP15 treatment resulted in the formation of multiple K48-ubiquitin/p62 positive aggregates in cytoplasmic and peri-nuclear regions that were not characteristic aggresome structures.

Aggresome formation is accompanied by redistribution of the intermediate filament protein vimentin, which forms a cage surrounding the nascent aggresome at the peri-nuclear region [183, 195, 196]. These observations made us investigate the vimentin localization in cells treated with b-AP15 and bortezomib. Both drugs induced vimentin re-localization, evident from the appearance of condensed vimentin localized at the peri-nuclear region. In agreement with previous results, bortezomib treated cells displayed vimentin cage structures surrounding the aggresome, which were absent in b-AP15 treated cells. Results so far suggest that poly-ubiquitinated conjugates induced by b-AP15 form aggregates but are incapable of forming aggresome structures.

Transport of aggregates along microtubules to MTOC is required for aggresome formation. It has been shown that depolymerization of microtubules with microtubule inhibitors prevents the formation of peri-nuclear aggresomes, resulting in the production of aggregates dispersed throughout the cytoplasm. [183]. The observation that treatment with b-AP15 also generated multiple aggregates in the absence of typical aggresome formation, made us wonder whether b-AP15 disrupted components of the aggresomal pathway. When we co-treated cells with b-AP15 and bortezomib a lower number of aggresomes was observed compared to bortezomib treatment, suggesting that b-AP15 partially inhibited aggresome formation. To further investigate this we utilized a GFP chimera GFP250 that readily forms peri-nuclear aggresomes that are dependent on the tubulin network but independent of the ubiquitin-proteasome system [196]. Treatment with both bortezomib and b-AP15 further enhanced the formation of aggresomes implying that b-AP15 inhibits aggresome formation in a microtubule-independent, but ubiquitin-dependent, manner.

We sought to investigate if b-AP15 interfered with another crucial component of the aggresomal pathway, the deacetylase HDAC6. HDAC6 is responsible for recruiting aggregates, through its ubiquitin binding domain, for the transport to aggresomes [198]. HDAC6 is known to be ubiquitinated and has also been shown to associate with DUB activity [199]. We hypothesize that b-AP15 might interfere with the ubiquitination profile of HDAC6 resulting in non-functional HDAC6 for aggresome formation. To investigate this we transfected cells with His-tagged ubiquitin and HDAC6. We observed increased levels of poly-ubiquitinated HDAC6 in b-AP15 treated cells (6-fold induction) compared to bortezomib treated cells. This difference may be due to inhibition of DUBs by b-AP15 involved in the regulation of HDAC6.

Our results show that b-AP15 blocks aggresome formation in an ubiquitin-dependent manner evident from our experiments with co-treatment with b-AP15 and bortezomib and also our experiments with GFP-250 chimera. We hypothesize that b-AP15 may inhibit HDAC6 mediate aggresome formation but whether this depends on the poly-ubiquitination of HDAC6 by b-AP15 or some other mechanism needs further investigation. Since it has been shown that HDAC6 inhibitors effectively synergized with bortezomib leading to disrupted aggresome formation and induced cytotoxicity, experiments using RNAi or pharmacological inhibitors to block HDAC6 would be informative for future studies [213]. Also we have not directly shown that the disruption of aggresomes by b-AP15 is due to inhibition of UCHL5 and USP14, which needs further investigation.

CONCLUSIONS & FUTURE PERSPECTIVES

The thesis identifies a novel small molecule inhibitor of the proteasome, b-AP15. b-AP15 blocks proteasome function by the inhibition of deubiquitinating enzymes, UCHL5 and USP14. For the future development of b-AP15 it is important to understand the exact specificity of b-AP15 to UCHL5 and USP14, given the notion that these two DUBs belong to different families of this class of enzymes. So far, we can only speculate that b-AP15 inhibition of UCHL5 and USP14 could be related to the unique confirmations of these enzymes or could be because of drug-induced alterations of the structure of the 19S RP.

We also show that b-AP15 elicits anti-tumor effects in a number of tumor models representing both hematological and solid tumor malignancies. The anti-tumor activity of b-AP15 both *in vitro* and *in vivo* is correlated to the inhibition of proteasome function. The potent induction of apoptosis by b-AP15 was associated with strong induction of oxidative stress. However, in an attempt to rescue cells from b-AP15 induced cytotoxicity by scavenging ROS we observed no change in the overall cell survival, suggesting that there are other mechanisms that will eventually cause cell death. In contrast to b-AP15, scavenging bortezomib-mediated ROS production resulted in increased cell survival. We propose a model for the potent anti-tumor activity of b-AP15 (Figure 5). Inhibition of deubiquitinating activity of the proteasome by b-AP15 results in the accumulation of higher molecular weight ubiquitin-substrate complexes compared to the induction of these complexes by bortezomib targeting the 20S enzymatic activities, resulting in the stronger proteotoxicity by b-AP15. The high molecular weight ubiquitin conjugates induced by b-AP15 are either sequestered into aggregates or they associate with membranes of different organelles via the exposed hydrophobic patches on the surface of protein substrates causing organelle dysfunction (depolarization and structural collapse) and eventually cell death.

Overexpression of Bcl-2 is involved in bortezomib-mediated resistance. In contrast, b-AP15 is insensitive to the overexpression of Bcl-2 suggesting that b-AP15 could be used as a second line therapy in patients to overcome bortezomib resistance. Indeed, a study on b-AP15 and multiple myeloma has recently been published, in collaboration with our group, where it is shown that b-AP15 significantly decreased cell viability of multiple myeloma cells derived from patient who have relapsed from prior therapies with bortezomib [270].

Furthermore, b-AP15 is not a genotoxic agent. The use of DNA-damaging agents in clinics has been limited by the adverse side effects and increased risk of secondary cancers as a consequences of agents' genotoxicity.

The findings in this thesis provide a basis for evaluation of b-AP15 as a potential therapy for cancer treatment.
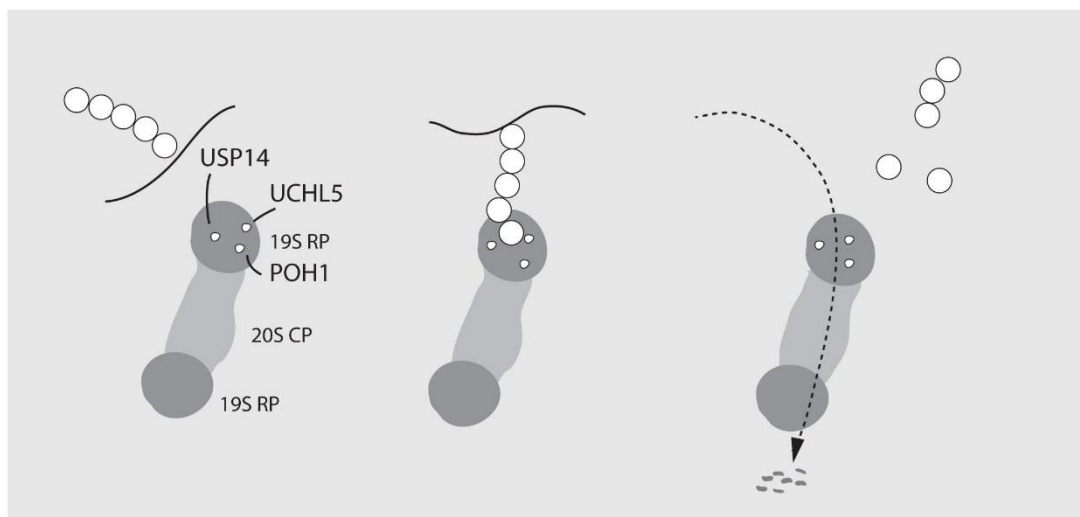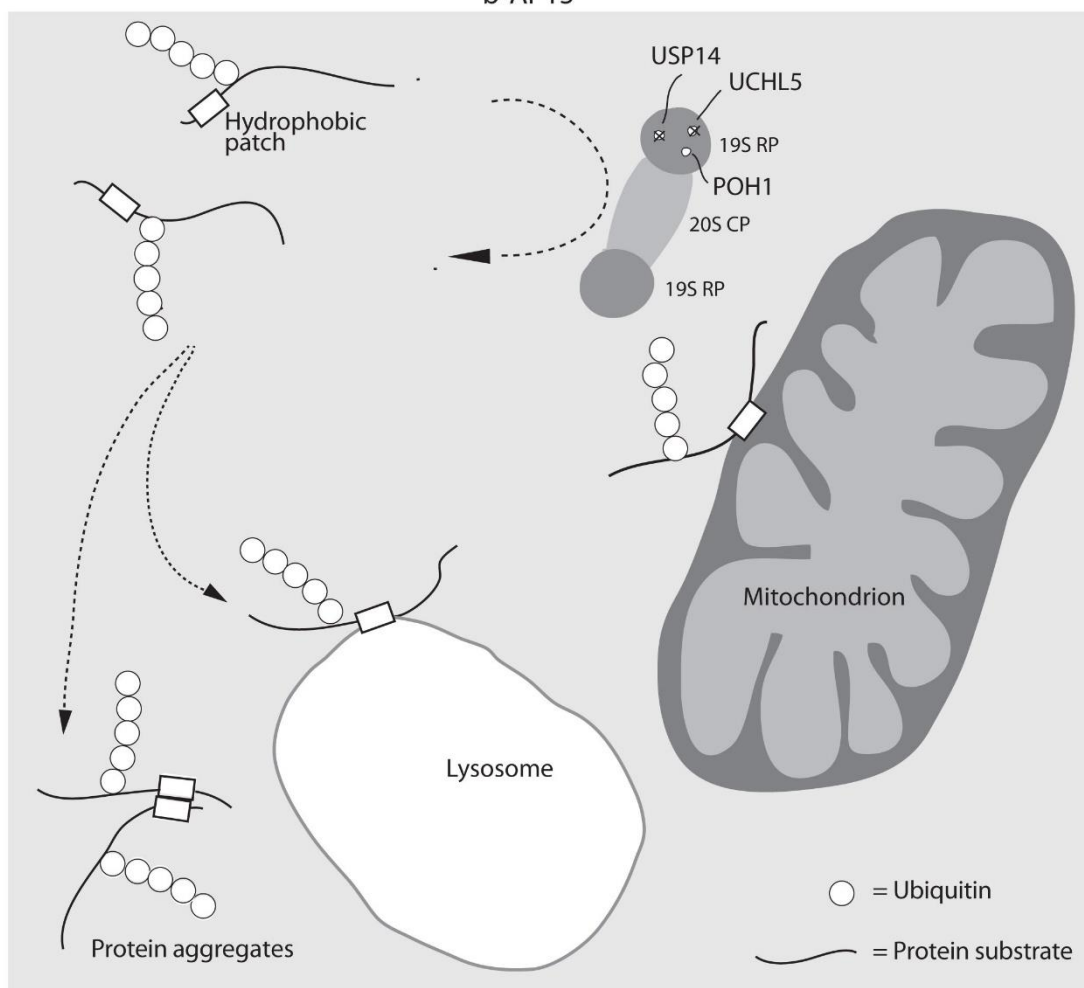
Figure 5. The effect of b-AP15 treatment on cancer cells.

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